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THE PHYSICAL, CHEMICAL, AND STRUCTURAL CHARACTERISTICS OF MoO$_x$
CATALYSTS SUPPORTED OVER THE BINARY OXIDE OF SILICA/TITANIA
AND RELATIONSHIPS TO THEIR BEHAVIOR IN THE OXIDATIVE
DEHYDROGENATION OF PROPANE AND ETHANE

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
The Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By
Rick Bruce Watson, B.S.

* * * *

The Ohio State University
2001

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ABSTRACT

The research drive to develop an oxidative dehydrogenation (ODH) process for propane and ethane comes from the fact that the chemical industry depends heavily on propylene and other alkene feedstocks. Research efforts on ethane ODH have led to catalytic systems with yields approaching those that could compete with the current non-oxidative technology, indicating the high probability of a viable process. However, in propane ODH, the development of catalysts able to achieve higher yields is of current interest in the literature and warrants further investigation. The use of a silica-titania mixed-oxide-supported molybdenum catalysts has been studied in regard to their activity for the oxidative dehydrogenation (ODH) of propane and ethane. By varying the K/Mo molar ratio a maximum in activity was obtained for propane ODH.

The effect of modifiers (alkali and halide) on the catalyst surface characteristics and, in turn, on the catalytic performance in ethane and propane ODH has been examined. The catalysts used in this study have been synthesized by a "one-pot" sol gel/co-precipitation technique. The main focus of the work has been characterization of the surface molybdena species, physical-chemical properties of the Si:Ti support, surface acidity, reducibility, adsorption/desorption behavior, and surface intermediates present during the reaction. Catalysts were characterized by BET surface area measurements, X-
ray diffraction (XRD), laser Raman spectroscopy, X-ray photoelectron spectroscopy (XPS), Diffuse Reflectance Infrared Fourier Transform Spectroscopy (DRIFTS), Transmission Electron Microscopy (TEM), Electron-Spin Resonance (ESR), $^{29}$Si CP-MAS NMR, transient/ steady-state isotopic labeling studies using $^{18}$O, and propane temperature programmed desorption (TPD)

Insight into the supported MoO$_x$ structure is obtained. This allows characterization of the surface molybdena species, physical-chemical properties of the Si:Ti support, surface acidity, reducibility, adsorption/desorption behavior, and surface intermediates present during the reaction, and correlation of these characteristics with the level of alkali or halide doping. It was found that potassium suppresses the reducibility of the molybdenum species at low K/Mo ratios while possibly stabilizing the Mo(V) oxidation state during reaction. The trends seen in XPS, and X-ray diffraction patterns indicate that there are additional factors due to the interaction of the support that control catalytic behavior. Further characterization has also been performed over un-promoted Mo/Si:Ti catalysts with various Mo loadings to elucidate the effects of surface coverage.
Dedicated to my grandparents, Curtis and Flora Evans
ACKNOWLEDGMENTS

I wish to thank my grandparents, Curtis and Flora Evans. By dedicating their lives to my advancement, they have enabled me to achieve what may once have been envisioned as just a dream.

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CHAPTER 1

INTRODUCTION

Over the past 15 years, many industrial and academic research efforts have been focused on the conversion of lower alkanes (C1-C5) to petrochemical feedstocks. These are catalytic reactions that include: methane, partial oxidation to formaldehyde and oxidative coupling to C₂ hydrocarbons; ethane and propane, oxidative dehydrogenation to olefins and oxygenates (acetic acid, acrolein, acrylic acid; butane and pentane, oxidative dehydrogenation to maleic and phthalic anhydrides). Thus far, there are no industrially practical operations for such applications except for the production of maleic anhydride and acetic acid from butane. However, the incentive for such processes is high given the vast amounts of natural gas available containing significant ethane, propane, and butane in addition to methane.

There exists a strong research drive for propylene production by a supplemental route, other than petrochemical cracking and non-oxidative dehydrogenation. Propylene is one of the top 10 organic chemicals produced every year (>25,000Mlbs) and the commercial demand is on the rise through the year 2000 [1]. Propylene is an industrial starting material for polyethylene and polypropylene production while propane is
relatively cheap and readily available. Thermal dehydrogenation suffers from several drawbacks from the industrial standpoint. Since the dehydrogenation reaction is highly endothermic, there exists a thermodynamic limitation that requires operation at 700-800°C for appreciable yields of propylene to be obtained.

\[
\begin{align*}
\text{C}_3\text{H}_8 & \rightarrow \text{C}_3\text{H}_6 + \text{H}_2 \\
\text{maximum thermodynamic yield} & \end{align*}
\]

Propylene Yield

\[
\begin{array}{c|c|c|c|c|c|c|c|c|c|c|c}
& 300 & 400 & 500 & 600 & 700 & 800 & 900 \\
0.1 & 0.2 & 0.3 & 0.4 & 0.5 & 0.6 & 0.7 & 0.8 & 0.9 & 1 \\
\end{array}
\]

Temp(°C)

Figure 1.1 : Thermodynamic limitation of propane dehydrogenation

The thermodynamic dependence of propane dehydrogenation was obtained from a Gibb's free energy calculation and is presented in Figure 1.1. Operation at such high temperatures also makes reactions difficult to control. Furthermore, industrial high pressures shift equilibrium towards reactants.
The oxidative dehydrogenation of lower alkanes offers several advantages to the previously mentioned limitations. Oxidative dehydrogenation (ODH) is a thermodynamically favored exothermic reaction that can allow operation at lower temperatures (300-500°C). Furthermore, in an oxygen atmosphere, the likelihood of catalyst coking is greatly reduced. In the oxidative dehydrogenation of alkanes ($C_nH_{2n+2} + \frac{1}{2}O_2 \rightarrow C_nH_{2n} + 2H_2O$) hydrogen is abstracted from the alkane by oxygen to form an olefin and water. Over many catalysts, the abstraction of the first hydrogen is usually the rate-determining step. However, because of the high activation energy barrier associated with this step, the activation of the alkane requires rather severe reaction conditions. The conditions are termed severe because at conditions conducive to olefin formation the catalyst can readily oxidize the partially oxidized or dehydrogenated product and intermediates to carbon oxides and water. Thus, this practically limits the yield of olefins due to the subsequent reactivity of the olefin.

Over selective catalysts, [2-4], propane ODH is overwhelmingly accepted to proceed via an overall Mars-van Krevelen red-ox mechanism [5] in which gas phase propane is activated by lattice oxygen of the catalyst to ultimately form propylene through several possible intermediate surface structures. Gas phase oxygen then serves the role of re-oxidizing the catalyst surface. This mechanism is presented in Figure 1.2, in which $O_1$ represents lattice oxygen and $V$ a surface vacancy.
Although it is accepted that propane ODH undergoes an *overall* Mars van Krevelen red-ox mechanism, there are many unselective mechanistic steps and roles of the catalyst surface that can break down the mechanism in a quite complicated manner. In propane ODH there are many paths down which the reaction can proceed, depicted in Scheme 1.1.
One of the most characterized unselective paths in propane ODH is the sequential reaction of the formed propylene to oxygenated compounds (e.g. acrolein) and then to ultimate combustion ($\text{CO}_x$). In fact, many routes exist for each step of the reaction to lead to C-C bond breaking and combustion. It is the goal of research to concisely illustrate the selective pathway over selective and active catalyst. The most often studied catalytic systems include vanadium or molybdenum. Some non-selective pathways and the characteristics of the catalytic sites are also research goals. A particular focus is quite often made on the relation of mechanistic behavior to specific surface sites and physical/chemical reaction requirements.
CHAPTER 2

LITERATURE REVIEW

2.1 The Catalytic Oxidative Dehydrogenation (ODH) of Lower Alkanes

The research drive to develop an oxidative dehydrogenation process for propane and ethane comes from the fact that the chemical industry depends heavily on propylene and other alkene feedstocks. For example, propylene demand is estimated to grow 4.5% per year between 1991 and 2000 [1]. Catalytic oxidative dehydrogenation (ODH) is an attractive alternate route for the production of alkenes compared to conventional cracking and dehydrogenation processes. This is because ODH is thermodynamically favored at lower temperatures and usually does not lead to the formation of coke and smaller hydrocarbons. Recent literature has focused on catalysts active below 823K that can limit the amount of carbon oxides formed.

Kung et al. has published a review of ODH on C₂ to C₄ alkanes summarizing the most significant results up to 1994 [2]. This review mentioned the important role of the homogeneous reactions. Since propylene contains a weak allylic C-H bond, its reactivity can be several times higher than propane on any given catalyst. Furthermore, reactor dead-volume minimization is necessary in this reaction to isolate catalytic activity for
propane ODH. This is because radicals, formed on the surface of the catalyst, can desorb during reaction and contribute to the gas phase reaction above the catalyst bed. A quartz packing can both provide a short residence time for propylene and has been shown to effectively quench desorbed radicals in the gas phase. This “surface-assisted gas phase reaction” that can occur in ODH of propane has been studied by Burch et al. [6] who has shown that above ~550°C the homogeneous reaction of propane becomes significant. Therefore, meaningful results are obtained below this temperature. This effect is less important for ethylene, which contains stronger vinylic C-H bonds. In fact, on ethane ODH catalysts a classic catalyst red-ox cycle is not present and the catalyst is only involved in C-H bond scission forming radicals. Nonetheless, researchers must take these factors into consideration in order to obtain accurate data on catalytic systems.

Some of the most effective catalysts studied in ODH reaction over the past five years include vanadium-magnesium, vanadia supported on niobium, other vanadium based catalysts, and nickel molybdates for propane ODH and Mo-V-Nb, Mo-V-Sb, and several alkali or halide-doped catalysts for ethane ODH. Furthermore, our research has shown that alkali-molybdate catalysts supported over sol-gel silica-titania mixed oxide are also a highly effective catalyst for propane and pentane ODH to the corresponding olefin. The development of catalysts able to achieve higher than 20% yield of propylene at high selectivities is of current interest in the literature and warrants further characterization. For ethane ODH, high ethylene yields have been obtained at high selectivities. Generally, the research aim in ethane ODH is to achieve a yield higher than 50% at appreciable selectivity. In another alkane activation review, published by Baerns
et al. [7], the fact was stated that ethane ODH results are in the range of current non-oxidative technology, indicating the high probability of a viable process. However, since the reactions are often operated at higher temperatures than propane ODH, the role of the homogeneous reaction remains a matter of study for any of these catalysts, also shown by Burch et al. [8].

2.2 Propane ODH

Common theories have risen from the literature that applies to many different catalysts studied for Propane ODH. These issues include role of homogeneous reaction, propylene residence time/reactivity, reducibility of the active metal, surface acidity, and propylene adsorption. Pantazidis et al. have noted that a good acid-red-ox balance is needed over VMgO catalysts in the ODH of propane [9]. Many other studies on different catalysts have shown selectivity-acidity-redox relationships. Abello et al. have reported that the stronger the acid strength on their potassium-molybdenum oxo catalyst in the ODH reaction the stronger propylene and propylene intermediates would be held thus creating a less-selective catalyst [10]. They have also proposed a Mo(V) stabilization effect related to acidity. These observations were confirmed over Mo/Si:Ti catalysts studied by Ozkan et al. [11]. However, when we discuss a balance in reducibility and acidity on catalysts in the ODH of propane it is necessary to think in terms of the relative rates of the kinetic steps occurring on oxide catalysts. As discussed by Kung [2], one needs to consider the two reactions propane→propylene and propylene→COx. It was
shown that, on perhaps all catalysts, the rate for the second reaction could be five to ten times higher than that of the first reaction. Work by Khodakov et al. has begun to answer these fundamental questions by measuring the relative rates of propylene and COx formation by residence time and isotopic tracer experiments [12]. Furthermore, they have commented on the likelihood of similar active sites being responsible for both reactions. When the selectivity is considered in terms of these two competing reactions, it becomes clearer that both the reducibility and oxygen mobility characteristics of the catalyst, and its acidity, need to be simultaneously taken into account. While the surface oxygen species affect the rates of both reactions, it is conceivable that a catalyst with a strong Lewis character will have a higher affinity for the electron-rich double bond of propylene, leading to its further oxidation before it desorbs from the surface.

2.3 Ethane ODH

The volume of literature on ethane oxidation is limited, especially in comparison to methane oxidation. However, studies exist although some of which are extensions of methane oxidation work. One of the earliest studies reported in the literature was from Lunsord's laboratories [13] where they studied oxidative dehydrogenation of ethane to ethylene over molybdenum supported on silica using nitrous oxide as the oxidizing agent. Later, they also reported obtaining high yields of acetaldehyde over the same catalysts in the presence of water vapor [14]. In more recent studies, Lunsford and co-workers reported use of lithium/magnesium [15] and chlorine-promoted lithium magnesium
catalysts [16] in oxidative dehydrogenation of ethane and noted a significant increase in ethylene selectivity due to chlorine promotion. The authors proposed a mechanism that involved an ethyl radical formation through a first hydrogen abstraction followed by subsequent oxidation of the alkyl species. They also mentioned the possibility of ethylene formation via surface ethoxide species.

Other studies on ethane oxidation include work by Thorsteinson et al. [17] over mixed oxides of molybdenum and vanadium, work by Erdohelyi and Solymosi [18] over potassium-promoted vanadia catalysts, work by Seshan et al. [19] on orthovanadates of alkaline and alkaline earth metal catalysts. While Iwamoto et al. [20] reported rather high selectivities for ethylene using N2O over metal oxides, an attempt to reproduce the same selectivity levels by another research group was not successful [14]. More recent studies include use of a manganese substituted molecular sieve catalyst by Wang and Huang [21] and use of calcium-nickel-lithium oxide by Zhang et al. [22]. Photocatalytic oxidation of ethane, with some partial success, has also been reported in the literature [23, 24].
Research on ethane ODH catalysts usually fall into two operating temperature categories, above or below around 600°C. Lower temperature catalysts consist of reducible transition metal ions such as V, Mo, and Sb. The higher temperature catalysts do not contain easily reducible ions and consist of Mg, Li, or other group IA or IIA ions or oxides. Furthermore, as stated by Cavani and Trifiro [25] in an ethane and propane ODH review, several catalysts active for the oxidative coupling of methane (OCM) also work well for ethane ODH. Ji et al. [26] have studied the OCM catalyst Li/CaO in the ODH of ethane with excellent ethylene selectivity when promoted with group IIIA elements.

Several researchers propose a high temperature mechanism that involves ethyl radical formation on surface oxygen and the subsequent degradation to ethylene through a surface ethoxide intermediate. Whether this oxygen species is adsorbed from the gas phase or part of the lattice is still a matter of investigation on different types of catalysts. For instance, Kaddouri et al. [27] claim that, on their NiMoO₄ catalysts, activation of ethylene takes place from adsorbed oxygen species (O'). The authors also make mention of the ability of Mo(V) sites important for the adsorption of such species. However, this theory refutes the older theory proposed by Thorsteinson et al.[28] on mixed molybdenum oxides (Mo/V/Nb) that stated the lattice oxygen of Mo(VI) sites participates in the intermediate ethoxide formation. Kung [2] has stated that the role of adsorbed oxygen probably varies depending on the catalyst. Our research has shown ESR to be well suited to characterize the presence of oxygen anion species present on the catalyst surface before and after a reaction.
Wan et al. [29][30] have studied the ODH of ethane over BaF$_2$-LaOF catalysts at high temperature (680-720°C). The authors comment on the surface acidity/basicity relationship to activity/selectivity present on many ethane ODH catalysts. In addition to acidity issues, \textit{in situ} Raman investigations were able to show that activity may be also related to the abundance of di-oxygen species adsorbed on the catalysts ($O_2^{2-}$, $O^-$) during reaction and this adsorption may be influenced positively by $F^-$ ions. Indeed, these oxygen species are crucial in the radical mechanism of ethylene formation. Conception et al. [31] have studied the activity of V and Co containing aluminophosphates in ethane ODH. Here, the researchers see the positive effects on ethylene selectivity of both surface acidity and redox function of the active transition metal (VO$_x$ species).

Several ethane ODH catalysts are successful at lower temperatures, <550°C. Schuurman et al. [32] have studied group IIIA metals in the ODH of ethane. They have also given evidence for involvement of irreversibly adsorbed oxygen species (possibly $O^-$) in the reaction to ethylene at lower temperatures (200-300°C). The authors have also shown that formed CO$_2$ results from an irreversibly adsorbed ethane mechanism. Furthermore, Wan et al. [29] have stated that the surface of a strongly basic catalyst can be, in effect, poisoned by CO$_2$ formation. Several of the cited researchers have stated that the addition of halide ions to the catalyst or feed (for instance Cl$^-$) can have the effect of both making the catalyst less basic to desorb the poisoning CO$_2$ molecules and to promote radical decomposition of the ethylene intermediate to give a vast improvement in selectivity [2][25][29].

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2.4 Studies Common to Propane and Ethane ODH

As mentioned by Kung [1], oxidative dehydrogenation of lower alkanes share many common features. An alkane molecule can react on the surface of a catalyst to produce and alkyl radical or proceed through a β-hydrogen elimination to form an alkene. Thereafter, the radical or alkene can either desorb from the surface or stay bound for further reaction. If the radical desorbs from the surface it can undergo homogeneous reaction in a desirable or undesirable way. If the radical stays on the surface it may go through an alkoxide or other intermediate to eventually be transformed either to an oxygenate, a lower hydrocarbon, or a carbon oxide. Which one of these is favorable depends on the alkane and the desired product. Thus, there are different catalysts capable of completing different functions.

The Lewis/Brønsted acidity of ODH catalysts is frequently characterized. Often a moderately basic catalytic site is required for the first hydrogen abstraction step. Consequently, the acidity will also play a role in the adsorption/desorption/reaction behavior of the formed product. The consecutive reaction of the formed olefin is a matter of study in both ethane and propane reactions. These acidity features can be studied in various ways. By studying the adsorption/desorption behavior of probe bases (i.e. ammonia, pyridine) one may calculate acid strength or site density. Furthermore, in-situ IR and thermo-gravimetric studies combined with adsorption of a probe molecule can prove very beneficial in determining the nature of acid sites.
With the use of atomic-scale characterization and in situ methods, it is becoming easier to formulate mechanisms for catalytic reactions and deactivation processes. With the use of an active transition metal, such as Mo, it is essential to study the red-ox behavior, interaction with a support, or actual bonding of intermediate during a reaction process. With the aid of XPS, AES, Raman, ESR, TPR, and DRIFTS one can study the oxidation states before and after a reaction, see intermediates on the surface of the catalyst, see what structural changes have taken place, and study red-ox behavior. Besides coking, a metal oxide catalyst (most often used for ODH reactions) can deactivate by a reduction of the active metal.

2.5 Catalyst Functionality For ODH Reactions

2.5.1 Molybdenum and Vanadium-Based Catalysts

An instance where surface-reactant information obtained can be obtained from isotopic labeling experiments is from the oxidative dehydrogenation (ODH) of propane. Chen et al. [33] have used $^{18}\text{O}_2$ and $\text{CH}_3^{13}\text{CH}_2\text{CH}_3$ tracer techniques to answer questions fundamental to propane ODH surface chemistry. Using $^{18}\text{O}_2/\text{C}_3\text{H}_8$ mixtures over vanadium based supported catalyst several mechanistic observations were made. First, $^{16}\text{O}$-containing products were observed from the beginning of the reaction. Secondly, cross-labeled $^{16}\text{O}^{18}\text{O}$ species were not observed in the product stream. The observations indicated that lattice oxygen does indeed initiate the activation of propane (the rate-
limiting step). Furthermore, the absence of cross-labeled oxygen in the products indicated, in the mechanism over vanadium oxide based catalysts, the dissociative adsorption of gas phase oxygen (for re-oxidation) is irreversible. In the same set of experimental results, C$_3$H$_8$-C$_3$D$_8$-O$_2$ reactants underwent ODH without forming C$_3$H$_{8-x}$D$_x$, suggesting that the bond activation of C-H is also an irreversible one. The researchers proceeded to describe an overall mechanism for the selective route of propane ODH present over vanadium and molybdenum (MoO$_x$ or VO$_x$) based catalysts, Scheme 2.1. Strong experimental evidence was presented [34,35], to validate various reaction steps. Using a series of deuterated propanes and propylenes, the experiments provided strong evidence, through Kinetic Isotope Effects (KIE), that the activation of propane for propane ODH takes place on the central methylene C-H bond (at the C$_2$ carbon) and the sequential activation of the product propylene takes place at propylene’s allylic C-H bond. Intuitively, these are the weakest bonds in each molecule. Further, the results indicated a different lattice oxygen site for each activation.

\begin{align*}
1) & C_3H_8 + O^* \leftrightarrow C_3H_8O^* \\
2) & C_3H_8O^* + O^* \rightarrow C_3H_7O^* + OH^* \\
3) & C_3H_7O^* \rightarrow C_3H_6 + OH^* \\
4) & OH^* + OH^* \leftrightarrow H_2O + O^* + ^* \\
5) & O_2 + 2^* \rightarrow 2O^* \\
\end{align*}

\textit{Weak adsorption of propane on lattice oxygen}  \\
\textit{C-H bond cleavage with neighboring lattice oxygen forming a surface 2-propoxy species}  \\
\textit{Desorption of propylene}  \\
\textit{Recombination of OH groups forming a reduced metal center}  \\
\textit{Re-oxidation of the reduced metal center}

Scheme 2.1: Propane ODH reaction sequence
It is important to note that in this kinetic analysis, no differentiation is made, at this point, as to which type of oxygen species at the surface are involved in C-H bond activation. Conceivably, several may exist in the form of M=O, M-O-M, or M-O-Support bonds; where M is the transition metal of interest. Furthermore, the role of adsorbed oxygen species at the surface has not been elucidated in this scheme. Nonetheless, certain aspects of the mechanism were proven with various quantitative experiments. By co-feeding water at various concentrations, it was shown [35] that increased water concentrations inhibit propylene formation. This indicated the reversibility of step (3) in Scheme 2.1. This fact has greater importance, overall, because the presence of –OH group on the catalyst surface implies a relationship between acidity and reactivity. This subject will be discussed in a later section.
Examining the equations of Scheme 2.1, one can visualize the nature of the active oxygen species and red-ox properties playing a detrimental role. For an optimum combination of activity and selectivity there should exist an optimum between the oxidation of the hydrocarbon and the ease of removal of oxygen from the catalyst surface. Oxygen too tightly bound will result in low activity. While, a catalyst with oxygen that is too mobile will be very active, but not selective. A quantitative technique used to characterize red-ox properties is Temperature Programmed Reduction/Oxidation (TPR/TPO) (reduction/oxidation rates of the catalyst as a function of temperature). TPR/TPO yields information over the temperature range where the reduction/oxidation takes place, the amount of reducible oxygen (or oxidizable material), the oxidation state of the metal, the presence of various surface phases, possible interactions between these species, interaction with the support, and factors that affect dispersion.

2.5.2 Unselective/Selective Mechanisms in Propane ODH

Busca et al. [35-40] have presented, in a several research articles, studies of cracking and combustion pathways of C3 compounds over catalytic surfaces. The research provides the most direct evidence available pointing to the initial "activated complex" of propane activation being the 2-propoxy species, \( C_3H_7O^* \). Furthermore, the selective and non-selective pathways the proposed 2-propoxy may undergo were also investigated. By using representative compounds (propane, propylene, isopropanol, and acetone) in combination with FTIR spectroscopy, the researchers found that propylene
and iso-propanol react to form the same surface species and gas phase reaction products, by way of a surface 2-propoxy species. The activation of propane to form propylene occurs at a much higher temperature (by more than 200°C) at which the 2-propoxy groups are already unstable toward the decomposition to propylene. Hence, the formation of the 2-propoxy groups from propane is much slower than their sequential transformation and the 2-propoxide species is not expected to be long-lived on the surface during propane ODH. This work is in agreement with the reaction scheme proposed by [37].

As for the non-selective pathways, the 2-propoxide species is found to degrade on catalytic surfaces to formate and acetate species. Indeed, these species are also found to be present on the surface from propane. Watson and Ozkan [11] have found a strong relation between the formation of such species and the combustion route of propane ODH from in-situ IR measurements over MoOx/Si:Ti catalysts. The most selective ODH catalysts show a lack of intensity from the acetate and formate species in comparison to non-selective catalysts. This agrees well with reaction results as these intermediates lead to the formation of COx products.

Culminating selective and non-selective pathways of propane ODH together can allow for an "overall" mechanism. This is presented in Scheme 2.2. Here, we see the formation of the surface 2-propoxy species and it's subsequent hydride elimination to form propylene. The non-selective pathway can proceed from two directions, either from the re-adsorption of formed propylene, or by the decomposition of 2-propoxide species. In Figure 4, the reaction path that begins with the re-adsorption of propylene followed by
attack of the electron-rich double bond has been omitted. This brings in the issue of Lewis acidity and the presence of acid centers on the catalyst, which if present, may preferentially prefer the re-adsorption of the formed propylene through the double bond.

When we discuss a balance in reducibility and acidic/basic characteristics of catalysts in the ODH of propane, it is necessary to think in terms of the relative rates of the kinetic steps occurring on oxide catalysts. As discussed by Kung in a review article [1], one qualitatively needs to consider the two reactions, namely propane→propylene and propylene→CO\textsubscript{x}. It was shown that, perhaps on all catalysts, the rate for the second reaction could be five to ten times higher than that of the first reaction. When the selectivity is considered in terms of these two competing reactions, it becomes clearer that both the reducibility and oxygen mobility characteristics of the catalyst, and its acidity, need to be simultaneously taken into account.

Scheme 2.2: Overall propane oxidation mechanism

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While the surface oxygen species affect the rates of both reactions, it is conceivable that a catalyst with a strong Lewis character will have a higher affinity for the electron-rich double bond of propylene, leading to its further oxidation before it desorbs from the surface.

The acidic nature of solid catalysts are often characterized, *ex-situ*, and related to reactivity. This can be accomplished by acid-base probe reactions or the IR spectra of ammonia species formed on catalysts. Quantitative Lewis and Brønsted characteristics of the catalysts are made using probe reaction selectivities and peak areas/intensity correlations, respectively.

The exact nature of the participation of the oxidic catalyst surface seems to be the most illusive in propane ODH studies. The scheme presented in Scheme 2.2 makes many assumptions about the M-O bond participation. The difficulty arises from the fact that metal oxide systems commonly posses numerous possible crystalline phases and/or dispersed species (Scheme 2.3), and the active and selective catalyst compositions often are reported to contain several of these. Much effort has been expended trying to identify the single active and selective crystalline phase or species in these systems, and conflicting conclusions have been drawn. Synergism has been reported in several of these systems and offered as a possible explanation for apparently conflicting results. This synergism is most commonly attributed to remote communication of the phases, e.g. through spillover.
Determination of the crystal structure and identification and quantification of phases can be achieved using X-ray diffraction (XRD). Furthermore, study of X-ray line broadening can give a measure of particulate size. Raman spectroscopy is also exceptional at determining surface species present on a catalyst before and after a reaction. With the use of Of particular interest is the nature of metal-ligand and metal-oxygen bonds encountered in many catalysts. Moreover, the real strength of catalyst characterization lies in the availability of highly surface-sensitive instrumental techniques. X-ray photoelectron spectroscopy (XPS) can be used to determine the surface chemical composition of heterogeneous samples and the oxidation state of various elements contained therein. It is very effective in determining quantitative and qualitative surface/bulk structural and oxidation state information. The relative concentrations of each element can also be determined.
Through XRD, Raman, and XPS results, it has been found that both Mo-O-Mo and Mo=O bonds are involved in propane, and other hydrocarbon, conversion [41-43]. Generally speaking, a balance between mono dispersed species (Mo=O) and 2-dimensional structures (Mo-O-M) have given the optimal performance, i.e. lowest k₁/k₂ (where k₁/k₂=propane conversion to propylene/propylene combustion). The formation of 3-D crystalline structures is generally not beneficial for propylene selectivity. This is because of the increased combustion reactivity of crystalline lattices due to high lattice oxygen mobility. This was found to be the case in Mo/Si:Ti catalysts [44]. Increasing molybdenum loadings are seen to provide a rather constant propylene formation up to 10wt.% that declines with increasing Mo content. The decrease in activity was explained in terms of increasing crystalline MoO₃ or 3-dimenionality of the MoOₓ surface units as indicated by XPS and Raman results.

Characterization after the propane ODH reaction [45] has revealed that the surface of MoOₓ based catalysts contains a reduced state of molybdenum oxide. This correlates well with Electron Spin Resonance work in which a linear relationship was established between the presence of Mo⁵⁺ and propylene formation [41]. While these data do not explicitly differentiate between the activity of Mo-O-Mo and Mo-O bonds (or reduced M⁺-O centers for that matter), they do indicate a reduced coordination site that could be intimately interacting with the support material. Obviously, the state of bound oxygen during operating conditions is a dynamic one.
Differences observed in the ODH performance of catalysts cannot be explained solely in terms of the direct changes of the surface M-O species with coverages. Moreover, the role of a Mo-O support interaction species in the activation of propane remains unclear. Research has continued to focus on several keys to unlock the nature of the active sites. Some of the most commonly studied (i.e. most important) parameters of interest in the determination of the active sites are:

- The relationship between metal-oxygen bond strength and selectivity
- The oxidation state of metal-oxide necessary for the activation
- Interaction between an oxide support and “active” oxide
- Selectivity determining step and the relation to active sites
- Understanding the roles of acid sites, molecular adsorption, and diffusion

Wachs [42] has extensively studied the nature of M-O-M and Mo=O bonds under a variety of conditions. Through ex-situ and in-situ Raman spectroscopies, it was found that adsorption of water greatly effects the location (i.e. strength) of the Raman bands associated with the M=O bonds of vanadium and molybdenum compounds. At high conversions of propane, where much water would be formed and re-adsorbed, it is conceivable that the decrease in selectivity observed, in the presence of water, is to the blocking of Mo=O sites. This could be the site responsible for initial propane adsorption. However, many contradictions to that statement exist in the literature.
Also, the characterization of the re-oxidation step in the Mars van-Krevelen mechanism is also an important interest in propane ODH. Upon dissociative adsorption of molecular oxygen, anionic species (O²⁻, O⁻) can form. These can then be inserted into propane in a non-selective manner through electrophillic addition. It has been shown [46-47], that C-C bond breaking prevails when there is an abundance of adsorbed oxygen present at the surface during propane ODH.

The relationship between gas phase, adsorbed, and lattice oxygen can be obtained from Steady State Transient Isotopic Kinetic Analysis (SSITKA). The SSITKA technique involves abrupt switches in the isotopic concentration of one of the reactant molecules at steady state accompanied by the monitoring of the relaxation and evolution of labeled reactant and products [48]. Analysis of these transients can yield surface turnover frequencies and surface coverage. In one of the most common use of SSITKA, the isotopic exchange of oxygen, \(^{18}\text{O}_2\) or a mixture of \(^{18}\text{O}_2\) and \(^{16}\text{O}_2\) is contacted with an oxide surface and the reaction is monitored for the presence of \(^{16}\text{O}^{18}\text{O}\). The shapes and ultimate relaxation or stable value of the transients obtained depend on the type of oxide catalyst studied. The lattice oxygen diffusivities can be calculated by making a few assumptions. Furthermore, with certain assumptions made about the catalyst surface, the equivalent number of exchange-participating surface layers may be calculated. This is an exceptional way of comparing oxidation catalysts and relating bulk oxide properties to the overall reaction behavior.
In short, the surface of the catalyst, at high temperature and high conversion rates, is a dynamic one. Adsorption/desorption processes are occurring. Reduction/oxidation processes are occurring. Lattice oxygen diffusion is taking place. Hence, there may be considerable surface re-structuring occurring over the course of reaction. Although the search for a single active site is on going, the determining factor for selectivity may be a combination of the several competing phenomenon, involving several “active sites”.

2.5.3 The use of the sol-gel/co-precipitation technique for catalysts

The preparation of materials using sol-gel science offers many benefits over a wide range of applications. Originally focused of the preparation of homogeneous glasses and advanced ceramics, sol-gel processing has emerged as an advantageous route to fuel powders, nano-composites, membranes, protective coatings, thermal insulators, thin films, chemical sensors, and high-surface area and super-acid catalysts. Although the use of sol-gel based chemistry has not found wide industrial application, the amount of scientific publications in the area has grown exponentially within the past ten years in a variety of areas [49]. There is a need for research in the area of industrially interesting materials in terms of new materials, high-temperature resistance, low-cost processing, control of preparation parameters, and prediction of final properties.
The ultimate structural, textural, and chemical nature of sol-gel derived materials can be related to careful control of one or many of a number of preparation parameters. Depending on the materials of interest, the influential parameters include: concentrations, types of solvent, pH, temperature, control of gelling rate, addition of modifier/catalyst, and drying/aging methods. Final properties of interest can include but are not limited to: homogeneity of composition on a nano-scale dimension, temperature and chemical resistance, surface area, and porosity/pore volume. The years of research on the subject have established a strong relationship between preparation and final properties for ceramics and glasses and have lengthened to include catalyst preparation. The preparations of sol-gel catalysts can directly or indirectly affect catalytic properties such as: active metal dispersion, resistance to sintering, red-ox behavior, and adsorption/desorption. Sol-gel chemistry allows materials to be mixed on an atomic-scale and thus crystallization to be accomplished at a much low temperature. However, an atomic level homogeneity in a multiple component system requires detailed focus on the chemistry of formation, the difficulty arising from the varying chemical reactivity from precursor to precursor.

Sol-gel science, while becoming quite mature, can often become quite complex as applied to novel materials and applications. We know what materials are put in and we may analyze the results of what comes out, but the steps in between are often poorly illuminated. In order to create systems to perform specific functions for new applications, a number of parameters can be defined and studied to help shed some light onto the matter. While ceramic and glass applications of sol-gel processing parameters are
numerous and quite expanded in nature, the use of sol-gel chemistry as a basis of catalyst preparation is far scarcer and focused in a much narrower application field. However, there is a recent emergence of preparation techniques in the literature for a variety of catalytic systems and this trend is expected to grow. Preparative approaches for catalytic materials and glasses share many common features, but the desired properties of the sol-gel product are very different [50].

Preparation of catalytic supports using sol-gel chemistry can provide advantages that may otherwise be unobtainable with other preparation methods (i.e. wet-impregnation, co-precipitation). These benefits include unique chemical properties (often acidic) resulting from the formation of M-O-M' bonds; where M'≠M, strong metal-support interactions (SMSI) through molecular-scale mixing, hindering reduction and agglomeration (sintering) of the active metal, suppression of crystalline phase change, high mechanical strength, and smaller particle size that leads to better dispersion and higher surface area. Careful control of sol-gel parameters can also enable one to synthesize a catalyst with a desired surface area, pore structure and volume. The challenging task of research in this area is to then relate the preparation parameters involved, in an often heterogeneous system, to the resultant effect on the catalysts' physico-chemical properties. In the continuing research of catalytic science, one tries to prepare catalysts with desired chemical and physical properties and to establish the relations between synthesis parameters and catalytic performance.
The control of sol-gel preparation parameters allows the researcher to investigate the affect of several characteristics that are important in catalytic reactions:

- surface area, pore size
- particle size/shape/morphology/dispersion
- crystal structure/symmetry
- chemical composition
- oxidation state
- molecular structure
- surface chemistry
- adsorption/desorption characteristics
- oxidation/reduction characteristics
- acidity/basicity
- oxygen mobility
- catalyst deactivation

There is an increasing amount of literature available on sol-gel synthesis effective for the preparation of multi-component oxides, generally yielding materials with better homogeneities and higher surface areas than materials prepared using more conventional methods [52-53]. However, the incorporation of an active metal or metal oxide into a sol-gel matrix, by various means, is a matter of recent university study.

Mixed oxides, prepared using sol-gel chemistry, have been used in a variety of reactions, such as hydrotreating, selective oxidation, reforming, dehydrogenation, dealkylation, and isomerization. As catalytic supports, the mixed oxides including binary mixtures of alumina, zirconia, silica, alumina, or titania have received the most attention. The beneficial properties of the preparation is usually related to enhanced acid strength due to the formation of M-O-M' linkages where M=metal. A rather recent emergence of data has become available on the use of silica-titania mixed oxides as catalytic supports for active metals and has been studied extensively at The Ohio State University.
Study of silica-titania mixed oxides has gained much attention because of their high activity for epoxidation reactions of olefins with hydroperoxides. It has been cited [54-55], and shown in our work, that TiO₂ in mixed oxides of silica and titania can be present not only as anatase, but in the form of very small domains in which the normal octahedral coordination of TiO₂ has changed to tetrahedral. This leads to the unique structural and chemical properties of this material. Silica-titania mixed oxide supports, through sol-gel preparations, can provide advantages that the respective single oxides cannot. These benefits include stronger metal-support interactions, hindering reduction of the active metal, and smaller particle size that leads to better dispersion and higher surface area. Silica-titania mixed oxides have been studied extensively [54] for attributes such as acidity, porosity, Ti-O-Si connectivity, and phase separations. However, few studies have been done on their use as active metal supports. Baiker [56] et al. and Vogt [57] et al. have used vanadia supported on silica-titania mixed oxides for the reduction of nitric oxide with ammonia. Baiker has shown that the addition of titania causes an interaction that prevents agglomeration of surface vanadia species. Udomsak et al. [58] have shown a significant difference in isobutane dehydrogenation activity on chromia/silica-titania catalyst with different preparation methods. Hydrogen and carbon monoxide interaction with titania promoted palladium on silica was studied by Rieck and Bell [59]. Here, it was shown that TiOₓ species decorate the palladium, causing a notable difference in the CO adsorption behavior. Feng et al. [60] have shown the hydrogen abstracting ability of the weakly acidic silica titania mixed oxide supported palladium catalysts was the dominating factor for non-oxidative dehydrogenation of propane over
these types of catalysts. Ko et al. and Kumbhar [61-62] have shown that TiO$_2$/SiO$_2$ mixed oxides exert both direct and indirect support effects when used as supports for Ni catalysts. Furthermore, Baiker et al. [63] has proposed that by varying the TiO$_2$ content in the mixed oxides, one can "tune" the interaction with VOx species to form an optimal deNOx catalyst. Understanding of the structural characteristics of these mixed oxides and their relationship to physico-chemical properties is of great importance over a wide range of applied science [54]. Furthermore, the unique support effects on active metal oxides are a recent matter of study.
CHAPTER 3

EXPERIMENTAL

3.1 Catalyst Synthesis

The catalysts used in this study have been synthesized by a "one-pot" sol gel/co-precipitation technique. This sol-gel process involves the formation of inorganic networks through the formation of a colloidal suspension (sol) and gelation of the sol to form a network solid skeleton filled with a solvent (gel). When the solvent is removed, the wet gel becomes a xerogel through ambient pressure drying or an aerogel through supercritical drying. The precursors for synthesis consist of a metal element surrounded by various reactive ligands. These can include metal chlorides, hydroxides, oxides, and alkoxides. Metal alkoxides are have received the most attention because they react readily with water. The most characterized metal alkoxides are the alkoxides of silica, such as tetramethyloorthosilicate (TMOS) and tetraethylorthosilicate (TEOS). However, other alkoxides (i.e. aluminates, titanates) are also commonly used in the sol-gel possessing.
The method focused on here involves the reactions of metal alkoxide precursors in an alcohol solvent when contacted with water. Three groups of reactions are generally used to describe the sol-gel process: hydrolysis, alcohol condensation, and water condensation (shown in Scheme 3.1). During hydrolysis, the alkyl group leaves and forms an alcohol by nucleophillic addition of water. The next three types of reactions are condensation reactions; alcoxolation, oxolation, and olation.

\[ \text{Hydrolysis:} \quad H_2O + M - OR \leftrightarrow M - OH + ROH \]

\[ \text{Alcoxolation:} \quad M - O - H + M - OR \leftrightarrow M - O - M + ROH \]

\[ \text{Oxolation:} \quad M - O - H + M - O - H \leftrightarrow M - O - M + H_2O \]

\[ \text{Olation:} \quad M - OH + M * H_2O \leftrightarrow M - O - M + H_2O \]
\[ M - OH + M * ROH \leftrightarrow M - O - M + ROH \]

Scheme 3.1: Sol-Gel Reactions (alkoxides) in alcohol solvent (M=metal)

The types of reaction alkoxide precursors condense by depend on the charge of the metal, the leaving group R, and the type of solvent used. In general, the hydrolysis reaction replaces alkoxide groups (OR) with hydroxyl groups (OH). Subsequent condensation reactions involving the M-OH bond produce M-O-M linkages plus the by-
products water or alcohol. Under most conditions, condensation commences before hydrolysis is complete. However, alteration of the afore mentioned conditions (such as pH or water/precursor molar ratio) can force completion of hydrolysis before condensation begins [51]. Additionally, because water and alkoxides are immiscible, a mutual solvent such as an alcohol is utilized. With the presence of alcohol, hydrolysis is made possible due to the miscibility of the alkoxide and water. As the number of M-O-M bonds increases, the individual molecules are bridged and can join in 2 and 3 dimensional structures in the sol. When the sol particles aggregate the gel is formed. Upon drying, the water and alcohol are driven off and the network shrinks as further condensation can occur. At this point, it should be stressed that the addition of solvents and certain reaction conditions may promote esterification and depolymerization reactions according to the reverse of the reaction equations.

There are many parameters that affect relative rates of these reactions and thus determine the final properties of the material. These include: charge on metal (iso-electric point), coordination, composition, electronegativity, pH, nature of R, water/precursor ratio, time of reaction, addition of catalyzing agent, concentration, solvent, temperature, and drying /aging methods. An attempt is made in Table 3.1 to convey the general goals in alteration of preparation parameters to a specific end.
Table 3.1
Preparation parameters in the sol-gel chemistry of metal alkoxides

<table>
<thead>
<tr>
<th>Preparation Parameter</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time of reaction</td>
<td>Allowing reactions time to complete or halting reactions at a certain desired level without the occurrence of reverse reactions</td>
</tr>
<tr>
<td>Prehydrolysis</td>
<td>Allows a slower reacting alkoxide to get a &quot;head-start&quot; during hydrolysis</td>
</tr>
<tr>
<td>Acidic pH</td>
<td>Condensation of linear or randomly branched polymers, smaller particles, lower porosity</td>
</tr>
<tr>
<td>Basic pH</td>
<td>Condensation of highly branched discrete clusters, larger particles, higher porosity</td>
</tr>
<tr>
<td>Temperature</td>
<td>Increase rate of reaction and drive off solvent</td>
</tr>
<tr>
<td>Water/precursor ratio</td>
<td>Influence hydrolysis and, in-turn, condensation reaction rates</td>
</tr>
<tr>
<td>Addition of Catalyst</td>
<td>Speed hydrolysis or condensation reactions</td>
</tr>
<tr>
<td>Addition of complexing agent</td>
<td>Attenuate the reactivity of a precursor or bridge certain sites to prevent 3D branching</td>
</tr>
<tr>
<td>Drying/Ageing methods</td>
<td>Allow pore structure to strengthen and develop, re-dispersion of partially reacted precursors</td>
</tr>
<tr>
<td>Supercritical Drying</td>
<td>Obtain an aerogel (preservation of pore structure) with comparably high surface area and high pore volume and enhanced thermal properties</td>
</tr>
</tbody>
</table>
Furthermore, a more intricate situation is met when one synthesizes a multi-component system using two or more alkoxides or by placing another form of precursor (such as a metal salt) into the already complex chemistry. Thus, seemingly endless possibilities can exist. The behavior of gels during the different steps of preparation (forming, drying) is now well characterized. It is presently possible to select starting molecules to tailor physical and chemical properties of the resultant xerogels and aerogels.

Catalysts were prepared using a modified sol-gel/co-precipitation technique. Ammonium heptamolybdate (AHM) and alkali (Li, Na, K, Cs) hydroxides were used for molybdenum and alkali precursors, respectively. For silica-titania mixed oxides, tetraethylorthosilicate (TEOS) (Aldrich) and titanium(IV)isopropoxide (TIPG) (Aldrich) were used. The solvent was isopropyl alcohol. In this modified sol-gel method, calculated amounts of the silica and titania alkoxide precursors were placed in solvent to yield, after calcination, SiO$_2$-TiO$_2$ mixed oxides with the desired molar Si:Ti ratio. This solution was left stirring while an aqueous solution containing the necessary amount of AHM of molybdenum (2-20%Mo loading) was then added drop-wise with a syringe pump. The aqueous solution added contained a slight excess of the stoichiometric amount of water necessary to hydrolyze all of the alkoxide precursors. The aqueous solution was added at a rate of 0.5cm$^3$/min. for all catalysts with one exception. For the catalyst prepared with fast addition, the aqueous solution was added at a rate of 2cm$^3$/min.
For catalysts containing alkali, the alkali hydroxide was added to the aqueous solution to give the desired K/Mo molar ratio. For catalysts denoted as pre-hydrolyzed, the stoichiometric amount of water, necessary to hydrolyze the entire silica precursor, was added to the silica precursor only and stirred for 15 minutes before proceeding. For acidic and basic preparations, the effective pH of the alcohol solution was maintained at pH=3 (acidic) and pH=12 (basic) during preparation using HNO$_3$ and NH$_4$OH, respectively. For catalysts modified with chlorine, a calculated amount of HCl (Fisher) or NH$_4$Cl (Mallinkrodt) was included in the aqueous solution to give the desired Cl/Mo ratio. Resulting gels were stirred for an additional 15 minutes after all of the aqueous solution had been added and dried at room temperature for less than 3 hours. They were then placed into an oven at 110°C for overnight drying and solvent removal. After drying, the catalysts were ground to a fine powder and calcined under oxygen at 550°C for 5 hours. This method is referred to as a "one-pot" sol-gel/co-precipitation because as the silica and titania precursors are hydrolyzed and precipitate out of solution, alkali-molybdate species, that are insoluble in alcohol, also precipitate.
3.2 Catalyst Characterization

Knowledge of the chemical, structural and surface characteristics of a catalyst is essential for understanding the catalytic phenomena taking place on its surface. The rapid evolution of catalysis from an empirical practice to an interdisciplinary science in the last two decades is, for the most part, due to development of highly sensitive and sophisticated characterization techniques. Some of these techniques are aimed at measuring the physical properties. Others seek an understanding of the chemical characteristics. Chemical characterization refers to techniques used to examine the compositional, structural, morphological and surface properties of catalysts. Quantitative analysis of the elements present in the catalyst is one of the first steps in chemical characterization.
3.2.1 BET Surface Area Measurement

When the reaction at the surface of the catalyst is the controlling step in a reaction, the rate of reaction may be directly proportional to surface area. Thus, the measurement of surface area and pore volume is the first step in characterizing catalyst behavior. Surface area is largely determined using gas adsorption techniques. The well-known BET method, developed by Brunauer, Emmett, and Teller in 1938, is a technique that is still used today. By relating the rate of condensation of an adsorbing gas to its rate of evaporation they developed a linearized BET equation:

\[
\frac{P}{V(P_o - P)} = \frac{1}{V_mC} + \frac{(C-1)P}{V_mC P_o}
\]

where \(V\) is the volume of gas adsorbed at pressure \(P\), \(V_m\) is the volume of gas adsorbed as a monolayer, \(P_o\) is the saturation pressure of the adsorbing gas at temperature \(T\), and \(C\) is a constant. In the BET method, an inert gas, usually nitrogen or krypton, is physically adsorbed onto a clean catalytic surface to yield an adsorption isotherm graph. An isotherm shows the amount of gas adsorbed versus the pressure of adsorbing gas at a constant temperature. From the isotherm and the area occupied by the adsorbing molecule, one can calculate the surface area of the catalyst by plotting the above equation and determining \(V_m\). Information about pore size, shape and volume can also be extracted from adsorption-desorption isotherms. BET surface area measurement and nitrogen adsorption-desorption isotherms were recorded at liquid nitrogen temperatures (~77K) using a Micrometrics AccuSorb 2100E instrument.
3.2.2 X-Ray Diffraction (XRD)

Determination of the crystal structure (i.e., the geometry of the way the atoms are positioned in the solid) and identification and quantification of phases can be achieved using X-ray diffraction (XRD). XRD is particularly useful for the determination of structure for metal oxides, the catalysts studied most in oxidative dehydrogenation and other partial oxidation reactions. When electromagnetic radiation (X-rays) is incident upon a periodic array of scattering centers (lattice atoms), there are certain discrete directions for the incident ray that result in strong reflections. This is because of constructive interference of the radiation scattered from each of the centers. The directions for which these strong reflections occur are related through the Bragg law to the geometry of the arrangement. Therefore, measurements of the angles and intensities of the Bragg reflections can be used to deduce the arrangement and spacing of the scatterers. Important catalytic features such as dispersion, quantification, identification, and distortion of phases can be related to catalytic activity when XRD is used in combination with extensive reference patterns from a data base. Line broadening of x-ray diffraction peaks are inversely proportional to crystallite size and therefore can be used as a method to measure crystallite size in the range ~5-50nm. X-ray diffraction patterns of the catalysts studied were obtained with a Scintag PAD-V diffractometer using Cu-Kα radiation.
3.2.3 Laser Raman Spectroscopy (LRS)

The Raman effect arises when the incident light (laser radiation) excites molecules in the sample which subsequently scatter the light. While most of this scattered light is at the same wavelength as the incident light, some is scattered at a different wavelength. This inelastically scattered light is called Raman scatter. It results from the molecule changing its molecular motions. The energy difference between the incident light ($E_0$) and the Raman scattered light ($E_r$) is equal to the energy involved in changing the molecule's vibrational state, $E_v$. This energy difference is called the Raman shift. Several different Raman shifted signals will often be observed; each being associated with different vibrational or rotational motions of molecules in the sample. A plot of Raman intensity vs. Raman shift is a Raman spectrum.

Raman spectra of prepared catalysts were recorded with a Dilor spectrometer using the 514.5nm line of an Innova 300 Ar Laser. Spectra were taken in the range 200-1800cm$^{-1}$ in 180° back-scattering mode with a Spectrum One CCD detector. Raman spectroscopy under dehydrated conditions was performed using a quartz in-situ flow-cell that was capable of bringing the catalysts in contact with a gas flow at high temperatures. A portion of prepared samples were re-calcined at 550°C for thirty minutes under pure O$_2$ and transferred into the Raman cell. Additional dehydration occurred at 350°C for thirty minutes under a flow of dry 10% O$_2$/He after which the cell was sealed. Spectra were taken under 10%O$_2$/He atmosphere at room temperature.
3.2.4 X-ray Photoelectron Spectroscopy (XPS)

For every chemical element, there will be a characteristic binding energy associated with each core atomic orbital. Each element will give rise to a characteristic set of peaks in the photoelectron spectrum at kinetic energies determined by the photon energy and the respective binding energies. In XPS, a sample is irradiated by monochromatic X-rays under ultrahigh-vacuum conditions. Photoelectrons are ejected from various atomic shells with characteristic kinetic energy, which gives their binding energy according to the following equation:

\[ KE = h\gamma - BE - \phi_{sp} \]

where \( KE \) is the kinetic energy, \( h\gamma \) is the incident X-ray energy, \( BE \) is the binding energy, and \( \phi_{sp} \) is the spectrometer work function. The presence of peaks at particular energies therefore indicates the presence of a specific element in the sample under study - furthermore, the intensity of the peaks is related to the concentration of the element within the sampled region. Thus, the technique is capable of yielding a quantitative analysis and is sometimes known by ESCA (Electron Spectroscopy for Chemical Analysis). The exact binding energy of an electron depends not only upon the level from which photoemission is occurring, but also upon:

1. the formal oxidation state of the atom
2. the local chemical and physical environment

Changes in either (1) or (2) give rise to small shifts in the peak positions in the spectrum, called chemical shifts.
XPS of catalysts was performed with a VG Scientific ESCALAB MKII ESCA/Auger Spectrometer operated at 15kV, 20mA, and using Mg-Kα radiation. Spectra were charge-shift corrected using the C 1s signal, located at 284.6eV. Relative percentages of K₂MoO₄ and MoO₃ in the samples were calculated using the integrals of the de-convoluted Mo 3d spectra. The de-convolution of Mo 3d spectra was accomplished using linked doublets of equal FWHM, an intensity ratio of 2/3, and a splitting of 3.15eV. Surface concentrations were obtained by taking into account the atomic, instrumental sensitivity factors.

3.2.5 Temperature Programmed Reduction/Desorption (TPR/TPD)

Temperature-Programmed Reduction (TPR) determines the number of reducible species present on the catalyst surface and reveals the temperature at which the reduction of each species occurs. An important aspect of TPR analyses is that the sample need not have any special characteristics other than containing reducible metals. The TPR analysis begins by flowing an analysis gas (typically hydrogen in an inert carrier gas such as He) through the sample, usually at ambient temperature. While the gas is flowing, the temperature of the sample is increased linearly with time and the consumption of hydrogen by adsorption/reaction is monitored. Changes in the concentration of the gas mixture downstream from the reaction cell are determined. This information yields the volume of hydrogen uptake.
TPR of catalysts was performed using a laboratory-made gas flow system pictured in Figure 3.1. Catalyst samples (100mg) were placed in a 1/4-in.-i.d. U-tube quartz reactor and pre-treated under oxygen flow at 550°C for thirty minutes followed by cooling to room temperature under nitrogen. The reduction was performed with 10% hydrogen in nitrogen (25cm³/min.). The thermal conductivity detector (TCD) was operated in differential mode and the signal transferred to a data acquisition computer. The outlet of the reactor was passed through a column of silica gel to remove moisture formed during the reduction. The temperature program was as follows: 10 minutes at room temperature, 10°/min. ramp rate to 850°C, and holding at 850°C for 10 minutes.
Figure 3.1: Temperature programmed reduction/desorption (TPR/TPD) system
Temperature programmed desorption involves adsorption of one or more molecular species onto the sample surface at low temperature (room-temperature or sub-ambient). The sample is then either flushed with an inert gas, such as He, or vacuumed, to facilitate the desorption of physically adsorbed species. The sample is then heated in a controlled manner (usually a linear temperature ramp) whilst monitoring the evolution of species from the surface back into the gas phase. In modern implementations of the technique the detector of choice is a small, quadrupole mass spectrometer and the whole process is carried out under computer control with simultaneous monitoring of a large number of possible products. The data obtained from such an experiment consists of the intensity variation of each recorded mass fragment as a function of time / temperature. The area under a peak is proportional to the amount originally adsorbed, i.e. proportional to the surface coverage. The position of the peak (the peak temperature) is related to the enthalpy of adsorption, i.e. to the strength of binding to the surface. One implication of the peak temperature is that if there is more than one binding state for a molecule on a surface (and if these have significantly different adsorption enthalpies) then this will give rise to multiple peaks in the TPD spectrum.

Propane TPD of catalysts was performed using the same laboratory-made gas flow system described for TPR experiments. Catalyst samples (100mg) were placed in a 1/4-in.-i.d. U-tube quartz reactor and pre-treated under oxygen flow at 550°C for thirty minutes, followed by cooling to room temperature under helium. Samples were flushed with helium for 1h followed by 1h of propane adsorption. After adsorption, desorbed species were monitored by a mass spectrometer (HP5890GC-MS) under helium carrier
gas. For these studies, the G.C. columns were replaced by an empty capillary column. The mass spectrometer was equipped with a quadrupole mass analyzer that allows tracking of up to 20 mass-to-charge ratios (m/z) simultaneously in the selected ion mode. Identification of species with equal m/z ratios was accomplished by following characteristic mass fragments of the species. The temperature program was as follows: 10 minutes at room temperature, 10°/min. ramp rate to 700°C, and holding at 700°C for 20 minutes.

Ethane TPD was performed using the same laboratory-made gas flow system. Catalyst samples (150mg) were placed in a 1/4-in.-i.d. U-tube quartz reactor and pre-treated under oxygen flow at 550°C for thirty minutes, followed by cooling to room temperature under helium. Samples were flushed with helium for 1h following 1h of propane adsorption. Desorbed species were monitored by a mass spectrometer (HP5890GC-MS) under helium carrier gas. For these studies, the G.C. columns were replaced by an empty capillary column. The mass spectrometer was equipped with a quadrupole mass analyzer that allows tracking of up to 20 mass-to-charge ratios (m/z) simultaneously in the selected ion mode. Identification of species with equal m/z ratios was accomplished by following characteristic mass fragments of the species. The temperature program was as follows: 10 minutes at room temperature, 10°/min. ramp rate to 800°C, and holding at 800°C for 10 minutes.
3.2.6 Steady-State and Transient Isotopic Labeling Switch

Both steady state and transient kinetic analysis methods were used for isotopic labeling studies. Figure 3.2 shows a simplified schematic of the system that consists of a 4-way port valve, a reactor, and a mass spectrometer. A* indicates the feed to the reactor before the switch and A indicates the feed to the reactor after the switch. The first technique involves application of a step change to the system at steady state, where one feed stream is abruptly replaced by a feed stream in which one or more of the reactants are isotopes of their counterparts in the previous stream. The steady state is not perturbed during the step change and the transients of various isotopes are monitored continuously using a mass spectrometer. The second transient technique used involves abruptly removing one or more of the reactants from the feed stream after the steady state is established and letting the reaction subside at its own pace while simultaneously monitoring the decay profiles of various reactant and product species with a mass spectrometer.
The reactor system used for the isotopic transient studies consists of a feed system with mass flow controllers (Tylan model FC-280), a fixed-bed reactor, and a GC-MS. The reaction experiments were carried out on a fixed bed of catalyst in a tubular reactor with 6.4 mm O.D. x 4.6 mm I.D. The catalyst loading was kept constant at 100mg for different reactions.
Oxygen isotopic exchange flow experiments were performed the same gas-flow system described for TPR/TPD experiments. Catalyst samples (100mg) were placed in a 1/4-in.-i.d. U-tube quartz reactor and pre-treated under oxygen (^{16}O_{2}) flow at 550°C for one hour. A mixture of 10% ^{16}O_{2} in helium with a small amount of argon present (~1%) was then passed over the samples and allowed to equilibrate. After constant MS signals were obtained, the flow was switched to that containing 10% ^{18}O_{2} (isotec) in helium and the exchange was carried out for 10 minutes at 500°C. Oxygen species (^{16}O_{2}, ^{16}O^{18}O, ^{18}O_{2}) and argon were monitored by a mass spectrometer (HP5890GC-MS) under helium carrier gas. The normalized concentration of each isotope for a given species was calculated by dividing the signal for that isotope by the sum of the signals for all the isotopes of that species.

For transient isotopic oxygen exchange, catalyst samples (100mg) were pre-treated in 10% ^{16}O_{2} for 1 hour at 500°C followed by 1 hour of evacuation at 10^{-7} Torr in a quartz U-tube reactor. The samples were then flushed under flowing helium for 1 hour at 500°C. A flow of 10% ^{18}O_{2} (isotec) in helium with a small amount of argon (~1%) was introduced and the exchange was carried out for 10 minutes at 500°C.

Isotopic oxygen exchange, in the presence of reaction, was performed by establishing two gas-flow mixtures. The first contained 5% propane, 2.5% ^{16}O_{2}, 91.5% He, and 1% Argon. The second flow contained 5% propane, 2.5% ^{18}O_{2}, and 92.5% He. Experiments were performed in the same apparatus using 100mg of sample starting with a pre-treatment under oxygen (^{16}O_{2}) flow at 550°C for one hour. The first reaction mixture was introduced and reaction was carried out in the U-tube reactor for
one hour at 500°C at a flow rate of 25 cm³/min. The flow was subsequently switched to that containing the oxygen isotope and the exchange was carried out for 10 minutes at 500°C. During the exchange, no detectable change in MS signals associated with propane or propylene were detected indicating that the steady-state of the reaction was not perturbed by the switch.

In the two sets of oxygen isotopic exchange experiments conducted at steady-state it was necessary to determine if there was a significant contribution from a homogeneous mechanism for the mixing of \(^{16}\text{O}_2\) and \(^{18}\text{O}_2\) to form \(^{16}\text{O}^{18}\text{O}\) in the gaseous phase. These “blank” experiments were performed in a U-tube reactor containing under the same conditions using a quartz wool plug roughly the same size as catalyst samples. In these experiments there was no detectable formation of cross-labeled oxygen. Furthermore, during the blank experiment for isotopic oxygen exchange in the presence of reaction there was no detectable formation of cross-labeled oxygen as well as no propane conversion associated with gas-phase reaction.
3.2.7 Diffuse Reflectance Infrared Fourier Transform Spectroscopy (DRIFTS)

Among all the characterization techniques available to the catalysis researcher, the \textit{in-situ} techniques provide the most powerful tools. A technique, which allows characterization of catalysts and/or adsorbed species under reaction conditions, includes Fourier Transform Infra-red Spectroscopy. DRIFT is a surface sensitive technique for the analysis of trace quantities of materials. Unlike traditional solid sampling FTIR techniques, the sample does not need to be pelletized with KBr. Diffuse reflectance occurs when light impinges on the surface of a material and is partially reflected and transmitted. The types of reflectance are depicted in Figure 3.3. Light that passes into the material may be absorbed or reflected out again. Hence, the radiation that reflects from an absorbing material is composed of surface-reflected and bulk re-emitted components, which summed are the diffuse reflectance of the sample.

\begin{figure}
\centering
\includegraphics[width=0.5\textwidth]{SpecularDiffuseAbsorptionReflection.png}
\caption{Three types of reflections that occur at a powdered surface}
\end{figure}
Diffuse Reflectance Infrared Fourier Transform Spectroscopy (DRIFTS) of catalysts was performed using a Bruker IFS66 instrument equipped with a DTGS detector and a KBr beamsplitter. The system is pictured in Figure 3.4. For IR measurements of solid samples, samples (2wt% in KBr) were placed in a sample cup inside a Spectratech diffuse reflectance cell equipped with KBr windows and a thermocouple mount that allowed direct measurement of the surface temperature. Heating was performed under a flowing, dry helium atmosphere. KBr was used as reference. Spectra were averaged over 1000 scans in the mid-IR range (400-4000 cm\(^{-1}\)) to a nominal 2 cm\(^{-1}\) resolution. Spectra were converted to Kubelka-Munk units. Deconvolution and area measurement of hydroxyl bands was performed using the GRAMS 32 software package. For in-situ spectra, background spectrum was taken at room temperature and at 450°C sample surface temperature. Gas phase spectra were taken at 450°C. In-situ spectra were taken after 15 min. to 1 h of exposure to reaction mixture (%N\(_2\)/C\(_3\)/O\(_2\): 61%/26%/13%). An additional spectrum was recorded once the reaction chamber was quenched to room temperature under nitrogen flow. For propane and propylene adsorption experiments, background spectra was taken under a 1%O\(_2\) in helium flow at various temperatures (350, 300, 250, 200, and 150°C). Following background measurement, the samples were purged for one hour under helium after which propane or propylene adsorption was performed for one hour at 150°C. The samples were then flushed for one hour under helium flow at 150°C. A 1%O\(_2\) in helium flow was introduced and spectra were taken at each successive temperature after an equilibration time of 15 minutes.
Figure 3.4: The DRIFTS System
3.2.7 Magic Angle Spinning Nuclear Magnetic Resonance Spectroscopy with Cross-Polarization

The presence of strong dipole coupling between low-concentration nuclear spins (such as $^{29}$Si) and abundant spins (such as $^1$H) in solids can enhance the sensitivity of the low-concentration spin observation under appropriate conditions. Cross Polarization or Polarization Transfer is a modification to traditional NMR to observe very insensitive nuclei coupled to proton, such as the hydroxyl groups (OH) in close proximity of the Si nuclei. The key to this type of experiment is that the signal of the nucleus that we observe is modulated by the chemical shift of one or more of the other nuclei through the polarization transfer. A schematic representation of this process is shown in Figure 3.5.

CP-MAS $^{29}$Si NMR spectroscopy can be used to characterize the structure of silica networks and has been used to characterize SiO$_2$:TiO$_2$ mixed oxides. In single pulse $^{29}$Si NMR, both -OTi and -OH groups are reported to effect the silicon nucleus in a similar manner. However using $^1$H-$^{29}$Si cross-polarization, the most likely candidates for signal enhancement are the SiOH groups. These sites are depicted with the corresponding Q$^n$ notation, where n represents the number of Si-O ligands around each Si nucleus. The presence of adsorbed water may affect the signal enhancement by increasing the relaxation time of the protons involved in cross-polarization therefore samples were re-calcined at 550°C for 30 minutes and maintained in the dehydrated state before analysis.
• Magic Angle Spinning for solid samples

removing the anisotropy of crystalline/amorphous solids

• Cross Polarization

rf Pulse

\(^1\text{H}\)

\(^{29}\text{Si}\) acquire signal

Polarization transfer between H and Si nuclei (4-5 times larger signals for Si nuclei with bonded or nearby \(-\text{OH}\))

Figure 3.5: Schematic representation of CP-MAS-NMR
The $^1\text{H}^{29}\text{Si}$ CP-(MAS)-NMR spectra were obtained on a Bruker 400DMX Wide Bore FT/NMR. A portion of prepared samples were re-calcined at 550°C for thirty minutes under pure O$_2$ and maintained in the dehydrated state at 110°C for 2 days prior to analysis. The data were acquired using MAS of 5kHz in a ZrO$_2$ rotor. The chemical shifts are reported relative to tetramethylsilane (TMS).

3.2.8 Electron Spin Resonance

ESR spectroscopy is a widely used method for categorizing most paramagnetic, free-electron, or paramagnetic radical-based chemical compounds. The theory behind these reactions depends on the promotion/demotion of an unpaired electron in a chemical system. When a compound is placed into a magnetic field, the energy levels between different spin states (i.e. $+1/2$, $-1/2$) that would normally be of equal energy separate, mainly due to how the magnetic moment of the spins relate to the overall magnetic moment of the field it is placed in; this is known as the Zeeman effect. The energy consumed by such a jump can also be defined as: $E = g\mu_B B_0 m_s$, where $B_0$ is the strength of the magnetic field the electron is in (in Gauss), $m_s$ is the spin number on the electron (which can differ depending on the atom's environment), $g$ is a "free electron constant" that is normally equal to 2.002, and $\mu_B$ is the Bohr magneton, a conversion value that is usually equal to $9.724 \times 10^{-24}$ J/T.
Since it is easier to propagate a magnetic field through a substance and make changes to it instantly rather than doing the same with a frequency of a source of energy, most ESR experiments are done by applying a fixed frequency of energy (usually in the GHz range, where individual electrons are affected) to a small amount of sample while changing the strength of the magnetic field. At the point where the magnetic field reaches the correct intensity to cause the electron to split itself between the two possible states, a spike in the energy consumed would occur. To minimize the noise from the mw diode inside the spectrometer, a magnetic field modulation scheme with phase sensitive detection is usually employed. This gives the spectra the appearance of a first-derivative of the adsorption spectra. To complicate the spectra further, the adsorption depends upon orientation the 3-dimensions. This can separate the characteristic absorption into 3 values depending on the symmetry of the powder sample studies. A schematic representation of these features is shown in Figure 3.6.
Zeeman effect

![Zeeman effect diagram]

- energy absorbed when constant microwave frequency matches to the energy difference between two states of the *unpaired*-electrons in the sample

\[ H \rightarrow \text{energy absorbed when constant microwave frequency matches to the energy difference between two states of the unpaired-electrons in the sample} \]

- \( \Delta E \)

\[ H = \text{external magnetic field} \]

\[ g (\text{free electron}) = 2.0023 \]

\[ \beta = \text{Bohr magneton} \]

**Figure 3.6**: Schematic representation of ESR for powdered samples
Electron Spin Resonance (ESR) spectra were acquired on a Bruker ESP300 electron spin resonance spectrometer. For ESR spectrum taken under dehydrated conditions, a portion of prepared samples were re-calcined at 550°C for thirty minutes under pure O₂ and maintained in the dehydrated state at 110°C for 2 days prior to analysis. The spectra were obtained at room temperature with a Klystron frequency of 9.76GHz at 2mW power and 100kHz magnetic field modulation.

A portion of prepared samples was also placed inside a quartz ESR tube (Wilmad) capable of sealing the sample under a gas atmosphere or vacuum. Dehydrated samples were subsequently purged at 10⁻³ Torr for twelve hours after which 100 Torr of propane was introduced. ESR spectra were recorded after 15 minutes at room temperature under the propane atmosphere. Another set of propane “reduction” experiments were performed in which the samples were contacted with 500 Torr of propane at 400°C for thirty minutes followed by evacuation at 10⁻³ Torr. ESR spectra were recorded under vacuum.

3.2.10 Transmission Electron Microscopy (TEM)

Transmission electron microscopy (TEM) is dedicated to micro structural analysis of solid materials down to the sub-nanometer scale. TEM is analogous to optical microscopy in that the photons are replaced by high-energy (>100 keV) electrons and the glass lenses replaced by electromagnetic lenses. The electron beam passes an electron-transparent sample and an enlarged image is formed using a set of lenses. Since electrons
interact strongly with matter, electrons are attenuated as they pass through a solid requiring the samples to be prepared in very thin sections. The image is projected onto a Charged Coupled Device (CCD) camera for imaging. Image contrast is obtained by the interaction of the electron beam with the sample: a part of the electrons will be diffracted. By means of an aperture, one or more diffracted beams are selected for the formation of the image. In this way, one can distinguish between different materials, as well as image individual crystals and crystal defects.

TEM images and quantitative elemental analysis were performed on a Philips CM-300 FEG with a 1.7 Å point-to-point resolution. The instrument was equipped with a Gatan 1k x 1k Multiscan Digital Camera and a light element EDS X-ray detector. A small portion of catalyst samples were emulsified in a solution of isopropyl alcohol and a small drop of this solution was placed on a copper/FORMVAR grid for analysis.
3.2.11 Thermal Gravimetric Analysis (TGA)

TGA is the quantitative measurement of an analyte weight as a function of temperature. This method can be used to determine weight loss associated with a variety of processes including water loss, phase and crystallographic change, and decomposition. Thermogravimetric analysis was performed with a Perkin-Elmer TGA 7 thermogravimetric analyzer. Samples were pre-treated in dry air at 500°C for thirty minutes before analysis. The weight-loss of ambient-exposed samples (50 mg) versus temperature was recorded under a dry helium flow of 30 cm³/min. The differential weight loss curves (DTG) were analyzed with the accompanying Perkin-Elmer software package. The temperature program was as follows: 10 minutes 30°C, 5°/min. ramp rate to 800°C, and holding at 800°C for 15 minutes.

3.2.12 Differential Scanning Calorimetry (DSC)

Differential scanning calorimetry independently measures the rate of heat flow to a sample and a standard that are at the same temperature. Data is taken by monitoring the differential heat flow as a function of temperature. DSC is useful to make the same measurements as TGA and has the added capability to measure heat capacities. Differential Scanning Calorimetry was performed using a Perkin-Elmer DSC-7 differential power unit. The unit consisted of aluminum sample and reference pans. Samples (50mg) were placed in the sample aluminum pan and pretreated in 10%O₂ in
nitrogen at 500°C for thirty minutes followed by cooling to 100°C under nitrogen. The experimental procedure involved switching between dry nitrogen and 5% propylene/nitrogen flows using a 4-port valve to measure the heat associated with both irreversible and reversible propylene adsorption. DSC thermograms were obtained at 100°C. To account for the heat capacity difference between the flow of nitrogen and propylene/nitrogen, a series of switches were carried out under the same experimental conditions using an empty sample pan. The amount of measured heat corresponding to this heat capacity difference was then subtracted from that obtained during the experiments. The instrument was calibrated individually for each sample studied by the heat of fusion of indium metal (5mg) as measured by placing the metal on top of the catalyst sample placed in the sample pan. This method corrects for the heat conduction of the catalyst samples and heat loss to the gas phase. An experimental diagram with setup is given in Figure 3.7.
**Mass Flow Controllers**

- Water Trap
- Perkin-Elmer DSC-7 differential power unit

**Method**

- \( N_2 \) to 5\%C\(_3\)=/N\(_2\) switch
- Adsorption/Desorption

Figure 3.7 : Schematic representation the DSC system and set-up
3.3 Steady-State Reaction

The reactor system, shown in Figure 3.4, is such that gaseous feed may be directed through 1/4" heated stainless steel lines and into a reactor and furnace assembly. The furnace and reactor assembly consists of a quartz reactor that may be packed with a catalyst. Both the feed and the product stream may be analyzed a mass spectrometer or by an on-line gas chromatograph with both a FID and a TCD (HP 5890 series II).

The lower hydrocarbons considered in the reaction system are methane, ethane, and propane. The feed to the reactor will consist of less than 5%, on a volume basis, of the hydrocarbon, from 0 to 10 % oxygen, and the balance nitrogen. These gases will combine to give a total flowrate of 100 cc/min. Other gases included for GC operation are helium, air, and hydrogen.

The gas chromatograph has been modified to provide the best performance for the reaction studied. The FID packed sample inlet is a 1/8" OD eight foot long HayeSep D® column and the TCD packed sample inlet is a 1/8" OD six foot long Porapak Q® column. The pneumatic valve #2 inside the GC, shown in the diagram, is configured in such a way as to allow separation of similar molecular weight gases such as oxygen, nitrogen, and carbon oxides. This is accomplished by passing the gaseous stream through a 1/8" OD, six foot long column packed with 5Å molecular sieves (Zeolite) before the TCD inlet. The sample loops of the GC are 1/16" OD columns. The TCD sample loop is 1cc, while the FID sample loop is 0.1cc.
Figure 3.8: ODH reactor system
Steady state reaction experiments were carried out in a fixed-bed, quartz reactor, operated at ambient pressure. Catalyst samples, ranging from 0.1 g to 1.5 g, were held in place by a quartz frit. The dead volume of the quartz microreactor was filled with quartz wool and/or ceramic beads to minimize effects from any homogeneous reaction/surface-assisted gas phase reaction and to provide a short residence time for propylene formed. Reaction temperatures ranged from 723 K to 823 K. The quartz reactor, both empty and filled with quartz wool/ceramic beads, showed no activity up to 823 K for ethane and propane experiments. The feed consisted of hydrocarbon/oxygen/nitrogen at flows between 10-200 cm³/min., usually at a flowrate of 25 cm³/min. The amount of nitrogen was varied for some runs. However, the hydrocarbon/oxygen molar ratio was held constant at 2. The concentration of the feed stream was maintained outside the flammability limits of the hydrocarbon-oxygen-nitrogen mixtures for all runs.
CHAPTER 4

CHARACTERIZATION OF MOLYBDENUM OXIDE STRUCTURE OVER THE SILICA/TITANIA MIXED OXIDE SUPPORT

4.1 Introduction

Because alkanes are relatively inexpensive and are abundant due to side products of refineries and natural resources such as natural gas, one of their potential uses is in the conversion to alkenes [7]. Alkenes are heavily used as feedstock in industry but are more expensive and less available than alkanes. A method that has been widely used to achieve this conversion is steam cracking of alkanes at high temperatures. This method is constrained in many ways, namely, thermodynamic limitations, endothermic reactions that require a large input of heat, and formation of coke on the catalyst [3]. Because of these limitations, many research studies have focused on the oxidative dehydrogenation (ODH) of alkanes. ODH is a desirable alternative because, when a proper catalyst is selected, the reaction occurs at lower temperatures and is exothermic, preventing heat input requirements.
Common theories have risen from the literature that applies to many different catalysts studied for Propane ODH. These issues include role of homogeneous reaction, propylene residence time/reactivity, reducibility of the active metal, surface acidity, and propylene adsorption. Pantazidis et al. have noted that a good acid-red-ox balance is needed over VMgO catalysts in the ODH of propane [9]. Many other studies on different catalysts have shown selectivity-acidity-redox relationships. Abello et al. have reported that the stronger the acid strength on their potassium-molybdenum oxo catalyst in the ODH reaction the stronger propylene and propylene intermediates would be held thus creating a less-selective catalyst [10]. They have also proposed a Mo(V) stabilization effect related to acidity. These observations were confirmed over Mo/Si:Ti catalysts studied by Ozkan et al. [11]. However, when we discuss a balance in reducibility and acidity on catalysts in the ODH of propane it is necessary to think in terms of the relative rates of the kinetic steps occurring on oxide catalysts. As discussed by Kung [2], one needs to consider the two reactions propane→propylene and propylene→COx. It was shown that, on perhaps all catalysts, the rate for the second reaction could be five to ten times higher than that of the first reaction. Work by Khodakov et al. has begun to answer these fundamental questions by measuring the relative rates of propylene and COx formation by residence time and isotopic tracer experiments [12]. Furthermore, they have commented on the likelihood of similar active sites being responsible for both reactions. These properties will depend on transition metal loading. When the selectivity is considered in terms of these two competing reactions, it becomes clearer that both the reducibility and oxygen mobility characteristics of the catalyst, and its acidity, need to be
simultaneously taken into account. While the surface oxygen species affect the rates of both reactions, it is conceivable that a catalyst with a strong Lewis character will have a higher affinity for the electron-rich double bond of propylene, leading to its further oxidation before it desorbs from the surface.

Research on ethane ODH catalysts usually fall into two operating temperature categories, above or below the temperature at which significant gas-phase reaction mechanisms participate. Lower temperature catalysts usually consist of reducible transition metal oxides, such as vanadium or molybdenum, and are considered to proceed via a surface mechanism. The higher temperature catalysts do not contain easily reducible ions and consist of Mg, Li, or other group IA or IIA ions or oxides. Several researchers proposed a high temperature surface/gas-phase mechanism that involves ethyl radical formation by surface oxygen and the subsequent degradation to ethylene through a surface ethoxide intermediate. Whether this oxygen species is adsorbed from the gas phase or part of the lattice is a current matter of investigation on different types of catalysts that operate by a non-red-ox mechanism [64].

Over reducible metal oxides at the lower temperatures of operation (<600°C), a red-ox mechanism exists. The red-ox mechanism of ethane ODH is generally accepted to occur by a Mars Van-Krevelen mechanism [5]. The rate-determining step is the abstraction of one H from a C-H bond by lattice-oxygen. It is important to note that, in this scheme, often no differentiation is made as to which type of lattice oxygen species at the surface are involved in C-H bond activation. Over supported transition-metal oxides, several species may exist in the form of M=O, M-O-M, or M-O-support bonds, where M
is the transition metal of interest. The nature of the active oxygen species and red-ox properties will play a critical role in catalyst performance and will certainly depend upon transition metal loading, dispersion, and support effects. For an optimum combination of activity and selectivity there should exist a balance between the activation of the hydrocarbon and the ease of removal of oxygen from the catalyst surface. Oxygen that is too tightly bound will result in low activity, while a catalyst with oxygen that is too labile will be very active, but not selective. Examples from literature indicate that most supported transition metal-oxide based catalysts (Mo and V in particular) achieve ethylene yields in the range 15-30% [2-3].

It is generally accepted that impregnation methods to support MoOx over high surface area silica are limited by several factors such as choice of precursor and low reactivity of surface silanols [65]. Thus, in literature, detailed preparation methods have been employed to enhance silica affinity for supported MoOx species. Several methods have been adopted such as preparation methods to increase the concentration or reactivity of surface silanol groups, Mo-complexes that directly react with the surface hydroxyl groups of silica, gas-phase grafting, non-aqueous impregnation, or sol-gel synthesis. Over the mixed oxides of silica and titania, a much better dispersion of MoOx is obtained compared to the silica supported catalyst. Addition of TiO2 into the silica matrix has been shown to lead to unique structural and chemical properties that can provide advantages that the respective single oxides cannot [54,56-57,66-70]. These benefits include stronger metal-support interactions, hindering reduction and segregation of the active metal, and smaller particle size that leads to better dispersion and higher surface
area. Additionally, Klimova et al. [71] have achieved an enhanced dispersion of MoO$_x$ over silica (MCM-41) by incorporating titania into the silica network. Much of these discussed properties are related to changes in surface acidity, porosity, Ti-O-Si connectivity, and phase segregations.

Gao, et al. [72] have prepared and characterized vanadium oxide species dispersed on TiO$_2$/SiO$_2$ (5-15% TiO$_2$). Studies have shown that these catalysts are selective for the partial oxidation of ethane to ethylene due to the lower availability of oxygen atoms for total oxidation [73]. The authors have concluded that the VO$_x$ species preferentially interact with titanium oxide species. On the same samples however, addition of VO$_x$ to TiO$_2$/SiO$_2$ was found to consume some of the surface Si-OH hydroxyls indicating that an interaction of VO$_x$ with silica is not necessarily absent and several supported species may be present with different Ti-O$^-$ and Si-O$^-$ ligands. This led the authors to describe the samples as a bi-layered surface metal oxide catalyst consisting of vanadium oxide and titanium oxide on silica. Research from the same laboratory [74], has reached the same conclusion for VO$_x$ supported on Al$_2$O$_3$/SiO$_2$. Since the oxygenated ligands present around transition-metal centers play a detrimental role in determining the reactivity of the surface species on mixed-oxide supports, the characterization of surface layers and support structure is crucial for understanding catalytic behavior.

In this work, a series of molybdena catalysts (0-20% wt. loadings) supported on the mixed oxides of silica and titania have been prepared by a "one-pot" sol-gel synthesis that distributes the molybdenum precursor through the Si:Ti support network as it forms.
A review of literature suggests that research on mixed oxides used as supports for active red-ox metal oxides have usually followed impregnation, incipient-wetness, or grafting methods [75-80]. A dispersed mixed-oxide support is usually pre-formed to reach certain surface characteristics, such as dispersion or crystallinity. In our sol-gel method, we believe the one-pot preparation can allow for a greater possibility and a more even distribution of hetero-linkages $M$-$O$-$M'$, where $M$ and $M'$ can be Si, Ti, or Mo.

Prepared catalysts have been studied in regard to their activity for the oxidative dehydrogenation (ODH) of ethane. X-ray diffraction (XRD), Raman spectroscopy (ambient and dehydrated conditions), and X-ray photoelectron spectroscopy (XPS) were used to determine the nature of the molybdena species at various weight loadings. To demonstrate the structural differences obtained on a mixed oxide support, catalysts with single oxide supports of silica and titania have also been prepared. Temperature Programmed Desorption has been used to study changes in desorption behavior as a function Mo wt. Loading.

Synthesized catalysts are listed in Table 4.1. Catalysts numbered 1 through 6 are a series of Si:Ti 1:1 supported Mo catalysts (0-20wt.%). Catalysts 7 and 8 are 10wt.% loading of Mo over SiO$_2$ and TiO$_2$ respectively. For characterization purposes, pure oxides of silica and titania were also prepared by the same method.
Table 4.1
Sol-gel prepared catalysts with different Mo loadings

<table>
<thead>
<tr>
<th>#</th>
<th>Composition</th>
<th>Surface Area (m²/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Si:Ti 1:1</td>
<td>320</td>
</tr>
<tr>
<td>2</td>
<td>2%Mo/Si:Ti 1:1</td>
<td>343</td>
</tr>
<tr>
<td>3</td>
<td>5%Mo/Si:Ti 1:1</td>
<td>246</td>
</tr>
<tr>
<td>4</td>
<td>10%Mo/Si:Ti 1:1</td>
<td>210</td>
</tr>
<tr>
<td>5</td>
<td>15%Mo/Si:Ti 1:1</td>
<td>188</td>
</tr>
<tr>
<td>6</td>
<td>20%Mo/Si:Ti 1:1</td>
<td>116</td>
</tr>
<tr>
<td>7</td>
<td>10%Mo/SiO₂</td>
<td>548</td>
</tr>
<tr>
<td>8</td>
<td>10%Mo/TiO₂</td>
<td>91</td>
</tr>
</tbody>
</table>

4.2 Structural Changes With Molybdenum Loading

From 2 through 20% wt. loading of molybdena, the catalysts show a general decrease in surface area with increasing amounts of molybdenum added. X-ray diffraction patterns of the Mo loaded catalysts are shown in Figure 4.1. X-ray diffraction of the Si:Ti 1:1 support yielded a pattern characteristic of a silica-titania mixed oxide [81]. One broad peak with center located at a d spacing of 3.59 Å was observed, which is indicative of a finely dispersed, small X-ray particulate anatase structure supported over amorphous silica. For comparison, the X-ray diffraction pattern of TiO₂ (anatase structure) is included in the figure. With the addition of molybdenum, this peak becomes narrower, indicating a change in the dispersion and segregation of titania in the Si:Ti matrix with the addition of Mo.
Figure 4.1: X-ray diffraction patterns of Mo/Si:Ti catalysts with different Mo loadings
Molybdena species are finely dispersed showing no MoO$_3$ diffraction patterns up to 15% wt. loading on the mixed oxide support. However, it may be possible for MoO$_3$ to exist as microcrystalline material below the approximately 40 Å XRD detection limit [82]. Features from crystalline MoO$_3$ become detectable when the Mo loading level is increased to 20%. For 10%Mo on silica, only one peak associated with the (021) plane of crystalline MoO$_3$ is resolved. For 10%Mo on titania, no feature associated with crystalline MoO$_3$ is detected. It is interesting to note that although the surface area of silica-supported sample is much larger than that of Mo/TiO$_2$, the titania support appears to provide a better dispersion of the metal.

Raman spectra under ambient and dehydrated conditions were recorded for catalysts with different Mo loadings as well as for the Si:Ti 1:1 support. Figure 4.2 shows the change in the Raman spectra (focused in the range 540-1120 cm$^{-1}$) when the Si:Ti 1:1 support is dehydrated. For comparison, Raman spectra (ambient and dehydrated) of pure silica are included. The dehydrated Si:Ti 1:1 surface have Raman bands at 605, 800, 916, and 1080 cm$^{-1}$. The band at 605 cm$^{-1}$ is assigned to tricyclosiloxane rings of the silica produced via the condensation of surface hydroxyls upon dehydration while the band at 800 cm$^{-1}$ is attributed to the symmetric mode of Si-O-Si stretching [83]. On pure silica, a distinct band can be observed at 976 cm$^{-1}$ under ambient conditions. This band is ascribed to surface silanols and is seen to disappear upon dehydration of the sample.
Figure 4.2: Raman spectra of Si:Ti 1:1 support under ambient and dehydrated conditions.
In the literature, the bands located at 916 and 1080 cm\(^{-1}\) have been assigned to perturbed silica vibrations that are indicative of Ti-O-Si bonds [83-85]. However, the broad band at 1080 cm\(^{-1}\) can be convoluted with the expected asymmetric mode of Si-O-Si stretching at ~1050 cm\(^{-1}\) [86]. When compared to silica, the 1050 cm\(^{-1}\) band can be seen to sharpen and shift to 1080 cm\(^{-1}\). An important feature of these spectra is the dispersed nature of TiO\(_x\) species. The most intense Raman band from TiO\(_2\) (anatase) cannot be distinguished around 640 cm\(^{-1}\).

Thermogravimetric analysis of the SiO\(_2\), TiO\(_2\), and Si:Ti 1:1 supports reveals that all physically adsorbed water leaves the supports between 150 and 200°C. Analysis of the DTG curves can show the temperature at which dehydroxylation begins and thus give insight as to the origin (i.e. TiO\(_2\) or SiO\(_2\)) and thermal stability of chemically bound hydroxyl groups. The dehydroxylation was found to occur in a single-step process beginning at a single temperature (TiO\(_2\) - 303°C, SiO\(_2\) - 325°C, Si:Ti 1:1 - 325°C) as summarized in Figure 4.3. Based on the surface area of the samples and the total weight loss after dehydroxylation begins, the concentration of hydroxyls was calculated. The temperature of dehydroxylation is the same for SiO\(_2\) and Si:Ti 1:1. This, together with the fact that there was no lower temperature peak observed in the DTG curve which may correspond to hydroxyls on titania, suggests that the hydroxyls on Si:Ti are largely from silica. Furthermore, there is no significant change in the strength of hydrogen bonding of clustered hydrolyzed Si-OH—HO-Si bonds since the temperature of dehydroxylation (Si-OH—HO-Si \(\rightarrow\) Si-O-Si +H\(_2\)O) for pure SiO\(_2\) is roughly the same for Si:Ti 1:1.
Figure 4.3: De-hydroxylation temperatures and hydroxyl concentrations of support materials as determined by TGA analysis.
Quaranta et al. [87] have found that on TiO$_2$/SiO$_2$ samples prepared by homogeneous precipitation, a substantial amount of the silica surface remains uncovered even above monolayer coverage. Furthermore, Armaroli et al. [85] have suggested that in TiO$_2$/SiO$_2$ aerogels of up to 40% TiO$_2$, silica tends to form an overlayer on a TiO$_2$ core and the surface is dominated by silanol groups similar to those of pure silica. The authors detected no increase in the acidity (strength) of the surface silanols after incorporation of titania to silica, through IR studies of adsorbed acetonitrile.

When compared to SiO$_2$ and TiO$_2$, the overall concentration of hydroxyls is increased in Si:Ti 1:1. Since the hydroxyl concentration of SiO$_2$ and TiO$_2$ are almost equal, the possibility of hydroxyls resulting from TiO$_x$ species in Si:Ti 1:1 cannot be completely ruled out, even though not detected in TGA experiments. For a pure titania support, it has been shown that preparation of high surface area titania of small particle size, such as that obtained from sol-gel methods, can alter surface acidity and lead to several types of surface hydroxyls [88]. Nonetheless, Si:Ti 1:1 was found to have an increased hydroxyl concentration compared to that of SiO$_2$ which can conceivably arise from the perturbation of the surface by Ti-O-Si interactions.

The Raman spectra of 10%Mo catalysts supported on TiO$_2$ and SiO$_2$ are shown in Figure 4.4 (ambient and dehydrated conditions). These samples are prepared in the same manner as the catalysts which are supported on the mixed oxide Si:Ti. Bands arising from poorly dispersed crystalline MoO$_3$ are present over the SiO$_2$ supported catalysts at 671, 820, and 996 cm$^{-1}$ and do not change upon dehydration.
Figure 4.4: Raman spectra of 10%Mo/SiO₂, (a) ambient conditions (b) dehydrated conditions and 10%Mo/TiO₂, (c) ambient conditions (d) dehydrated conditions. Spectra taken under 10%O₂/He at room temperature.
Small bands located at 781, 854, 877, and 957 cm$^{-1}$, that are assigned to MoO$_x$ species in polymolybdate structures [65], are present in the ambient Raman spectra but disappear upon dehydration. In general, bands in the range of 750 to 950 cm$^{-1}$ are attributed to antisymmetric stretching of Mo-O-Mo bonds or the symmetric stretch of (-O-Mo-O-)$_n$ bonds, while bands in the range 950 to 1050 can be attributed to the stretching mode of Mo=O bonds [36]. In particular, the broad Mo=O band at 957 can be assigned to Mo$_8$O$_{26}^{4-}$ and Mo$_7$O$_{24}^{6-}$ polymolybdate species [65, 89]. In the Raman spectra of 10%Mo/SiO$_2$ before and after dehydration, a distinct band at 976-979 cm$^{-1}$ is present which could be assigned to Mo-O-Si surface bonds [65, 90]. However, a Raman band of surface silanols is also reported to occur around 970-976 cm$^{-1}$ [81, 91]. Also visible in the spectrum taken from the dehydrated sample are the bands that are typical of the SiO$_2$ support (607, 800 and 1050 cm$^{-1}$).

For 10%Mo/TiO$_2$, no crystalline MoO$_3$ is observed and a band arising from surface-supported MoO$_x$ appears at 1001 cm$^{-1}$ that shifts to 1006 cm$^{-1}$ and becomes more intense upon dehydration. Furthermore, there is a broad feature at 986 cm$^{-1}$ that completely disappears upon dehydration. However, bands arising from polymolybdate species become slightly more visible (850 and 932 cm$^{-1}$) when 10%Mo/TiO$_2$ is dehydrated. The results indicate significant differences in the way titania and silica can support MoO$_x$. The segregation of crystalline MoO$_x$ over silica is commonly observed and arises from the low reactivity of the surface hydroxyls of silica toward MoO$_x$ causing the formation of bulk MoO$_3$ crystallites [92-93]. However, a more intriguing difference is the change of bands arising from molybdenum poly-oxo structures upon dehydration.
Over the silica-supported sample, these bands disappear, while over titania, bands at 850 and 932 cm\(^{-1}\) become more pronounced as the broad band at 986 cm\(^{-1}\) disappears. In literature, several researchers have observed the disappearance of polymolybdate bands over Mo/SiO\(_2\) catalysts upon dehydration. Furthermore, hydration can result in the hydrolysis of Mo-O-Si bonds to create surface hydroxyls. Upon dehydration these hydroxyls re-interact with the surface MoO\(_x\) species [94]. It appears that titania is able to accommodate stable poly-oxo molybdenum species while silica supports only a small amount of MoO\(_x\) species as much is present as crystalline MoO\(_3\).

Raman spectra of ambient-exposed Mo/Si:Ti 1:1 catalysts are shown in Figure 4.5 covering the Mo=O and Mo-O-Mo vibrational frequency range together with the most intense Raman band from TiO\(_2\) (anatase). An important feature of these spectra is that there is no evidence of crystalline MoO\(_3\) up to 15% wt. loading. At 20wt% loading, formation of crystalline MoO\(_3\) is apparent by the bands located at 663, 819, and 996 cm\(^{-1}\). The broad bands associated with isolated terminal Mo=O stretching vibrations were observed to shift with Mo loading from 973 cm\(^{-1}\) at 2wt.% to a maximum of 998 cm\(^{-1}\) at 15wt. %.

Shifts in the Mo=O Raman frequency are related to changes in bond length [37] and can be ascribed to a decreasing interaction with the support and the formation of 3-dimensional structures. The Raman band of TiO\(_2\) is observed in all samples (640 cm\(^{-1}\)) except for 2%Mo/Si:Ti 1:1 where it has shifted to 632cm\(^{-1}\). This feature indicates that TiO\(_x\) is well dispersed on this sample, whereas with increasing wt. loading of molybdenum, larger domains of TiO\(_2\) (anatase) are readily formed.
Figure 4.5 : Raman spectra (ambient conditions) of Mo/Si:Tl 1:1 catalyst with different Mo loadings
A pronounced change is observed in the Raman spectra of Mo/Si:Ti 1:1 catalysts under dehydrated conditions, Figure 4.6. Perhaps the most important feature is that the isolated terminal Mo=O stretching vibrations shift to higher wavenumbers for all samples and several shoulders can be observed in the broad vibration. At the same time, changes in the silica network with Mo loading can be readily observed. As under ambient conditions, there is no evidence of crystalline MoO₃ up to 15% wt. loading. The main Mo=O Raman frequency remains essentially unchanged with weight loading (1004-1006 cm⁻¹) until crystalline MoO₃ appears at 20%. Small, yet discernable, shoulders are observed in the main Mo=O Raman band for 2% wt. loading at 977 and 997 cm⁻¹. With increasing wt. loading, the shoulder at 977 cm⁻¹ is no longer observed while higher wavenumber shoulders are still observed up to 15wt.% (5%Mo - 990 cm⁻¹, 997 cm⁻¹, 10%Mo - 982, 998 cm⁻¹, 15%Mo - 987, 1001 cm⁻¹). The band present on 2%Mo/Si:Ti 1:1 at 977 cm⁻¹, as stated previously, could arise from Si-O-Mo bonds corresponding to a silicomolybdic acid species.
Figure 4.6: Raman spectra (dehydrated conditions) of Mo/Si:Ti 1:1 catalyst with different Mo loadings. Spectra taken under 10%O₂/He at room temperature.
Examination of the bands from the Si:Ti 1:1 support present in the Raman spectra of Mo/Si:Ti 1:1 catalysts under dehydrated conditions reveal changes with molybdenum wt. loading that are not resolved under ambient conditions. On 2%Mo/Si:Ti 1:1, bands are present from the silica support at 605, 800 cm\(^{-1}\), from TiO\(_2\) (anatase) at 640 cm\(^{-1}\), and from Si-O-Si and/or Si-O-Ti bonds centered at \(-1080\) cm\(^{-1}\). In general, the features from the silica support are decreasing with Mo loading. The silica bands at 605 cm\(^{-1}\) is seen to decrease with 5% wt. loading and become unresolved at 10% wt. loading. The band at \(-1080\) cm\(^{-1}\) shows the same trend. The broad band at 800 cm\(^{-1}\) disappears altogether at 5% wt. loading. This could be an indication that, as Mo loading is increased, there is increased coverage over the silica on the support. Concurrent with these trends is a relative increase in the TiO\(_2\) band at 640 cm\(^{-1}\).

A question arises as to whether it can be established if MoO\(_x\) species are preferentially supported over SiO\(_x\) or TiO\(_x\) domains and if so, to what extent. Wachs \[42\] has summarized the Raman spectroscopy results of Mo/SiO\(_2\) and Mo/TiO\(_2\) prepared by several preparation methods and stated that Mo=O Raman bands of surface supported MoO\(_x\) on titania appear in the range 998-1001 cm\(^{-1}\). On silica, these bands are reported to be a function of wt. loading and appear in the range 975-990 cm\(^{-1}\). Considering the results in this context, MoO\(_x\) species attached to titania (Raman bands present 1004-1006 cm\(^{-1}\)) and species attached to silica (Raman bands present as shoulders in the range 977-998 cm\(^{-1}\)) appear to co-exist. However, Gao et al. \[72\] describe the VO\(_x\)/TiO\(_2\)/SiO\(_2\) (5-15% TiO\(_2\)) system as a bi-layered surface metal oxide catalyst consisting of vanadium oxide and titanium oxide on silica with several supported species containing different Ti-
O⁻ and Si-O⁻ ligands. While the extent of MoOₓ coverage on each of Si:Ti 1:1 is not determined from the Raman spectra, considering the similarity of the spectrum of 10%Mo/TiO₂ to that of the Si:Ti 1:1 supported catalyst, it would appear that more MoOₓ species are interacting with titania. However, the decrease in the Raman bands of silica observed with increasing wt. loading seems to suggest an increased interaction of MoOₓ with silica, possibly leading to perturbation of the Si-O-Si matrix or even formation of Si-O-Mo linkages.

To further study the dispersion of MoOₓ over the Si:Ti 1:1 support, XPS was performed over samples with various Mo loadings. XPS (Mo3d region) of Si:Ti 1:1 supported catalysts exhibited one linked doublet corresponding to Mo 3d₃/₂ and 3d₅/₂ binding energies. The variation in molybdenum 3d₅/₂ binding energies with Mo loading over Si:Ti 1:1 is shown in Figure 4.7. The binding energy is seen to shift from 232.0 eV (2%Mo) to 232.9 eV (20%Mo) approaching to that of bulk MoO₃ (Mo 3d₃/₂ B.E. = 233.4 eV). An additional feature (Mo 3d₅/₂ B.E. = 229.1 eV) was observed within the spectrum of the 2%Mo/Si:Ti 1:1 catalyst. This lower B.E. feature was ascribed to the presence of Mo-O-Si bonds [94] and is not present in the spectra of the catalysts with higher loading.
Figure 4.7: Variation in Mo3d₅/₂ B.E. of Mo/Si:Ti 1:1 catalysts with different Mo loadings.
Additional XPS characterization (Si 2p, Ti 2p, and O 1s) is shown in Figures 4.8, 4.9, and 4.10 respectively. The Ti 2p3/2 binding energy (Figure 4.8) is seen to shift to lower values with increasing Mo loading, from 459.2 eV for the Si:Ti 1:1 support to 458.3 eV for 20%Mo. The Ti 2p3/2 binding energy of pure anatase is around 458.0 eV. There is also a pronounced feature in the Ti 2p spectrum of the Si:Ti support located at 456.5 eV. Although less pronounced, this feature is seen in the spectrum of 2%Mo/Si:Ti 1:1. This feature may be associated with Ti atoms present in Ti-O-Si. Fernandez et al. [95] have assigned an XPS feature at 456.4 eV to a reduced titania site (Ti⁴⁺). While this remains as a possibility, it is more likely that the Ti site responsible for the XPS feature in Figure 8-a may be an electron rich species produced by integration of Ti into the silica network at boundaries, and not a fully reduced site. Notari [97], points out that in TiO₂ rich samples of Si:Ti, the Ti⁴⁺ in Ti-O-Si shares more negative charges from the oxygen in SiO₂ than from the oxygen of the bulk. The large difference between the BE of the pure anatase phase and that of the Si:Ti support is indicative of TiOₓ closely interacting with the silica surface [90]. The fact that the difference becomes smaller, i.e., the BE of the catalyst approaches that of pure anatase with increasing Mo loading, suggests that the presence of Mo may be interfering with the Si-Ti interaction, as will be discussed further. The Ti/Si surface atomic ratio, as determined from XPS, indicates that there is less Ti at the surface (Ti/Si=0.6-0.4 for all catalysts) than in the bulk (Ti/Si=1). This indicates that silica is preferentially concentrated at the top layers (10-50 Å) of the surface.
Figure 4.8: XPS spectra of Mo/Si:Ti 1:1 catalysts with different Mo loadings, Ti2p
The Si 2p XPS spectra (Figure 4.9) shows a peak located at ~102.3 eV characteristic of SiO$_2$. There is an additional feature in the Si2p spectrum of the Si:Ti 1:1 support and the 2%Mo/Si:Ti 1:1 sample. For the Si:Ti support, there is a shoulder present at ~99.0 eV. This feature is assigned to the Si atoms of Ti-O Si. Additionally, there is a shoulder present in the spectrum of 2%Mo/Si:Ti 1:1, B.E. ~99.8 eV. Because only a weak signal Si-O-Ti was detected in the Ti 2p spectrum of the 2% wt. loading sample, this pronounced change is likely to be due to another species and can be considered further evidence of a Mo-O-Si species. The O1s XPS spectra, shown in Figure 4.10, reveal oxygen contributions from TiO$_x$, SiO$_x$, and MoO$_x$. O1s from SiO$_x$ is located at ~533.4 eV, while the signal from TiO$_x$ and MoO$_x$ coincide around 530 eV. Furthermore, there is evidence of Si-O-Ti species, which can exhibit an O1s band in between those of SiO$_2$ and TiO$_2$ making the peak significantly broadened. The O1s spectrum of 2%Mo/Si:Ti 1:1 appears substantially different than that of the Si:Ti support. Again, it appears there is an additional feature present in the spectrum for 2%Mo/Si:Ti 1:1 located at ~527 eV that could be ascribed to the same Mo-O-Si interaction species.
Figure 4.9: XPS spectra of Mo/Si:Ti 1:1 catalysts with different Mo loadings, Si2p
Figure 4.10: XPS spectra of Mo/Si:Ti 1:1 catalysts with different Mo loadings, Si2p
The surface concentrations, as determined by XPS, are shown in Figure 4.11. As expected, there is an increase in Mo surface concentration with wt. loading that approximately corresponds to the bulk percentage. At the lowest wt. loading studied, 2 wt.%, the percentage of Ti at the surface decreases while that of silica increases compared to that of bare Si:Ti 1:1. This could indicate that low loadings of MoO\textsubscript{x} preferentially cover TiO\textsubscript{x} domains. However, as Mo wt. loading increases, the surface concentration of Ti remains constant with increasing Mo loading. The change in the Si surface concentration on the other hand, exhibits a mirror image of the change in the Mo surface concentration where the increase in one is accompanied by an equal decrease in the other. Reddy et al. [97] have used XPS to quantify Mo dispersion on their 12%Mo samples supported on various 1:1 ratio mixed oxides (Si, Ti, Zr, and Al). XPS surface compositions indicated that MoO\textsubscript{x} is more dispersed on SiO\textsubscript{2} than on TiO\textsubscript{2} over the Si:Ti 1:1 support. However, the dispersion of MoO\textsubscript{x} characterized in this manner, depends on the surface concentrations of the mixed oxides before molybdenum incorporation (i.e. the Si and Ti content before introduction of MoO\textsubscript{x}).
Figure 4.11: Variation of Mo/Si:Ti 1:1 surface concentrations (XPS) with Mo loading
In our study, the data suggest that MoO$_x$ is supported on both silica and titania domains with an increasing affinity for silica as wt. loading is increased from 2 to 15 wt.%. This is in agreement with the Raman spectroscopy results presented. Raman data indicate that MoO$_x$ is supported preferentially on titania; however, the interaction with silica, present even at the lowest wt. loading, increases with wt. loading of molybdenum until the formation of crystalline MoO$_3$ occurs. The Raman data also suggests that polyoxo molybdenum species may be more stable on titania than on silica. Thus, it is plausible that there are significant structural differences in the MoO$_x$ species supported on silica versus titania and that these differences may give rise to the observed differences in surface compositions. However, it has been stated [54] that Ti atoms in TiO$_2$/SiO$_2$ may be located inside channels or pores of silica that may be out of the range of XPS. Thus, it may be possible that in this "one-pot" preparation method in which components are dispersed together during gelation, all the MoO$_x$ and TiO$_x$ species may not be preferentially located at the surface. Furthermore, there may be a significant amount of molybdena species located inside larger titania aggregates or deeper inside the silica network. Combined with the fact that MoO$_x$ species may share several types of support ligands with differing electronegativity, it seems inadequate to describe MoO$_x$ species as being supported exclusively on SiO$_2$ or TiO$_2$ domains.
The presented results indicate that, using this preparation method, Mo loading affects not only the structure of the surface MoO$_x$ species, but also influences the nature of TiO$_x$/MoO$_x$ concentrations over the silica. As molybdenum loading is increased, there is a greater degree of crystalline titania segregation. At the same time, there appears to be an increased interaction of the silica with MoO$_x$.

4.3 Performance in Propane ODH

For the oxidative dehydrogenation of propane (and ethane), the contribution of homogeneous reaction due to propyl and ethyl radicals formed at the surface and desorbed into the gas phase can be significant. Thus, it is necessary to distinguish catalytic activity from gas-phase activity in order to study the effectiveness of the catalysts. Burch and Crabb have studied homogenous contributions to the catalytic ODH of propane and ethane and have confirmed that filling the dead space of the reactor with quartz inhibits gas-phase radical reactions [6,98]. To assure that the homogenous reaction is indeed eliminated by this technique, reaction tests were run using an empty-volume quartz reactor. The results of this experiment showed no conversion of propane and ethane up to 650°C under the feed conditions studied. During catalytic reaction experiments, the dead volume of the quartz reactor was packed with quartz wool and ceramic beads to minimize any gas-phase effects that may occur in the presence of a catalyst.
Reaction with 200 m² reactor loading of the Si:Ti 1:1 support (no Mo component) showed a level of 6% ethane conversion at 600°C, in which ethylene and carbon dioxide were the only observed reaction products. The support performance in propane ODH will be discussed in a subsequent chapter on the effect of potassium.

Steady-state reaction experiments were carried out in a fixed-bed, quartz reactor, operated at ambient pressure. Catalyst samples, roughly 65 m² of surface area in the reactor, were held in place by a quartz frit. To minimize effects from any homogeneous reaction or surface-initiated gas phase reaction and to provide a short residence time for propylene formed, the dead volume of the quartz microreactor was filled with quartz wool and/or ceramic beads. Reaction temperature was 500°C. The feed consisted of propane (26%), oxygen (13%), and nitrogen (61%) at a flowrate of 25 cm³/min. The product distributions maintained a carbon balance of 100% (± 5%). Mo/Si:Ti catalysts were compared in the ODH reaction using equal surface area loading in the reactor and at a temperature of 500°C.
The formation rates of propylene and CO\textsubscript{x} and the depletion rates of propane during the first hour of reaction are presented in Figure 4.12. The propylene formation rates, normalized to Mo atoms, show a decrease with Mo loading under these reaction conditions. The observed decrease in propylene formation can be ascribed to the rising formation of 3-dimensional molybdate species and/or microcrystalline MoO\textsubscript{3}. Observing the Raman spectra (Figure 4.5) of these catalysts, the decrease in propane depletion and propylene formation is accompanied by a shift to higher frequency of the Mo=O terminal bonds. Similar Mo=O shifts under “hydrated” conditions have been related to an increase in the strength of the bond that lowers ODH activity [99].
Figure 4.12: Formation Rates (■ propylene, ▲ CO₂) and Depletion Rates (◆ propane) for Equal Surface Area (65m²) Reaction Experiments (μmol/min/m²). ◆ Normalized Propylene Formation Rate (mol/min/Mo atom × 10¹²)
Mo/Si:Ti catalysts are found to be active for the oxidative dehydrogenation of propane. Increasing molybdenum loadings are seen to provide a rather constant propylene formation up to 10wt.% followed by a decline with increasing Mo content. The decrease in activity can be explained in terms of increasing crystalline MoO$_3$ or 3-dimenionality of the MoO$_x$ surface units as indicated by XPS and Raman results. However, differences observed in the ODH performance of these catalysts cannot be explained solely in terms of the direct changes of the surface MoO$_x$ species with molybdenum loadings. Furthermore, the role of a Mo-O support interaction species in the activation of propane remains unclear. Other structural and surface characteristics, which may be affected by the sol-gel parameters, can also change the catalytic behavior. The effects of oxygen mobility, support structure, and the presence of Mo-support interactions that may occur through surface hydroxyls or in Si:Ti surface interfaces will be discussed further. These studies will help correlate the surface and structural characteristics of this group of novel catalysts with their catalytic properties for lower alkane activation.
4.4 Performance in Ethane ODH

Mo/Si:Ti catalysts were compared in the ODH reaction using equal surface area loading (200 m²) in the reactor and at temperatures of 550°C and 600°C. The feed percentages for these experiments were N₂/C₂/O₂: 85/10/5. Reaction data was taken after steady-state was reached. The main products of the dehydrogenation reaction were ethylene, methane, carbon dioxide, carbon monoxide, and water. Acetaldehyde was only observed to form over 10% Mo/SiO₂. The product distributions maintained a carbon balance of 100% (+/- 5%) except for 10% Mo/SiO₂ due to inadequate separation/quantification of acetaldehyde from water.

The influence of support composition on catalytic performance is shown in Table 4.2, Figure 4.13, and Figure 4.14. By comparing the performance of Si:Ti 1:1, TiO₂, and SiO₂ supports for 10% wt. loading, we see that the titania-supported catalyst behaves quite similarly to that supported on Si:Ti 1:1 at both 550 and 600°C. However, the titania-supported catalyst shows an increased selectivity to carbon monoxide and a decreased selectivity to methane when compared to the Si:Ti 1:1 support. At 550°C, the SiO₂-supported catalyst exhibits much lower ethane conversion than TiO₂-supported one. A similar difference in activity, although less pronounced, is also observed at 600°C.
Table 4.2

Ethane ODH reaction data - Support comparison

<table>
<thead>
<tr>
<th>Catalyst</th>
<th>Temp.</th>
<th>Conversion (%)</th>
<th>Selectivity (%)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(°C)</td>
<td>C\textsubscript{2}H\textsubscript{4}</td>
<td>C\textsubscript{2}H\textsubscript{4}</td>
<td>CO\textsubscript{2}</td>
<td>CO</td>
<td>CH\textsubscript{4}</td>
</tr>
<tr>
<td>10% Mo/SiO\textsubscript{2}</td>
<td>550</td>
<td>5.1</td>
<td>49.6</td>
<td>12.1</td>
<td>38.3</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>600(*)</td>
<td>21.0</td>
<td>31.7</td>
<td>11.3</td>
<td>34.1</td>
<td>0.6</td>
</tr>
<tr>
<td>10% Mo/TiO\textsubscript{2}</td>
<td>550</td>
<td>25.8</td>
<td>38.5</td>
<td>19.5</td>
<td>38.9</td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>33.6</td>
<td>47.9</td>
<td>15.8</td>
<td>32.3</td>
<td>4.0</td>
</tr>
<tr>
<td>10% Mo/Si:Ti 1:1</td>
<td>550</td>
<td>29.3</td>
<td>39.3</td>
<td>19.0</td>
<td>30.4</td>
<td>12.6</td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>39.2</td>
<td>44.1</td>
<td>17.8</td>
<td>12.3</td>
<td>28.8</td>
</tr>
</tbody>
</table>

*ethylene oxide formation observed
Figure 4.13: Comparison of support materials for 10% Mo loading. Conversion (ethane) rate for equal surface area (~200 m²) reaction experiments (μmol/m²/s)
Figure 4.14: Comparison of support materials for 10%Mo loading. Formation (ethylene) rate for equal surface area (~200m$^2$) reaction experiments ($\mu$mol/m$^2$/s).
The Raman spectra of 10%Mo/SiO₂ indicates that MoOₓ is largely present as crystalline MoO₃ with only slight evidence for surface supported species. This could possibly explain the very different reaction behavior of this catalyst. Acetaldehyde was observed to form in an appreciable amount over 10%Mo/SiO₂ at 600°C. Banares [100] has stated in a recent review, that water vapor is essential for acetaldehyde formation over MoOₓ/SiOₓ from ethane/oxygen reactants and that surface molybdenum oxide species interacting with silica are active for acetaldehyde formation. Acetaldehyde formation could possibly result from a surface reaction involving adsorbed water (as -OH) or oxygen that could easily be formed on the high surface area silica. However, further characterization would be needed to make a distinction.

As the Raman spectra and XRD patterns indicate, the surface-supported species over titania and Si:Ti 1:1 are rather similar while those on SiO₂ are quite different. It is generally accepted that surface metal oxide species primarily anchor to the support by "titration" of the surface hydroxyl sites [42]. As previously mentioned, silica is unable to stabilize large amounts of supported MoOₓ species, even well below monolayer coverage and crystalline MoO₃ segregation occurs. However, titania has been shown to disperse Mo much more effectively than silica. [102]. Therefore it is conceivable that MoOₓ is supported over pure TiO₂ in a dispersed manner.

Comparing the performance in ethane ODH at different Mo loadings, reaction data indicate that ethane conversion increases to a maximum at 15wt.%Mo both at 550°C and 600°C declining at 20wt.%Mo. The ethylene yield increased to a maximum at 10%Mo/Si:Ti 1:1 giving 11.5% and 17.3% at 500°C and 600°C respectively (Table 4.3).
Table 4.3

Ethane ODH reaction data - Mo loading comparison

<table>
<thead>
<tr>
<th>Catalyst</th>
<th>Temp.</th>
<th>Conversion (%)</th>
<th>Selectivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(°C)</td>
<td>C\textsubscript{2}H\textsubscript{6}</td>
<td>C\textsubscript{2}H\textsubscript{4}</td>
</tr>
<tr>
<td>2%Mo/Si:Ti 1:1</td>
<td>550</td>
<td>20.9</td>
<td>38.3</td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>27.6</td>
<td>36.7</td>
</tr>
<tr>
<td>5%Mo/Si:Ti 1:1</td>
<td>550</td>
<td>26.8</td>
<td>40.1</td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>37.0</td>
<td>34.9</td>
</tr>
<tr>
<td>10%Mo/Si:Ti 1:1</td>
<td>550</td>
<td>29.3</td>
<td>39.3</td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>39.2</td>
<td>44.1</td>
</tr>
<tr>
<td>15%Mo/Si:Ti 1:1</td>
<td>550</td>
<td>30.1</td>
<td>34.4</td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>43.8</td>
<td>26.5</td>
</tr>
<tr>
<td>20%Mo/Si:Ti 1:1</td>
<td>550</td>
<td>24.5</td>
<td>33.8</td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>31.9</td>
<td>34.3</td>
</tr>
</tbody>
</table>
The trend in ethane conversion and ethylene formation, normalized to catalyst surface area, is illustrated in Figures 4.15 and 4.16 respectively. It is important to note that increased ethylene yields are accompanied by an increase in methane selectivity for these reaction conditions. This may possibly be a contribution from the surface-assisted pyrolysis of ethane, which is reported to occur at temperatures as low as 500°C [102], even though no ethane conversion was detected in blank reactor experiments up to 600°C. It is less likely, but also possible, that a mechanism exists for methane formation to take place from the decomposition of an ethoxide or other oxygenated surface intermediate to ultimately form two methane molecules. This would also cause the observed decrease in CO\textsubscript{x} selectivity.
Figure 4.15: Comparison of Mo/Si:Ti 1:1 catalysts with different Mo loadings. Conversion (ethane) rate for equal surface area (~200m²) reaction experiments (µmol/m²/s).
Figure 4.16: Comparison of Mo/Si:Ti 1:1 catalysts with different Mo loadings. Formation (ethylene) rate for equal surface area (~200m\(^2\)) reaction experiments (\(\mu\text{mol/m}^2\text{s}\))
Ethane Temperature programmed Desorption was performed to gain insight into the interaction of ethane with the surface oxygen of the catalysts with different Mo loadings. Analysis of the desorbed species after ethane adsorption on the Mo/Si:Ti 1:1 catalysts showed ethane, ethylene, water, methane, carbon dioxide, carbon monoxide, and trace acetaldehyde desorbing from the surface. The temperatures of desorption for various species are summarized in Table 4.4 where an attempt has been made to group peaks that correspond to the same temperature region. Although ethylene creates the same fragmented species as ethane in the mass spectrometer, by following specific fragments created by each, we determined that the first desorption peaks (<150°C) are associated with ethane desorption and the remainder of the profile belongs to ethylene alone.
Table 4.4

Desorbed species during ethane TPD over Mo/Si:Ti 1:1 catalysts

<table>
<thead>
<tr>
<th>catalyst species</th>
<th>desorption temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>235 343 587 660</td>
</tr>
<tr>
<td>2% Mo ethylene</td>
<td>methane</td>
</tr>
<tr>
<td></td>
<td>284</td>
</tr>
<tr>
<td></td>
<td>carbon dioxide</td>
</tr>
<tr>
<td></td>
<td>504-655</td>
</tr>
<tr>
<td></td>
<td>carbon monoxide</td>
</tr>
<tr>
<td>5% Mo ethylene</td>
<td>methane</td>
</tr>
<tr>
<td></td>
<td>422w</td>
</tr>
<tr>
<td></td>
<td>carbon dioxide</td>
</tr>
<tr>
<td></td>
<td>422w</td>
</tr>
<tr>
<td></td>
<td>carbon monoxide</td>
</tr>
<tr>
<td></td>
<td>292</td>
</tr>
<tr>
<td>10% Mo ethylene</td>
<td>methane</td>
</tr>
<tr>
<td></td>
<td>257</td>
</tr>
<tr>
<td></td>
<td>carbon dioxide</td>
</tr>
<tr>
<td></td>
<td>328</td>
</tr>
<tr>
<td></td>
<td>carbon monoxide</td>
</tr>
<tr>
<td></td>
<td>321</td>
</tr>
<tr>
<td>15% Mo ethylene</td>
<td>methane</td>
</tr>
<tr>
<td></td>
<td>305</td>
</tr>
<tr>
<td></td>
<td>carbon dioxide</td>
</tr>
<tr>
<td></td>
<td>314</td>
</tr>
<tr>
<td></td>
<td>carbon monoxide</td>
</tr>
<tr>
<td></td>
<td>373</td>
</tr>
<tr>
<td>20% Mo ethylene</td>
<td>methane</td>
</tr>
<tr>
<td></td>
<td>296</td>
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<td>carbon dioxide</td>
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<tr>
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<td>320</td>
</tr>
<tr>
<td></td>
<td>carbon monoxide</td>
</tr>
<tr>
<td></td>
<td>377</td>
</tr>
</tbody>
</table>

sh=shoulder; w=weak
Ethylene desorption over 2 and 5%Mo/Si:Ti 1:1 were quite low in intensity and are multiplied by four in Figure 4.17 for comparison to the other catalysts. Two lower temperature desorption features of ethylene take place over 2%Mo/Si:Ti 1:1 at 235 and 343°C. Also, two, higher temperature desorption features occur with lower intensity at 587 and 660°C. At higher wt. loadings of molybdenum, only two ethylene desorption features are present, at low and high temperatures. This indicates that an additional site is present for ethylene on 2%Mo/Si:Ti 1:1 that is missing in the other catalysts. This could possibly be related to the higher interaction of MoOx in this catalyst with silica, as observed in Raman and XPS experiments. The amount of ethylene desorption is seen to be directly related to Mo content as the lower temperature desorption maxima increase in intensity with Mo loading. Also, the overall desorption of ethylene is seen to shift to lower temperatures with increasing Mo loading (from 235 to 176°C in the low temperature region and 587 to 473°C in the high temperature region).
Figure 4.17: Ethylene desorption profiles from ethane temperature programmed desorption, over 10%Mo/Si:Ti 1:1 catalysts with different Mo loadings, (□ ethane desorption, ○ ethylene desorption)
As indicated in Figure 4.18, methane desorption from the Mo/Si:Ti catalysts also appear to correlate with Mo loading as the overall intensity is increasing. No methane desorption was detected over 2 and 5 wt.% Mo catalysts. As can be seen from Table 4.4, each low temperature desorption of methane coincides with a desorption of ethylene over 10, 15, and 20 wt.% Mo catalysts and experiences a corresponding shift to lower temperature. As the loading is increased from 10%, a second, higher temperature methane desorption appears at 15 wt.% (303°C) and becomes two convoluted desorption features at 20 wt. loading (273°C, 304°C). This higher temperature desorption does not shift in temperature and must be related to the formation of crystalline MoO₃.

The desorption of carbon dioxide and carbon monoxide are shown in Figures 4.19 and 4.20, respectively. For 2 and 5% Mo catalysts, carbon dioxide and carbon monoxide formation is weak and accompanies the high temperature desorption of ethylene over these catalysts (see Table 4.4). Whereas methane accompanies the low temperature desorption of ethylene over 10wt.% and higher Mo loadings, carbon monoxide is seen to form only after the low temperature ethylene desorption for 2 and 5%wt. loadings. At 10% wt. loading of molybdenum several sites for COₓ formation are observed that do not coincide with the desorptions of other species, indicating several types of adsorbed surface complexes exist. Different oxygen species at differing activities become present on the catalyst surface at this loading. At the 15 and 20 wt.% levels, COₓ desorption become much more intense and is accompanied by methane formation.
Figure 4.18: **Methane** desorption profiles from ethane temperature programmed desorption, over 10%Mo/Si:Ti 1:1 catalysts with different Mo loadings.
Figure 4.19: Carbon monoxide desorption profiles from ethane temperature programmed desorption, over 10%Mo/Sl:Ti 1:1 catalysts with different Mo loadings
Figure 4.20: Carbon dioxide desorption profiles from ethane temperature programmed desorption, over 10%Mo/Si:Ti 1:1 catalysts with different Mo loadings.
While TPD results cannot be directly related to behavior under reaction conditions, several important characteristics are found. The results indicate that there is a change in the catalyst surface occurring between 5 and 10 wt.% loading of molybdenum which is responsible for the formation of several active oxygen-containing sites. However, the sites are not as active as the ones corresponding to crystalline MoO₃, present on 15 and 20 wt.% samples. This could be related to an even distribution of MoOₓ over silica and titania portions of the Si:Ti support present over 10%Mo/Si:Ti 1:1. The fact that ethylene desorption shifts to lower temperatures indicates that ethylene resides longer on the lowest loading catalyst. However, the formation of carbon oxides and methane is not only due to the conversion of formed ethylene but also arises from the transformation of the surface ethoxide intermediates into acetaldehyde, which readily converts to CO and CO₂ [103]. Thus, as mentioned previously, during reaction conditions there may be several sites responsible for ethylene formation and further conversion.

4.5 Summary

The Raman spectra of Mo/Si:Ti 1:1 catalysts taken under ambient conditions have shown that terminal Mo=O stretching vibration shifts to higher wavenumber with increasing Mo loading. Chen et al. [99] have related a similar increase in the Mo=O Raman frequency (ambient conditions) of supported MoOₓ to a decrease in propane ODH reactivity over Mo/ZrO₂ catalysts. Here, the propane conversion rate, normalized per Mo
atom, decreased steadily with increasing MoO$_x$ loading over ZrO$_2$. However, this trend seems highly dependent on the support as research from the same laboratory [104] has shown that propane conversion rate, normalized per Mo atom, went through a maximum at intermediate MoO$_x$ loading over Al$_2$O$_3$. The difference is likely to arise from the nature of the supported species, which results in changes in Mo=O accessibility, activity of Mo-O-support bonds, if formed, and the reducibility of increasing domain sizes over the specific support. Calculation of MoO$_x$ surface densities, assuming all Mo is located at the surface, while applicable for impregnated Si:Ti supports, may be inaccurate for the "one-pot" preparation method employed for these catalysts. As discussed previously, there may be a significant amount of molybdena species located inside larger titania aggregates or deeper inside the silica network.

Faraldos et al. [105] have linked the activity of MoO$_3$/SiO$_2$ catalysts for partial oxidation of methane to the MoO$_x$ dispersion, noting a maximum in the rate of reducibility of the catalysts at an intermediate loading before crystalline MoO$_3$ forms. When supported on Si:Ti 1:1, there is XPS evidence of Mo-O-Si linkages present at the lowest Mo loading studied, 2wt.%. This, considered with Raman results, suggests that the "spreading" of MoO$_x$ over Si:Ti begins with the formation supported poly-oxo species on TiO$_x$ and isolated, monomeric MoO$_x$ on SiO$_x$. At higher loadings, poly-oxo molybdena domains either cover or replace the isolated species and the amount of supported MoO$_x$ on silica sites is increased. Reiche et al. [106], have observed a similar trend with vanadia grafted onto a TiO$_2$/SiO$_2$ support, in which increased loading provided a more equalized covering of Si and Ti constituents of the mixed oxide. The size of the
MoO\textsubscript{x} domains grow in size until, eventually, crystalline MoO\textsubscript{3} begins to form in appreciable amounts, as indicated by the XRD patterns. While these data do not explicitly differentiate between the ODH activity of Mo-O-Mo and Mo=O bonds (or reduced M\textsuperscript{2+}-O centers for that matter), they do show that there is an optimum coverage of MoO\textsubscript{x} in which several supported MoO\textsubscript{x} species could exist together. The best catalyst may be formed at the intermediate wt. loading of Mo, where MoO\textsubscript{x} species may be more equally distributed between the domains and sharing mixed ligands with both silica and titania, which consequently provides the highest ethylene yield over 10\% Mo/Si:Ti 1:1. Furthermore, the state of bound oxygen during ODH operating conditions is certainly a dynamic one and several "active-sites" may be responsible for favorable selectivity to ethylene during the reaction.

The "one-pot" preparation method in which components are dispersed together during gelation, provided dispersed MoO\textsubscript{x} species over Si:Ti 1:1. However, there may be a significant amount of molybdena species located inside larger titania aggregates or deeper inside the silica network. Raman spectroscopy, under ambient and dehydrated conditions, provides insight into the nature of supported MoO\textsubscript{x} species over Si:Ti 1:1. Together with XPS data, it is revealed that at the lowest wt. loading studied, 2\% Mo, MoO\textsubscript{x} may be supported preferentially on titania. However, at this same wt. loading, the presence of Mo-O-Si bonds is detected. The interaction with the silica component of the support increases with wt. loading of molybdenum until the formation of crystalline MoO\textsubscript{3} occurs above 15wt.\%. Combined with the fact that MoO\textsubscript{x} species may share several types of support ligands with differing electronegativity, it seems inadequate to
describe MoO\textsubscript{x} species as being supported exclusively on SiO\textsubscript{2} or TiO\textsubscript{2} domains, however Raman results would indicate a mix of both species. Using this preparation method, Mo loading affects not only the structure of the surface MoO\textsubscript{x} species, but also influences the nature of TiO\textsubscript{x}/MoO\textsubscript{x} concentrations over the silica. As molybdenum loading is increased, there is a greater degree of crystalline titania segregation. At the same time, there appears to be an increased interaction of the silica with MoO\textsubscript{x}.

The ethylene yield obtained in ethane ODH experiments increases with Mo loading over Si:Ti 1:1 to a maximum at 10\%Mo/Si:Ti 1:1 giving 11.5\% and 17.3\% at 500\degree C and 600\degree C respectively. The trend in ethane conversion and ethylene formation is accompanied by an increase in methane selectivity. In propane ODH experiments, a slightly different behavior is observed. Propylene formation rates remain rather constant up to 10\% wt. loading of Mo where they fall at higher wt. loadings. The decrease in propylene formation is accompanied by an increase in CO\textsubscript{x} formation. The best catalyst is formed at the intermediate wt. loading of Mo for both reactions, where MoO\textsubscript{x} species may be more equally distributed between silica and titania domains or contain an optimum mix of silica and titania ligands, which consequently provides the highest ethylene yield over 10\%Mo/Si:Ti 1:1. Ethane TPD results agree, suggesting that different oxygen species at differing activities become present on the catalyst surface at this loading.
CHAPTER 5

THE STRUCTURAL EFFECTS OF ALKALI PROMOTION ON MoO₃ CATALYSTS
FOR PROPANE ODH

5.1 Introduction

Current chemical industry depends heavily on propylene and other alkene feedstocks. Propylene demand is estimated to grow 4.5% per year between 1991 and 2000. The catalytic oxidative dehydrogenation (ODH) of propane is an attractive alternate route for the production of propylene compared to the conventional cracking and dehydrogenation processes. This is because ODH is thermodynamically favored at lower temperatures and usually does not lead to the formation of coke and smaller hydrocarbons. Recent literature has focused on selective, high surface area catalysts active below 823K. The most selective catalysts reported in recent literature consist of vanadium-magnesium, vanadia supported on niobium, and nickel molybdates. In particular, promising results have been obtained when molybdate-based catalysts are promoted or supported.
A major challenge in heterogeneous catalysis is the design and development of new catalysts for selective transformation of lower hydrocarbons [107]. The oxidative dehydrogenation (ODH) of lower alkanes to form alkenes has been extensively studied [2, 7, 25, 108] for such purpose. In particular, the ODH of propane is attractive as a process to produce propylene, given its high demand for the production of polypropylene, acrylonitrile, and propylene oxide. However, ODH selectivity is limited considering the high reactivity of propylene toward further oxidation. As discussed by Kung in a review article [2], the limitation arises from the two consecutive reactions propane→propylene and propylene→COx. It was shown that, perhaps on all catalysts, the rate for the second reaction could be five to ten times higher than that of the first reaction. In fact, more recent work by Khodakov et al. [43,109] has determined the relative rate of propylene combustion to be twenty times as high as the rate of propylene formation over vanadium based catalysts. Chen et al. has also measured the same rate to be ten to twenty times higher on molybdenum-based catalysts [99,104]. Furthermore, the authors have commented on the likelihood of similar active sites being responsible for both reactions.

This limitation in propane ODH has led to, at best, a general description of the qualities that a selective catalyst must possess. Selectivity requires careful control of the abundance of active oxygen and of the adsorption/desorption characteristics to avoid re-adsorption of the formed propylene. There is a joint influence between the red-ox and acid-base properties of the surface of the oxides used for propane ODH. Intermediate reducibility, weak Lewis acid centers, and high oxygen mobility represent the essential requirements for selective ODH; as they are consistent with the trends in ODH rates
observed on VO₂, MoOₓ, and WOₓ-based catalysts [110]. However, quantitative correlations between these properties and catalytic performance cannot easily be obtained and these characteristics are therefore usually expressed in literature as a "good-mix" or "favorable balance" between acid-base characteristics and red-ox behavior [9, 111-112]. The "favorable" oxygen that can provide this requirement is that which binds strongly enough to the surface to have attenuated oxidizing strength but weakly enough to oxidize the reactant molecule selectively. Over supported transition-metal oxides, the species of interest exist in the form of M=O, M-O-M, or M-O-support bonds, where M is the supported transition metal. The nature of the active oxygen will certainly depend upon transition metal loading, dispersion, support effects, and the addition of modifiers, such as potassium.

The accompanying increase in activity and/or selectivity in partial oxidation reactions with the use of alkali (Li, Na, K, Rb, and Cs) doping has been widely studied and offers a way to adjust the red-ox and acidic properties of supported transition metal oxides [113-121]. The positive effects of alkali promotion arise from the alkali’s ability to alter oxidation/reduction behavior, affect surface acidity, and/or cause a synergism between alkali and transition metal oxide phases. Recent work from our laboratory has examined the structural changes associated with alkali promotion at low levels [122]. The presence of potassium significantly alters the electronic structure of the surface MoOₓ domains supported over the binary oxide of silica/titania. Data suggested that surface supported species, present as distorted octahedral MoOₓ, become the most distorted at low levels of alkali (K/Mo=0.07). The MoOₓ species at this level of alkali promotion,
experiencing a decrease in Lewis acidity, could tend to be more reactive toward 
electronnegative Si-O' support ligands on the Si:Ti 1:1 support. 

The interaction of MoO₅ domains with the binary oxide support, and consequently 
reactivity, can be altered with the addition of potassium. It was shown that small levels of 
alkali suppress the reducibility and Lewis acidity of the molybdenum species and 
possibly stabilize a lower oxidation state of Mo during the propane ODH reaction [11]. 

For example, Ni-Co-Mo [123], V-Nb-Mo/TiO₂ [124], K-MnMoO₄ [125], and 
K₂MoO₄ [126] have shown promise in ODH and other partial oxidation reactions. The 
positive effect of alkali dopants (Li, Na, K, Rb, and Cs) has long been known in many 
reactions and is becoming more and more applicable to different catalysts. However, the 
effect is still not well characterized. Alkali doping can have the effect of increasing 
selectivity and activity while preventing phase transformations, inhibiting sintering, and 
creating basic centers on the catalyst surface. Abello et al. [119] have shown a 
significant increase in selectivity on Mo/MgO-γ-Al₂O₃ with the addition of potassium. 
On this catalyst, an interesting trend was noticed in catalyst activity, red-ox behavior, and 
surface acidity. Furthermore, past work from our group has shown that potassium can 
largely enhance oxygen exchange between bulk MnMoO₄ catalysts and gas phase oxygen 
as well as adsorption/desorption behavior of the catalyst. These parameters are the most 
common features used to describe ODH catalysts. 

Although it is generally accepted that the selective mechanism of propane ODH 
follows a Mars van Krevelen red-ox mechanism, there are many unselective mechanistic 
steps that can possess several structural requirements. This can develop the observed
reaction behavior in a quite complicated manner. Non-selective mechanisms can exist in which oxygen, from the lattice or activated from the gas phase, can be inserted into the hydrocarbon and several reaction steps advance to ultimately form carbon oxides. For selective catalysts, there should exist an optimal balance in the oxygen activity, acid-base characteristics, and lattice diffusivity. It is therefore essential for a catalyst to possess a certain degree of structural complexity for an optimum combination of activity and selectivity. This differing metal-oxygen bonding interactions and the oxides of V and Mo exhibit a rich structural complexity for such purpose [111]. Over supported molybdenum and vanadium oxides, several isolated species may exist in the form of M=O, M-O-M, or M-O-support bonds, where M is the transition metal of interest. The nature of the active oxygen species and red-ox properties will play a critical role in catalyst performance and will certainly depend upon transition metal loading, dispersion, and support effects.

One way to achieve the complex functionality and site isolation of a partial oxidation catalyst is to support the active metal oxide on a binary oxide support. Binary mixed oxides have been studied as catalyst supports in a variety of reactions including hydrotreating, selective oxidation, and the SCR of nitrogen oxides [127-134]. The mixed oxides including binary mixtures of alumina, zirconia, silica, or titania have received the most attention. In particular, the binary TiO$_2$/SiO$_2$ system is considered an advanced material to replace TiO$_2$ that possesses enhanced thermal/mechanical stability, high surface area, and is economically attractive [74,96]. In spite of their application potential, few studies exist on the structural aspects of supported vanadium or molybdenum on these binary oxide supports. Ko et al. and Kumbhar [61,62] have shown
that TiO$_2$/SiO$_2$ mixed oxides exert both direct and indirect support effects when used as supports for Ni catalysts. Furthermore, Baiker et al. [63] has proposed that by varying the TiO$_2$ content in the mixed oxides, one can “tune” the interaction with VO$_x$ species to form an optimal deNOx catalyst. Catalysts comprised of vanadium oxide species dispersed on TiO$_2$/SiO$_2$ (5-15% TiO$_2$) [135] and Al$_2$O$_3$/SiO$_2$ (0-10% Al$_2$O$_3$) [136] have recently been prepared and thoroughly characterized. It was concluded that the vanadium-oxo species preferentially interact with the titania or alumina portions of the support. On the same samples however, addition of VO$_x$ to the support was found to consume some of the surface Si-OH hydroxyls indicating that an interaction of VO$_x$ with silica is not necessarily absent and several supported species may be present with different Ti-O$^-$ (or Al-O$^-$) and Si-O$^-$ ligands. Furthermore, it was the varying ratio of these ligands that was ascribed to significant changes in the reactivity of isolated vanadia oxide units. Since ODH reaction pathways on molybdenum and vanadium containing catalysts are similar [137], it is plausible that the nature of dispersed MoO$_x$ domains supported on a binary oxide will exhibit characteristics similar to that of VO$_x$ domains when supported on a binary oxide.

The structural characteristics inherent over the high TiO$_2$ content silica-titania mixed oxide support, combined with the structural effects that are induced by an alkali promoter, is the primary focus of this investigation. Alkali doping can also influence surface properties by preventing phase transformations, inhibiting sintering, inducing coordination changes, decreasing reducibility, and creating basic centers on the catalyst surface [113, 138-139]. Our previous work has shown that the Mo/Si:Ti 1:1 catalysts are
effective for the oxidative dehydrogenation (ODH) of propane. When promoted with alkali (K), the catalysts have shown a broad maximum in activity and selectivity with the amount of alkali (K) promoter added. Preparation of multiple batches of catalysts and testing under different analytical conditions has revealed that the activity trend with K/Mo ratio, that is an initial increase in activity at the low alkali content followed by a sharper decline, is unambiguous.

In this study, a series of potassium-promoted molydena catalysts (Alkali/Mo molar ratio<1) have been characterized by probing the surface molybdena species and physical-chemical properties of the Si:Ti support. The effect of alkali level on the surface characteristics of the support has been examined. Surface areas were measured using the BET N₂ adsorption method. Characterization has been performed using laser Raman spectroscopy, X-ray photoelectron spectroscopy (XPS), Transmission Electron Microscopy (TEM), and Diffuse Reflectance Infrared Fourier Transform Spectroscopy (DRIFTS) to elucidate surface/bulk structure, and the nature of Si:Ti surface hydroxyl species. Electron-Spin Resonance (ESR) was performed to probe the existence and nature of reduced or distorted MoO₃ structures. ²⁹Si CP-MAS NMR was performed to observe the change in the silica network cross-linkage with the level of alkali doping.
5.2 The Choice of Akali Dopant

Catalysts were prepared using a modified sol-gel/co-precipitation technique. The method used here involves the reactions of metal alkoxide precursors in an alcohol solvent when contacted with water. Ammonium heptamolybdate (AHM) and alkali (Li, Na, K, Cs) hydroxides were used as molybdenum and alkali precursors, respectively. For silica-titania mixed oxides, tetraethylorthosilicate (TEOS) and titanium(IV)isopropoxide (TIPO) were used. The solvent was isopropyl alcohol. This method is referred to as sol-gel/co-precipitation because as the silica and titania precursors are hydrolyzed and precipitate out of solution, active metal species present in the aqueous solution, that are insoluble in alcohol, also precipitate. The catalysts reported in this paper are listed in Table 5.1. Our previous results have shown that a silica-titania molar ratio of 1:1 support performed the best in the ODH reaction. Therefore, all catalysts were supported over Si:Ti 1:1. These include a series of alkali-doped molybdate catalysts with alkali/Mo molar ratio of 0.1 at constant 10% weight loading of Mo.
Table 5.1
Alakli-containing catalysts for propane ODH

<table>
<thead>
<tr>
<th>Composition</th>
<th>BET Surface Area (m²/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Si:Ti 1:1</td>
<td>320</td>
</tr>
<tr>
<td>10%Mo/Si:Ti 1:1</td>
<td>229</td>
</tr>
<tr>
<td>10%(Li/Mo=0.1)/Si:Ti 1:1</td>
<td>170</td>
</tr>
<tr>
<td>10%(Na/Mo=0.1)/Si:Ti 1:1</td>
<td>323</td>
</tr>
<tr>
<td>10%(K/Mo=0.1)/Si:Ti 1:1</td>
<td>191</td>
</tr>
<tr>
<td>10%(Cs/Mo=0.1)/Si:Ti 1:1</td>
<td>188</td>
</tr>
</tbody>
</table>

Alkali-doped molybdate catalysts show no specific trend in BET surface area with differing alkalis or amounts of potassium added. All catalysts containing alkali exhibited lower surface area than the "molybdenum only" catalyst with the exception of the sodium-doped catalyst. The nitrogen adsorption-desorption isotherm of the Si:Ti 1:1 support indicated a micro to meso-porous structure. The pore size distribution was calculated using the desorption isotherm. This yielded an average pore diameter of 2.1 nm and a pore volume of 0.34 cm³/g. X-ray diffraction of the Si:Ti 1:1 support yielded a pattern typical of a silica-titania sample with one broad peak located at a d spacing of 3.59 Å, which is the most intense diffraction line from the anatase structure. Molybdenum species were not detected in the 10%(alkali/Mo) loaded Si:Ti 1:1 catalysts. This suggests that molybdena species on the mixed oxide supports are more finely dispersed than on a silica or titania support alone.
Raman spectra of the Si:Ti 1:1 support showed that the bands associated with the anatase structure were shifted to lower wavenumbers than those of pure anatase. Evidence for Si-O-Ti connectivity also was observed in the Raman spectra. Bands associated with terminal Mo=O stretches around 950 cm\(^{-1}\) were observed on all catalysts studied except for the 10%(Na/Mo=0.1)/Si:Ti 1:1 catalyst (Figure 5.0). Considering the higher surface area of this catalyst, surface molybdate species may be undetectable. Broad bands arising from Mo-O-Mo vibrations were observed around 850 cm\(^{-1}\). From the Raman spectra, there is no evidence of crystalline MoO\(_3\). Molybdenum in these catalysts is in the state of surface coordinated molybdena species.

![Raman spectra of alkali promoted catalysts](image)

Figure 5.0: Raman spectra of alkali promoted catalysts
Alkali/Mo catalysts were tested in the ODH reaction using equal surface area loading (65m²) in the reactor and at temperatures of 450°C and 550°C. The feed percentages for these experiments were N₂/C₃/O₂: 61/26/13. The results are presented in Table 5.2.

Table 5.2

<table>
<thead>
<tr>
<th>Reaction Results for Alkali/Mo Catalysts</th>
<th>Yield(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C₃H₆</td>
</tr>
<tr>
<td><strong>10% (Mo)/Si:Ti 1:1</strong></td>
<td></td>
</tr>
<tr>
<td>T (°C)-450</td>
<td>5.2</td>
</tr>
<tr>
<td>T (°C)-550</td>
<td>10.0</td>
</tr>
<tr>
<td><strong>10% (Li/Mo =0.1)/Si:Ti 1:1</strong></td>
<td></td>
</tr>
<tr>
<td>T (°C)-450</td>
<td>5.6</td>
</tr>
<tr>
<td>T (°C)-550</td>
<td>9.2</td>
</tr>
<tr>
<td><strong>10% (Na/Mo=0.1)/Si:Ti 1:1</strong></td>
<td></td>
</tr>
<tr>
<td>T (°C)-450</td>
<td>7.3</td>
</tr>
<tr>
<td>T (°C)-550</td>
<td>10.1</td>
</tr>
<tr>
<td><strong>10% (K/Mo=0.1)/Si:Ti 1:1</strong></td>
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</tr>
<tr>
<td>T (°C)-450</td>
<td>8.6</td>
</tr>
<tr>
<td>T (°C)-550</td>
<td>12.1</td>
</tr>
<tr>
<td><strong>10% (Cs/Mo=0.1)/Si:Ti 1:1</strong></td>
<td></td>
</tr>
<tr>
<td>T (°C)-450</td>
<td>2.6</td>
</tr>
<tr>
<td>T (°C)-550</td>
<td>10.8</td>
</tr>
</tbody>
</table>

Conditions: equal surface area (65m²), %N₂/C₃/O₂: 61%/26%/13%, 25cc/min.
Results indicate that the alkali promoted catalysts behave rather similarly under these conditions. However, the Cs-promoted catalyst shows a considerably lower activity at 450°C, whereas the potassium-promoted catalyst exhibits the highest yield of propylene. The propylene yield of these catalysts increases in the order Cs, Li, Na, K at 450°C and Li, Na, Cs, K at 550°C. There appears to be no correlation between catalyst activity and atomic weight of the alkali promoter. However, the differences in performance may be due to not only the size of the alkali but other parameters such as dispersion and interaction of the support with alkali and molybdena species. While the differences were small, the potassium-promoted catalyst gave a better ODH performance, warranting its further investigation. It appears that molybdena catalysts supported over Si:Ti mixed oxides has ODH activity, but the incorporation of small quantities of an alkali promoter significantly improves the product distribution in favor of propylene. It should also be kept in mind, however, that the differences observed in the ODH performance of these catalysts cannot be explained solely in terms of the direct modification of the surface by the alkali promoters. Other structural and surface characteristics, which may be affected by the sol-gel parameters, can also change the catalytic behavior.
5.3 Structural Modification of MoO\textsubscript{x} Domains and the Si:Ti Support with the Addition of Potassium

Synthesized catalysts are listed in Table 5.3. Catalysts numbered 1 through 8 are a series of molybdate catalysts with increasing K/Mo molar ratio at constant (10 weight %) loading of Mo and a Si:Ti molar ratio of 1. Catalysts 9 through 12 were prepared keeping K/Mo constant at 2 and varying the silica and titania content. Since TEOS hydrolyzes much slower than TIP0, a series of pre-hydrolyzed catalysts were also prepared. Catalysts 13 through 16 were synthesized using different hydrolysis methods (pre-hydrolysis of silica precursor, fast or slow addition of the aqueous solution, acidic or basic conditions). Catalysts 17 and 18 refer to a bare silica-titania support and a potassium-doped silica-titania support, respectively. Molybdate catalysts show a general decrease in surface area with increasing amounts of potassium added. The catalyst with the K/Mo ratio of 2, however, is somewhat outside this trend. Furthermore, all catalysts containing potassium exhibited lower surface area than the “molybdenum only” catalyst. Comparing catalysts of different Si:Ti contents, it is seen that the highest surface area is achieved with a silica-titania molar ratio 1:1. Pre-hydrolyzed catalysts all show higher surface areas than the catalyst prepared using stoichiometric hydrolysis of both precursors. The effect of potassium on the support is shown to increase the surface area from 320 to 380 m\textsuperscript{2}/g. The nitrogen adsorption-desorption isotherm of the Si:Ti 1:1 indicated a micro to meso porous structure. The pore size distribution was calculated using the desorption isotherm. This yielded an average pore diameter of 2.1nm and a pore volume of 0.34cm\textsuperscript{3}/g.
### Table 5.3

**Sol-gel Catalysts and Supports**

<table>
<thead>
<tr>
<th>#</th>
<th>Composition</th>
<th>Preparation</th>
<th>Surface Area (m²/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10%Mo/Si:Ti 1:1</td>
<td>co-precip., sol-gel</td>
<td>229</td>
</tr>
<tr>
<td>2</td>
<td>10%(K/Mo=0.07)/Si:Ti 1:1</td>
<td>co-precip., sol-gel</td>
<td>136</td>
</tr>
<tr>
<td>3</td>
<td>10%(K/Mo=0.14)/Si:Ti 1:1</td>
<td>co-precip., sol-gel</td>
<td>121</td>
</tr>
<tr>
<td>4</td>
<td>10%(K/Mo=0.3)/Si:Ti 1:1</td>
<td>co-precip., sol-gel</td>
<td>166</td>
</tr>
<tr>
<td>5</td>
<td>10%(K/Mo=0.6)/Si:Ti 1:1</td>
<td>co-precip., sol-gel</td>
<td>65</td>
</tr>
<tr>
<td>6</td>
<td>10%(K/Mo=1)/Si:Ti 1:1</td>
<td>co-precip., sol-gel</td>
<td>17</td>
</tr>
<tr>
<td>7</td>
<td>10%(K/Mo=2)/Si:Ti 1:1</td>
<td>co-precip., sol-gel</td>
<td>106</td>
</tr>
<tr>
<td>8</td>
<td>10%(K/Mo=2)/SiO2</td>
<td>co-precip., sol-gel</td>
<td>156</td>
</tr>
<tr>
<td>9</td>
<td>10%(K/Mo=2)/TiO2</td>
<td>co-precip., sol-gel</td>
<td>43</td>
</tr>
<tr>
<td>10</td>
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</tr>
<tr>
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<td>sol-gel KOH (for K/Mo=2)</td>
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X-ray diffraction patterns were obtained on all catalyst samples. X-ray diffraction of the Si:Ti 1:1 support yielded a pattern characteristic of a silica-titania mixed oxide containing extra-framework anatase [81]. One broad peak with center located at a d spacing of 3.59 Å was observed, which is the most intense diffraction line from anatase. This is indicative of a nano-sized anatase structure supported over amorphous silica. When the size of individual crystals is below 50nm, a reasonably good measure of particle size can be determined from the broadening of diffraction lines measured at half of their maximum intensity [140]. Results of this measurement are shown in Figure 5.1. With the addition of molybdenum, the anatase diffraction peak becomes narrower, indicating a change in the dispersion and segregation of titania in the Si:Ti matrix with the addition of Mo. With the addition of potassium, the peak continues to narrow indicating a higher degree of TiO\(_2\) (anatase) segregation with K/Mo molar ratio. With the introduction of molybdenum to the Si:Ti 1:1 support, the particulate size increases from approximately 2nm to 9nm. The particulate size then slowly increases with the addition of K until a sharper increase is observed at K/Mo=0.6. This agrees with previous XPS data which has shown a continuous shift of the Ti 2p\(_{3/2}\) binding energy from that of TiO\(_x\) closely interacting with silica toward that of pure anatase [11]. This bulk structural change may be due to the higher pH of the “one-pot” synthesis medium with increasing potassium doping. The increasing basic conditions during preparation can have the effect of increasing condensation rates leading to larger particle size [51]. The pH of the preparation medium not only affects the support structure, but it also has a strong effect on the type of molybdena species that are formed on the surface.
Figure 5.1: TiO$_2$ (anatase) particulate size as determined from X-ray diffraction line broadening
To observe the bulk structural changes associated with potassium addition, several TEM images of the samples were obtained. EDX analysis of the Si:Ti 1:1 indicated a homogeneous dispersion of Si and Ti that closely matched the bulk atomic concentrations based on Si, Ti and Mo atoms ("as-prepared" 46.25% Si and Ti). One of the TEM images obtained for Si:Ti 1:1 is shown in Figure 5.2.

Figure 5.2: TEM micrograph of Si:Ti 1:1
On all images obtained, an average of 2 nm particle sizes was observed with evidence of crystalline TiO$_2$ homogeneously distributed throughout the sample. However, with the addition of molybdenum to the Si:Ti 1:1 support, the larger size of TiO$_x$ domains in the sample was more readily observed as indicated by the lattice fringes from crystalline TiO$_2$. These lattice fringes, with an average spacing of ~3.3 Å, were more prominent than in the Si:Ti 1:1 support. On average, the overall particle size of the sample was observed to increase to 4.5-6nm. EDX analysis over different regions revealed atomic concentrations fairly close to the “as-prepared” bulk values with only a few observations of TiO$_2$-rich regions. One such observation is shown in Figure 5.3-a; Si 38%, Ti 54%, Mo 8% and Figure 5.3-b; Si 45%, Ti 46%, Mo 9%. EDX analysis over several regions also revealed a homogeneous distribution of molybdenum.

With the addition of potassium (K/Mo=0.07), further increase in the size of TiO$_x$ domains is apparent by the presence of anatase lattice fringes as well as the formation of hexagonal TiO$_2$ chips observed in the TEM image for an average particle size of 5-6nm. EDX analysis provided evidence that Mo rich regions exist in this sample. In Figure 5.44, the atomic concentrations are fairly close to the bulk values with (Figure 4-a; Si 48%, Ti 40%, Mo 12% and Figure 4-b; Si 46%, Ti 47%, Mo 7%).
Figure 5.3: TEM micrograph of 10%Mo/Si:Ti 1:1; EDX analysis yielded: region (a) Si 38%, Ti 54%, Mo 8%; region (b) Si 45%, Ti 46%, Mo 9%.
Figure 5.4: TEM micrograph of 10%(K/Mo=0.07)/Si:Ti 1:1; EDX analysis yielded:
region (a) - Si 48%, Ti 40%, Mo 12%;
region (b) Si 46%, Ti 47%, Mo 7%.

However, there were several small, dark-contrast regions indicative of Mo segregation over 10%(K/Mo=0.07)/Si:Ti 1:1. Figure 5.5-a depicts such a region with atomic concentrations: Si 26%, Ti 27%, Mo 47%. Figure 5.5 is representative of the complete analysis of the K/Mo=0.07 particles, in which all Mo-rich regions analyzed showed an equal distribution of Si and Ti. Figure 5.5-b illustrates that much of the sample maintained a distribution of components close to the bulk values: Si 44%, Ti
50%, Mo 6%. In Figure 5.5-c, analysis was performed at an interface between a SiO$_2$-rich region and a TiO$_2$-rich region. The white rift apparent in the figure is damage to the sample caused by several repeated EDX analysis and the image was taken post-EDX analysis. Atomic concentrations at this SiO$_2$:TiO$_2$ interface did not show segregation of molybdenum (Si 45%, Ti 47%, Mo 8%).

Figure 5.5: TEM micrograph of 10%/(K/Mo=0.07)/Si:Ti 1:1; EDX analysis yielded: region (a) - Si 26%, Ti 27%, Mo 47%; region (b) Si 44%, Ti 50%, Mo 6%; region (c) Si 45%, Ti 47%, Mo 8%. White rift in region (c) caused by extended EDX analysis.
When the level of alkali doping is increased the K/Mo=0.3, a dramatic change was observed in particle size. Particles of differing geometries and sizes were observed with the largest particles ranging about 40 nm in size. Figure 5.6 shows one set of the several SiO$_2$ and TiO$_2$ agglomerates observed, where the TiO$_2$-rich agglomerate is the oval-shaped particle on the right in the figure. However, analysis over SiO$_2$-rich regions, TiO$_2$-rich regions, and SiO$_2$:TiO$_2$ interface regions did not show an increase in molybdenum concentration in Figure 5.6 and during the entire analysis (Figure 6-a; Si 59%, Ti 37%, Mo 4% and Figure 6-b; Si 44%, Ti 51%, Mo 5%). It is apparent from Figure 5.7 that the bulk nature of the catalyst at alkali level K/Mo=0.3 is much different than the K/Mo=0 catalyst by the very large degree of TiO$_2$ segregation observed.

Figure 5.6: TEM micrograph of 10%(K/Mo=0.3)/Si:Ti 1:1; EDX analysis yielded: region (a) - Si 59%, Ti 37%, Mo 4%; region (b) Si 44%, Ti 51%, Mo 5%.
Results from TEM analysis agree with the XRD patterns showing how TiO$_x$ domains that are initially evenly dispersed over silica increase in size and crystallinity with K/Mo molar ratio. At the alkali level of K/Mo=0.3, a very large segregation of titania is observed. However, even though EDX analysis indicated that a high agglomeration of Ti exists, there was a substantial amount of silica present in TiO$_2$ regions. Furthermore, a substantial amount of titania was also found to exist in silica-rich regions. Within experimental error, the bulk characterization provided by EDX analysis did not provide evidence that molybdenum is preferentially located over silica-rich,
titania-rich, or Si:Ti interfacial regions of the support on any of the samples studied. Thus, the analysis suggests that any preferential interaction of Mo with TiO$_2$ or SiO$_2$ that may exist would have to be a surface effect.

Raman spectra have been obtained over the Si:Ti 1:1 support and compared to different preparations of pure silica in Figure 5.8. It was found that the dehydrated Si:Ti 1:1 surface possess Raman bands at 605, 800, 916, and 1080 cm$^{-1}$. The bands at 605 cm$^{-1}$ 800 cm$^{-1}$ were attributed to the silica surface while the bands located at 916 and 1080 cm$^{-1}$ have been assigned to perturbed silica vibrations that are indicative of Ti-O-Si bonds [83,85]. It is concluded that the Si:Ti 1:1 support is in the state of nano-crystalline TiO$_2$ dispersed over silica.
Figure 5.8: Raman spectra of SiO$_2$ and Si:Ti 1:1 support. Ambient conditions: (a) SiO$_2$ (Cab-O-Sil), (c) SiO$_2$ (sol-gel), (e) Si:Ti 1:1; Dehydrated conditions: (b) SiO$_2$ (Cab-O-Sil), (d) SiO$_2$ (sol-gel), (f) Si:Ti 1:1. Spectra of dehydrated samples taken under 10%O$_2$/He atmosphere at room temperature.
To differentiate between the Raman bands arising from the Si:Ti support and those from surface supported molybdate species, Raman spectra was performed under ambient and dehydrated conditions over silica (sol-gel), prepared in the same way the catalysts were, fumed silica (Cab-O-Sil), and the Si:Ti 1:1 support and compared in Figure 5.8. On a pure silica sample, a distinct band has been reported to occur between 971 and 976 cm\(^{-1}\) that corresponds to surface silanols [81,91]. Although not expected to interfere at the somewhat high Mo loading of 10 wt.%, this band can be convoluted with the Mo=O band of MoO\(_x\) species supported on silica at low Mo loadings. On 6.4%Mo/SiO\(_2\), Williams et al. [141] have observed this Raman band on dehydrated samples at room temperature. After heating at 550°C under oxygen, the Mo=O stretching frequency was observed at 994 cm\(^{-1}\) together with the broader silanol band at 971 cm\(^{-1}\). After cooling to room temperature under oxygen, the Mo=O and silanol peaks shifted to 998 and 977 cm\(^{-1}\) respectively. The authors stated that, over 0.1 to 10% wt. loading of Mo over silica prepared by a variety of precursors, the terminal Raman band was shifted to 994-998 cm\(^{-1}\) upon dehydration for those samples that did not display the Mo-O-Mo bridging mode associated with crystalline MoO\(_3\).
Bands associated with surface silanols are not observed in the Si:Ti 1:1 support under ambient or dehydrated conditions. However, in the spectrum of SiO₂ (sol-gel) under ambient conditions, the band is present as a small and sharp feature at 976 cm⁻¹ that disappears upon dehydration. In contrast, the Cab-O-Sil sample exhibits a much broader silanol band at the same location that also disappears upon dehydration. It is therefore concluded that there should be no Raman bands arising from the silanols that would interfere with Mo=O vibrations present on the dehydrated Si:Ti 1:1 support. The importance of this conclusion will be illustrated in the subsequent text.

Raman Spectra of catalysts with different K/Mo ratios under ambient conditions are presented in Figure 5.9. Present in the spectra are the 3 bands associated with anatase at ~643, ~523, and ~404 cm⁻¹. These bands are shifted to lower wavenumbers than those of pure anatase and seen to grow in intensity with increasing K/Mo ratio. An important feature of these spectra is that there is little or no evidence of crystalline MoO₃ since the most intense band characteristic of Mo-O-Mo stretching vibrations in MoO₃ is not present, except as a very weak band on the catalyst with K/Mo=0.6. The bands associated with isolated terminal Mo=O stretching vibrations are visible in the 970-999 cm⁻¹ region. Broad bands arising from surface-coordinated Mo-O-Mo vibrations are located around 850 cm⁻¹. For catalysts K/Mo=0.6 and 1, there is evidence for higher crystallinity of potassium molybdate species (K₂MoO₄, K₂Mo₂O₇) indicated by sharper bands around 900-950 cm⁻¹[42,138].
Figure 5.9: Raman Spectra of 10%Mo/Si:Ti 1:1 catalysts with different K/Mo ratios under ambient conditions
Raman spectra of the alkali promoted Mo/Si:Ti 1:1 catalysts, taken under dehydrated conditions, are shown in Figure 5.10 in the molybdenum-oxygen (Mo=O and Mo-O-Mo) vibration region. Broad bands associated with crystalline TiO₂ limit the detection of MoOₓ species below 640 cm⁻¹. Present in all the samples is a broad feature at 800 cm⁻¹ that it attributed to the silica of the support. There are several discernable vibrations observed for each sample in the range 950-1005 cm⁻¹. Additionally, there are features present in the 780-850 cm⁻¹ region for K/Mo=0.14 and 0.6 catalysts. In general, bands in the range of 750 to 950 cm⁻¹ are attributed to antisymmetric stretching of Mo-O-Mo bonds or the symmetric stretch of (-O-Mo-O-)₂ bonds, while bands in the range 950 to 1050 can be attributed to the symmetric stretching mode of Mo=O bonds [89]. There was no evidence of crystalline MoO₃ on any of the samples. However, as the alkali level reaches K/Mo=0.6 there is evidence of the crystallinity of alkali molybdate species, possibly K₂MoO₄ and K₂Mo₂O₇, indicated by several bands between 840-960 cm⁻¹ [143-144]. In fact, previous XPS data has indicated that much of the MoOₓ of this catalysts is present in tetrahedral, K-molybdate matrix [11]. Contributions to the Mo=O stretching frequency of 10%Mo/Si:Ti 1:1 appear at 982, 998, and 1005 cm⁻¹. With increasing K/Mo molar ratio, the band at ~1005 cm⁻¹ is seen to diminish in intensity relative to the lower frequency bands and only appear as a shoulder in the K/Mo=0.14 and 0.3 catalysts. In parallel with the decrease of the ~1005 cm⁻¹ band with K/Mo ratio is the increase of the band located at 996-998 cm⁻¹ up to K/Mo=0.3. In addition, a broad feature observable in K/Mo=0 around 980 cm⁻¹ is seen to grow in intensity with K/Mo molar ratio until a broad peak at 974 cm⁻¹ is observed at K/Mo=0.3.
Figure 5.10: Raman spectra of 10%Mo/Si:Ti 1:1 catalysts with differing K/Mo molar ratios. Spectra taken under 10%O₂/He atmosphere at room temperature under dehydrated conditions.
Overall, the multiple bands associated with isolated terminal Mo=O stretching vibrations are observed to shift with K/Mo ratio to lower wavenumbers. Shifts in the Mo=O Raman frequency are related to changes in bond length [42] and could result from an enhanced interaction with the support or from the introduction of K-containing species. Similar Mo=O band shifts were ascribed, on dehydrated Cs-promoted Mo/ZrO₂ catalysts, to the altering of electronic properties of MoOₓ domains with the addition of small amounts of alkali that did not form detectable quantities of alkali molybdates [121]. However, for similar Raman spectra observed for a set of dehydrated, sodium-doped Mo/SiO₂ catalysts [144], it was stated that the observed increase in intensity in the wavenumber region ~940-990 cm⁻¹ was due to the formation new molybdate species. Thus, it can be envisioned that the observed changes in Mo=O vibrations can arise from both an electronic interaction with potassium at small alkali content and the formation of alkali-molybdates at higher K/Mo ratios.

There is a scarcity of data in the literature on Raman bands arising from surface supported molybdenum species under dehydrated conditions, especially for those arising from molybdate species supported on the binary oxides of silica-titania. However, Wachs [42] has summarized the Raman spectroscopy results, under dehydrated conditions, of Mo/SiO₂ and Mo/TiO₂ prepared by several preparation methods and stated that Mo=O Raman bands of surface supported MoOₓ on titania appear in the range 998-1001 cm⁻¹. On silica under dehydrated conditions, these bands are reported to be a function of wt. loading and appear in the range 975-990 cm⁻¹. However, in another study [91], bands arising from dehydrated surface-supported MoOₓ species on silica were
observed up to 998 cm\(^{-1}\). The data present in literature suggest that it may be possible to interpret Raman spectrum of MoO\(_x\) supported over binary oxides to gain qualitative information as to whether MoO\(_x\) preferentially interacts with one of the oxide domains by incorporating different support-O bonds. Therefore, in parallel with the observed changes in Raman spectra being assigned to an electronic interaction with potassium, the Raman spectra of Figure 11 can also be interpreted in terms of the MoO\(_x\) interaction with the support. In these terms, an increasing interaction with silica (increase in the bands 970-998 cm\(^{-1}\) with a decrease in the band ~1005 cm\(^{-1}\)) can be envisioned with addition of potassium. These bands appear to be in equal proportion on the K/ Mo=0.035 and 0.07 catalysts. Further support for the proposal that MoO\(_x\) could be increasingly interacting with silica comes from the fact that the bands located at 998 and 977cm\(^{-1}\) at higher K/Mo molar ratios are virtually identical to those reported for dehydrated β-silicomolybdic acid supported on silica [92,144].

It appears that with the electronic interaction of potassium, the MoO\(_x\) species experience a greater interaction with silica, while that with titania decreases. Raman bands that could be associated with both types of species become overshadowed by the formation of new molybdate species at higher K/Mo ratios (0.3 and higher). However, exact structure cannot be assigned solely on the basis of Raman Mo=0 positions because several phenomena are taking place at the same time that must be considered. XRD and TEM results have shown the nature of TiO\(_x\) dispersion with K/Mo molar ratio. Together with the interaction of potassium with the supported molybdate species, the question arises as to how the combined phenomenon effects the dispersion over the binary oxide.
Furthermore, it has been pointed out that when supported on binary oxides, transition metal oxides are capable of sharing more than one type of support-O bond [135-136]. At this point, the effect of potassium on the number and kind of support-O-Mo ligands remains unclear. However, withstanding these complications, Raman spectroscopy does provide information that supported K-MoO\(_x\) species are existing in several types of structural configurations simultaneously and rather uniformly over Si:Ti 1:1.

To characterize the surface of the Si:Ti 1:1 support, a series of XPS experiments was performed to compare the support to SiO\(_2\) (sol-gel), TiO\(_2\) (sol-gel), and their physical mixtures. The Ti 2p XPS spectra for the support materials are shown in Figure 5.11. The Ti 2p\(_{3/2}\) binding energy of the sol-gel prepared TiO\(_2\) is 458.0 eV (Figure 5.11-a). The spectrum obtained from the physical mixture is very similar to pure TiO\(_2\) although it appears that a slight interaction may exist between mixtures of SiO\(_2\) and TiO\(_2\) as this binding energy is seen to shift to a slightly higher value (458.4) in the physical mixture (Figure 8-b). In Figure 5.11-c, it can be seen that the Ti 2p\(_{3/2}\) B.E. shifts much more pronouncedly, to 459.2eV, for the Si:Ti 1:1 support. The large difference between the BE of TiO\(_2\) (sol-gel) and that of the Si:Ti support is indicative of TiO\(_x\) closely interacting with the silica surface [90].
Figure 5.11: Ti 2p XPS spectra for Ti containing support materials; (a) TiO$_2$ (sol-gel), (b) SiO$_2$:TiO$_2$ 1:1 molar (physical mixture), (c) Si:Ti 1:1 support.
There is also a pronounced feature in the Ti 2p spectrum of the Si:Ti support located at 456.5 eV. This feature may be associated with Ti atoms present in Ti-O-Si. Fernandez et al. [95] have assigned an XPS feature at 456.4 eV to a reduced titania site (Ti$^{3+}$). While this remains as a possibility, it is more likely that the Ti site responsible for the XPS feature in Figure 5.11-c may be an electron rich species produced by integration of Ti into the silica network at boundaries, and not a fully reduced site. In TiO$_2$-rich samples of Si:Ti, the Ti$^{4+}$ in Ti-O-Si shares more negative charges from the oxygen in SiO$_2$ than from the oxygen of the bulk [96]. Furthermore, it has been stated that, in phase-separated SiO$_2$-TiO$_2$ samples such as those under investigation here, Si-O-Ti bonds are likely to form at interface boundaries between SiO$_2$ and TiO$_2$ [145].

The Si 2p XPS spectra (Figure 5.12) shows a peak located at ~102.3 eV characteristic of SiO$_2$ that does not shift positions between the support samples studied. There is an additional feature in the Si 2p spectrum of the Si:Ti 1:1 support (Figure 5.12-c) present as a shoulder around ~99.0 eV. This feature is assigned to the Si atoms of Ti-O-Si. Further evidence for Si-O-Ti bonds is obtained by examining the O1s XPS spectra of the support samples presented in Figure 5.13. O1s from SiO$_x$ is located at ~533.4 eV, while the signal from TiO$_x$ is located around 530 eV. Si-O-Ti species can exhibit an O1s band in between those of SiO$_2$ and TiO$_2$ around 532 eV making the peak significantly broadened. The O1s spectra of the Si:Ti 1:1 support is very similar to that reported in literature by Stakheev et al. [66], who have observed an identical spectrum over a 50% TiO$_2$/SiO$_2$ sample.
Figure 5.12: Si 2p XPS spectra for Si containing support materials;
(a) TiO$_2$ (sol-gel), (b) SiO$_2$:TiO$_2$ 1:1 molar (physical mixture),
(c) Si:Ti 1:1 support.
Figure 5.13: O 1s XPS spectra for support materials; TiO₂ (sol-gel), SiO₂:TiO₂ 1:1 molar (physical mixture), Si:Ti 1:1 support
To further examine the surface, XPS was performed over several K/Mo samples. Molybdenum 3d$_{5/2}$ binding energies of catalysts with different K/Mo ratios are presented in Table 5.4. In potassium-containing catalysts, molybdenum exists in two distinct coordination environments, one that corresponds to an octahedral MoO$_3$ matrix and the other to a tetrahedral K$_2$MoO$_4$ matrix. Binding energies of bulk MoO$_3$ and K$_2$MoO$_4$ are presented for comparison. When on a Si:Ti 1:1 support, our work has shown that Mo 3d binding energies shift to a lower value when compared to bulk MoO$_3$. The Mo3d$_{5/2}$ peak for the K/Mo=0.6 shows the nearest binding energy to that of bulk MoO$_3$ possibly indicating the presence of three-dimensional MoO$_3$ regions on this catalyst. The percentages of molybdenum in the MoO$_3$ matrix, calculated using the deconvoluted peak areas, closely match the “as prepared” compositions. It appears that all of the potassium added to these catalysts exists in a K$_2$MoO$_4$ type matrix. Furthermore, K 2p$_{3/2}$ spectra show one peak at an average location of 292.5eV corresponding to that of K$_2$MoO$_4$. 
To study the change in the nature of the support with K/Mo ratio, Ti 2p$_{3/2}$ XPS were taken for catalysts with differing K/Mo ratios. The variation of Ti 2p$_{3/2}$ binding energies with increasing K/Mo ratio is presented in Figure 5.14. Here, the Ti 2p$_{3/2}$ binding energy is seen to shift to lower values with the addition of potassium. The binding energy for the K/Mo=0 catalyst appears at 459.7eV indicating that titania is closely interacting with silica and in a state of very small anatase domains [66]. The peaks shift to lower binding energies with increasing K/Mo ratios, reaching 458.2eV for the K/Mo=2 catalyst. The Ti 2p$_{3/2}$ binding energy of pure anatase is around 458.0eV.
Figure 5.14: Variation of titania 2p₃/₂ binding energies for 10%Mo/Si:Ti 1:1 catalysts with different K/Mo ratios.
CP-MAS $^{29}$Si NMR spectroscopy can be used to characterize the structure of silica networks and has been used to characterize SiO$_2$:TiO$_2$ mixed oxides [146-148]. In single pulse $^{29}$Si NMR, both -OTi and -OH groups are reported to effect the silicon nucleus in a similar manner. However using $^1$H-$^{29}$Si cross-polarization, the most likely candidates for signal enhancement are the SiOH groups [148]. These sites are depicted in Figure 5.15 with the corresponding Q$^n$ notation, where n represents the number of Si-O ligands around each Si nucleus. The presence of adsorbed water may affect the signal enhancement by increasing the relaxation time of the protons involved in cross-polarization [149], therefore samples were re-calcined at 550°C for 30 minutes and maintained in the dehydrated state before analysis.
Figure 5.15: Silica species responsible for contributions to observed $^1\text{H-}^{29}\text{Si}$ CP (MAS)-NMR signals.
Figure 5.16 shows the $^1$H-$^{29}$Si CP (MAS) NMR spectrum of the K/Mo catalysts. Contributions from the silicon nuclei show Gaussian peaks at ~ -109ppm($Q^4$), -101ppm($Q^3$), -92ppm($Q^2$), -84ppm($Q^1$). The relative contributions of these structural sites were calculated from deconvoluted, integrated areas and are presented in Table 5.5. As the data show, $Q^4$ sites are the dominant species present in all samples indicating a well-developed silica network. However, 30-40% of the silicon nuclei are seen to contribute to the $Q^3$ sites of the silica network.

The ratios among different $Q^q$ sites are often used to ascertain the degree of silica polymerization [149]. To examine the change in the silica network, the percentage contribution of $Q^3$ sites (free-hydroxyls) and the ratio $Q^2/(Q^2+Q^3)$ are plotted in Figure 5.17 as a function of K/Mo molar ratio. As Figure 5.17 indicates, there is a pronounced minimum in the signal corresponding to free hydroxyl groups ($Q^3$) that occurs at the K/Mo=0.07 sample. Furthermore, the ratio of geminal hydroxyl groups ($Q^2$) to the total hydroxyl content is found to reach a maximum over the same sample. The NMR data show that with K/Mo molar ratio, the nature of the silica surface is changing.
Figure 5.16: $^{1}H-^{29}Si$ CP-(MAS)-NMR spectra of 10%Mo/Si:Ti 1:1 catalysts with differing K/Mo molar ratios.
Table 5.5

Chemical Shifts of (K/Mo)/Si:Ti 1:1 Catalysts

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Figure 5.17: Variation in Q3 percentage (free-hydroxyls) and Q2/(Q2+Q3) percentage (percentage geminal hydroxyls) with K/Mo molar ratio.
Previous data have shown that TiO\textsubscript{x} species continuously segregate from the surface with increasing K/Mo ratio and reach a relatively large particle size at K/Mo=0.3. Thus, it is expected that the interaction of TiO\textsubscript{x} domains with silica decrease continuously with K/Mo ratio. It is therefore most likely that the change in the silica surface arises from an interaction of MoO\textsubscript{x} with the surface hydroxyl groups. The type of molybdate species responsible for such an interaction and weather this species shares different support-O ligands remains unclear. However, the data agrees with the Raman results presented previously which have suggested an increased interaction of molybdenum with silica before the onset of alkali-molybdate formation.

High resolution IR spectra can be used to ascertain the presence and relative quantity of the same isolated and geminal hydroxyl groups detected in the \textsuperscript{1}H-\textsuperscript{29}Si CP-(MAS)-NMR spectra [150]. To corroborate the information obtained from NMR spectroscopy, a series of DRIFTS experiments was performed over the K/Mo catalysts. The IR bands from surface hydroxyls were recorded for all samples, in the range 3000-3900 cm\textsuperscript{-1}, at successive temperatures of 25, 50, 100, 150, and 200°C. For the sake of conciseness, only the results for K/Mo=0, 0.07, and 0.3 are shown in Figures 5.18, 5.19, and 5.20 respectively.
Figure 5.18: The IR bands from surface hydroxyls for K/Mo=0 at varying temperatures
Figure 5.19: The IR bands from surface hydroxyls for K/Mo=0.07 at varying temperatures
Figure 5.20: The IR bands from surface hydroxyls for K/Mo=0.3 at varying temperatures.
For each sample, the IR bands associated with free hydroxyl groups were located 3745-3747 cm\(^{-1}\), while those from geminal hydroxyl groups are resolved at 3739-3740 cm\(^{-1}\) [150-151]. Broad and convoluted bands in the region 3730 cm\(^{-1}\) and lower arise from weakly adsorbed water and are observed in the room temperature spectrum. Hydroxyl species arising from Ti-OH were not observed over any of the samples at any temperature and are reported to occur at a maximum wavenumber of 3716 cm\(^{-1}\) [152].

Upon examination of Figure 5.19, it can be seen that the K/Mo=0.07 possesses a relatively weaker contribution from free hydroxyls than the other samples. As temperature is raised to 200\(^{\circ}\)C from room temperature, trends can be established in the relative quantities of free and geminal hydroxyl groups present on the samples. To compare with the NMR data, the hydroxyl bands were deconvoluted and relative ratios (free/geminal) of the hydroxyl content was calculated for the K/Mo catalysts both at room temperature and 200\(^{\circ}\)C. Results are plotted in Figure 5.21 along with the ratio obtained from the NMR data (Q\(^{3}/Q^{2}\)). As can be seen, DRIFTS data is in relatively close agreement with the ratios obtained from NMR at both room temperature and 200\(^{\circ}\)C, suggesting a minimum amount of free hydroxyl species present at the low alkali level of K/Mo=0.07.
Figure 5.21: Comparison of NMR and DRIFTS data: ratio of free/geminal hydroxyls on 10%Mo/Si:Ti 1:1 catalysts with differing K/Mo molar ratios.
ESR techniques have been widely used to obtain insight into many aspects of the catalysis and surface chemistry of metal oxide surfaces [153]. In particular, calcination of MoO$_x$ catalysts at high temperatures can lead to the formation of paramagnetic Mo(V) centers [154] which can be studied to probe the local environment of the surface supported species. ESR spectra were recorded, at room temperature, on ambient-exposed and dehydrated K/Mo catalysts and the Si:Ti 1:1 support (Figures 5.22 and 5.23). A small signal from the superoxide ion (O$_2^-$) was the only signal detected on the Si:Ti 1:1 support with a characteristic average g-value of 2.011 [155]. The structural assignment of this species cannot directly be determined as the superoxide ion can exist and be stabilized both in extra-framework TiO$_2$ [155] and in silica domains [153].
Figure 5.22: ESR spectra of 10%Mo/Si:Ti 1:1 catalysts with differing K/Mo molar ratios under ambient conditions

H (Gauss)
Figure 5.23: ESR spectra of 10%Mo/Si:Ti 1:1 catalysts with differing K/Mo molar ratios under dehydrated conditions. (1) Variation of $g$ (perpendicular) with K/Mo molar ratio. (2) Variation of normalized signal intensity with K/Mo molar ratio.
All ambient-exposed samples reveal asymmetric ESR signals associated with Mo(V) and, to a lesser extent, the superoxide ion \( \text{O}_2^- \) (Figure 5.22). The associated g tensor for Mo(V) is seen to change slightly among the ambient-exposed K/Mo catalysts (Table 5.6), while that of \( \text{O}_2^- \) remains fairly constant at \( g=2.011 \). The spectrum of Figure 5.22 is characteristic of Mo(V) in a state of non-axial symmetry. The adsorption of atmospheric water can have a significant effect on the dispersion and coordination of MoO\(_x\) domains and thus, the ESR spectrum. Therefore, the same experiment was performed over the K/Mo catalysts under dehydrated conditions (Figure 5.23). A noticeable broadening of the signal appears upon dehydration without substantial loss of intensity. The removal of adsorbed water can cause a decrease in the separation between Mo(V) ions and a consequent broadening of the ESR linewidths [156]. The broad spectrum of Figure 5.23 is indicative of Mo(V) in a dehydrated oxide matrix [156-157]. Since the spectra in Figure 5.23 are reasonably symmetric (or quasi-axial [157]), parallel and perpendicular parameter values can be defined as \( g_{\perp} = (g_x + g_y) / 2 \) and \( g_{\parallel} = g_z \). The \( g_{\perp} \) and \( g_{\parallel} \) values of Mo(V) in the K/Mo samples are presented in Table 5.6. The \( g_{\perp} \) values are seen to go through a maximum at K/Mo=0.07 and this trend is depicted as insert 1 in Figure 5.23. The integral intensity of the spectrum in Figure 5.23 is included as insert 2.
Table 5.6

g-tesor Mo(V) in (K/Mo)/Si:Ti 1:1 catalysts

<table>
<thead>
<tr>
<th>Catalyst</th>
<th>Ambient Conditions</th>
<th>Dehydrated Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>gx</td>
<td>gy</td>
</tr>
<tr>
<td>10%Mo/Si:Ti 1:1</td>
<td>1.956</td>
<td>1.930</td>
</tr>
<tr>
<td>10%(K/Mo=0.035)/Si:Ti 1:1</td>
<td>1.955</td>
<td>1.929</td>
</tr>
<tr>
<td>10%(K/Mo=0.07)/Si:Ti 1:1</td>
<td>1.956</td>
<td>1.930</td>
</tr>
<tr>
<td>10%(K/Mo=0.14)/Si:Ti 1:1</td>
<td>1.956</td>
<td>1.929</td>
</tr>
<tr>
<td>10%(K/Mo=0.3)/Si:Ti 1:1</td>
<td>1.954</td>
<td>1.929</td>
</tr>
<tr>
<td>10%(K/Mo=0.6)/Si:Ti 1:1</td>
<td>1.949</td>
<td>1.928</td>
</tr>
</tbody>
</table>
The g-values for different coordination environments of Mo(V) are reported in literature as follows: 6-coordinate Mo(V), \( g_\perp = 1.944 \), \( g_\parallel = 1.892 \); 5-coordinate Mo(V), \( g_\perp = 1.957 \), \( g_\parallel = 1.866 \); and 4-coordinate Mo(V), \( g_\perp = 1.926 \), \( g_\parallel = 1.755 \) [153, 159-161]. Since the perpendicular component is expected to be the most sensitive to the coordination of the isolated MoO\(_x\) species, the data indicate that with the addition of potassium, there is a change in the coordination sphere (six-coordinate distorted toward 5-coordinate) of the Mo(V) species that reaches a maximum at K/Mo = 0.07. Furthermore, the integral signal intensity follows the same trend. The g-values of the K/Mo = 0.3 and 0.6 samples indicate a distortion in the opposite direction toward that of a 4-coordinate Mo(V) species that can arise from the formation of K-molybdates. Furthermore, the symmetry of the spectrum for the K/Mo = 0.6 sample is significantly altered compared to the sample with no K component. This is a likely result of the formation of more tightly packed K\(_2\)MoO\(_4\) and K\(_2\)Mo\(_2\)O\(_7\) crystalline domains observed to be present on this sample.
It cannot be expected that information regarding the coordination of MoO$_x$ species obtained from ESR data on Mo(V) centers corresponds to the vast majority of Mo(VI) present in the fully oxidized catalysts. However, some important characteristics are found that can be related to changes that may be occurring to the Mo(VI)O$_x$ domains with the addition of alkali. It has been stated that surface MoO$_x$ species, when supported on silica, are highly distorted hexa-coordinate species, irrespective of the preparation method [91]. ESR data indicate that over the Si:Ti 1:1 support, this distortion is maximized at the alkali level of K/Mo=0.07. This distortion could be a result of an increased number of Si-O$^-$ ligands attached to the MoO$_x$ supported species, as would be in agreement with the NMR and DRIFTS results.

5.4 Summary

Several studies over MoO$_3$/SiO$_2$ catalysts have suggested the specificity of Mo=O bonds for the partial oxidation of methane to formaldehyde while Mo-O-Mo bonds contribute to total oxidation products [164-166]. However, other studies have proposed that Mo=O bonds do not play a critical role in partial oxidation reaction and it is the nature of the Mo-O-Mo bond that determines reactivity[100]. This naturally leads to the fact that the oxygen environment around molybdenum will be the decisive factor for oxidation reactions. It has been reported that the addition of K to Mo-based catalysts can affect this oxygen environment by causing the cleavage of Mo-O-Mo bonds [167]. Kantschewa et al. [167] have found that, over Ni-Mo/Al$_2$O$_3$ catalysts, the addition of
potassium brings about a decrease in reducibility and transforms the coordination of Mo\(^{6+}\) from octahedral to tetrahedral at high alkali content. However, surface polymolybdate domains were eliminated, even before evidence of K-molybdate formation was observed. This phenomenon was accompanied by the stabilization of Mo in the +5 oxidation state. It has also been reported that, below the level where new compounds are formed, the addition of alkali can significantly reduce the Lewis acidity of Mo(VI) ions without drastically changing the structure [11,121]. Poor dispersion of MoO\(_x\) over silica is usually ascribed to the low reactivity of surface silanols. However, results presented here indicate that MoO\(_x\) surface species are interacting with both silica and titania domains of the support. Furthermore, with addition of low levels of potassium, distorted MoO\(_x\) units, experiencing a decreased Lewis acidity, could tend to be more reactive toward electronegative Si-O\(^-\) support ligands on the Si:Ti 1:1 support. This helps explain the concurrent trend of the ESR results on the coordination of Mo(V) species with that of the decrease in available free-hydroxyls obtained in the NMR and DRIFTS experiments.
The ESR results presented herein do not indicate a loss of oxygen but rather a significant electronic interaction of K with the MoO\textsubscript{x} domains. This interaction could be strong enough to "force" MoO\textsubscript{x} domains to interact with silica by forming more Mo-O-Si bonds. While the exact nature of this interaction has not been determined, a highly distorted structure sharing both titania and silica ligands may account for the observed increase in reactivity at the low K/Mo molar ratios that will be discussed in subsequent chapters. Raman data suggest that the best dispersion over the Si:Ti 1:1 support is obtained before the onset of K-molybdate formation. Furthermore, analysis of the bulk structure, which shows continuous segregation of titania as anatase crystallites, shows that the best MoO\textsubscript{x} dispersion may be obtained over a support where titania remains as nano-dispersed TiO\textsubscript{x} regions.
CHAPTER 6

THE INDUCED EFFECTS OF ALKALI PROMOTION ON MoOx CATALYSTS FOR PROPANE ODH

6.1 Introduction

A major challenge in heterogeneous catalysis is the design and development of new catalysts for selective transformation of lower hydrocarbons [107]. The oxidative dehydrogenation (ODH) of lower alkanes to form alkenes has been extensively studied for such purpose. In particular, the ODH of propane is attractive as a process to produce propylene, given its high demand for the production of polypropylene, acrylonitrile, and propylene oxide. However, ODH selectivity is limited considering the high reactivity of propylene toward further oxidation. It was shown that, perhaps on all catalysts, the rate for the second reaction could be five to ten times higher than that of the first reaction. In fact, more recent work by Khodakov et al. [43,109] has determined the relative rate of propylene combustion to be twenty times as high as the rate of propylene formation over vanadium based catalysts. Chen et al. has also measured the same rate to be ten to twenty
times higher on molybdenum-based catalysts [104,110]. Furthermore, the authors have commented on the likelihood of similar active sites being responsible for both reactions. This limitation in propane ODH has led to, at best, a general description of the qualities that a selective catalyst must possess.

Selectivity requires careful control of the abundance of active oxygen and of the adsorption/desorption characteristics to avoid re-adsorption of the formed propylene. There is a joint influence between the red-ox and acid-base properties of the surface of the oxides used for propane ODH. Intermediate reducibility, weak Lewis acid centers, and high oxygen mobility represent the essential requirements for selective ODH; as they are consistent with the trends in ODH rates observed on VO_x, MoO_x, and WO_x-based catalysts [168]. However, quantitative correlations between these properties and catalytic performance cannot easily be obtained and these characteristics are therefore usually expressed in literature as a "good-mix" or "favorable balance" between acid-base characteristics and red-ox behavior [9,11-112]. The "favorable" oxygen that can provide this requirement is that which binds strongly enough to the surface to have attenuated oxidizing strength but weakly enough to oxidize the reactant molecule selectively. Over supported transition-metal oxides, the species of interest exist in the form of M=O, M-O-M, or M-O-support bonds, where M is the supported transition metal. The nature of the active oxygen will certainly depend upon transition metal loading, dispersion, support effects, and the addition of modifiers, such as potassium. The accompanying increase in activity and/or selectivity in partial oxidation reactions with the use of alkali (Li, Na, K, Rb, and Cs) doping has been widely studied and offers a way to adjust the red-ox and
acidic properties of supported transition metal oxides. The positive effects of alkali promotion arise from the alkali's ability to alter oxidation/reduction behavior, affect surface acidity, and/or cause a synergism between alkali and transition metal oxide phases. Recent work from our laboratory has examined the structural changes associated with alkali promotion at low levels. The presence of potassium significantly alters the electronic structure of the surface MoO\textsubscript{x} domains supported over the binary oxide of silica/titania. Data suggested that surface supported species, present as distorted octahedral MoO\textsubscript{x}, become the most distorted at low levels of alkali (K/Mo=0.07). The MoO\textsubscript{x} species at this level of alkali promotion, experiencing a decrease in Lewis acidity, could tend to be more reactive toward electronegative Si-O\textsuperscript{-} support ligands on the Si:Ti 1:1 support. The interaction of MoO\textsubscript{x} domains with the binary oxide support, and consequently reactivity, can be altered with the addition of potassium. It was shown that small levels of alkali suppress the reducibility and Lewis acidity of the molybdenum species and possibly stabilize a lower oxidation state of Mo during the propane ODH reaction.

In this investigation, we continue to examine the induced effects of low-level alkali promotion in order to obtain a better understanding of the way the MoO\textsubscript{x} reactivity, altered by the presence of potassium, relates to the adsorption and reactivity of propane and propylene under various conditions. Furthermore, the use of non-steady-state and steady-state isotopic transient kinetic analysis (SSITKA) was used to extend our discussion to changes in oxygen mobility cause by the presence of potassium. Differential Scanning Calorimetry was used to determine the heat effects associated with
propylene adsorption. Diffuse Reflectance Infrared Fourier Transform Spectroscopy was used to characterize adsorbed species present in non-selective transformations of propane/propylene in an oxygen atmosphere. Electron Spin Resonance was used to investigate changes in Mo(V) species upon contact with propane under different conditions as a probe for the electronic properties of the supported MoO₅ species. A summary of the observed effects is provided with relation catalyst structure.
6.2 Propane ODH Performance

To determine the optimal support composition and preparation conditions, initial experimentation has focused on a series of K/Mo=2 catalysts. This work comes from initial screening of K promoted MoO_3, or bulk K_2MoO_4 catalysts. Bulk, precipitated potassium molybdate catalysts were 60% selective to propylene formation but at a conversion less than 6%. Furthermore, these samples showed considerable selectivity to cracking products, i.e. methane, ethane, and ethylene. Un-supported potassium molybdate catalysts had seriously low surface areas, because of the precipitation and calcination procedures. A well-known method for increasing the surface area of a catalyst is to disperse the active component on a support. In an attempt to promote support-metal interactions between molybdenum and the support, the mixed oxides of silica and titania were used because of the strong Lewis acidity these materials are known to exhibit. Reaction evaluation followed to determine the optimal support composition for the K_2MoO_4 (or K/Mo=2) catalysts.

A set of pre-hydrolyzed catalysts with a constant K/Mo ratio of 2 were compared to the "stoichiometrically" hydrolyzed catalyst (As listed in Table 6.1) in regard to their ODH behavior. Selectivities obtained at an equal conversion of ~3% and a temperature of 500°C are presented in Table 6.1.
Table 6.1
Effect of hydrolysis conditions for 10%(K/Mo=2)/Si:Ti 1:1 catalysts

<table>
<thead>
<tr>
<th></th>
<th>Selectivity</th>
<th>C₃H₆(%)</th>
<th>CO₂(%)</th>
<th>CO(%)</th>
<th>C₂H₄(%)</th>
<th>CH₄(%)</th>
<th>C₂H₆(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrolyzed</td>
<td></td>
<td>83.2</td>
<td>15.5</td>
<td>0.0</td>
<td>1.1</td>
<td>0.1</td>
<td>0.0</td>
</tr>
<tr>
<td>Prehydrolyzed</td>
<td></td>
<td>90.0</td>
<td>8.6</td>
<td>0.0</td>
<td>1.4</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Prehydrolyzed Fast Addition</td>
<td></td>
<td>73.5</td>
<td>24.2</td>
<td>0.0</td>
<td>2.2</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Prehydrolyzed Acidic(pH=3)</td>
<td></td>
<td>66.9</td>
<td>28.2</td>
<td>0.0</td>
<td>4.9</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Prehydrolyzed Basic(pH=11)</td>
<td></td>
<td>77.3</td>
<td>13.3</td>
<td>7.1</td>
<td>2.0</td>
<td>0.3</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Conditions: ~3% propane conversion, 500°C, %N₂/C₃/O₂ 92.5/5/2.5, 25cc/min.

When the silica precursor is prehydrolyzed keeping the other synthesis parameters the same, we observe an appreciable increase in propylene selectivity compared to the catalysts prepared without any prehydrolysis step. However, changing the preparation parameters, such acidity or the speed of addition of the aqueous solutions for the prehydrolyzed catalyst does not appear to have much effect on propylene selectivity.

Another point worth noting about these comparisons is the fact that catalysts prepared without pH control (with or without prehydrolysis) show the lowest CO₂ and ethylene selectivities.

To investigate the effect of the support composition, catalysts with different Si:Ti ratios were compared using 10% Mo loading and a K/Mo ratio of 2. The comparison was based on equal mass of Mo metal in the reactor. The temperature for these reactions was
550°C. The yields of different reaction products obtained are presented in Figure 6.1. There was no C2H6 observed in these runs. The highest propylene yield was obtained over the catalysts that had a Si:Ti ratio of 1:1. This catalyst showed no CO or CH4 formation and very little CO2 and C2H4 formation. The propylene yield for the Si-rich catalysts also appear to give relatively high yields for propylene, but these are accompanied by high yields for CO, CO2, C2H4 and CH4.

![Figure 6.1: Effect of support Si:Ti molar ratio of 10%/(K/Mo=2) loaded catalysts on yield, constant Mo loading (0.1g), 550°C, %N2/C2/O2: 92.5%/5%/2.5%, 25cc/min.](image-url)
Homogeneous volume minimization downstream from reactor or free-radical quenching is necessary in propane ODH reaction to isolate catalytic activity from gas phase activity. Radicals, once formed on the surface of the catalyst, can desorb during reaction and contribute to gas phase pyrolysis downstream from the catalyst bed. To examine the contribution of homogeneous reaction, experiments were performed with and without dead volume packing downstream from the reactor. Our results showed this effect to be important even at 400°C. Experiments performed using the K/Mo=0.07 catalyst and a feed stream ( (%N₂/C₃/O₂: 92.5%/5%/2.5%).) of 25 cm³/min flow rate showed that the propane conversion increased from 13 to 16% when packing downstream from the catalyst bed was removed. In all our runs, a great deal of attention was paid to minimizing the gas phase reaction contribution. The measures we took include raising the catalyst bed, using a quartz wool packing to minimize the homogeneous volume downstream from the reactor and providing rapid quenching in the post-catalyst bed region. Care was also taken to eliminate "hot spots" or temperature effects by using very small catalyst particles, small catalyst bed volumes and large percentages of the inert gas (N₂) compared to propane and oxygen concentrations. As for the contribution from the non-oxidative dehydrogenation, the very low yields we saw for methane, and the balances for carbon, hydrogen and oxygen, which were ~100% for the reactions, lead us to think that this contribution is negligible.
The findings of Burch and co-workers [8,98], which showed that the non-oxidative dehydrogenation became appreciable only at temperatures close to 700°C, also support this assumption. Although care was taken to isolate the catalytic activity for the oxidative reaction, contributions from gas phase reactions cannot be completely ruled out.

(K/Mo)/Si:Ti 1:1 catalysts were compared in the propane ODH reaction using equal surface area loading (65m²) in the reactor and at a temperature of 550°C. The feed percentages for these experiments were N₂/C₃/O₂: 61/26/13. Reaction data was taken after steady-state was reached. The variation of propylene formation rate with K/Mo ratio for these equal surface area tests is presented in Figure 6.2. The propylene formation rate reported here is the "observed" rate that results from formation and further reaction of propylene. The rate is seen to slowly increase through a maximum between K/Mo=0.07 and 0.14 and fall as K/Mo is increased to 0.3 and 0.6...

In previous studies [11,162], it has been found that alkaline promoters decrease the acidity and increase the basicity of MoOₓ catalysts, this affect being maximized at low alkali/Mo ratios. Consequently, the alkali can decrease the adsorption of formed propylene thus facilitating more to desorb as product. Furthermore, it is likely that, when supported on a binary oxide, favorable interaction of MoOₓ domains with both Si:Ti support can increase not only reactivity, but also the accessibility of surface supported domains. These factors help to explain the initial increase of propylene formation rate during propane ODH at the level of alkali promotion where the favorable interaction was found to exist in the characterization experiments.
Figure 6.2: Conversion (□ propane) and formation (△ propylene, ○ CO₂) rate for equal surface area (~65 m²) reaction experiments (μmol/m²/s). Insert: Propylene formation rate vs. Mo(V) ESR intensity (dehydrated conditions).
It is interesting to note that a linear relationship is obtained when propylene formation rate is plotted versus the Mo(V) signal intensity obtained in ESR experiments under dehydrated conditions (insert of Figure 6.2). MoOₓ based catalysts are often believed to operate effectively under reduced conditions where the presence of Mo(V) is essential [111,154]. Furthermore, it has been claimed that Mo(V) is the active site in propane ODH and other oxidation reactions over MoOₓ based catalysts [45,100,111,163]. Although, many of the correlations regarding Mo(V) as active sites are based on ex-situ measurements, such as the experiments presented in this study. While Mo(V) is, in all probability, related to the active sites of MoOₓ catalysts, it seems more likely that the electronic structure of MoOₓ domains (explicitly the nature of the oxygen atoms) is the underlying factor in determining "activity" over the Mo/Si:Ti 1:1 catalysts. This electronic structure influences their preferential interaction with the binary oxide support and, thus, influences the reactivity.

Figure 6.3 shows the effect of residence time on propylene formation over the K/Mo=0.07 catalyst. Experiments were performed at 550°C and the residence time was varied from 0.6 to 10 seconds. The formation of propylene increased with increasing residence time at a residence time of ~5 seconds, where it leveled off and remained essentially unchanged with further increases in the residence time, while the yields of CO₂, C₂H₄, C₂H₆ and CH₄ all increased with increasing residence time. This could be an indication of the reactivity of propylene as compared to propane. As residence time is increased, propane conversion would be higher. However, the longer propylene would reside in the reactor, the more likely is its subsequent conversion
Figure 6.3: Effect of feed residence time on propylene formation, 550°C, %N₂/C₃/H₂O₂: 92.5%/5%/2.5%
6.3 The Changes in Adsorption Properties with the Addition of Potassium

Propane Temperature Programmed Desorption (TPD) of the K/Mo catalysts was performed. Catalyst samples (100mg) were placed in a 1/4-in.-i.d. U-tube quartz reactor and pre-treated under oxygen flow at 550°C for thirty minutes, followed by cooling to room temperature under helium. Samples were flushed with helium for 1h followed by 1h of propane adsorption. After adsorption, desorbed species were monitored by a mass spectrometer (HP5890GC-MS) under helium carrier gas. Identification of species with equal m/z ratios was accomplished by following characteristic mass fragments of the species. The temperature program was as follows: 10 minutes at room temperature, 10°/min. ramp rate to 700°C, and holding at 700°C for 20 minutes.

Analysis of the desorbed species after propane adsorption showed propane, propylene, water, CO₂, CO, O₂, and trace amounts of methane, ethane, and acrolein desorbing from the surface. Propylene desorption profiles for the Si:Ti 1:1 support, 10%Mo/Si:Ti 1:1, and potassium containing catalysts of K/Mo=0.07 and 0.3 are plotted in Figure 6.4.
Figure 6.4: Propane temperature programmed desorption, propylene desorption profiles over bare support and 10%Mo/Si:Ti 1:1 catalysts with K/Mo ratios of 0, 0.07, 0.03
Focusing on the “molybdenum only” catalyst, there is one desorption feature with peak maximum temperature around 200°C. An important aspect of these profiles is that the first desorption feature shifts to lower temperatures with increasing potassium loading. The water desorption profiles follow the propylene desorption closely, indicating that propylene formation takes place oxidatively, using the lattice oxygen. The shift of the propylene desorption peaks to lower temperatures also points to an ease of desorption from the surface for the potassium containing catalysts. With the addition of potassium, two additional sites for propylene desorption are formed on the K/Mo=0.3 catalyst at higher temperatures (~260 and ~300°C). The desorption profiles for carbon monoxide for the same catalysts are plotted in Figure 6.5. Although ethylene has the same molecular weight as carbon monoxide, by following fragments created by both, we determined that the first desorption peaks (<200°C) are associated with ethylene desorption and the remainder of the profile belongs to carbon monoxide alone. There are three significant desorption features present on the “molybdenum only” catalyst at 360, 465, and 650°C. With the addition of potassium, these desorption features appear to be suppressed and less pronounced in a broad profile.
Figure 6.5: Propane temperature programmed desorption, carbon monoxide desorption profiles over bare support and 10%Mo/Si:Ti 1:1 catalysts with K/Mo ratios of 0, 0.07, 0.03 (* =ethylene desorption)
DSC thermograms were obtained over the Si:Ti 1:1 support and molybdate catalysts with K/Mo molar ratios of 0, 0.07, and 0.3 during propylene adsorption. The DSC thermogram for 10%(K/Mo=0.07)/Si:Ti 1:1 is shown in Figure 6.6. The first peak is associated with a combination of the irreversible and reversible adsorption of propylene. After a steady baseline is obtained. The flow was switched to nitrogen only and that propylene which was reversibly adsorbed is allowed to desorb. This switching process was continued until the adsorption and desorption peaks matched indicating that the surface was saturated with irreversible adsorbed propylene. The thermogram obtained for 10%Mo/Si:Ti 1:1 was of similar form, whereas those for the Si:Ti 1:1 support and 10%(K/Mo=0.3)/Si:Ti 1:1 showed only reversible adsorption. Correcting for the heat capacity difference during the flow differences, as described in the experimental section, and integrating each individual peak allows the calculation of the heat associated with both the reversible and irreversible adsorption of propylene over the samples. Results are shown in Table 6.2. As can be seen, there was only reversible adsorption associated with the Si:Ti 1:1 support.
Figure 6.4: DSC thermogram obtained for 10%(K/Mo=0.07)/Si:Ti 1:1 during propylene adsorption/desorption at 150°C. Flow switch: 5%propylene/nitrogen \(\rightarrow\) nitrogen (25cc/min.).
Table 6.2  
Heat associated with propylene adsorption of Mo/Si:Ti 1:1 catalysts with different K/Mo molar ratios

<table>
<thead>
<tr>
<th>Sample</th>
<th>$Q_{rev}(\text{mJ/m}^2 \text{ sample})(10^4)$</th>
<th>$Q_{rev}(\text{mJ/m}^2 \text{ sample})(10^4)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>10%Mo/Si:Ti 1:1</td>
<td>-3.193</td>
<td>-0.126</td>
</tr>
<tr>
<td>10%(K/Mo=0.07)/Si:Ti 1:1</td>
<td>-1.323</td>
<td>-0.065</td>
</tr>
<tr>
<td>10%(K/Mo=0.3)/Si:Ti 1:1</td>
<td>-</td>
<td>-0.036</td>
</tr>
<tr>
<td>Si:Ti 1:1</td>
<td>-</td>
<td>-0.020</td>
</tr>
</tbody>
</table>

The Mo-only catalyst exhibited the largest heat value associated with the irreversible adsorption of propylene. With the introduction of potassium, K/Mo=0.07, this value is reduced by roughly a third. At K/Mo=0.3, there is no detectible irreversible adsorption of propane. It can furthermore be noted that the heat associated with the reversible adsorption of propylene is seen to decrease with the addition of potassium. This information further corroborates the relationship between potassium addition and the decreased reactivity for propylene. The addition alkali additives lead to electron transfer to the Mo$^{6+}$ cation in MoO$_x$ domains, which become less acidic and shows smaller $\Delta H_{ads}$ and reactivity rates for propylene [121]. Even though propylene is more basic than propane, it is also expected that the addition of alkali will effect the adsorption properties of propane during ODH reactions. To explain the observed increase in propylene formation rates with the introduction of low amounts of alkali in our ODH experiments, it
is also necessary to also consider the structure of MoOx domains. Specifically, the interaction of the MoOx domain with the support can also change affect the reactivity of the catalysts and must be taken into account together with the observed affects of alkali on the surface acidity of the catalysts.

To further corroborate the observed effects on the reactivity and adsorption of propylene, a series of propylene oxidation experiments were performed under identical ODH conditions. The (K/Mo)/Si:Ti 1:1 catalysts were compared in the propane ODH reaction using equal surface area loading (65 m²) in the reactor and at a temperature of 550°C. The feed percentages for these experiments were N₂/C₃/O₂: 92.5/5/2.5. Reaction data was taken after steady state was reached. The variation of propylene formation rates with K/Mo ratio for these equal surface area tests are presented in Figure 6.7. These are observed rates for propylene formation in that they are the summation of both formation from propane and its successive depletion that are observed during the reaction. The rates are seen to increase through a maximum between K/Mo=0.07 and 0.14 and fall as K/Mo is increased to 0.3. Propylene oxidation experiments run under the same conditions (N₂/C₃/O₂: 92.5/5/2.5; 550°C) revealed a conversion rate that steadily decreased with K/Mo molar ratio. When propane and propylene oxidation experiments are compared under these identical conditions a maximum exists in the ratio of propane to propylene conversion rates.
Figure 6.7: Propylene formation rates (□) of Mo/Si:Ti 1:1 catalysts with different K/Mo molar ratios. Reaction Conditions: equal surface area reactions (65 m$^2$), %N$_2$/C$_3$/O$_2$: 92.5%/5%/2.5%, 25 cc/min. Propane/Propylene conversion ratio (O) obtained under identical conditions. Reaction conditions: equal surface area reactions (65 m$^2$), %N$_2$/C$_3$/O$_2$: 92.5%/5%/2.5%, 25 cc/min.
This suggests that the increase in propylene formation at the low K/Mo molar ratios (0.07 and 0.14) during ODH experiments is due to a decreased reactivity toward propylene caused by the addition of alkali. In previous work, we have related this tendency to the secondary effect induced by alkali promotion [11]. The surface oxygen species are affected by the presence of potassium and this, in turn, affects the rates of both propane activation and propylene combustion. The reactivity trends are related to a combination of the decrease in Lewis acidity and the slight suppression of the reducibility at these low K/Mo molar ratios. However, the deeper nature of the electronic and structural effects on the active oxygen has been investigated throughout this dissertation.

ESR techniques have been widely used to obtain insight into many aspects of the catalysis and surface chemistry of metal oxide surfaces [153]. In particular, calcination of MoOx catalysts at high temperatures can lead to the formation of paramagnetic Mo(V) centers [171] which can be studied to probe the local environment of the surface supported species. The adsorption of atmospheric water can have a significant effect on the dispersion and coordination of MoOx domains and thus, the ESR spectrum. The presence of adsorbed water can cause separation between Mo(V) ions and a consequent sharpening of the ESR linewidths [156]. In this investigation, all samples were maintained in the dehydrated state prior to analysis. Previously dehydrated samples were subsequently purged at 10⁻³ Torr for thirty minutes after which 100 Torr of propane was introduced. ESR spectra were recorded after 15 minutes at room temperature under the propane atmosphere. The before and after ESR spectra are shown in Figure 6.8 for the 10%Mo/Si:Ti 1:1 and the 10%(K/Mo=0.07) catalysts.
Figure 6.8: ESR spectra of 10%Mo/Si:Ti 1:1 and 10% (K/Mo=0.07)/Si:Ti 1:1 catalysts under dehydrated conditions (before) and after room temperature propane adsorption.
(100 Torr propane, spectra taken under propane at room temperature)
A small signal from the superoxide ion (O$_2^-$) is present in the spectra both before and after contact with propane with a characteristic average g-value of 2.011. The structural assignment of this species cannot directly be determined as the superoxide ion can exist and be stabilized both in extra-framework TiO$_2$ and in silica domains as well as being associated with the formation of Mo(V) species [156]. The rather broad spectrum are indicative of Mo(V) in a dehydrated oxide matrix [156-157]. The spectra in figure 6.8, before contact with propane, are reasonably symmetric (or quasi-axial [157]). Thus, parallel and perpendicular parameter values can be defined as $g_\parallel = (g_x + g_y)/2$ and $g_\perp = g_z$. The g-values for different coordination environments of Mo(V) are reported in literature as follows: 6-coordinate Mo(V), $g_\perp = 1.944$, $g_\parallel = 1.892$; 5-coordinate Mo(V), $g_\perp = 1.957$, $g_\parallel = 1.866$; and 4-coordinate Mo(V), $g_\perp = 1.926$, $g_\parallel = 1.755$ [153,159-161]. The $g_\parallel$ and $g_\perp$ values of Mo(V) in the samples are presented in Table 6.3. The $g_\perp$ values before propane contact are indicative of hexa-coordinated Mo(V). After contact with propane, the ESR spectra for 10%Mo/Si:Ti 1:1 remains essentially unchanged. However, the spectra for 10%(K/Mo=0.07)/Si:Ti 1:1 is no longer quasi-axial after adsorption of propane.
Table 6.3  
g-tensor of Mo(V) in (K/Mo)/Si:Ti 1:1 Catalysts

<table>
<thead>
<tr>
<th>Before Adsorption (vacuum)</th>
<th>Catalyst</th>
<th>$g_\perp$</th>
<th>$g_{\parallel}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>10%Mo/Si:Ti 1:1</td>
<td>1.943</td>
<td>1.900</td>
<td></td>
</tr>
<tr>
<td>10%(K/Mo=0.07)/Si:Ti 1:1</td>
<td>1.946</td>
<td>1.895</td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>After Adsorption*</th>
<th>Catalyst</th>
<th>$g_\perp$</th>
<th>$g_{\parallel}$</th>
<th>$g_x$</th>
<th>$g_y$</th>
<th>$g_z$</th>
</tr>
</thead>
<tbody>
<tr>
<td>10%Mo/Si:Ti 1:1</td>
<td>1.942</td>
<td>1.890</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10%(K/Mo=0.07)/Si:Ti 1:1</td>
<td>1.927</td>
<td>1.956</td>
<td>1.891</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*under 500 Torr propane atmosphere

Keeping in mind that the spectra was taken under a propane atmosphere, it is realized that several species formed from propane could be responsible for the reduction in symmetry of the Mo(V) domains. Since only Mo(V) is detectable in the ESR technique, we are not able to directly observe transformation between Mo(VI), Mo(V), and Mo(V). The integral intensity of Mo(V) in these experiments was not observed to change. Therefore, a reduction process was not observed. However, the symmetry change observed in the K/Mo=0.07 sample is very similar to previous results for sample that where exposed to atmospheric water. It is possible that the formation of –OH groups may have occurred from the adsorption of propane. Furthermore, oxygen-containing adsorbates could be formed from the same process. Whatever the cause of the symmetry...
change, it is clear that propane interacts more strongly with the lattice oxygen in the potassium containing catalyst in these experiments. This interaction occurs without a specific reduction of MoO\textsubscript{x} species but a definite interaction with the lattice oxygen thereof. It is therefore concluded that potassium can not only effect the transformation of propylene, but also change the interaction of the catalyst with propane.

To ascertain the differences in reaction intermediates present on the catalyst during reaction conditions, IR spectra of adsorbed species were obtained at 450°C surface temperature. After gas phase spectra subtraction, the results are plotted in Figure 6.9 in the range 1800-1300 cm\textsuperscript{-1}. Similar bands are observed on the K/Mo catalysts studied. Bands observed around 1689 cm\textsuperscript{-1} is associated with adsorbed acetone. Bands located around 1540, 1503, 1430, 1360, and 1328-1336 cm\textsuperscript{-1} are associated with acetate, formate, \pi-allyl, and acrolein type species [172-179]. Olefinic CH\textsubscript{x} stretches are located at 1458 and 1390 cm\textsuperscript{-1}. Two interesting features to note in Figure 6.9 are that the highest yield ODH catalyst of this study, K/Mo=0.07, shows the most intense olefinic CH\textsubscript{x} stretching bands. Furthermore, one of the most selective ODH catalysts, K/Mo=0.3, shows a lack of intensity from the acetate and formate species present on the other catalysts. This agrees well with the reaction results as these intermediates may lead to the formation of CO\textsubscript{x} products.
Figure 6.9: In situ DRIFTS spectra of over bare support and 10%Mo/Si:Ti 1:1 catalysts with K/Mo ratios of 0.07, 0.3, 0.5°C surface temp, 2% N/C/O.
In Figure 6.10, the IR spectra are shown after the reaction has been quenched to room temperature under nitrogen. It is apparent that olefinic species are still present on the "molybdenum only" catalyst, indicated by the bands at 1441 and 1375 cm\(^{-1}\), which have shifted to lower wavenumbers with the decrease in temperature of the sample surface. Over the potassium-containing catalysts, on the other hand, these bands have essentially disappeared. Again, this suggests a weaker adsorption of propylene on the surface, leading to easier desorption.

Figure 6.10: DRIFT spectra after quenching under N\(_2\)
6.4 The Changes in Acid-Base and Red-Ox properties with the Addition of Potassium

Temperature Programmed Reduction experiments were performed on catalysts with different K/Mo ratios. The results are compared to bulk MoO\textsubscript{3} and K\textsubscript{2}MoO\textsubscript{4} samples prepared from the same precursors as the synthesized catalysts. The results are plotted in Figure 6.11. The profiles for supported K/Mo catalysts are similar, consisting of one major temperature maximum in the 400-500°C range. As the K/Mo ratio increases, the maxima begin to shift to higher temperatures and the peaks show considerable broadening. At K/Mo ratios of 0.6 and higher, we begin to see peaks becoming very noticeably asymmetrical, possibly representing two different reduction sites. When these profiles are compared to that of bulk K\textsubscript{2}MoO\textsubscript{4}, we see that the temperature for this secondary feature, which appears as a large shoulder, coincides with the major reduction peak observed over the bulk K\textsubscript{2}MoO\textsubscript{4}. Table 6.4 summarizes the temperature maxima and the FWHM (Full Width at Half Maximum) of these major reduction peaks.
Figure 6.11: Temperature programmed reduction profiles for 10%Mo/Si:Ti 1:1 catalysts with different K/Mo ratios, 10%H₂/N₂
Table 6.4

Temperature Programmed Reduction of 10%(K/Mo)/Si:Ti 1:1 Catalysts With Different K/Mo Ratios

<table>
<thead>
<tr>
<th>Catalyst</th>
<th>1st peak Maxima(°C)</th>
<th>FWHM(°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MoO₃</td>
<td>459</td>
<td>34</td>
</tr>
<tr>
<td>K/Mo=0</td>
<td>436</td>
<td>58</td>
</tr>
<tr>
<td>K/Mo=0.07</td>
<td>450</td>
<td>46</td>
</tr>
<tr>
<td>K/Mo=0.14</td>
<td>456</td>
<td>53</td>
</tr>
<tr>
<td>K/Mo=0.3</td>
<td>512, 560sh</td>
<td>75</td>
</tr>
<tr>
<td>K/Mo=0.6</td>
<td>531, 566sh</td>
<td>104</td>
</tr>
<tr>
<td>K/Mo=2</td>
<td>520, 570sh</td>
<td>118</td>
</tr>
<tr>
<td>K₂MoO₄</td>
<td>581</td>
<td>54</td>
</tr>
</tbody>
</table>

sh=shoulder

To further investigate the effect of potassium on the interaction of propane, a set of propane "reduction" ESR experiments were performed in which the sample were contacted with propane at 400°C for 30 minutes and any adsorbed species were evacuated for thirty minutes at 10⁻³ Torr. The spectra of a series of K/Mo molar ratios (0, 0.035, 0.07, 0.14, and 0.3) are shown in Figure 6.12 before and after the reduction.
Figure 6.12: ESR spectra of 10%Mo/Si:Ti 1:1 catalysts with differing K/Mo molar ratios under dehydrated conditions (before) and after 400°C propane “reduction”. (500 Torr propane, spectra taken after 30 min. evacuation at 10^{-3} Torr)
At first glance, it is obvious that an increase in Mo(V) signal intensity has occurred after the reduction, corresponding to Mo(VI)\( \rightarrow \)Mo(V). After the reduction with propane, the spectra remains quasi-axial and the perpendicular and parallel values of the g-tensor are given in Table 6.5. It can be seen that, after the reduction, the g values changes and match that of a penta-coordinated Mo(V) species as described previously \((g = 1.957)\). This indicated that the reaction of propane with the surface of the catalysts, in the absence of gas phase oxygen, removes one oxygen atoms from individual MoO\(_x\) domains existing in the Mo(V) state. It cannot be expected that the ESR data obtained for coordination of Mo(V) centers corresponds to the vast majority of Mo(VI) present in the fully oxidized catalysts. However, some important characteristics are found that can be related to some of the changes that may be occurring to the Mo(VI)O\(_x\) domains with the addition of alkali. The integral intensity of the Mo(V) signal is seen to increase differently depending on K/Mo molar ratio. The percentage change in the signal is given in Figure 6.13. It is revealed that the K/Mo=0.07 catalyst experiences the smallest change in Mo(V) signal. MoO\(_x\) based catalysts are often believe to operate effectively under reduced conditions where the presence of Mo(V) is essential. Furthermore, as previously stated, it has been claimed that Mo(V) is the active site in propane ODH and other oxidation reactions over MoO\(_x\) based catalysts. It may be possible that potassium is able to stabilize the MoO\(_x\) domains in a more “reduced” state and that Mo(V) is able to participate in the observed “one-oxygen loss” more effectively that that Mo(VI) species. This would agree with the observed reaction behavior if this distinction is responsible for the selective and unselective transformations of propane and propylene.
Table 6.5
g-tensor of Mo(V) in (K/Mo)/Si:Ti 1:1 Catalysts

| Dehydrated Conditions                                | Catalyst | $g_\perp$ | $g_{||}$ |
|-------------------------------------------------------|----------|----------|----------|
|                                                        | 10%Mo/Si:Ti 1:1    | 1.943    | 1.900    |
|                                                        | 10%(K/Mo=0.035)/Si:Ti 1:1 | 1.945    | 1.900    |
|                                                        | 10%(K/Mo=0.07)/Si:Ti 1:1 | 1.946    | 1.895    |
|                                                        | 10%(K/Mo=0.14)/Si:Ti 1:1 | 1.944    | 1.907    |
|                                                        | 10%(K/Mo=0.3)/Si:Ti 1:1    | 1.940    | 1.905    |

| After Propane (Reduction)*                             | Catalyst | $g_\perp$ | $g_{||}$ |
|-------------------------------------------------------|----------|----------|----------|
|                                                        | 10%Mo/Si:Ti 1:1    | 1.956    | 1.868    |
|                                                        | 10%(K/Mo=0.035)/Si:Ti 1:1 | 1.955    | 1.867    |
|                                                        | 10%(K/Mo=0.07)/Si:Ti 1:1 | 1.955    | 1.867    |
|                                                        | 10%(K/Mo=0.14)/Si:Ti 1:1 | 1.953    | 1.866    |
|                                                        | 10%(K/Mo=0.3)/Si:Ti 1:1    | 1.953    | 1.866    |

* 500Torr Propane contact at 400 °C for 30 minutes followed by vacuum at 10⁻³ for 30 min spectra taken under vacuum
Figure 6.13: %Change in Mo(V) signal intensity associated with 400°C propane “reduction” for the 10%Mo/Si:Ti 1:1 catalysts with differing K/Mo molar ratios.
Isotopic oxygen exchange experiments were performed over the Si:Ti 1:1 support and 10%Mo/Si:Ti 1:1 catalysts with K/Mo molar ratios of 0, 0.07, and 0.3. There was no appreciable exchange detected over the Si:Ti 1:1 support. The transients obtained under steady-state conditions for the remaining samples are shown in Figure 6.14, 6.15, and 6.16. As can be seen, under steady-state conditions, there is only a small amount of cross-labeled oxygen (m/z=34) observed over the 10%Mo/Si:Ti 1:1 catalyst (Figure 6.14). This transient does not relax back to zero during the course of the experiment, indicating there is a source of oxygen available for exchange besides that in the gas phase. Furthermore, the $^{16}\text{O}_2$ and $^{18}\text{O}_2$ response lines take much longer to relax than the corresponding gas-phase hold up line of argon (m/z=40). This indicated that there is a considerable interaction of oxygen with the surface of the catalyst. With the addition of potassium at K/Mo=0.07 (Figure 6.15) there is no detectable cross-labeled oxygen species. However, by comparing the transients for $^{16}\text{O}_2$ and $^{18}\text{O}_2$ it can be seen that there is still an interaction of oxygen with the surface that lasts through the course of the experiment. When the potassium level is increased to K/Mo=0.3 (Figure 6.16) the interaction is markedly decreased. There is no cross-labeled oxygen observed and the transient for doubly exchanged oxygen relaxes back to zero. However, some interaction still exists as the oxygen transients relax slower than the argon inert from the beginning of the switch.
Figure 6.14: Oxygen isotopic exchange experiments under steady-state conditions 
(10%$^{16}$O$_2$/1%Ar/He$\rightarrow$10%$^{18}$O$_2$/He) for 10%M_0/Si/Ti 1:1
Figure 6.15: Oxygen isotopic exchange experiments under steady-state conditions (10% $^{16}$O$_2$/1%Ar/He $\rightarrow$ 10% $^{18}$O$_2$/He) for 10% (K/Mo=0.07)/Si:Ti 1:1.
Figure 6.16: Oxygen isotopic exchange experiments under steady-state conditions 
(10%^{16}O_2/1%Ar/He → 10%^{18}O_2/He) for 10%(K/Mo=0.3)/Si:Ti 1:1
On metal oxides, oxygen may be exchanged by two mechanisms, depending on weather oxygen exchanges one or both of its atoms with the surface of the catalyst. The results presented here indicate that both mechanisms are present, to a certain extent, over the supported molybdate catalysts and that these mechanisms are affected by the addition of potassium. With the addition of potassium, the mechanism for the formation of cross-labeled oxygen appears to be suppressed. There are two ways in which oxygen from the surface can participate in double-exchange as described by Doomkamp et al [180]. Two vacancies are required for the exchange and they may be present on the catalyst surface. Additionally, only one vacancy may be present that is responsible for the adsorption of a gas phase oxygen molecule. Then the double-exchange can proceed when the second vacancy is able to quickly diffuse from the bulk to the surface of the catalyst. Thus, the rate of diffusion of such vacancies is expected to influence the relation of the oxygen transients. Thus, potassium addition to the MoO₅ catalyst appears to be decreasing lattice diffusivity as the mechanisms of oxygen exchange are suppressed. Addition of alkali to V₂O₅ catalysts has led to the same conclusion [180]. Additionally, it is possible that the decrease in lattice oxygen mobility is inter-related to the commonly observed effects on reducibility in alkali promoted MoO₅ catalysts. Martin and Duprez [181] have found a good correlation between oxygen mobility and metal-oxygen bond strength, which in part, can determine reducibility.

Oxygen exchange experiments were performed over the same catalysts under different conditions. After a pre-calcination step, the samples were evacuated for 1 hour at 10⁻⁷ Torr. The samples were then flushed under flowing helium for 1 hour at 500°C.
A flow of $10\%^{18}\text{O}_2$ in helium with a small amount of argon (~1%) was introduced. Exchange under these conditions showed slightly different results indicating that the oxygen mobility of the catalysts is sensitive to pre-treatment conditions. However, the Si:Ti 1:1 support showed no significant exchange or holdup of oxygen species. The transient responses obtained are plotted in Figure 6.17, 6.18, and 6.19. As Figure 6.17 indicates, cross-labeled exchange on 10%Mo/Si:Ti 1:1 is not observed under these conditions. However, the double-exchange mechanism is present and participates throughout the course of the reaction. It is possible that the double vacancies required for the double-exchange are more easily formed during the one our vacuum treatment, making it possible for this mechanism to dominate. On all catalysts studied the $^{18}\text{O}_2$ signal did not grow as fast as the argon inert, indicating an adsorption of oxygen on the surface of the samples. Weather the adsorption of oxygen will lead to exchange depends on the nature and mobility of the oxygen vacancies. Weather the mobility of these vacancies can be related to selective or non-selective routes in propane ODH remains to be characterized. However, it is found that the oxygen of the lattice participating in the exchange can be significantly influenced by the presence of potassium.
Figure 6.17: Oxygen isotopic exchange experiments under transient conditions (He $\rightarrow$ 10%$^{18}$O/He) for 10%Mo/Si:Ti 1:1
Figure 6.18: Oxygen isotopic exchange experiments under transient conditions 
(He$\rightarrow$10%$^{18}$O/He) for 10%(K/Mo=0.07)/Si:Ti 1:1
Figure 6.18: Oxygen isotopic exchange experiments under transient conditions
The oxygen exchange experiments give an indication of the influence the presence of potassium has on the mobility of oxygen in the catalysts. However, these results can not be expected to translate to the behavior of the catalysts under reaction conditions. In the presence of propane, under ODH conditions, the catalysts may operate in a slightly reduced state, thereby altering the behavior of the oxygen of the surface.

To investigate this behavior, a series of oxygen exchange experiments were performed in the presence of the ODH reaction over the 10%Mo/Si:Ti 1:1 catalysts with K/Mo molar ratios of 0, 0.07, and 0.3. It has been found that, during propane ODH experiments, the dissociative chemisorption of gas phase oxygen is irreversible giving rise to no exchange during the reaction [34]. This information is corroborated in our experiments in which no significant exchange of cross-labeled oxygen was observed under ODH conditions (e.g. after the switch 5% propane, 2.5%^{16}O_{2}, 91.5%He, 1% Argon \rightarrow 5\%\text{ propane}, 2.5\%^{18}O_{2}, 92.5\%\text{He}. However, there was a significant interaction of diatomic oxygen with the catalysts surface, Figures 6.20, 6.21, and 6.22. During the course of the exchange experiment, 10%Mo/Si:Ti 1:1 shows about a 50\% of the oxygen exiting the reactor is still^{16}O_{2} (Figure 6.20). Similar to the exchange experiments without reaction, the addition of potassium suppresses the interaction of gas phase oxygen with the catalysts, Figures 6.21 and 6.22. However, the interaction is not eliminated, even at the level K/Mo=0.3, indicating the catalyst must be partially reduced in the presence of propane causing an increase in the exchange as compared to experiments performed with no propane present.
Figure 6.20: Oxygen isotopic exchange experiments in the presence of reaction; oxygen transients (2.5%$^{16}$O$_2$, 91.5%He, 1% Argon $\rightarrow$ 5% propane, 2.5%$^{18}$O$_2$, 92.5%He) for 10%Mo/Si:Ti 1:1
Figure 6.21: Oxygen isotopic exchange experiments in the presence of reaction; oxygen transients (2.5% $^{16}$O$_2$, 91.5%He, 1% Argon $\rightarrow$ 5% propane, 2.5% $^{18}$O$_2$, 92.5%He) for 10%(K/Mo=0.07)/Si:Ti 1:1
Figure 6.22: Oxygen isotopic exchange experiments in the presence of reaction: oxygen transients (2.5% $^{16}$O, 91.5% He, 1% Argon $\rightarrow$ 5% propane, 2.5% $^{18}$O, 92.5% He) for 10%(K/Mo=0.3)/Si:Ti 1:1.
Additionally, the presence of potassium appears to reduce the time gas phase oxygen spends on the surface. Adsorbed oxygen species are often ascribed to unselective transformation of propane during ODH experiments [182]. It is therefore possible that the addition of potassium prevents desorbable oxygen species that may be associated with unselective conversion of propane or propylene. However, it is known that the addition of potassium decreases the reducibility. This returns the discussion to a "balance" of effects in which the decreased reducibility of the catalyst may cause the activation of propane to be hindered, but at the same time, unselective transformations are also hindered. This would certainly contribute to the maximum in activity observed with K/Mo molar ratio.
Since it is generally accepted that reducibility and surface acidity are inter-related for supported metal oxides. Experiments were performed to determine the acidity changes with K/Mo molar ratio. Following background DRIFTS measurement, NH$_3$ adsorption (0.5%NH$_3$/He) was performed for 1 hour over the K/Mo samples. Spectra were taken after evacuation for thirty minutes under helium. The IR spectra of ammonia species formed on 10%Mo/Si:Ti 1:1 catalysts with different K/Mo ratios, are shown in Figure 6.23. The bands listed in Table 6 characterize the spectra. Bands commonly used to characterize Lewis and Brønsted acidity are those located at ~1607cm$^{-1}$ (Lewis) and ~1448cm$^{-1}$ (Brønsted). In Table 6.6, an attempt to quantify the Lewis and Brønsted characteristics of the catalysts is made using these peak areas and intensities. Compared to the Si:Ti support, the K/Mo=0 catalyst has considerably more Brønsted acid character and similar Lewis acid character. With the addition of potassium to the catalyst, the Lewis acid character decreases to a minimum at K/Mo=0.07 and sharply increases at K/Mo=2.
Figure 6.23: Adsorbed NH₃ IR bands over 10%Mo/Si:Ti 1:1 catalysts with different K/Mo ratios and bare support.
Table 6.6

NH₃ IR Adsorption Bands

<table>
<thead>
<tr>
<th>Band (cm⁻¹)</th>
<th>Acidity</th>
<th>Assignment</th>
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</thead>
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<tr>
<td>1074-1082</td>
<td>H-Bonded NH₃</td>
<td>NH₂ Rocking</td>
</tr>
<tr>
<td>1220-1248</td>
<td>Lewis</td>
<td>N-N Stretching</td>
</tr>
<tr>
<td>1450-1434</td>
<td>Bronsted</td>
<td>NH₂ Wagging</td>
</tr>
<tr>
<td>1604-1607</td>
<td>Lewis</td>
<td>NH₂ Scissoring</td>
</tr>
<tr>
<td>1670-1680</td>
<td>Bronsted</td>
<td>NH₂ Scissoring</td>
</tr>
</tbody>
</table>

### baseline corrected peak areas

<table>
<thead>
<tr>
<th>K/Mo Ratio</th>
<th>Lewis ~1607 cm⁻¹</th>
<th>Bronsted ~1448 cm⁻¹</th>
<th>% Lewis*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Si:Ti 1:1</td>
<td>6.4</td>
<td>26.1</td>
<td>20</td>
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<tr>
<td>0</td>
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<tr>
<td>0.07</td>
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<td>4</td>
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<td>2</td>
<td>5.7</td>
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<td>27</td>
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*calculated with peak areas
6.5 Structure/Activity Relationships and Summary

Catalyst comparisons done using equal surface area experiments or equal conversion experiments showed a maximum in propylene formation with increasing K/Mo ratios. The performance of 10%(K/Mo)/Si:Ti 1:1 catalysts in ODH exhibited the alkali doping effect reported for several types of catalysts, that is, an initial increase in selectivity or activity and a sharper decrease as larger quantities of alkali are added. Jiang et al. have seen the same K-Mo interaction on a γ-Al₂O₃ supported catalyst for alcohol synthesis from syngas [183]. They found a parallel between the activity for alcohol formation and the formation of K-Mo species (K-containing Mo₇O₂₄⁶⁻ units). This effect was maximized at a K/Mo ratio of 0.8. They have also shown, from TPR and Raman results, that several types of Mo(VI) oxidic species are present on their K/Mo catalyst (K/Mo=0.35). With interaction of Mo(VI) with K, supported Mo species can lose coordinated oxygen during calcination and be converted to Mo(V). This effect can also take place on a TiO₂ support [184]. This theory is consistent with Mo(V) being most active for molybdate catalysts in ODH reactions frequently cited in the literature. Furthermore, Erdoheleyi et al. have also seen the presence of Mo(V) on K-promoted Mo/SiO₂ catalysts for the partial oxidation of ethane [142]. Kantschewa et al. [167] have shown a strong interaction between K and Mo where the reducibility of a NiMo/γ-Al₂O₃ catalyst was suppressed with the addition of K. Our TPR results, which show a shift of the reduction maxima to higher temperatures with the addition of potassium, are consistent with these findings in the literature, which point to a strong relationship between the reduction behavior of the catalyst and the extent of alkali doping. It is
conceivable that potassium is suppressing the reducibility of the molybdenum species at low K/Mo ratios and stabilizing the Mo(V) oxidation state. This is supported by the ESR data which states that the K/Mo=0.07 catalyst experience the least amount of reduction.

In addition to affecting the reducibility of molybdenum species, it is possible that the presence of alkali dopants also influence the acidity of the catalysts. Abello et al. have reported an acidity-adsorption strength relation on their K-Mo catalyst in the ODH reaction [10]. In our ammonia adsorption experiments, it should be noted that the catalyst that gives the highest propylene yield (K/Mo=0.07) exhibits the lowest Lewis acidity as determined by IR bands of coordinated NH$_3$ species. The Brønsted centers, on the other hand, are quantified by the IR bands associated with ammonium ions (NH$_4^+$), which form on M-OH sites, where M is a metal center. It is interesting that the catalyst which gives the highest propylene yield has also the highest Brønsted-to-Lewis ratio. Another interesting point about these results is that this ratio is higher on the bare support than it is over the high K-content catalysts (K/Mo>1). If the Brønsted centers can activate the alkane and if the Lewis sites provide strong adsorption sites for the olefin, then, this result could help to explain why the bare Si:Ti support gave a higher propylene yield than catalysts with high K/Mo ratios. The fact that this ratio is lower over the support than it is over the Mo-only catalyst is consistent with this argument, since the propylene yield is lower over the former than it is over the latter.

Pantazidis et al. have noted that a good balance between acidity and reducibility is needed over VMgO catalysts in the ODH of propane [9]. O'Young has studied the reducibility of Mo/γ-Al$_2$O$_3$ catalysts with the addition of potassium and cesium [185] and
has shown two levels of alkali/Mo interaction. One appears at alkali/Mo<1, where the reducibility of Mo is only slightly affected. The other interaction type occurs at alkali/Mo>1, where the reducibility of the catalyst is significantly suppressed. This could offer an explanation for the very low activity observed over the catalysts with K/Mo ratios greater than 1, especially for the K/Mo=2 catalyst. When explaining the very low activity observed over the K/Mo=2 catalyst, one needs to consider the fact that all of the Mo over this catalyst exists in a K₂MoO₄ matrix, and, as seen in the XPS data, there are no surface-coordinated Mo-oxide species. This catalyst is likely to behave quite differently than a catalyst with both molybdenum oxide and molybdate species present on its surface.

There is also evidence in the literature pointing to electronic properties of molybdenum being sufficiently altered by the presence of K in close proximity to Mo to affect its adsorption behavior. In this study, in situ IR spectra of K/Mo catalysts together with propane TPD and DSC results indicate that the addition of potassium is affecting how strongly propylene is held on the surface of the catalyst. The experiments show propylene desorbing from the K-containing catalyst surfaces much more easily compared to the potassium-free catalysts. Especially worth noting is the fact that, during reaction, the K-containing catalysts exhibit the highest intensity for olefinic species in the DRIFTS spectra, but once quenched and flushed, lose these species completely. On the other hand, the potassium-free catalysts show that these species continue to exist on the surface, even after quenching and flushing. Another interesting observation from our studies is related to the oxidation behavior of the catalysts. In situ IR spectra show a
much weaker intensity for the oxygen-containing intermediates over the K-containing catalysts than Mo-only catalyst. This is in good agreement with the reaction data, which show a product distribution favoring ODH products more than complete oxidation products over the potassium-doped catalysts. Propane TPD experiments also show that the lattice oxygen availability for carbon monoxide formation is suppressed with the addition of potassium to the catalyst.

The catalyst characterization studies indicate that there are additional factors that control catalytic behavior besides the alkali dopants present. The effect of the alkali is intertwined with the structural nature of the MoO\textsubscript{x} species over the binary oxide support. The Si:Ti 1:1 mixed oxide support is in a state of nano-dispersed titania (anatase) over silica. With the introduction of molybdenum and successive levels of alkali promoter, the dispersion of titania decreases as does the interaction of TiO\textsubscript{x} species with silica. Concurrent with this change is the observation of an enhanced interaction of the MoO\textsubscript{x} species with silica. Raman spectroscopy suggests that low levels of alkali (K/Mo= 0.035 to 0.14) promote the formation of more isolated surface species interacting with silica, while higher alkali content leads to the formation of K-molybdate. In agreement, the silica network of the support experiences a loss in free hydroxyls at the same level of alkali.

The presence of potassium significantly alters the electronic structure of the surface MoO\textsubscript{x} domains even before the onset K-molybdate formation. This electronic interaction is observed readily in the ESR spectrum of dehydrated samples. Data suggest that surface supported species, present as distorted octahedral MoO\textsubscript{x}, become the most
distorted at low levels of alkali (K/Mo=0.07). When tested in propane ODH, there is an initial increase in activity at the same low alkali content followed by a sharper decline. A linear relationship is obtained when propylene formation rate is plotted versus the Mo(V) signal intensity obtained in ESR experiments for the K/Mo/Si:Ti 1:1 catalysts. While the concentration of Mo(V) is not specifically assigned as the active site of propane ODH, it is indicative of the electronic interaction of K resulting in the observed structural effects on the oxygen environment of MoO₅ domains. It was found that with addition of low levels of potassium, distorted MoO₅ units, experiencing a decreased Lewis acidity, could tend to be more reactive toward electronegative Si-O⁻ support ligands on the Si:Ti 1:1 support. While the exact structure of this interaction species has not been determined, a highly distorted structure sharing both titania and silica ligands may account for the observed increase in propane ODH reactivity at the low K/Mo molar ratios.
7.1 Introduction

Because alkanes are relatively inexpensive and are abundant due to side products of refineries and natural natural gas, one of their potential uses is in the conversion to alkenes [7]. Specifically, ethane is the second major component in natural gas, which makes it an abundant source of chemicals such as light olefins, oxygenates, and aromatic hydrocarbons [186]A method that has been widely used to utilize ethane is steam cracking to form alkenes. This method is constrained in many ways, namely: thermodynamic limitations, endothermic reactions that require a large input of heat, and formation of coke on the catalyst [3]. Because of these limitations, recent studies have focused on the oxidative dehydrogenation (ODH) of ethane. ODH is a desirable alternative because, when a proper catalyst is selected, the reaction occurs at lower temperatures and is exothermic, preventing heat input requirements.
According to Cavani and Trifiro [3], catalysts that are active in the ODH of ethane can be grouped into two main categories. Catalysts based on ions and oxides of Group IA and IIA metals are active at a temperature above 600°C. The reaction occurs through the formation of ethyl radicals that react in the gas phase to form ethylene. Thus, the catalyst chiefly participates in directing the gas-phase reaction.

The activity of the catalysts based on Group IA and IIA metals can be significantly increased by the addition of small amounts of chlorine-containing materials in the feed or directly into the catalyst as part of the preparation method [187-191]. Operating at high temperatures, the increase in activity would seem to arise from chlorine radicals, present in the gas-phase, favoring the gas-phase decomposition of ethyl radicals to ethylene. However, Burch et al. [192] have concluded that, during tri-chloromethane treatment, Cl- mainly modifies surface sites and does not participate in gas phase reactions. The same conclusion was reached after tri-chloromethane addition to Mg₃(PO)₄ catalysts [189]. Thus, it also seems possible to improve catalyst performance by incorporation of chlorine during catalyst preparation or by a subsequent treatment after preparation. Au et al. have achieved a higher yield of ethylene by adding BaCl₂ to Ho₂O₃ catalysts [193]. Also, inclusion of Cl' ions in La/Sr/Fe oxide catalysts increased ethylene selectivity and surface oxygen mobility in studies performed by Dai et al. [194]. While many studies have shown the positive effects of modifying catalysts with halides in order to enhance the homogeneous radical reaction in the ODH of ethane, there are significant environmental implications associated with the use of chlorinated compounds as feed materials. Furthermore, the stability of chlorine phases on the catalyst surface at
high temperature can also contribute to the release of chlorine. It has been stated [100] in a recent review on ethane conversion that the role of these additives on supported metal oxides is still poor in regard to structural, red-ox, and acid-base modifications. Therefore, further research is needed to develop halide-modified catalysts that operate at lower temperatures and exhibit stable and active structures.

The second class of ethane ODH catalysts includes those based on transition metal oxides and, in the absence of gas-phase contributions, are typically less active than those operating at the higher temperatures. One such catalyst, Mo/V/Nb, is active in ethane ODH at much lower temperatures, ~350°C [195]. The reaction is heterogeneous and follows a red-ox cycle, with homogeneous reactions occurring only at high temperatures. Examples from literature indicate that most supported transition metal-oxide based catalysts (Mo and V in particular) operating by a red-ox mechanism achieve ethylene yields in the range 15-30% [2,3].

Our previous work has shown that the addition of alkali, in small amounts, to Mo/Si:Ti catalysts can beneficially alter the electronic structure of the surface MoOx domains for the ODH of propane. It was found that with addition of low levels of potassium, distorted MoOx units, experiencing a decreased Lewis acidity, could tend to be more reactive toward electronegative Si-O\(^-\) support ligands on the Si:Ti 1:1 support and a highly distorted structure sharing both titania and silica ligands may be ascribed to an observed increase in propane ODH reactivity at low K/Mo molar ratios. In the current investigation, these results are extended to the ODH of ethane in order to compare their effectiveness.
In addition, several catalysts are modified with chlorine to determine if the addition of halide over transition metal oxide based catalysts, operated at lower temperatures (<650°C), are able to modify the nature of MoO₅ domains and, thus, the red-ox mechanism of ethane ODH.

A series of chlorine-modified molydena catalysts (Cl/Mo molar ratio=0.1) have been tested in ethane ODH and characterized by several methods. These catalysts have been prepared by a “one-pot” sol-gel/co-precipitation technique with the exception of one chlorine-containing catalyst prepared by wet impregnation for comparison purposes. Surface areas were measured using the BET N₂ adsorption method. Characterization has been performed using Temperature Programmed Reduction (TPR), laser Raman spectroscopy, X-ray Diffraction (XRD), and X-ray photoelectron spectroscopy (XPS) to elucidate surface structure, the nature of MoO₅ species, and their interaction with Si and Ti of the support.
Catalysts were prepared using a modified sol-gel/co-precipitation technique. For the molybdenum precursor, ammonium heptamolybdate (AHM) (Mallinkrodt) was used. Tetraethylorthosilicate (TEOS) (Aldrich) and titanium(IV)isopropoxide (TIPO) (Aldrich) were used for the mixed-oxide supports. Isopropyl alcohol was used for the solvent. The modified sol-gel technique uses calculated amounts of silica and titania alkoxide precursors placed in solvent to yield SiO$_2$-TiO$_2$ mixed oxides with a 1:1 ratio after calcination. The solution was stirred while adding an aqueous solution with the desired amount of AHM of molybdenum (10% Mo loading) using a syringe pump. The stoichiometric amount of water needed to hydrolyze all of the alkoxide precursors was contained in the aqueous solution with one exception explained in the subsequent text. The gels formed from this preparation were stirred an additional 15 minutes following the complete addition of the aqueous solution and then dried overnight in an oven at 110°C. Following drying, the catalysts were ground to a powder and calcined under oxygen at 550°C for 5 h. For catalysts modified with chlorine, a calculated amount of HCl (Fisher) or NH$_4$Cl (Mallinkrodt) was included in the aqueous solution to give the desired Cl/Mo ratio. For catalysts containing alkali, KOH, or LiOH was added to the aqueous solution to give the desired alkali/Mo molar ratio.

A list of the prepared catalysts is given in Table 7.1. Catalyst 1 is a 10 wt% molybdate catalyst on a mixed oxide support of Si:Ti in a molar ratio of 1. Catalyst 2 is a molybdate catalyst with a Cl/Mo ratio of 0.1 prepared by co-precipitation using HCl.
Catalyst 3 has the same composition, also prepared by co-precipitation using HCl. However, for Catalyst 3, the amount of hydrolyzing water was doubled from the amount used for Catalyst 2. Catalyst 4 also has a Cl/Mo molar ratio of 1, but was prepared using NH₄Cl. Catalyst 5 has the same composition as Catalysts 2, 3 and 4, but was prepared using wet impregnation of a molybdate catalyst (Catalyst 1) in powder form by dropwise addition of HCl. Catalysts numbered 6 and 7 are alkali-modified catalysts, prepared by coprecipitation using KOH and LiOH, respectively, to give an alkali/Mo molar ratio of 0.1.

Table 7.1
Sol-gel catalysts for the ODH of Ethane

<table>
<thead>
<tr>
<th>No.</th>
<th>Composition</th>
<th>Preparation</th>
<th>Surface Area (m²/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10% Mo/Si:Ti 1:1</td>
<td>Coprecip., sol-gel</td>
<td>133</td>
</tr>
<tr>
<td>2</td>
<td>10% (Cl/Mo=0.1)/Si:Ti 1:1</td>
<td>Coprecip.-NH₄Cl</td>
<td>223</td>
</tr>
<tr>
<td>3</td>
<td>10% (Cl/Mo=0.1)/Si:Ti 1:1</td>
<td>Wet Impregnation HCl</td>
<td>139</td>
</tr>
<tr>
<td>4</td>
<td>10% (Cl/Mo=0.1)/Si:Ti 1:1</td>
<td>Coprecip. HCl</td>
<td>190</td>
</tr>
<tr>
<td>5</td>
<td>10% (Cl/Mo=0.1)/Si:Ti 1:1</td>
<td>Coprecip. HCl a</td>
<td>246</td>
</tr>
<tr>
<td>6</td>
<td>10% (K/Mo=0.1)/Si:Ti 1:1</td>
<td>Coprecip., sol-gel</td>
<td>188</td>
</tr>
<tr>
<td>7</td>
<td>10% (Li/Mo=0.1)/Si:Ti 1:1</td>
<td>Coprecip., sol-gel</td>
<td>170</td>
</tr>
</tbody>
</table>

a double the stoichimetric amount of hydrolyzing water used
Comparing the surface area of the prepared catalysts (Table 7.1), it is seen that the all alkali and chloride modified catalysts exhibit higher surface area than the Mo-only catalysts. Furthermore, wet impregnation of the Cl\(^-\) modifier results in the smallest increase in surface area. Comparing the chloride-containing catalysts prepared by co-precipitation, it is seen that preparation with twice the necessary hydrolyzing water (catalyst no. 3) caused a 56 m\(^2\)/g increase in surface area, which was the purpose of this preparation.

X-ray diffraction patterns were obtained on the Cl-modified catalysts. X-ray diffraction of the Si:Ti 1:1 support yielded a pattern characteristic of a silica-titania mixed oxide containing extra-framework anatase. One broad peak with center located at a d spacing of 3.59 Å was observed, which is indicative of a finely dispersed, nano-dispersed anatase structure supported over amorphous silica. When the size of individual crystals is below 50nm, a reasonably good measure of particle size can be determined from the broadening of diffraction lines measured at half of their maximum intensity [140]. Results of this measurement, together with the diffraction patterns, are shown in Figure 7.1. With the addition of molybdenum, the anatase diffraction peak becomes narrower, indicating a change in the dispersion and segregation of titania in the Si:Ti matrix with the addition of Mo. With the introduction of molybdenum to the Si:Ti 1:1 support, the particulate size increases from approximately 2nm to 9nm. Introduction of chlorine by the co-precipitation technique does not appear to increase the segregation of crystalline anatase as indicated by similar XRD patterns.
Figure 7.1: X-ray Diffraction patterns and average anatase particulate size for the Mo/Si:Ti 1:1 catalyst modified by chlorine (a) - co-precipitation with NH₄Cl; (b) - wet impregnation of HCl; (c) - co-precipitation with HCL; (d) - (c) using double the stoichiometric hydrolyzing water
With the addition of chlorine, the average anatase particulate size remains roughly the same as that of the Mo-only catalyst with the exception of that prepared by wet impregnation of HCl (Figure 7.1-b), which shows a slight increase. Furthermore, the XRD patterns indicate that molybdena species are finely dispersed as no MoO₃ diffraction patterns were observed for all samples studied.

Raman Spectra of 10%Mo catalysts, modified with chlorine, are presented in Figure 7.2 (ambient conditions). Bands associated with Mo=O terminal stretches and Mo-O-Mo structures are presented with the ~640 cm⁻¹ band from TiO₂ (anatase). An important feature of these spectra is that there is little or no evidence of crystalline MoO₃ since the most intense band characteristic of Mo-O-Mo stretching vibrations in MoO₃ is not present as a sharp peak. In general, bands in the range of 750 to 950 cm⁻¹ are attributed to antisymmetric stretching of Mo-O-Mo bonds or the symmetric stretch of (-O-Mo-O-)ₙ bonds, while bands in the range 950 to 1050 cm⁻¹ can be attributed to the symmetric stretching mode of Mo=O bonds present in supported species [89]. The Raman spectra of the 10%Mo/Si:Ti 1:1 catalyst contains a broad Mo=O stretching band centered at 996 cm⁻¹. Compared to the Mo-only catalyst, the addition of chlorine by co-precipitation methods brings about a sharpening of the terminal Mo=O bands. The bands associated with isolated terminal Mo=O stretching vibrations are visible in the 970-1005 cm⁻¹ region and the contributions to this band can arise from MoOₓ supported solely on silica or titania domains of the support, or from species sharing mixed support-O ligands.
Figure 7.2: Raman spectra (ambient conditions) of Mo/Si:Ti 1:1 catalyst modified with chlorine. (notation as in Figure 7.1)
The catalysts modified with chlorine exhibit similar Raman spectra under ambient conditions with the exception of that prepared by wet impregnation of HCl (Figure 7.2-b). Additionally, the wet impregnated catalyst shows small features indicative of polymolybdate structures at 866 and 918 cm⁻¹ and is very similar to that of the Mo-only catalyst.

To better elucidate the nature of the supported MoOₓ species, a set of Raman spectra taken under dehydrated conditions was performed and is presented in Figure 7.3. Under dehydrated conditions, new Raman bands associated with the silica surface are resolved. The dehydrated Si:Ti 1:1 support surface reveals broad Raman bands at 605, 800, and ~1070 cm⁻¹. The band at 605 cm⁻¹ is assigned to a defect structure present in tricyclosiloxane rings produced via the condensation of surface hydroxyls upon dehydration while the band at 800 cm⁻¹ is attributed to the symmetric mode of Si-O-Si stretching [83]. Pure silica possesses a Si-O-Si stretching vibration located at ~1050 cm⁻¹ [86]. In figure 3, this band is shifted to 1070 cm⁻¹, which shows a perturbed vibration that is indicative of Ti-O-Si bonds [83-85]. Chlorine modified catalysts, prepared by co-precipitation, show a significant increase in the broad band associated with Si-O-Si centered at 800cm⁻¹ (Figure 7.3-a,c,d) when compared to the Mo-only and wet impregnated catalyst. This could be an indication of a decreased coverage of the MoOₓ species over silica and/or a significantly altered silica network due to the presence of non-bonded Cl⁻ ions.
Figure 7.3: Raman spectra (dehydrated conditions) of Mo/Si:Ti 1:1 catalysts modified with chlorine. Spectra taken under 10%O2/He at room temperature. (notation as in Figure 7.1)
Chlorine has been referred to as a “damage precursor” when present on silica glasses and has been found to significantly distort silica glass networks [196-197]. Furthermore, the presence of halide, Cl and F, over low Mo-content silica has been found to decrease the interaction of molybdenum oxide domains with silica by the elimination of Mo-O-Si bonds [198]. The presence of chlorine can also bring about the appearance of defects in the silica network as indicated by the band at ~605 cm\(^{-1}\) present in Figure 3-b.

Upon dehydration, the Raman bands associated with Mo=O stretching for the 10%Mo/Si:Ti 1:1 catalyst are seen to shift to higher frequencies and contain several contributions. However, for the chlorine-modified catalysts, the band locations do not experience a large shift indicating that the surface supported species are stable through the dehydration process. A similar effect was found on MoO\(_x\)/SiO\(_2\) catalysts, in which the interference of adsorbed water was inhibited with the introduction of chlorine [198]. Bands located at 982 and 998 cm\(^{-1}\) can be ascribed to MoO\(_x\) species experiencing a stronger interaction with silica while that located at 1005 cm\(^{-1}\) corresponds to a supported species interacting more strongly with titania. With the addition of chlorine to the catalyst, the band at ~982 cm\(^{-1}\) disappears on all catalysts except for one of the samples prepared by co-precipitation with HCl (Figure 7.3-c) while the two bands at higher frequency become sharper. A small shoulder present at 832 cm\(^{-1}\) provides weak evidence for polymolybdate structures present on the catalyst prepared by co-precipitation of HCl using excess hydrolyzing water.
Wachs [42] has summarized the Raman spectroscopy results, under dehydrated conditions, of Mo/SiO₂ and Mo/TiO₂ catalysts prepared by several methods and stated that Mo=O Raman bands of surface supported MoOₓ on titania appear in the range 998-1001 cm⁻¹. On silica under dehydrated conditions, these bands are reported to be a function of wt. loading and appear in the range 975-990 cm⁻¹. However, in another study [91], bands arising from dehydrated surface-supported MoOₓ species on silica were observed up to 998 cm⁻¹. The data present in literature suggest that it may be possible to interpret Raman spectrum of MoOₓ supported over binary oxides to gain qualitative information about the interaction of MoOₓ with the Si:Ti surface. Considering the spectra in Figure 7.3, it appears that different preparation methods for the introduction of chlorine changes the relative contribution of the supported species that experience a stronger interaction with silica or titania. While chlorine has been reported to cause a decrease in the interaction of MoOₓ with silica, there does not exist a significant decrease in the Mo=O bands for those species interacting with silica except for the Cl/Mo=0.1 catalyst prepared with excess hydrolyzing water. For this catalyst, the broad band at 800 cm⁻¹ and the defect band at 605 cm⁻¹ are more prominent indicating that, perhaps only under certain preparation conditions, can chlorine significantly distort the silica network so that a diminished interaction of MoOₓ with silica may exist.

To further study the dispersion of MoOₓ over the surface (~50 angstroms) of the Si:Ti 1:1 support, XPS was performed over the various chlorine-modified catalysts. XPS (Mo3d region) of Si:Ti 1:1 supported catalysts exhibited one linked doublet, indicative of molybdenum oxide, corresponding to Mo 3d₅/₂ and 3d₃/₂ levels at 232.6 and 235.7 eV
respectively, Figure 7.4. However, the Mo3d spectra of the catalysts prepared by co-precipitation with HCl become slightly broader as a convoluted feature at lower binding energy is present near 231.6 eV for Mo 3d_{5/2} indicating an additional Mo component. This feature, which is most positively related to chlorine introduction, is located at higher binding energy that what has been reported for molybdenum chlorides. The Mo 3d_{5/2} binding energy for molybdenum chlorides (MoCl₄ and MoCl₅) are reported to be located 230.4-230.7 eV [199-201]. However, a reduced Mo(V) species has been reported to have a binding energy between 231 and 231.5 eV on alumina-supported catalysts [202-204]. It is therefore conceivable that this extra Mo 3d_{5/2} feature in Figure 7.4 arises from the electronic interaction of Cl⁻ with MoOₓ surface species without forming a new molybdenum chloride compound.
Figure 7.4: Mo 3d XPS spectra of Mo/Si:Ti 1:1 catalysts modified with chlorine (notation as in Figure 7.1)
Additional XPS characterization (Si 2p, Ti 2p, and O 1s) was performed over the Cl-modified catalysts. The Si 2p spectra of all samples studied exhibited a peak located at 102.3 eV characteristic of SiO₂. The Ti 2p spectra of 10%Mo/Si:Ti 1:1 exhibited one linked doublet corresponding to Ti 2p½ and 3d½ levels at 458.4 and 464.1 eV respectively. The Ti 2p spectra remained unchanged in all Cl-modified catalysts. Since shifts in the Ti 2p binding energies are indicative of changes in the dispersion of TiOₓ over silica, it is concluded that the introduction of chlorine does not significantly alter the dispersed nature of TiOₓ over the silica surface. This is in agreement with the XRD patterns obtained for the Cl containing catalysts, which have shown a comparable anatase crystallite size. This effect of chlorine modification seems to be in contrast to the introduction of potassium to the 10%Mo/Si:Ti 1:1 catalyst in this aspect, in which the dispersion of titania decreases as does the interaction of TiOₓ species with silica. The O 1s XPS spectra of the chlorine-containing catalysts did exhibit some differences, however, and are shown in Figure 7.5.
Figure 7.5: O1s XPS spectra of Mo/Si:Ti 1:1 catalysts modified with chlorine. (notation as in Figure 7.1)
Each sample exhibits oxygen contributions from TiO\textsubscript{x}, SiO\textsubscript{x}, and MoO\textsubscript{y}. O1s from SiO\textsubscript{x} is located at \(~533.4\) eV, while the signal from TiO\textsubscript{x} and MoO\textsubscript{y} coincide around 531.5 eV. A subtle difference is noted in the spectrum for the catalyst prepared by co-precipitation of HCl (Figure 7.5 c and d). These samples have a broader O1s spectrum that tails out from the MoO\textsubscript{y}(O1s) peaks in the range 531-526 eV. These broad features could arise from the same Cl-Mo interaction species observed in the Mo3d spectra. The broadness of this feature shows that the interaction comes not from one but a distribution of chemical environments. This is evidence that the Cl-Mo interaction is restricted to an electronic one and can effect the environment of MoO\textsubscript{y} species, i.e. the oxygen atoms surrounding molybdenum atoms.

The surface concentrations, as determined by XPS, are shown in Table 7.2. As expected, there is an increase in Si surface concentration when compared to the as-prepared values for catalysts studied. A decrease in Ti concentration observed by XPS measurements is common for Si:Ti binary oxides prepared by a variety of methods and is due the challenging task of dispersing TiO\textsubscript{x} over silica [55,136]. It has also been suggested that a majority of Ti atoms may reside on the surface of inside channels or pores of silica, which can be out of the XPS detection sight [55]. Within the experimental limits of the technique, it is seen that the surface concentrations do not vary largely with the introduction of chlorine by the various methods employed. There is only a slight increase in the Ti concentration for the catalyst prepared by wet impregnation with HCl, which is in agreement with the information obtained from XRD patterns.
The data indicate that the introduction of chlorine by these methods do not significantly alter the state of the Si:Ti support and that the chlorine predominantly acts upon MoO\textsubscript{x} domains without significantly changing the surface concentrations (as determined by XPS). It may be possible that in the "one-pot" preparation method, in which components are dispersed together during gelation, all the MoO\textsubscript{x} and TiO\textsubscript{x} species may not be preferentially located at the surface of silica readily accessible for detection. However, the present data indicates that there is not a large change in homogeneity of the catalyst with preparation method. While Raman data suggest that, with certain preparations, the presence of chlorine may decrease the interaction of MoO\textsubscript{x} with silica, there were no large features from MoO\textsubscript{3} crystallites detected. It is likely that the presence of chlorine, by affecting the electronic structure of MoO\textsubscript{x} domains, can alter the nature of the support-O ligands at the surface without causing significant changes in MoO\textsubscript{x} dispersion or agglomeration. As the Raman data indicates, MoO\textsubscript{x} species may be supported at the surface through different types of support ligands with differing...
electronegativity. Furthermore, this distribution of oxygen environments can be affected by the presence of chlorine, as indicated by the XPS data. The electronic effect of chlorine can alter the nature of these ligands and thereby affect reactivity of the supported species as well as their interaction with the support surface, without a detectable change in dispersion.
7.3 Performance in Ethane ODH

(Cl/Mo)/Si:Ti catalysts were compared in the ODH reaction using equal surface area loading (200m$^2$) in the reactor and at temperatures of 550°C and 600°C. For comparison to the effects of alkali-promotion, the alkali promoted (K and Li) catalysts were also tested. The feed percentages for these experiments were $N_2/C_2/O_2$: 85/10/5. Oxygen conversion was nearly 100% in all runs. Reaction data was taken after steady-state was reached. Ethane conversion for these reaction tests is given in Figure 7.6. Compared to the Mo-only catalyst, all promoted catalysts exhibited a decrease ethane conversion at 550°C with the exception of the Li promoted catalyst, which showed a slight increase. At 600°C the highest conversion is achieved using the catalyst modified with lithium, followed by the potassium-modified catalyst with ethane conversions of 41 and 37%, respectively. While the alkali-modified catalysts increase ethane conversion at 600°C compared to the molybdenum-only catalyst, all chlorine-modified catalysts decreased ethane conversion.
Figure 7.6: Ethane conversion obtained in ODH experiments over Mo/Si:Ti 1:1 catalysts modified with chlorine and alkali (K, Li). Reaction conditions: equal surface area reactions (200 m²), %N₂/C₂/O₂: 85%/10%/5%, 25 cc/min
Table 7.3 shows the yield of the reaction products from each experiment. The data indicate that the highest yield of ethylene is ~19% obtained over the catalyst prepared by co-precipitation of HCl (using only the stoichiometric amount of hydrolyzing water necessary to form the support structure). Doubling the hydrolyzing water in the preparation of catalyst (d) in Table 7.2, was attempted to increase ethylene yield by increasing the surface area of this catalyst. As Table 7.3 indicates, this attempt was unsuccessful, as the yield of this catalyst is lower than that of the Mo-only catalyst. It is apparent that only certain preparation methods may be able to modify the catalyst for ethane ODH in a favorable way. Thus, co-precipitation with HCl was the only method that enhanced ethylene yield when compared to the Mo-only catalyst. The data indicate that this increase is accompanied by a decrease in the formation of CO₂.
Table 7.3

Reaction results for the ODH of ethane (equal surface area experiments, 200m²)

<table>
<thead>
<tr>
<th>T(°C)</th>
<th>Conversion(%)</th>
<th>Yield</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>C₂H₄</td>
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<tr>
<td>10% Mo/Si:Ti 1:1</td>
<td></td>
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</tr>
<tr>
<td>550</td>
<td>30.5</td>
<td>11.9</td>
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<tr>
<td>600</td>
<td>34.8</td>
<td>16.3</td>
</tr>
<tr>
<td>10% (Cl/Mo=.1)/Si:Ti 1:1 ³</td>
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<tr>
<td>550</td>
<td>25.1</td>
<td>9.1</td>
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<tr>
<td>600</td>
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<td>12.7</td>
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<tr>
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<td>25.8</td>
<td>9.6</td>
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<td>600</td>
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<tr>
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<td>14.1</td>
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<td>34.2</td>
<td>18.6</td>
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<tr>
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<tr>
<td>550</td>
<td>27.0</td>
<td>12.4</td>
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<tr>
<td>600</td>
<td>33.5</td>
<td>16.0</td>
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<tr>
<td>10% (K/Mo=.1)/Si:Ti 1:1</td>
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<td></td>
</tr>
<tr>
<td>550</td>
<td>27.4</td>
<td>12.8</td>
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<tr>
<td>600</td>
<td>37.1</td>
<td>15.0</td>
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<td>10% (Li/Mo=.1)/Si:Ti 1:1</td>
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<tr>
<td>550</td>
<td>31.3</td>
<td>11.0</td>
</tr>
<tr>
<td>600</td>
<td>40.6</td>
<td>12.8</td>
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</tbody>
</table>

a - co-precipitation with NH₄Cl; b - wet impregnation of HCl;
c - co-precipitation with HCL; d - c using double the stoichiometric hydrolyzing water
The Raman spectra of this catalyst, Figure 7.3-c, shows a higher contribution from bands that could be associated with MoO\textsubscript{x} species experiencing a stronger interaction with silica at 976, 987, and 998 cm\(^{-1}\). Previous results have also shown an increase in propylene formation in propane ODH experiments when the interaction of MoO\textsubscript{x} with the silica of the Si:Ti support is increased in such manner. Furthermore, the electronic interaction of Cl with MoO\textsubscript{x} species appears to be maximized over this Cl/Mo=0.1 catalyst as indicated by the XPS data.

It is interesting to note that, in contrast to propane ODH results, low levels of alkali (K or Li) do not increase the yield of olefin and form a much larger amount of methane during the ethane ODH experiments when compared to the Mo-only catalyst. Alkali promotion has been shown to increase olefin yields in lower alkane ODH by affecting the nature of the transition metal-oxygen bond and thusly affecting acid-base and red-ox characteristics of the surface [100, and references therein]. Concerning the Si:Ti 1:1 support, work from our laboratory has shown that the presence of potassium significantly alters the electronic structure of the surface MoO\textsubscript{x} domains even before the onset K-molybdate formation. Surface supported species, present as distorted octahedral MoO\textsubscript{x}, become the most distorted low levels of alkali (K/Mo=0.07) and exhibit an increase in propylene yield during propane ODH experiments. However, the positive effects of alkali doping for higher hydrocarbons has been reported to be reversed for ethane ODH [100,205].
This reversal is partially due to the fact that ethylene is more stable than the higher olefins. In attempts to decrease further oxidation of the formed olefin, a large benefit in ethane ODH would not be obtained by decreasing the Lewis acidity of the catalyst surface by introduction of alkali.

The reaction data presented in Table 7.3 can be normalized to molybdenum atoms in the form of turn over frequencies (TOF). Calculation of MoO₅ surface densities, assuming all Mo is located at the surface, while applicable for impregnated Si:Ti supports, may be inaccurate for the "one-pot" preparation method employed for these catalysts. As discussed previously, there may be a significant amount of molybdena species located inside larger titania aggregates or deeper inside the silica network. However, since the XPS data indicates that there are not large surface concentration changes present with the preparation method for the introduction of chlorine, the TOF calculation may give a fair comparison between the catalysts. The ethylene TOF is plotted in Figure 7.7 for the Cl-modified catalysts. It can be seen that all three of the co-precipitation catalyst increases the TOF of ethylene compared to the Mo-only catalyst. In particular, the co-precipitations using HCl obtain the largest TOFs at both temperatures of study.
Figure 7.7: Ethylene TOF ($s^{-1}$) obtained for Mo/Si:Ti 1:1 catalysts modified with chlorine. (notation as in Figure 7.1)
7.4 The Changes in Red-Ox properties with the Addition of Halide

Temperature Programmed Reduction experiments were performed on 10%Mo catalysts with the modifiers of chlorine. TPR profiles were obtained over the three Cl-containing catalysts prepared by co-precipitation. The results are plotted in Figure 7.8. The bare Si:Ti support showed no reduction under these conditions. Thus, the profiles for the Si:Ti 1:1 supported catalysts are similar, consisting of one major reduction feature in the temperature range 350-500°C due to the reduction of supported MoO\(_x\) species. The center of the major reduction peaks and shoulders present within this peak, are listed in Table 7.4. The major reduction peak for 10%Mo/Si:Ti 1:1 is centered at 431°C. With the addition of chlorine to the catalysts the major reduction peak become noticeably asymmetrical, representing two different reduction sites. The catalyst prepared using NH\(_4\)Cl shows the lowest temperature reduction peak, centered at 410°C with a shoulder at 446°C. When this profile is compared to the other Cl/Mo catalysts, we can see that the shoulder in the TPR profile of the NH\(_4\)Cl catalyst is also present there, but as the main reduction feature. Furthermore, the main reduction peak in the NH\(_4\)Cl catalyst is present in the HCl-prepared catalysts labeled 10%Cl/Mo (b) and (c) at 421°C and 403°C respectively. The major reduction feature for 10%Cl/Mo (b) and (c) catalysts have higher temperature centers at 455°C and 448°C respectively. All chlorine containing catalysts also show an increase in the reduction features in the temperature range 500°C to 600°C when compared to the Mo-only catalyst.

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Figure 7.8: Temperature Programmed Reduction (TPR) profiles obtained for Mo/Si:Ti 1:1 catalysts modified with chlorine by co-precipitation. (a) - co-precipitation with NH₄Cl; (b) - co-precipitation with HCl; (c) - (b) using double the stoichiometric hydrolyzing water.
### Table 7.4
Temperature Programmed Reduction of promoted Mo/Si:Ti 1:1 Catalysts

<table>
<thead>
<tr>
<th>Catalyst</th>
<th>1st peak Maxima(°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10%Mo/Si:Ti 1:1</td>
<td>431</td>
</tr>
<tr>
<td>10%(Cl/Mo=0.1) (a)</td>
<td>410, 446sh</td>
</tr>
<tr>
<td>10%(Cl/Mo=0.1) (b)</td>
<td>403sh, 448</td>
</tr>
<tr>
<td>10%(Cl/Mo=0.1) (c)</td>
<td>421sh, 455</td>
</tr>
</tbody>
</table>

a - co-precipitation with NH$_4$Cl; b - co-precipitation with HCl; c - b using double the stoichiometric hydrolyzing water
sh=shoulder

It is apparent that the introduction of chlorine to the catalyst enhances the number of reducible sites present in the catalysts. All chlorine-containing catalysts have shown two main reduction features while the Mo-only sample shows only one. The best performing catalyst in this study, Figure 7.8-b, has close to equal contributions from both a lower temperature reduction feature (421°C) and a higher temperature reduction feature (455°C). This mix of reducible species may provide an optimum balance of oxygen availability at differing strengths. As a result, the C-H bond activation step of ethane, which would require the reduction of a Mo$^{6+}$ site, may proceed more readily on a more reducible surface. The origin of these reducible sites could be related to the interaction of MoO$_x$ domains with the support. Wachs et al. [206] have reported a TPR spectrum that splits into two reduction peaks for the 1% V$_2$O$_5$/30% TiO$_2$/SiO$_2$ sample where both TiO$_2$ crystallites and the surface titanium oxide species coexist on silica. Furthermore, Arena and Parmaliana [207] have claimed that that the several surface Mo(VI) species of silica-
supported molybdena catalysts detected in TPR measurements arise from different interaction strengths with the underlying support. Thus, it is arguable that the two reduction sites, that are in somewhat equal proportion on (10%Cl/Mo=0.1) (b) in Figure 7.8, are due to MoOx domains strongly interacting with titania and silica respectively. Raman data presented in this study would be consistent with this argument, indicating that there is a more even distribution of MoOx interacting with the silica and titania of the support over this catalyst. This may be, in part, the explanation for the higher ethylene yield obtained over this catalyst when compared to the Mo-only catalyst. However, when reaction data is normalized per Mo atom, all Cl containing catalysts prepared by co-precipitation appear to perform better than the Mo-only catalyst. The differing performance amongst the preparations likely arises from the degree to which chlorine is able to modify the oxygen environment around MoOx species, thereby affecting their reactivity and interaction with the support structure.

It is believed that activity in the oxidative dehydrogenation of ethane is a strong function of red-ox characteristics of the catalyst. This is especially true for transition metal oxides on which reaction proceeds via a red-ox mechanism at low temperatures. When we compare the chlorine containing catalysts is seems apparent that through different preparations, we are altering the red-ox properties of the catalysts. All chlorine-containing catalysts have shown two distinct reduction features while the alkali promoted sample shows only one. It has previously been observed [17], from XPS and ESR data, that the low-level addition of alkali to MoOx-based is able to alter the electronic properties of supported MoOx species while significantly affecting their interaction with
the binary oxide support. This can naturally lead to positive effects in reducibility and acidity of those supported centers. Thus, it seems reasonable to conclude that, under the right preparation conditions, the addition of Cl to the MoO$_x$-based catalysts can alter the surface species in a similar manner.

7.5 Structure/Activity Relationships and Summary

This study has examined the effects of chlorine modification on molybdenum catalysts supported on the mixed oxide of SiO$_2$ and TiO$_2$. These catalysts were prepared using "one-pot" sol-gel/coprecipitation or wet impregnation techniques. Ethane ODH reaction experiments were performed with constant feed conditions at two different temperatures. The highest improvement in ethylene yield (−16% to −19% at 600°C) was achieved using a catalyst modified with chlorine by co-precipitation with HCl. However, reaction performance comparisons normalized per Mo atom, suggest that all Cl-modified catalysts prepared by co-precipitation were able to improve performance. Addition of alkali (Li, K) to the 10%Mo/Si:Ti 1:1 catalyst did not improve the ethane ODH performance compared to the un-modified catalyst, in contrast to pervious results obtained for propane ODH.

The introduction of chlorine by the co-precipitation technique does not appear to increase the segregation of crystalline anatase in the Si:Ti 1:1 support, as indicated by X-ray Diffraction results. Similarly, XPS shows that the surface concentrations of Si, Ti, and Mo remain relatively constant with different preparation methods for the introduction of chlorine to the 10%Mo/Si:Ti 1:1 catalyst. The data suggest that, without forming a
specific Mo-Cl phase or compound, and without causing detectable MoO$_x$ aggregation, chlorine is able to significantly alter the reducibility of the supported species. Furthermore, Raman and XPS data suggest that, under certain preparation conditions, this can lead to an optimal interaction of the oxygen environment surrounding the Mo atoms to that of the Si:Ti binary support.

While the addition of halide modifiers is known to positively affect the gas phase mechanisms of ethane ODH at higher temperatures (>650°C), current work indicates that the effect for surface red-ox mechanisms over MoO$_x$-based catalysts may be somewhat less-pronounced but still beneficial, at lower temperatures of operation.
RECOMMENDATIONS

- A novel preparation method was developed for supporting dispersed K-Mo dehydrogenation catalysts. This method takes advantage of sol-gel chemistry, or in other words, inorganic polymerization. Sol-gel science, while becoming quite mature, can often become quite complex as applied to novel materials and applications. In order to create systems to perform specific functions for new applications, a number of parameters can be defined and studied to help shed light onto the matter. The effect of sol-gel parameters (i.e. pH, water content, type of precursor, addition of modifying agents) could be systematically studied and related to catalyst performance through such properties as pore size/distribution, metal oxide dispersion, and nature of the interactions present in the binary oxide support material.

- Alkali doping was investigated using different molar ratios (K/Mo) ranging from 0.01 to 2 over molybdate based catalysts. By varying the K/Mo molar ratio a maximum in selectivity and yield of propylene was obtained over 10wt%Mo loaded catalysts. Limited studies have also been performed on other alkali metals (Li, Cs, and Na). The nature of the alkali-effects on the MoOx domains of the supported catalyst may be better understood by further systematically studying the effects of other alkali metals in relation to electronegativity, atomic size, and Lewis acidity of the alkali
metal. Furthermore, modifications to the sol-gel method can be performed to determine the best incorporation of the dopant to provide the most active catalyst. Finally, incorporation of the common ODH metal oxide, vanadium, into the catalyst may give rise to a superior catalytic material.

- The MoO$_x$-based catalyst was modified with halide for ethane ODH with much success. In ethane ODH reaction comparisons, strong correlations between Mo surface coverage and catalytic activity are observed. To improve the performance further, the addition of alkali-halide into the sol-gel preparation should be attempted. The effects of many preparation parameters and choice of halide precursor remain unknown. Furthermore, the use of other halides is a further matter of study.

- With the success of molybdate based Si-Ti mixed oxide catalysts in the ODH of propane there is incentive to further develop these catalysts for the ODH of ethane. Since the mechanisms of both reactions share common characteristics, there may exist some common features of the catalyst. Previously mentioned, there are notable difference is the activation of ethane and propane. It is conceivable that with modification of the sol-gel matrix one may be able to change the way molecular oxygen is activated for the ODH reaction. Adsorbed oxygen species, while causing the negative effect of combusting and cracking the propylene intermediates, may be detrimental in the activation of ethane to form ethylene intermediates. Furthermore, the surface acidity of the catalyst plays a different role for each reaction. Considerable effort is needed in order to identify the surface species and active sites present of a mono-disperse material. In this project, the full potential Electron Spin
Resonance (ESR), laser Raman spectroscopy, X-ray diffraction (XRD) and NMR techniques should be utilized by in determining the types of sites involved in various oxygen insertion steps. Considerable work needs to be performed to further examine the structure of non-amorphous surface layers or defects, and determining chemical and electronic structures at the surface.

- With advanced preparation methods, nano-scale preparation of catalysts is becoming more common. This is the manufacture of catalysts engineered to have the correct active phase at the atomic level. Atomic level catalyst characterization is becoming more widely accessible with advances in X-ray adsorption techniques and analytical spectroscopies. Preparation at the atomic level is becoming more prevalent with increased understanding of unique “organometallic” precursors and sol-gel techniques that can decompose to give a more controlled catalyst. This information can be used not only for ODH catalysts. With nano-scale preparation as the driving force for catalyst preparation, the effects of many catalytic parameters, such as precursor, thermal treatments, sol-gel parameters, effect of promoter, metal oxide dispersion, and ultimate activity can be combined for a complete study.
BIBLIOGRAPHY


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APPENDIX

SAMPLE CALCULATIONS

Response factors, relative to the highest percentage component, for product P relative to nitrogen (N₂) and propane (C₃) are defined as:

For TCD: \( \text{RF}(P/N₂) = \frac{\text{mol}\% P}{\frac{\text{Area}_P}{\text{mol}\% N₂}} \)

For FID: \( \text{RF}(P/C₃) = \frac{\text{mol}\% P}{\frac{\text{Area}_P}{\text{mol}\% C₃}} \)

Composition of the product stream, for TCD, can be determined as follows:

\[ \text{mol}\% P = \text{RF}(N₂) \times \text{RF}(P/N₂) \times \text{Area}_P \]

The areas of impurities in the feed stream that are also produced as reactants are subtracted from the product area. The standard definition of conversion is the moles of propane reacted divided by the moles of propane in the feed, or:

\[ \left( \frac{\text{flow}}{\text{mol}\% C₃} \right)_{\text{in}} - \left( \frac{\text{flow}}{\text{mol}\% C₃} \right)_{\text{out}} \times 100 \]
The carbon based definition of conversion is defined as the moles of carbon reacted divided by the moles of carbon in the feed, or:

\[
\frac{\left(\text{flow}\sum_{\text{all}} (\text{mol}\%_p)\#C_p\right)_{\text{out}}}{\left(\text{flow}\sum_{\text{all}} (\text{mol}\%_c)\#C_c\right)_{\text{in}}} \times 100 \%
\] (100)

Selectivity to carbon containing product \( P \) is defined as the moles of carbon in \( P \) produced divided by the moles of carbon reacted, or:

\[
\frac{\left(\text{flow}\sum_{\text{all}} (\text{mol}\%_p)\#C_p\right)_{\text{out}}}{\left(\text{flow}\sum_{\text{all}} (\text{mol}\%_c)\#C_c\right)_{\text{in}}} \times 100 \%
\] (100)

When the selectivity is defined in this manner the selectivities of all carbon containing products add to 100%. The yield of product \( P \) is defined as the moles of carbon in \( P \) produced divided by the moles of carbon in the feed, or:

\[
\frac{\left(\text{flow}\sum_{\text{all}} (\text{mol}\%_p)\#C_p\right)_{\text{out}}}{\left(\text{flow}\sum_{\text{all}} (\text{mol}\%_c)\#C_c\right)_{\text{in}}} \times 100 \%
\] (100)

The rate of formation of product \( P \) is defined as the molar flowrate of product \( P \) \( (\mu\text{mol/min}) \) divided by the surface area of the catalyst \( (\text{m}^2) \) \( (\mu\text{mol/min/m}^2) \):

\[
\frac{\left(\text{flow}\times\text{mol}\%_p\right)_{\text{out}} \times (0.1) \times (10^{-6})}{\text{SA}}
\]

The rate of depletion of a reactant \( R \) is defined as the moles/min reacted divided by the surface area of the catalyst, for \( C_3 \) \( (\mu\text{mol/min/m}^2) \):

\[
\frac{\left(\text{flow}\times\text{mol}\%_{C_3}\right)_{\text{in}} - \left(\text{flow}\times\text{mol}\%_{C_3}\right)_{\text{out}} \times (0.1) \times (10^{-6})}{\text{SA}}
\]
The carbon, oxygen, and hydrogen balances; all defined similarly, are defined as the moles out divided by the moles in (of products P and reactant R):

\[
\left[ \frac{\text{flow} \left( \sum_{\text{all}} \text{mol}\%_{P\&R} \# C_{P\&R} \right)_{\text{out}}}{\text{flow} \left( \text{mol}\%_{C_{1\text{,in}}} \# C_{1\text{,in}} \right)} \right]_{100} (100)
\]

\[
\left[ \frac{\text{flow} \left( \sum_{\text{all}} \text{mol}\%_{P\&R} \# H_{P\&R} \right)_{\text{out}}}{\text{flow} \left( \text{mol}\%_{C_{1\text{,in}}} \# H_{C_{1\text{,in}}} \right)} \right]_{100} (100)
\]

\[
\left[ \frac{\text{flow} \left( \sum_{\text{all}} \text{mol}\%_{P\&R} \# O_{P\&R} \right)_{\text{out}}}{\text{flow} \left( \text{mol}\%_{O_{2\text{,in}}} \# O_{2\text{,in}} \right)} \right]_{100} (100)
\]
For species with non-linear detector responses, a series of gas mixtures were used to determine the Gas-chromatograph response with concentration. The concentration of species with non-linear responses during reaction experiments was determined by applying the appropriate concentration-concentration equation as determined from the calibration experiments. An example of this calibration follows.

For TCD, all responses were linear (example-nitrogen).

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For FID, propane, propylene, ethane, and ethylene, responses were not linear and functions were applies by calibrating with various concentrations,

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PHRAGMITES AUSTRALIS: RESPONSE TO WAVE EXPOSURE GRADIENTS, SUBSTRATE CHARACTERISTICS, AND ITS INFLUENCE ON PLANT SPECIES DIVERSITY IN A LAKE ERIE COASTAL MARSH

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

Bradley A. Welch

The Ohio State University
2001

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Adviser
School of Natural Resources
ABSTRACT

The expansion of *Phragmites australis* throughout the marshes of East Harbor State Park, Ohio, over the past 50 years has been associated with a perceived decline in floristic diversity. Few studies exist in the literature to confirm this relationship. Fewer studies explore the autecology or the limits to the aggressive nature of *Phragmites*, especially in the context of freshwater marshes on Lake Erie. This study attempts to explore the interactions between *Phragmites* and floristic diversity following substrate disturbance, the effects of *Phragmites* on species density in three proximate freshwater marshes, and *Phragmites'* response to key environmental and biotic variables.

The results from this study suggest that mechanical substrate disturbance successfully created an environment conducive to the proliferation of a variety of wetland plant species but did not differentially promote rare and endangered species over aggressive species. While the abundance of *Phragmites* was reduced in the short term, this single disturbance event and subsequent hydrologic stress was insufficient or too infrequent to maintain extant species richness. It was also inadequate to maintain seed bank richness and did not limit the richness and abundance of aggressive species over an eight-year period.
Vegetation surveys conducted in 2000 in three proximate East Harbor marshes indicate that the abundance of *Phragmites* negatively affected species diversity, most effectively at a high standing crop (1400-2000 g/m²). Species density was unrelated to the presence or absence of *Phragmites* within each marsh, suggesting that other factors such as nutrient availability, stress and disturbance, and topographic heterogeneity may be directly responsible for species diversity and *Phragmites* abundance.

The relationship between species data and environmental variables was investigated by using canonical correspondence analysis (CCA). Sediment chemistry, wave exposure, distance to shoreline, elevation, light interference, species density, and *Phragmites* standing crop were considered as possible predictor variables. Two gradients combined to explain 62% of the variation in the species-environment relationships: a gradient representing elevation (35.7%) and a gradient representing *Phragmites* standing crop and soil fertility (26.2%). Elevation was instrumental in describing species composition, distinguishing wet meadow species from marsh species. The *Phragmites* standing crop-soil fertility gradient was more closely associated with species density. Species density was highest at low to moderate soil fertility for both wet meadow and marsh species. The association of *Phragmites* with elevated wave exposure and elevated soil fertility was attributed to the plant’s ability to withstand physical disturbance and dissipate wave energy, thereby contributing to increasing soil fertility via accumulation of soil organic matter.
This study suggests that the species diversity-*Phragmites* abundance relationship is driven by a complex interaction of environmental and biotic factors. The mere presence of *Phragmites* does not beget low plant species diversity in the freshwater, non-tidal marshes that I studied. Future research should investigate specific combinations of these variables, particularly water depth and soil fertility, and the effects these combinations have on the species diversity-*Phragmites* abundance relationship.
Dedicated to my family
for their love and trust
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CHAPTER 1

INTRODUCTION

What would the world be, once bereft
Of wet and wildness? Let them be left,
O let them be left, wildness and wet;
Long live the weeds and the wilderness yet.
—from Inversnaid, Gerard Manley Hopkins (1881)

Historically, Lake Erie wetlands have been considered exceptionally diverse ecosystems (Moseley, 1899; Pieters, 1901; Herdendorf, 1987 and 1992; Stuckey, 1988) in terms of both species and functional diversity. Maintenance of diversity within these wetlands has been attributed to the oscillating environmental conditions that characterize Lake Erie (Herdendorf, 1987 and 1992; Bedford, 1992; Stuckey, 1988; Keddy and Reznicek, 1986). Fluctuating water levels, storm events, wave exposure, and seiches all have been considered a part of the Lake Erie disturbance regime.

After the arrival of European settlers, however, freshwater marshes along the coast of Lake Erie succumbed to agriculture, large-scale drainage projects, and, more recently, coastline development (Herdendorf, 1992; Keddy, 1990; Mitsch and Gosselink, 1986; Ohio Lake Erie Commission, 1998). Wetland loss resulted from landscape alterations, hydrologic manipulations, and from
disruption of marsh-lake interactions. Currently, those freshwater marshes that remain along Lake Erie are being colonized by aggressive hydrophytes, many of which are non-native species.

*Phragmites australis* (Cav.) Trin. ex Steud. (common or giant reed) is one of the most successful and prevalent representatives of these aggressive plants, making its presence known throughout the Great Lakes states and along the Atlantic seaboard (Marks et al., 1994; Pellegrin and Hauber, 1999). Once known as *Phragmites communis*, *Phragmites australis* (hereafter, *Phragmites*) has been categorized as a non-native, aggressive plant. *Phragmites* is currently considered a native species. Paleo-archaeological evidence indicates that *Phragmites* existed in North America long before the arrival of European settlers (Tucker, 1990). There is some speculation, however, that both aggressive and non-aggressive genotypes exist (Hauber et al., 1991; Chambers et al., 1999; Pellegrin and Hauber, 1999). Nevertheless, *Phragmites*, once a small component of marsh vegetation in North America, has rapidly expanded its range, most noticeably in the last 50 years (Chambers et al., 1999).

*Phragmites* is a hardy perennial grass that can reach heights of over 4 m. It reproduces predominantly from rhizomes or stolons, forming monodominant stands that exclude less-hardy species (Haslam, 1972). Stem densities have been noted in excess of 200 stems per m² (Haslam, 1971a). Very little else survives in dense colonies of *Phragmites*. Consequently, biodiversity typically decreases, usually in the form of a loss of rare and endangered native plants (McCormac, 1993; Gusewell and Klotzli, 1998). Ecosystem properties, such as
soil nutrient ratios and hydrologic patterns may be changed, sometimes drastically (Gusewell and Klotzli, 1998; Templer et al., 1998), and the aesthetic quality of an area can be compromised.

What makes *Phragmites* so pervasive, aside from vegetative reproduction, is the plant's tolerance of a wide variety of environmental conditions (Haslam, 1972; Ostendorp, 1989). *Phragmites* occurs in moist to inundated soils. It can tolerate acidic and calcareous conditions. *Phragmites* can even grow in areas with elevated saline concentrations (Hellings and Gallagher, 1992).

Understandably, there is increasing concern among wetland scientists and resource managers that *Phragmites* colonization is threatening plant species diversity and the functional integrity of Lake Erie wetlands (Keddy and Reznicek, 1986; Chambers et al., 1999; Galatowitsch et al., 1999). Thus, there is a need to understand *Phragmites* expansion in the context of its interactions with the Lake Erie ecosystem.

Despite a plethora of literature on *Phragmites* (Ostendorp, 1989; University of Aarhus, 1997; van der Putten, 1997; Brix, 1999), there are many basic questions without satisfactory explanations (den Hartog et al., 1989). Few studies have focused on the aggressive nature of the plant (Gusewell and Klotzli, 1998), its potential impacts on Great Lakes ecosystems (Pellegrin and Hauber, 1999), or the role disturbance plays in limiting its expansion and distribution (Weisner, 1991). In fact, most of the literature detailing the ecology of *Phragmites* concerns the die-back of reed beds throughout Europe (den Hartog et al., 1989). Many of these studies indicate that processes such as
eutrophication (Weisner, 1987; Weisner and Graneli, 1989), genetic homogeneity (Koppitz et al., 1997), and anthropogenic site disturbances (Ostendorp, 1989) are causing reed loss. Oddly enough, these same processes are being indicted for the proliferation of *Phragmites* throughout the Great Lakes and other areas in eastern North America (den Hartog et al., 1989; Pellegrin and Hauber, 1999).

Very little information has been published regarding the autecology of *Phragmites* in freshwater systems in North America (Meyerson et al., 2000).

Given the uncertainty and lack of understanding surrounding *Phragmites* ecology in Lake Erie marshes, this study will attempt to define some of the prominent interactions between this aggressive species and associated communities. More specifically, this study examines:

1) species richness, species composition, and *Phragmites* abundance in a diked marsh over a nine-year period following human-induced substrate disturbance;

2) variation in floristic diversity as it relates to *Phragmites* live standing crop in three proximate Lake Erie marshes; and

3) the response of *Phragmites* and other plant species found in East Harbor wetlands to substrate characteristics, wave exposure, and biotic variables.
These themes are presented as the three central chapters (Chapters 2, 3, and 4) of this dissertation. Each chapter is presented in journal format, with its own abstract, introduction, methods section, results, discussion, and list of references. Figures and tables relevant to a given chapter are included at the end of that chapter. Conclusions and implications for management have been gleaned from each of the central chapters and summarized in a final chapter (Chapter 5). A bibliography consisting of all cited references and other pertinent literature utilized but not referenced is included at the end. Citations for Chapters 1 and 5 are included in the final bibliography, not in a separate list of references at the end of each chapter.
CHAPTER 2

VEGETATION CHANGES FOLLOWING HUMAN-INDUCED SUBSTRATE DISTURBANCE

Abstract

Development activities over the past 50 years have disrupted the hydrologic disturbance regime associated with floristically diverse, freshwater marshes located in Lake Erie's Western Basin. Concurrently, native plant species richness has waned while aggressive species abundance has escalated. The substrate of a 0.6 ha section of a diked marsh was mechanically disturbed in 1992 to activate the seed bank and limit the abundance of aggressive species. The disturbance area was then reconnected to the surrounding harbor via a small culvert. Species richness increased by as much as 12% in the three years following disturbance. The number of state-listed species also increased from seven to as many as nine. The abundance of aggressive species, particularly Phragmites australis (Cav.) Trin ex Steudel, was reduced, although aggressive species richness remained unchanged.
Extant vegetation data obtained in 1999 and 2000 indicate, however, that species richness declined from pre-disturbance levels. Species richness also was lower in the disturbance site than in the remainder of the marsh. The number of state-listed species was similar to that observed in 1991-1994, although the number of aggressive species was lower than in previous surveys. Species composition varied from year to year, most likely in response to water level fluctuations. Additionally, fewer species were observed in the seed bank than in the extant vegetation, an unusual result for freshwater wetlands. While Phragmites re-colonized parts of the disturbance site, mean Phragmites stem density did not change between 1999 and 2000. The variability in stem density declined over this period, however. Similar results were obtained in both the disturbance site and the remainder of the marsh. These results suggest that Phragmites stem densities in the disturbance site and the remainder of the marsh may be equilibrating but at a lower density than existed during initial colonization.

While the initial disturbance activated seed bank reserves and reduced the abundance of Phragmites, it did not differentially affect the number of state-listed and aggressive species. Additionally, this single disturbance event and subsequent hydrologic stress was insufficient or too infrequent to maintain extant species richness, to maintain seed bank species richness, or to limit the richness and abundance of aggressive species over an eight-year period. Future restoration efforts may need to incorporate more frequent disturbance events or alternative management techniques.
Introduction

Historically, the freshwater marshes of Lake Erie's Western Basin have been considered exceptionally diverse ecosystems (Moseley, 1899; Pieters, 1901; Moore, 1973), harboring a number of rare and endangered plant species (Stuckey, 1988). Maintenance of floristic diversity within these wetlands has been attributed to the oscillating environmental conditions typical of Lake Erie (Herdendorf, 1987 and 1992; Stuckey, 1988; Bedford, 1992). Long- and short-term water level fluctuations, storm events, wave exposure (sensu Keddy, 1982 and 1984), and seiches have created a patchy community structure (Pickett and White, 1985). These forces also have altered the landscape via sediment deposition and removal (Middleton, 1999), and have selectively activated the seed bank (van der Valk and Davis, 1976, 1978, and 1979; Leck and Graveline, 1979; van der Valk, 1981; Keddy, 1982).

Dredging, diking and development activities along Lake Erie's shoreline in the past 50 years have altered marsh-lake interactions (Stuckey, 1988; Herdendorf, 1992). With the local disturbance regime modified, aggressive plant species, particularly Phragmites australis (Cav.) Trin. ex Steudel., have formed monodominant stands in many formerly diverse marshes (Galatowitsch et al., 1999). The diverse seed banks associated with these wetland plant communities (van der Valk and Davis, 1978; Pederson and van der Valk, 1984) often are adversely affected as well. Prolonged occupation of wetland sites by competitive dominants prevents seed bank renewal by desirable species, augments the seed
bank with aggressive species’ propagules, and allows for the continued decay and predation of the buried seeds of pre-existing species (Leck, 1989). By restoring or imitating the natural disturbance regime, however, historic plant populations and community structure can be re-established (Brown and Bedford, 1997), renewing seed bank reserves, and controlling aggressive species (Keddy and Reznicek, 1982 and 1986).

The substrate of a 0.6 ha section of a 6.4 ha diked marsh in East Harbor State Park, Lakeside-Marblehead, Ohio, was mechanically disturbed in 1992 to activate the seed bank. The marsh was reconnected to East Harbor via a culvert. Species richness increased by as much as 12% in the three years following disturbance (McCormac, 1994). The number of state-listed species also increased from seven to as many as nine. The abundance of aggressive species, particularly Phragmites, was curtailed, though aggressor richness remained unchanged.

While attempts have been made to re-establish species richness in Phragmites-dominated marshes through the removal of culms (Farnsworth and Meyerson, 1999), Phragmites is known to re-invade if treatments are not continued periodically (Marks et al., 1994). Most of these studies, however, have been conducted in brackish or freshwater tidal communities. Very little extant vegetation or seed bank data exist for Phragmites populations in freshwater, non-tidal marshes (Meyerson et al., 2000). Keller (2000) and Farnsworth and Meyerson (1999) emphasized the need to monitor Phragmites removal
treatments over time to characterize the dynamics of *Phragmites* reinvasion and to monitor associated plant community dynamics. The species richness and composition of Lake Erie marsh plant communities fluctuates from year to year depending on water levels and climatic conditions. Short-term monitoring programs, therefore, are unlikely to separate annual variation from changes produced by removal treatments.

Plans have been made recently to restore historic plant species diversity to the entire diked marsh in East Harbor. Plans include re-establishing the marsh-lake hydrologic connection and utilization of the existing seed bank. The goal of this study was to assess the long-term success of such human-induced disturbance activities. Pre- and post-disturbance vegetation data collected by McCormac (1994) were compared to extant vegetation data collected in 1999 and 2000. These data were used to characterize changes in plant species richness and species composition resulting from the 1992 disturbance. Current extant vegetation data collected in the disturbance site also were compared to data collected in the remainder of the marsh to evaluate the long-term efficacy of such a disturbance. Particular attention was given to rare species and to aggressive species. *Phragmites* stem density was related to species density in both sites to assess the impact of *Phragmites* on species richness and to compare this relationship between the two sites. Seed bank studies were conducted in both sites in 1998 and 1999 to evaluate the species richness potential of these sites.
Methods

Site description

East Harbor State Park is located in Ottawa County, Ohio, along the northern edge of the Lake Erie Marblehead peninsula (41°33'N, 82°48'W; Figure 2.1). The site has a temperate climate with a growing season that extends from mid-April to early September. East Harbor is separated from Lake Erie by a sand bar formed by lateral shoreline drift.

The study site is a 6.4 ha rectangular, freshwater, non-tidal marsh created from dredge spoils taken from East Harbor in 1967 and placed along the harbor side of the sand bar (Moore, 1973). The dredge material is predominantly sand. The site is separated from the harbor's surface hydrology by an earthen dike except for a stand pipe at the northwest corner and a culvert at the southwest corner of the wetland. Both pipes are less than 1 m in diameter. A nature trail runs parallel to and inside the dike wall (Figure 2.1).

At least a partial groundwater connection between the marsh and the harbor is suspected to exist (S. Mackey, ODNR Geologist, pers. comm.). Pockets of standing water exist in the marsh prior to July in low water years, and throughout the year in high water years. Portions of the marsh are never inundated.
Although several aggressive species are present, *Phragmites* has formed monodominant stands throughout the marsh over the past 30 years. Plans have been made to restore the natural hydrology to the site in an attempt to reduce the abundance of *Phragmites* and enhance floristic diversity.

**Vegetation surveys**

McCormac (1994) conducted a pre-disturbance, vegetation survey in 1991. The substrate of a 0.6 ha portion of the 6.4 ha marsh was disturbed in the spring of 1992 via a low-ground pressure bulldozer. Post-disturbance vegetation surveys were conducted in the spring and fall each year between 1992-1994. Pre- and post-disturbance surveys consisted of 2 belt transects 2 m wide, 50 m apart, and delineated perpendicular to the footpath. Plant species presence data were recorded. A visual estimation of species abundance also was made for the dominant species and other species of concern.

Recent vegetation surveys were conducted over the entire wetland restoration site, including the 0.6 ha, 1991-manipulated section, in early June and mid-August 1999 and 2000. A baseline was established along the nature trail and transects established perpendicular to this baseline. One transect was established every 18 m along the baseline. A 1 x 1 m quadrat was placed every 25 m along each transect. Identical quadrats were surveyed in 1999 and 2000.
Within each quadrat, plant species were identified, stems counted, percent cover estimated, and average stem height noted. Water depth and elevation also were measured within each quadrat.

Taxonomic nomenclature follows Gleason and Cronquist (1991). While McCormac (1994) referred to the species Carex garberi Nutt., Eleocharis obtusa (Willd.) Schultes., Eleocharis smallii Britt., and Scirpus purshianus Fern., Gleason and Cronquist list these names in synonymy with other species or as localized variants. For these species, the nomenclature used by McCormac is retained for consistency. Lists of rare, threatened, or endangered species (hereafter, state-listed species), and aggressive species are maintained by the Ohio Department of Natural Resources, Division of Natural Areas and Preserves (ODNR, 1999a and 1999b).

Elevations were measured using an auto-level and calibrated with both Lake Erie water levels and existing survey points established by the Ohio Department of Natural Resources, Geologic Survey Division. The mean elevation of the site is 174.59 m (IGLD, 1985), ranging from 173.94 to 175.17 m (Figure 2.2).

**Seed bank study**

Seed bank samples were collected in 1998 every 10 m along six randomly selected transects running perpendicular to the nature trail. Seed bank samples were collected in 1999 every 50 m along four randomly selected transects.
running parallel to the nature trail. In each case, the top 5 cm of soil were collected and placed in a sealed plastic bag, transported to the laboratory, and stored at 5°C until preparation time.

Each sample was homogenized manually. A 2.0 cm layer of each sample was placed in each of four 17.5 cm x 12.5 cm x 6 cm trays containing 3.5 cm of sterilized sand (after Siegley et al., 1988). Two replicates of each sample were labeled and placed in pools containing 10 cm (inundated) and 2 cm (saturated) of water, respectively. Control treatments of sterilized sand were placed in each pool as well. Samples were exposed to standard greenhouse conditions and water levels maintained for a period of six months. Individual plants were counted and removed as soon as identification was possible.

Two-tailed t-tests, regression analyses, and Pearson’s correlation coefficients (r) were calculated using Minitab® Release 10 Xtra (Minitab®, 1995). Confidence intervals (95%) were used with t-tests to differentiate mean *Phragmites* stem density (stems/m²) and mean species density (species/m²) among study years. Regression analysis and correlation coefficients were used to establish relationships between *Phragmites* stem density and species density for each year. Calculations were made for both the disturbance site (DS) and the remainder of the restoration site (RS).
Results

Lake Erie water levels

Lake Erie water levels declined appreciably in 1999, falling below levels recorded over the previous 30 years. Water levels continued to decline in 2000. Mean June Lake Erie water levels declined from 174.55 m in 1998 to 174.29 m in 1999 and then to 174.20 m in 2000 (USAGE, 2001). While these values were within the range of the long-term mean for Lake Erie water levels in June (USAGE, 2000), very few sample points in the East Harbor restoration marsh were located at elevations below the long-term mean (Figure 2.2). Based on these data, over half of the quadrats in the East Harbor marsh were inundated in June 1998, whereas only quadrats in the disturbance site (DS) were inundated in June 1999 and 2000.

Species richness

Figures 2.3 and 2.4 illustrate the year-to-year variation in total species richness, state-listed species richness, and aggressive species richness recorded in both the DS and the remainder of the restoration site (RS).

Total species richness varied between 1991 and 1994 in the DS. The wetland indicator status (WIS; USFWS, 1996) also varied from year to year following disturbance (Figure 2.5). In general, the percentage of obligate wetland species increased after disturbance while the percentage of obligate upland and facultative upland plants declined in 1992-1994. Substrate disturbance in 1991
lowered the average elevation of the DS. While total species richness and WIS seemed to respond to water level changes, water level fluctuations did not exceed critical values necessary to significantly alter vegetation within the DS (Figure 2.2; Harris and Marshall, 1963). Variation in total species richness between 1992 and 1994 may have been in response to substrate disturbance, but the magnitude of change was not beyond the range of yearly variation previously observed in these marshes (Stuckey, 1988).

Total species richness was much lower in 1999 and 2000 than it was in all years of the McCormac study, however. Water levels also declined, dropping below the threshold at which germination conditions switch from inundated to mudflat conditions. Accordingly, the percentage of obligate and facultative wetland species declined (Figure 2.5). The percentage of obligate and facultative upland species increased, particularly in 2000.

Total species richness was greater in the RS than in the DS in 1999 and 2000 (Figures 2.3 and 2.4). The distribution of species among WIS categories was very similar for the DS (Figure 2.5) and RS (Figure 2.6) in both 1999 and 2000, with one exception. The percentage of obligate wetland species in the RS was less than in the DS in 2000. This most likely is a result of declining water levels. The mean elevation of the RS is much greater than that of the DS (Figure 2.2). Declining water levels, therefore, may have had a more dramatic effect on the obligate wetland species in the RS than in the DS.
State-listed species richness remained relatively unchanged in the DS for all years that data were available (Figure 2.3). There also was very little difference in state-listed species richness between the DS and the RS in 1999 and 2000 (Figures 2.3 and 2.4).

Aggressive species richness was relatively constant within the DS throughout the McCormac study but was lower in the 1999 and 2000 surveys (Figures 2.3 and 2.4). Nevertheless aggressive species richness in the DS increased from five to ten species between 1999 and 2000, while the total species richness remained relatively the same. Concurrently, the percentage of obligate upland species increased in the DS (Figure 2.5). Because water levels declined sufficiently to open areas to colonization by aggressive species and because upland species increased in the DS, one also would expect the percentage of upland, aggressive species to increase. Aggressive species present in the DS in 2000 were mostly wetland species, however (Table 2.1). In fact, very few aggressive species associated with uplands were present in any of the surveys conducted. Further, the number of aggressive species recorded in 2000 was well within the range of aggressive species observed in the DS and the RS for all years for which data were available. While fluctuating water levels may have been associated with changes in aggressive species richness, there does not seem to be a direct relationship.

Despite a perceived relationship between water levels and variation in species richness, no relationship was established between water levels and total
species richness, state-listed species richness, or aggressive species richness in the DS or the RS (linear regression, $P >> 0.05$). In fact, water level fluctuations were significantly correlated only with the percentage of obligate upland species observed each year in the DS (linear regression, $F$-statistic= 8.37, $P = 0.034$). Water level explained 63% of the variation in the obligate upland species data. Only a few species exist in this category, though, so this relationship is of limited value, explaining only the variation for species with an extreme intolerance to inundated conditions.

Although the RS was at least twice as large as the DS, species richness in all categories was not dramatically different between the two sites (Figures 2.3 and 2.4). One would expect species richness to increase with sample size (Krebs, 1999), which was generally true for this study. The difference in species richness between sites, however, was small and comparable to the difference in species richness among years within each site. Consequently, these data suggest that the DS could be a species rich site relative to the RS, despite its smaller size.

**Total species composition**

Within the DS, eleven species present prior to substrate disturbance were not recorded in subsequent vegetation surveys (Table 2.1). Five of these species were associated with wetland habitats. While this seems counter-intuitive given that the elevation decreased on the whole throughout the DS
owing to the disturbance, three of these wetland species were rhizomatous or woody, perennial species: *Calamagrostis canadensis* (Michx.) P. Beauv, *Fraxinus pennsylvanica* Marshall., and *Salix discolor* Muhl. These species are more susceptible to large-scale, substrate disturbance (i.e.—bulldozing) than most of the herbaceous species typical of this marsh because they require more time and energy to regenerate. *Juniperus virginiana* L. was the only upland species present in 1991 that was not recorded after the disturbance. It, too, is a woody, perennial species.

On the other hand, 61 different species were recorded in the years following disturbance that were not present in the 1991 study (Table 2.1). Forty-one of these species were wetland species. Of course, not all of these species were recorded in 1992, so a simple comparison between pre-disturbance species and post-disturbance species would be misleading. These values, however, give some indication of the variation in species composition that is typical of this marsh over time. Because an equivalent sample of pre-disturbance data was not collected, it is difficult to make direct links to specific causal factors: substrate disturbance, fluctuating water levels, and/or increased water presence.

Plant species composition became less diverse in recent years and consisted of fewer wetland species than were present in the McCormac surveys (Table 2.1). In fact, 41 species (73% wetland species) recorded by McCormac were absent from 1999 and 2000 surveys. Conversely, 21 species were recorded in 1999 and 2000 but were not observed by McCormac. Twelve of the
species recently recorded were wetland species (Table 2.1). The total percentage of wetland species declined along with water levels in 1999 and 2000. Not only were fewer species present in recent surveys but a greater percentage of them were upland species. Current Lake Erie water levels may be more conducive to the proliferation of upland species than wetland species.

Differences in species composition also existed between the DS and the RS in 1999 and 2000 (Table 2.1). Fifty-two species were recorded in the DS that were not recorded in the RS. Thirty-one of these species were wetland species. Thirteen species were recorded in the RS that were not present in the DS. Seven of these species were wetland species. One would expect species composition to vary more in the RS than in the DS because there is greater variation in elevation in the RS and, therefore, more variation in habitat types. This was not the case, however. Additionally, total species richness was greater in the RS than in the DS in both 1999 and 2000. Together these data indicate that species composition varied more from year to year in the DS than in the RS. Consequently, there may have been greater variety in the extant and seed bank vegetation in the DS than in the RS. Alternatively, there may have been greater variation in environmental conditions affecting the DS than there were in the RS.

species over time suggests not only that they have a broad tolerance to a variety of environmental conditions but that they also have a widespread distribution of propagules throughout both study sites.

**State-listed species composition**

Seven state-listed species were observed after the disturbance event that were not recorded in 1991 (Table 2.1). All of these species were associated with wetland habitats. Only *Myriophyllum sibiricum* Komarov., a state-listed, submersgent species, was eliminated from the DS following the disturbance. Conversely, *Carex viridula* Michx., *Eleocharis palustris* L., and *Juncus alpinoarticulatus* Chaix. were recorded in nearly every study conducted in the DS, before and after the disturbance. *Eleocharis canabaea* (Rottb.) S. F. Blake., which was not recorded in 1991, was observed in all post-disturbance studies. *Cyperus diandrus* Torr. also was absent from the 1991 survey but was present intermittently in years following the disturbance. The absence of *C. diandrus* coincided with high water years. *Potentilla anserina* L. was absent from the McCormac study following disturbance but was observed in 1999 and 2000. Additionally, two species, *Carex garberi* and *Eleocharis smallii*, were not observed after 1994. One state-listed species, *Alisma triviale* Pursh., however, was added to the DS species list after 1994.

Five state-listed species were recorded in the DS that were not present in the RS. All five were wetland species. There were no state-listed species in the
RS that were not present in the DS. Although state-listed species composition changed from year-to-year, the 1992 disturbance seemed to provide environmental conditions conducive to the growth and reproduction of several state-listed species.

**Aggressive species composition**

Most aggressive species were present continuously throughout the post-disturbance portion of the McCormac study (Table 2.1). Six new aggressive species were recorded in the DS after disturbance. Only one aggressive species (*Hieracium pilosilloides* Villars.) was not recorded after the disturbance in the DS. Many of the species observed after the disturbance also were recorded in 1999 and 2000. Five of the six aggressive species present after 1991 were wetland species, suggesting that disturbance may have provided more favorable conditions for these species than existed prior to the manipulation. Many of these aggressive species could have been present in the DS prior to 1991 or as seed bank species in 1991, however.

Three aggressive species present in McCormac’s surveys were not recorded in the DS in 1999 or 2000—*Agrostis gigantea* Roth., *Butomus umbellatus* L., and *Typha x glauca* Godr. *Lythrum salicaria* L., a dominant component of many other Lake Erie wetlands, was observed in the DS and the RS in 2000. In contrast, aggressive species such as *Daucus carota*, *Phalaris arundinacea* L., and *Phragmites* were present in both sites for each of the extant
vegetation surveys. Interestingly, six aggressive species were present in the DS that were not recorded in the RS. Three of these species were wetland species.

Seed bank species richness

Total species richness for the 1998 and 1999 seed bank surveys was lower than extant species richness in 1999 and 2000 for both sites (Table 2.1). Over 75% of the species germinating from samples taken from each site in each year were wetland species. One or two species in each study were associated with upland conditions. Total seed bank species richness was similar between 1998 and 1999 for both the DS and the RS.

Six state-listed species were recorded in the DS seed bank in 1998 and eight were recorded in 1999 (Table 2.1). These figures were similar to state-listed, extant species richness recorded in the DS in 1999 and 2000 (Figure 2.3). Only five state-listed species were recorded in the RS seed bank studies (Table 2.1). This is marginally fewer than state-listed, extant species richness in the RS (Figure 2.4). Sampling intensity may not have been adequate to sample rare species within the RS. Nine different state-listed species were observed in the DS seed bank surveys, however. Only six different state-listed species were observed in the RS seed bank surveys.

Aggressive species richness for seed bank studies varied little between years or sites (Table 2.1). Six (1998) and five (1999) aggressive species were
recorded in the DS seed bank studies. Five aggressive species were recorded in both of the RS seed bank studies. Approximately twice as many aggressive species were recorded in each of the extant vegetation surveys as were observed in each of the seed bank studies (Figures 2.3 and 2.4). This was true in both the DS and the RS. Only extant vegetation data from the 1999 DS deviated from this pattern. Aggressive species richness for this study was equivalent to aggressive species richness in the 1998 and the 1999 DS seed bank studies. These results suggest that disturbance activities have had little effect over the long term on aggressive species richness in the seed bank.

Seed bank species composition

Seed bank species composition did not vary dramatically between years or between sites (Table 2.1). In total, 24 species were common to both sites for both years that seed bank samples were collected. Most of these species were the same species that were present consistently in the extant species surveys (Table 2.1). Only three species, *Eupatorium maculatum* L., *Myriophyllum spicatum* L., and *Najas gracillima* (A. Braun) Magnus., were present in the seed bank and not recorded in the extant vegetation. *Eleocharis obtusa*, *Najas minor*, and *Polygonum pensylvanicum* L. were the only seed bank species absent from the 1999 and 2000 vegetation surveys that were present in McCormac's extant...
vegetation records. Seed bank species richness and composition, though less than extant species richness and composition, were relatively uniform across the DS and the RS despite disturbance activities.

**Phragmites** stem density

While mean values were slightly higher in 1999 for both sites, *Phragmites* stem density did not differ between years for either the DS or the RS (Table 2.2). Similarly, *Phragmites* stem density did not differ between sites in either 1999 or 2000 (Table 2.3). If disturbance activities initially reduced *Phragmites* stem density within the DS, these results suggest that *Phragmites* stem density has returned to pre-disturbance levels. There is some evidence that *Phragmites* stem density actually may be declining in both sites (Tables 2.2 and 2.3).

**Species density**

Species density did not differ among years within each site (Table 2.4). There also were no differences in species density between sites in 1999 or 2000 (two sample t-test, $P>>0.05$; Table 2.4). Because there was little difference in species density between years and sites, these results suggest that species density also returned to pre-disturbance levels.
Phragmites stem density and species density

Phragmites stem density was negatively correlated with species density in the DS in 1999 and 2000 (Table 2.5). Phragmites stem density explained 70% and 34% of the variation in species density data in 1999 and 2000, respectively. Phragmites stem density was unrelated to species density in the RS. While there was no difference in Phragmites stem density or species density between sites, Phragmites stem density seems to have had a negative impact on species density in the DS but not in the RS. The strength of this relationship seems to be declining.

Discussion

Species richness

McCormac (1994) reported that substrate disturbance enhanced species richness, particularly state-listed species richness, in an East Harbor marsh. Disturbance simultaneously reduced the abundance of Phragmites, the dominant plant, within the disturbance site (DS). There is little doubt that Phragmites abundance was reduced directly following disturbance given that several cm of substrate were removed from the site (McCormac, 1994). Variation in species richness between 1991 and 1994, however, was within the range previously recorded for this site (Stuckey, 1988). Because of this, it is difficult to discern upon closer inspection of the data if any increase in species richness following
the disturbance was a result of substrate manipulation, a response to fluctuating water levels, a result of increased water presence, or a combination of all three factors.

The results from this study suggest that all three mechanisms were responsible for species richness patterns. Harris and Marshall (1963), van der Valk and Davis (1978), Keddy and Reznicek (1982 and 1986), and Wilcox and Meeker (1991) emphasized the important role that low water years play in regeneration of wetland plant species, and the role high water years play in reducing the abundance of dominant species.

Substrate disturbance in this study affected the regeneration of wetland species and reduced the abundance of dominant species. Substrate disturbance reduced the average elevation within the DS, thereby increasing the influence of hydrology on the plant community, and created gaps in Phragmites stands. A culvert connecting the DS with East Harbor provided a source of water, allowing water levels within the site to fluctuate with the harbor. Thus, all three mechanisms were a part of any changes that may have occurred after disturbance.

While the number of species may not have increased significantly after disturbance, the proportion of wetland species increased and was maintained between 1992 and 1994. Thus, substrate disturbance played a part in promoting, or, at least, maintaining wetland species richness within the DS.
Results from the 1999 and 2000 surveys indicate that this single disturbance event did not adequately maintain species richness over the subsequent eight-year period. Species richness in the DS was much lower in 1999 and 2000 than in 1992-1994. Consequently, more frequent disturbance events may be necessary to maintain species richness in the long term. As Keddy and Reznicek (1986) summarized, there is a very poor understanding of how frequent and intense disturbance events need to be to maximize diversity in a community despite an understanding among ecologists that disturbance is an integral part of plant community dynamics. In this particular wetland community, substrate disturbance or a similar technique may be necessary every 4-7 years to maintain plant diversity.

Alternatively, the decline in total species richness in 1999 and 2000 may be attributed to declining water levels. Mean Lake Erie water levels for June declined in 1999 to nearly the mean site elevation for the DS. At and below this level, drier soil conditions prevail, discouraging the presence of obligate wetland species and encouraging the presence of facultative and obligate upland species (USFWS, 1996). In fact, the number of wetland species declined in the DS while the presence of upland species increased in 1999 and 2000. But, these results do not account satisfactorily for the decline that occurred in total species richness between 1994 and 1999 in the DS. With a decline in water levels of this nature, one would expect the species composition to shift from species adapted to inundation to species adapted to mudflat and moist soil conditions. Species
 richness would not necessarily decline. In fact, species richness typically increases following a decline in water levels in freshwater wetlands (Keddy and Reznicek, 1982 and 1986, Stuckey, 1988). Further, plant species were present in the RS and DS seed bank surveys that could germinate in 1999 site conditions. Seed sources for these species exist in the extant vegetation in both sites. Factors other than declining water levels seem to be responsible for the decline in species richness observed between 1994 and 1999 in the DS.

Substrate disturbance and increased hydrologic activity did little to change the number of state-listed species found in the DS. State-listed species richness remained stable in the DS following disturbance. Additionally, state-listed species richness was the same in the DS and in the RS. These trends suggest that environmental conditions resulting from disturbance activities in the DS adequately promoted and maintained species richness equally as well as existing conditions in the RS. The RS is larger than the DS and has greater variation in elevation. If management objectives are aimed at increasing state-listed species richness in the East Harbor wetland, conditions in the DS may serve as a model for the RS.

Unfortunately from a management standpoint, aggressive species richness also was uniform among study years and did not change from its pre-disturbance value. In fact, aggressive species richness was at its lowest in the most recent surveys (1999 and 2000) in both the DS and the RS. Substrate disturbance, then, may have encouraged or, at least, maintained aggressive
species richness, in addition to fostering state-listed species richness. Brown and Bedford (1997), Havens et al. (1997), and Galatowitsch et al. (1999) also related human-induced substrate disturbance to the proliferation of aggressive wetland species, particularly *Typha angustifolia* L., *Phragmites*, and *Lythrum*. Managers using substrate manipulation as a wetland restoration technique, therefore, run the risk of promoting the same undesirable species they may be trying to control.

**Species composition**

Species richness, of course, does not address changes in composition that occur in a plant community. Indeed, the results from this study indicate that species composition varied more dramatically than species richness, particularly in the DS. While several species that existed in 1991 were not recorded in any of the post-disturbance surveys, many species were observed in the post-disturbance surveys that were absent in 1991. Most of these species were wetland species, indicating that wetland conditions were enhanced or, at least, maintained after disturbance. It is not equivalent, of course, to compare a single year's data to three year's data. But, this comparison illustrates the point that environmental conditions in the DS resulting from substrate disturbance were conducive to the regeneration of a wide variety of plant species. The variability in species composition over the years also indicates that disturbance activities
produced gaps in the vegetation suitable for colonization, and emphasizes the role hydrology plays in dictating which species germinate under a given set of conditions (van der Valk, 1981).

The role gap formation and hydrology play in fostering variation in species composition is further emphasized by the fact that there was greater variation in species composition in the DS than in the RS between 1999 and 2000. Most species absent from the RS but observed in the DS were wetland species. These patterns were observed despite the fact that species richness was greater in the RS than in the DS. Again, the RS is much larger than the DS. The elevation also is more heterogeneous in the RS. Additionally, hydrology was less of a factor in the RS than in the DS because the mean elevation in the RS was well above Lake Erie water levels during this period. The mean elevation of the DS was below these water levels. The reduction in site elevation resulting from substrate manipulations seems to have been an important factor determining species composition in the DS. More species were lost from species lists between the McCormac study and the 1999 and 2000 surveys in the DS than were gained, however. Coupled with declining species richness, these results indicate that disturbance activities have been too infrequent to maintain species richness and composition over an eight-year period.

Following disturbance, several state-listed species were observed for the first time. Only one state-listed species was not observed again after 1991. Additionally, more variation in the number of state-listed species was observed in
the DS than in the RS. All of the state-listed species found throughout the RS in 1999 and 2000 were also present in the DS. Given this overlap, disturbance activities in the DS seemed to have provided conditions suitable to the growth and establishment of a variety of state-listed species, in both time and space.

The same holds true for aggressive species composition. More new aggressive species were gained after the disturbance than were lost. It is important to note that most of these species were first found during the McCormac study and have persisted over time. Additionally, a greater variation in aggressive species was observed over time in the DS than in the RS. This is not surprising since aggressive species are defined, in part, by their ability to colonize disturbed sites (Galatowitsch et al., 1999). Once again, substrate disturbance and hydrology combined to encourage a variety of species to coexist in time and space. In this case, however, the presence of a number of aggressive species has been encouraged.

**Seed bank species richness and composition**

Unlike seed banks in many other freshwater wetlands (van der Valk and Davis, 1978; Leck and Graveline, 1979; Keddy and Reznicek, 1982 and 1986), seed bank species richness was lower than extant species richness in both the DS and the RS. This was true for total, state-listed, and aggressive species richness. In fact, only three species were observed in seed bank studies and not in the extant vegetation. Additionally, seed bank species composition did not
vary between the RS and the DS. Because variation in extant species composition was large between 1991-2000, one might assume that the seed bank was rich enough to provide for this variability. Unfortunately, no seed bank data exist for the McCormac study to say for certain. Nevertheless, if the seed bank was once rich, it no longer is.

Interspecific interactions, however, could have limited the number of species germinating from seed bank samples. It is widely accepted, for instance, that the decay of *Phragmites* litter releases organic acids that interfere with germination and growth of other species (van der Valk and Davis, 1976; Cizkova et al., 1999). Similarly, *Phragmites* litter reduces soil temperature and incident light, which further reduce germination rates of seeds (Haslam, 1971). With time, seed buried in the soil or under litter succumb to decay and predation, reducing seed stores (Leck, 1989). These processes undoubtedly have had some effect on seed bank richness and abundance over the past 30 years.

But this does not fully explain the relative paucity of the seed bank compared to the extant vegetation. McCormac (1994) noted that many of the species in the DS were present in small, isolated populations. Recent observations concur with this statement. These species may not have been included in substrate samples because their seeds were not evenly distributed throughout the site.
Additionally, Leck (1989) noted that seed bank methods such as those used in this study do not provide the germination conditions required by all species. Seed germination requires select temperature, light, and moisture conditions specific to each species. Certain species have wider tolerances than other species, and, thus, will germinate under a broader set of conditions. It is, therefore, possible that my methods did not provide a wide enough range of conditions to capture the entire array of seed bank species present in the DS and the RS. This may be true particularly for facultative and obligate species.

Despite its paucity, seed bank species richness in both the DS and the RS was greater than that for other freshwater wetlands (van der Valk and Davis, 1978; Leck and Graveline, 1979; Keddy and Reznicek, 1982 and 1986). While the East Harbor marsh can be considered a floristically diverse wetland, current trends indicate that the seed bank of this marsh may play a limited role in management plans designed to increase plant diversity.

*Phragmites abundance*

Qualitative, visual assessment of *Phragmites* abundance conducted in the DS during each site visit throughout the study period suggested that *Phragmites* was colonizing stands where it had been eliminated by disturbance. Concurrently, total species richness has declined in recent years from values
recorded by McCormac. Given the aggressive nature of *Phragmites*, the decline in species richness would seem to be associated with the perceived increase in *Phragmites* abundance.

Results from this study indicate, however, that *Phragmites* stem density did not change between 1999 and 2000 in either the DS or the RS. Though not statistically significant, mean *Phragmites* stem density actually declined over this period in both sites. Even more puzzling was the fact that *Phragmites* stem density did not differ between the DS and the RS for either study year.

At least one explanation may account for the inconsistency between visual assessments and actual quadrat counts. *Phragmites* rhizomes may be expanding into open sites in some areas, while they decline elsewhere. Haslam (1972) and Meyerson et al. (2000) noted that *Phragmites* stem density may reach a maximum determined by environmental conditions and genetic variation in a given site (Neuhaus et al, 1993). In a related sense, it is plausible that *Phragmites* stem densities are equilibrating in the DS and the RS. In other words, *Phragmites* stem density has reached its optimum at similar values in both sites. If this were occurring, the mean *Phragmites* stem density between years would be the same (which it is) and the variation among quadrats would decline. Indeed, the variation in *Phragmites* stem density data declined between years, suggesting that this argument is reasonable. Additionally, *Phragmites* was present in more quadrats in 2000 than in 1999, suggesting that *Phragmites*
rhizomes have expanded throughout the marsh. It seems likely, then, that
*Phragmites* stands have been expanding throughout the marsh while maintaining
a maximum stem density characteristic of the entire site.

*Phragmites* abundance and species density

Results from this study indicate that *Phragmites* stem density had a
negative impact on species density but only in the DS. *Phragmites* stem density
in the DS was strongly and negatively related to species density, particularly in
1999. *Phragmites* stem density was not related to species density in the RS.
These results may explain the decline in species richness observed over time in
the DS and explain the differences in species richness that exist between the DS
and the RS. These results also may account for the lack of a significant
relationship between Lake Erie water levels and species richness measures in
the DS. They also support the argument that the long-term presence of
*Phragmites* in East Harbor may have negatively affected seed bank species
richness. State-listed species and other aggressive species observed could
have coexisted with *Phragmites* during this period by occupying areas where
*Phragmites* was absent or stressed. These results also suggest that the
negative relationship existing between *Phragmites* stem density and species
density in the DS may be waning. This argument is supported by results
indicating that *Phragmites* stem density did not change, or may have declined,
If *Phragmites* stem density did not differ between sites in each year and species density also did not differ between sites, how can there be a significant negative relationship between *Phragmites* stem density and species density in the DS and not in the RS? *Phragmites* thrives in moderately and intermittently flooded environments. In these conditions, *Phragmites* stem height and diameter are greater than they are in stressed conditions (Haslam, 1972). Stem density does not necessarily increase in ideal environmental conditions. *Phragmites* biomass (live standing crop), therefore, can increase between years even though the number of stems remains the same. *Phragmites* biomass has been show to be negatively related to species density and better represents the plant's capacity to monopolize available resources than stem density (Lenssen et al., 1999; Welch, Chapter 3, 2001).

It is, therefore, possible that *Phragmites* biomass was greater in the DS than in the RS. The reduced elevation in the DS and declining water levels in 1999 and 2000 provided optimal conditions for *Phragmites* growth and expansion. Site conditions in the RS have been largely sub-optimal for this same period. One would expect *Phragmites* stems to be more robust in the DS than in the RS as a result. More robust stems would, in turn, have a greater impact on the surrounding vegetation. While the relationship between *Phragmites* biomass and species density was not explored in this analysis, the effects of *Phragmites* standing crop are addressed in Chapter 3.
In conclusion, McCormac's (1994) study was designed to test the practical application of disturbance theory in a freshwater marsh on the southern shore of Lake Erie, a marsh that has been disconnected from its original hydrologic disturbance regime. This wetland is known historically for its plant species diversity. Disturbance theory suggests species diversity is fostered and maintained by intermediate levels of disturbance and stress, intermediate in both frequency and intensity (Connell, 1978; Grime, 1979; Huston, 1979; Pickett and White, 1985; Keddy and Reznicek, 1982 and 1986). Stress and disturbance ultimately limit any particular species from dominating a given community. In freshwater marshes, disturbance takes the form of ice scour, wave action, sediment accretion and erosion, and periodic inundation. In the case of East Harbor, McCormac's intent was to replicate these actions using a bulldozer to scrape and turn the soil, thereby creating gaps in the existing vegetation. Presumably this activity would limit the dominant species, *Phragmites australis*, and simultaneously recruit species from the soil seed bank. While soil manipulation provided conditions suitable for the recruitment and maintenance of several species, both state-listed and aggressive were affected equally. This may present a conflict of interest for wetland managers, requiring them to implement additional control techniques to suppress newly established, aggressive species. Further, recent vegetation and seed bank surveys suggest that this single disturbance did not adequately maintain extant species richness and composition, promote an increase in seed bank species richness and
composition, or limit the spread of *Phragmites* in the long term. Post-disturbance colonization and establishment of *Phragmites* in the DS may have led to the decline in extant and seed bank species richness observed. The abundance of *Phragmites* and its associated impact on species richness seem to be waning with current low water levels.

**List of References**


Ohio Department of Natural Resources, Division of Natural Areas and Preserves. 1999. Rare native Ohio plants: 1998-1999 status list. Columbus, Ohio.


Table 2.1: List of plant species recorded for each extant and seed bank (SB) survey at East Harbor State Park, Ohio. Data recorded in the disturbance site (DS) and throughout the remainder of the restoration site (RS) are presented. Wetland indicator status (WIS) follows USFWS (1996) for the Northeast Region and definitions for estimated probabilities of occurrence in wetland habitats, where OBL= obligate wetland (99%), FACW= facultative wetland (67-99%), FAC= facultative (34-66%), FACU= facultative upland (1-33%), UPL= obligate upland (0-1%), NL= no indicator, and NL= not listed. The "+" indicates a frequency towards the higher end of the category. The "-" indicates a frequency towards the lower end of the category.
Table 2.1: (continued)

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<tr>
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<tr>
<td>Typha argutifolia*</td>
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<td>X</td>
<td>X</td>
<td>X</td>
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</tr>
<tr>
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<td>X</td>
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</tr>
<tr>
<td>Vicia sativa</td>
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<td>X</td>
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<tr>
<td>Xanthium strumarium</td>
<td>FACU</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
</tbody>
</table>

Species not listed as rare but were at time of McCollum (1994) study:

*Species considered non-native, aggressive species in Ohio.

**Species listed in Ohio as threatened, rare, or endangered.
<table>
<thead>
<tr>
<th>Site and Year</th>
<th>N</th>
<th>Mean (± SE)</th>
<th>T-statistic</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>DS 1999</td>
<td>16</td>
<td>23.3 (± 5.6) *</td>
<td>1.56</td>
<td>0.13</td>
</tr>
<tr>
<td>DS 2000</td>
<td>16</td>
<td>13.0 (± 3.2) *</td>
<td>-0.67</td>
<td>0.51</td>
</tr>
<tr>
<td>RS 1999</td>
<td>51</td>
<td>28.0 (± 4.2) *</td>
<td>1.69</td>
<td>0.094</td>
</tr>
<tr>
<td>RS 2000</td>
<td>55</td>
<td>19.7 (± 2.6) *</td>
<td>-1.56</td>
<td>0.13</td>
</tr>
</tbody>
</table>

Table 2.2: Comparison between years within each site of the mean (± one standard error) *Phragmites* stem density (stems/m²). Data were collected in both the disturbance site (DS) and the remainder of the restoration site (RS) in East Harbor State Park, Ohio, in 1999 and 2000. Within each site, different letters represent a significant difference between years (two-tailed t-test, $\alpha=0.05$).

<table>
<thead>
<tr>
<th>Site and Year</th>
<th>N</th>
<th>Mean (± SE)</th>
<th>T-statistic</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>DS 1999</td>
<td>16</td>
<td>23.3 (± 5.6) *</td>
<td>-0.67</td>
<td>0.51</td>
</tr>
<tr>
<td>RS 1999</td>
<td>51</td>
<td>28.0 (± 4.2) *</td>
<td>1.69</td>
<td>0.094</td>
</tr>
<tr>
<td>DS 2000</td>
<td>16</td>
<td>13.0 (± 3.2) *</td>
<td>-1.56</td>
<td>0.13</td>
</tr>
<tr>
<td>RS 2000</td>
<td>55</td>
<td>19.7 (± 2.6) *</td>
<td>-1.56</td>
<td>0.13</td>
</tr>
</tbody>
</table>

Table 2.3: Comparison between sites for each year of the mean (± one standard error) *Phragmites* stem density (stems/m²). Data were collected in both the disturbance site (DS) and the remainder of the restoration site (RS) in East Harbor State Park, Ohio, in 1999 and 2000. For each year, different letters represent a significant difference between sites (two-tailed t-test, $\alpha=0.05$).
<table>
<thead>
<tr>
<th>Site and Year</th>
<th>N</th>
<th>Mean (± SE)</th>
<th>T-statistic</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>DS 1999</td>
<td>16</td>
<td>10.6 (± 1.3)</td>
<td>0.46</td>
<td>0.650</td>
</tr>
<tr>
<td>DS 2000</td>
<td>16</td>
<td>10.2 (± 1.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RS 1999</td>
<td>51</td>
<td>9.2 (± 0.6)</td>
<td>-1.06</td>
<td>0.290</td>
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<tr>
<td>RS 2000</td>
<td>55</td>
<td>10.0 (± 0.5)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.4: Comparison between study years of the mean (± one standard error) species density (species/m²). Data were collected in both the disturbance site (DS) and the remainder of the restoration site (RS) in East Harbor State Park, Ohio, between 1999 and 2000. Within each site, different letters represent a significant difference between years (two-tailed t-test, α=0.05).

<table>
<thead>
<tr>
<th>Study and Year</th>
<th>Predictor</th>
<th>Response</th>
<th>F-statistic</th>
<th>P-value</th>
<th>Correlation</th>
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</thead>
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<td>stem density</td>
<td>species density</td>
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<td>-0.839</td>
</tr>
<tr>
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<td>species density</td>
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<td>0.017*</td>
<td>-0.586</td>
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<tr>
<td>RS 1999</td>
<td>stem density</td>
<td>species density</td>
<td>2.58</td>
<td>0.115</td>
<td>-0.224</td>
</tr>
<tr>
<td>RS 2000</td>
<td>stem density</td>
<td>species density</td>
<td>1.74</td>
<td>0.192</td>
<td>-0.179</td>
</tr>
</tbody>
</table>

Table 2.5: Relationship (linear regression) between species density (species/m²) and *Phragmites* stem density (stems/m²). Data collected between 1999-2000 in East Harbor State Park, Ohio are presented for both the disturbance site (DS) and the remainder of the restoration site (RS). "Correlation" is the correlation coefficient (r), indicating the directional nature of the relationship (positive or negative) as well as the fit of the data to the line. **" indicates that the slope of the regression line is significantly different than zero.
Figure 2.1: Site locations for the disturbance site (DS) and the remainder of the restoration site (RS) in East Harbor State Park, Ohio. Note that the scale is approximate.
Figure 2.2: Elevation profiles for all transects (T1 to T18) surveyed in the East Harbor State Park, Ohio, restoration site. Data for both the disturbance site (DS; dotted lines) and the remainder of the site (RS; solid lines) are presented. Transects progress in chronological order from southern end to the northern end of the marsh (Figure 2.1), running across its width. Along each transect, quadrats progress in chronological order from the harbor side to the lake side of the marsh. Data are based on existing elevation data points measured by the Ohio Department of Natural Resources, Geologic Survey Division, field measurements taken with an autolevel in 2000, and Lake Erie water level data (USACE, 2001). The thick, solid line represents the 100-year mean Lake Erie water level for June (USACE, 2001).
Figure 2.3: State-listed, aggressive, and total species richness recorded in the disturbance site (DS), East Harbor State Park, Ohio. Data from the McCormac (1994) study and the current study (1999-2000) are illustrated. Mean June Lake Erie water levels are also depicted for each study year.
Figure 2.4: State-listed, aggressive, and total species richness recorded in the remainder of the restoration site (RS) outside the disturbance site (DS), East Harbor State Park, Ohio. Only data from the current study (1999-2000) were available for this site. Mean June Lake Erie water levels are also depicted for each study year.
Figure 2.5: Plant indicator status relative to mean June Lake Erie water levels for vegetation data recorded in East Harbor State Park, Ohio. Data from all available years are depicted for the disturbance site (DS). Wetland indicator status follows USFWS (1996) definitions for estimated probabilities of occurrence in wetland habitats, where OBL = obligate wetland (99%), FACW = facultative wetland (67-99%), FACU = facultative upland (1-33%), UPL = obligate upland (0-1%). FACW and FACU categories include both "+" and "-" designations.
Figure 2.6: Plant indicator status relative to mean June Lake Erie water levels for vegetation data recorded in East Harbor State Park, Ohio. Data from all available years are depicted for the remainder of the restoration site (RS). Wetland indicator status follows USFWS (1996) definitions for estimated probabilities of occurrence in wetland habitats, where OBL = obligate wetland (99%), FACW = facultative wetland (67-99%), FACU = facultative upland (1-33%), UPL = obligate upland (0-1%). FACW and FACU categories include both "+" and "-" designations.
CHAPTER 3

FLORISTIC DIVERSITY AND THE ABUNDANCE OF PHRAGMITES AUSTRALIS IN THREE PROXIMATE LAKE ERIE MARSHES

Abstract

This study investigates the relationship between abundance of Phragmites australis and species density in three freshwater, non-tidal marshes located on the southern shore of Lake Erie. Species composition, stem density, and stem height were measured in June and August 2000 for each species in each quadrat. Plant measurements and above-ground biomass samples were used to estimate standing crop. For comparative purposes, total standing crop was also related to species density. Elevation as a dominant abiotic factor in wetlands was then related to each of these variables. Samples with and without Phragmites also were compared. Diversity indices other than species density were considered as well.

All measures of species diversity were lowest in the site (Site A), which contained the highest mean (± 95% confidence interval) Phragmites standing crop (1742 ± 338 g/m²). Phragmites standing crop was significantly lower
(α=0.05) and species density significantly higher (α=0.05) in the other two sites (Sites R and B). Total standing crop and Phragmites standing crop were negatively correlated (p<0.05) with species density in the two sites (Sites R and A) with the highest Phragmites standing crop values. These relationships explained <27% of the variation in species density, however. Only Phragmites standing crop in Site A accounted for a reasonable amount (59%) of the variation in species density data. Species density did not differ between quadrats with and without Phragmites for any of the three sites. Additionally, elevation was not significantly related to standing crop or species density (p>0.05), though heterogeneous basin morphology was observed in the most species rich site (Site R). Elevation was highly uniform in the other two sites. Nutrient availability, stress and disturbance, and topographic heterogeneity are suggested as key factors determining species diversity and Phragmites abundance in Lake Erie freshwater, non-tidal marshes. Future research and management efforts should target these factors to control aggressive species and foster diversity rather than focusing resources on control of a single species.
Introduction

Aggressive species have become dominant components of Lake Erie marsh plant communities over the past 100 years. Concurrently, species numbers have waned in Lake Erie marshes that were once known for their floristic diversity (Herdendorf, 1987). Rapid colonization of these marsh ecosystems by aggressive species has coincided with an increase in anthropogenic disturbance (Galatowitsch et al., 1999). Hydrologic patterns within wetlands have been disrupted and shorelines have been modified and reinforced. In addition to habitat reduction and direct physical changes, development also has led to nutrient enrichment and reduction of seed sources. In general, disruption of the ecological parameters to which Lake Erie flora have become adapted apparently has created circumstances advantageous to the proliferation of aggressive species.

*Phragmites australis* (Cav.) Trin. ex Steud. is one of the most prevalent of these aggressive colonizers. Native to North America (Tucker, 1990), *Phragmites* has expanded throughout the Great Lakes basin over the past century from historically isolated populations (Moseley, 1899; Pieters, 1901; Moore, 1973). Once established, *Phragmites* is capable of forming monodominant stands. Culm heights can reach four meters and stem densities have been noted in excess of 200 culms/m² (Haslam, 1971). Limited germination and growth of other plant species within reed stands is attributed to reduced light levels, limited nutrients, restricted space, and accumulated litter.
layers (Haslam, 1972; Marks et al., 1994; Cizkova et al., 1999). Consequently, *Phragmites* has been linked to diversity loss in Lake Erie freshwater marsh ecosystems (McCormac, 1994; Galatowitsch et al., 1999).

While *Phragmites* may be the most studied wetland plant in the world (Brix, 1999), the vast majority of information pertaining to the recent decline of *Phragmites* stands in Europe. Published information that does exist on the expansion of *Phragmites* in North America mostly pertains to salt marshes of the East Coast. There is little reference to *Phragmites* dynamics in freshwater, non-tidal marshes (Meyerson et al., 2000). Furthermore, there are few published studies addressing the relationship between *Phragmites* abundance and species diversity (richness and abundance) within and among freshwater wetlands in North America. Much of the information available concerning the effects of *Phragmites* on diversity in the United States is anecdotal (Marks et al., 1994). It is this type of information that is currently being used as the basis for *Phragmites* eradication and control efforts, most of which have met with limited success (Marks et al., 1994).

This study compares measures of plant species diversity among three proximate Lake Erie freshwater marshes and relates species density (number of species/unit area) to the abundance (standing crop) of *Phragmites* present in
each site. Gross levels of *Phragmites* abundance vary within and among sites as does the degree of natural and anthropogenic disturbance affecting the associated plant communities.

**Methods**

**Site descriptions**

The three sites surveyed in this study are located in East Harbor State Park, Ottawa County, Ohio, along the northern edge of the Lake Erie Marblehead peninsula (41°33'N, 82°48'W) (Figure 3.1). The Park has a temperate climate with a growing season that extends from mid-April to early September.

East Harbor State Park includes two harbors, East Harbor and Middle Harbor, separated by a causeway built in 1945. The surface hydrology of the two harbors is linked via a 2 m diameter culvert. Both harbors are separated from Lake Erie by a sand bar formed by lateral shoreline drift. Dredge material removed from East Harbor in 1967 was deposited on the harbor-side of the East Harbor sand bar, tripling the width of the sand bar. Middle Harbor, however, is a wildlife sanctuary and has experienced minimal anthropogenic disturbance. East Harbor is connected directly to Lake Erie by a boat channel cut through the sand bar. Middle Harbor is connected to Lake Erie indirectly by its connection to East Harbor. As a result, East Harbor and Middle Harbor both are subject to wave exposure, seiche activity, and water level fluctuations characteristic of the Lake Erie disturbance regime. The average depth of both harbors is < 2 m.
Site R is a 6.4 ha rectangular, freshwater, non-tidal marsh created from dredge spoils taken from East Harbor in 1967 and placed along the interior of the sand bar (Moore, 1973). The dredge material is predominantly sand. The site is separated from the harbor's surficial hydrology by an earthen dike except for a standing pipe at the northwest corner and a culvert at the southwest corner of the wetland. Both pipes are less than 1 m in diameter. At least a partial groundwater connection between the marsh and the harbor is suspected to exist (S. Mackey, ODNR Geologist, pers. comm.). Pockets of standing water exist in the marsh prior to July in low water years, and throughout the year in high water years. However, portions of the marsh are never inundated. The mean elevation of the site is 174.59 m (IGLD, 1985), ranging from 173.94 to 175.17 m. The substrate of 0.6 ha of Site R at the southern end was mechanically disturbed in 1992 by using a low ground pressure bulldozer. Sporadic mowing also has occurred throughout the site except in the disturbed substrate area. A nature trail runs parallel to and inside the dike wall. Although several aggressive species are present, *Phragmites* has formed monodominant stands throughout the marsh over the past 30 years. *Phragmites* stands are more robust in Site A than they are in Site R. Plans have been made to restore the natural hydrology to Site R in an attempt to reduce the abundance of *Phragmites* and enhance floristic diversity.

Site A is a 7.5 ha marsh located in East Harbor on the harbor-side of the sand bar and south of Site R. Site A is directly open to hydrologic interaction
with East Harbor except for a pile of rip-rap 20 m long and 1 m wide on the southern lip of the opening to the marsh. The rip-rap is submersed during seiche and storm events and was covered by water during the 1998 field season. The mean elevation of the site is 173.90 m (IGLD, 1985), ranging from 173.59 to 174.09 m. *Phragmites* has formed a vigorous, mature stand over the past 30 years across most of the site except for localized populations of *Typha angustifolia* L.

Site B is a 3 ha marsh located in the northwest corner of Middle Harbor. Site B is completely open to the harbor. The mean elevation of the site is 174.10 m (IGLD, 1985), ranging from 174.05 to 174.14 m. This site is fringed by sparse stands of *Phragmites* but was dominated by *Populus deltoides* Marshall., *Salix* spp. saplings, and mudflat annuals during this study. *Populus* and *Salix* species emerged following a drawdown in 1999. Woody species and mudflat conditions have persisted for >2 years. Site B is completely inundated during high water years.

**Vegetation surveys**

Vegetation surveys were conducted at each site in early June and mid-August 2000. A baseline was established along the nature trail in Site R, whereas baselines were established along the shoreline in Sites A and B. Each baseline was divided into 50-m intervals and transects established perpendicular to this baseline. One transect was located randomly within each interval. One
1 x 1 m quadrat was placed every 25 m along each transect. The compass direction for each transect was recorded. Within each quadrat, plant species were identified, stems counted, percent cover estimated, and average stem height noted for all species. Taxonomic nomenclature follows Gleason and Cronquist (1991). Percent cover was estimated to the nearest 5% increment. Cover estimates exceeded 100% in some cases because of the presence of multiple plant strata. Water depth and distance to the shoreline also were recorded. Elevations were measured using an auto-level and calibrated with both Lake Erie water levels and existing survey points established by the Ohio Department of Natural Resources, Geologic Survey Division. Elevation data were substituted for the inverse of water depth because water depth data were lacking from many quadrats as a result of declining Lake Erie water levels.

**Estimates of standing crop**

While stem density and percent cover are often used as measures of abundance, Magurran (1988) and Krebs (1999) have suggested that biomass measurements are more direct measures of resource use. Measurements such as standing crop also are more readily comparable across taxonomic levels and better emphasize the importance of a species in a community than count data (see also Hurlbert, 1971). Gaudet and Keddy (1995) pointed out that standing crop integrates both biotic factors such as competition and abiotic factors such as stress, disturbance, and resource availability within a community (see also
Grime, 1979 and Day et al., 1988). Standing crop is, therefore, a good predictor of variation in species richness and composition across communities (Lenssen et al., 2000). It is by this rationale that standing crop was used to estimate abundance for each species and used to calculate diversity indices.

Ten stems of each species were harvested at ground level adjacent to five random quadrats within each site. This technique ensured that representative samples were collected while avoiding harvesting samples from within quadrats. A minimum of 50 stems/species was harvested in both June and August surveys. A minimum of 100 stems/species was harvested for species <15 cm tall. In the case of rare, threatened, or endangered species, a species with similar growth form and size was used as an estimator. The height of each stem was recorded, and the specimens were bagged by species in paper sacks. Specimens were dried to a constant weight at 70°C and then weighed. Stem weights were classified by height for each species. A mean weight was calculated for each height class. The mean weight was divided by the height to obtain a height-adjusted weight (g/cm) for each species. The corresponding height-weight ratio was multiplied by the average height measured in the field for each species. Standing crop (SC) was estimated by multiplying this number by the stem density of each species within a given quadrat. This estimate was calculated for all species in all quadrats and is site-specific.
Data analysis

Continuing debate ensues over the use of diversity indices (Magurran, 1988; Krebs, 1999). Despite this debate, their use has become a convention such that inclusion is often necessary for comparisons with other studies. In this study, species density, the Shannon-Wiener function \( (H') \), and Simpson’s index \( (D) \) were calculated using quadrat data. The equations for Shannon’s and Simpson’s indices are, respectively:

\[
H' = \sum p_i \ln p_i \quad \text{and} \quad D = \sum p_i^2, p_i.
\]

In each equation, \( p_i \) represents the proportion of species \( i \) of the total sample. Both Shannon’s index and Simpson’s index are nonparametric measures of heterogeneity (species richness and evenness inclusive) that do not make assumptions about the shape of the species-abundance curves (Krebs, 1999). Shannon’s index emphasizes the role of rare species while Simpson’s index emphasizes common species.

Hill’s diversity indices, \( N_1 \) and \( N_2 \), were also calculated using the following equations:

\[
N_1 = e^{H'} \quad \text{and} \quad N_2 = D^{-1}.
\]
Both $N_1$ and $N_2$ are in units of species and reflect the number of abundant and very abundant species, respectively, found in a community (Krebs, 1999). A modified Hill's ratio (MHR) also was calculated to estimate evenness, where:

$$MHR = (N_2-1)(N_1-1)^{-1}.$$ 

MHR approaches zero as any one species dominates a community and is unaffected by species richness (Ludwig and Reynolds, 1988).

Statistical comparisons of diversity indices are even more controversial than the indices themselves. The variance associated with a given diversity index depends on the value of the index (Fritsch and Hsu, 1999). Variances of indices compared among sites are thus unequal. A comparison of the means and variances of these indices (e.g.—ANOVA), then, violates critical statistical assumptions of equal variances and normality. Further, there are no transformations that can be performed to rectify this disparity (J. Hsu, OSU statistician, pers. comm.). Replication and pseudo-replication (sensu Hurlbert, 1984) are ineffective as well.

There are, however, customized statistical techniques that have been developed for use with the Shannon-Wiener index and Simpson's index that allow multiple comparisons (Fritsch and Hsu, 1999). Similar techniques do not
exist for other diversity indices. Simple mathematical operations, such as a reciprocal Simpson’s index, complicate statistical models immensely (J. Hsu, OSU statistician, pers. comm.).

Hill’s diversity and evenness values are presented herein without the associated variance calculations because they are more intuitive to the ecologist than many other diversity indices (Ludwig and Reynolds, 1988).

Regression analysis was performed on standing crop (SC) data to identify relationships between total SC and species density, and Phragmites SC and species density for each site. Standing crop data were natural log-transformed to adjust for normality (Krebs, 1999). Linear relationships also were established between elevation and species density, and between elevation and SC data for each site. Two-tailed t-tests were used to compare data for quadrats with and without Phragmites within each site. In all cases, 95% confidence intervals (95% CI) were used to distinguish significantly different data sets.

Results

Gross differences among sites

Total species richness differed among all three sites (Table 3.1). Total species richness in Site R was double that of the other two sites, although it was intermediate in total area. Most species observed were classified as wetland indicator plants (USFWS, 1996); 59%, 83%, and 71% for Sites R, A, and B, respectively.
Species density (number of species/m²) differed significantly among all sites (two-tailed, \( t \)-test; \( p = 0.000, F = 68.12, df = 155 \)). Site R had the highest mean species density while Site A had the lowest (Table 3.2; 95% CI). Species densities were similar for Sites A and B.

Total SC in Site R was less than half the mean total SC calculated for both Sites A and B (Table 3.2; \( p = 0.000, F = 36.59, df = 155 \)). Total SC was similar for Sites A and B.

Differences in Phragmites SC existed among all sites, too (two-tailed, \( t \)-test; \( p = 0.000, F = 48.08, df = 155 \)). Site A had higher Phragmites SC than the other two sites (95% CI). Site B had the lowest Phragmites SC (Table 3.2).

Standing crops of non-Phragmites species were similar in Sites R and A, both of which differed from Site B (two-tailed, \( t \)-test; \( p = 0.000, F = 85.59, df = 155 \)). Site B quadrats contained much more non-Phragmites SC than was evident in the other two sites (Table 3.2; 95% CI). This large difference can be attributed in part to the presence of a young, dense stand of Populus deltoides and a quantity of Phalaris arundinacea L. occupying much of Site B. These two species accounted for 61% of the total SC in Site B, whereas Phragmites accounted for only 3% of the total SC (Figure 3.2). Conversely, Phragmites was the dominant species in Site R (Figure 3.3) and in Site A (Figure 3.4).
Elevation differed significantly among all sites (Table 3.2; two-tailed, t-test; \( p=0.000, F=118.52, \text{df}=148 \) ). Elevation data in Site R were heterogeneous (Table 3.2), while regression analyses indicated that elevation data within Sites A and B were nearly uniform.

Species diversity

Because of the statistical constraints associated with diversity indices, statistical significance was not evaluated for these indices (J. Hsu, OSU statistician, pers. comm.). But, diversity indices differed enough in this study to establish general trends. Species richness and density were both greater in Site R than in the other two sites (Figure 3.5; Table 3.1). The distribution of species, however, was not as equitable in Site R as it was in Site B as indicated by the value of other diversity indices. Site B contained the greatest number of abundant species (Shannon's index and Hill's \( N_1 \)) and the greatest number of very abundant species (Simpson's index and Hill's \( N_2 \)). Site B also had the greatest degree of evenness (modified Hill's ratio). With the exception of Simpson's index, which is indicative of dominant species, Site A had the lowest values for all diversity indices. Both Site R and Site A were dominated by *Phragmites* (Figures 3.3 and 3.4, respectively). Several species, however, shared the dominance role in Site B (Figure 3.2).
Elevation was not related to species density in Site R (linear regression; \( p=0.628, F=0.24, \text{df}=70 \)) or Site A (linear regression; \( p=0.323, F=1.00, \text{df}=50 \)). Elevation data were uniform in Site B.

**Standing Crop and Species Density**

Regression analysis indicated that total SC was negatively correlated with species density in Site R \( (p=0.000, F=16.57, \text{df}=70) \) and Site A \( (p=0.000, F=19.35, \text{df}=53) \). Nineteen percent and 27% of the variation in the data for Site R and Site A, respectively, were explained by the regression curves (Figure 3.6). No relationship was established between total SC and species density in Site B \( (p=0.415, F=0.68, \text{df}=30) \).

In those quadrats containing *Phragmites*, *Phragmites* SC was significantly and negatively correlated with species density in both Site R \( (p=0.000, F=23.41, \text{df}=64) \) and Site A \( (p=0.000, F=68.84, \text{df}=48) \). Fifty-nine percent of the variation in Site A data was explained by the regression relationship while 27% of the variation in Site R data was explained by the associated regression equation (Figure 3.7). There was no relationship between *Phragmites* SC and species density in Site B \( (p=0.469, F=0.61, \text{df}=6) \). Relationships between total SC and species density, and *Phragmites* SC and species density were similar in both Sites R and A because *Phragmites* was a large portion of the total SC in these sites.
No relationship was found between elevation and *Phragmites* SC in Site R ($p=0.993, F=0.00, df=64$). Elevation was related to *Phragmites* SC in Site A ($p=0.005, F=8.62, df=50$). Fifteen percent of the variation in Site A data was explained by this relationship.

**Quadrats with and without *Phragmites***

Total SC differed between quadrats with and without *Phragmites* in Site A ($p=0.000, T=-8.71, df=33$; Figure 3.8). No difference in total SC was found between quadrats with and without *Phragmites* in Site R ($p=0.035, T=2.40, df=11$) or Site B ($p=0.190, T=1.41, df=12$; Figure 3.8). The amount of non-*Phragmites* SC in each sample differed between quadrats with and without *Phragmites* in Site A ($p=0.010, T=3.70, df=6$) but not in Site R ($p=0.220, T=-1.40, df=5$) or Site B ($p=0.083, T=1.89, df=12$). There were no differences in species density between quadrats containing *Phragmites* and those that did not in any of the three sites ($p>0.05$; Figure 3.8). The same was true for elevation data ($p>0.05$; Figure 3.8).

**Discussion**

Regression analyses indicated that total SC and *Phragmites* SC both are negatively correlated with species density. These trends suggest that the commonly held perception that *Phragmites* negatively affects plant species diversity is applicable to two of the three (Sites R and A) Lake Erie freshwater
marshes investigated. Based on other results from this study, however, these relationships seem to be rather weak and lack a definite cause-and-effect relationship.

While negative relationships were established between total SC and species density in Sites R and A, there was no significant relationship between these variables in Site B. Similar quantities of total SC were measured in Sites A and B, however (Table 3.2). Additionally, total SC accounted for <27% of the variability in species density in each site. As was true of similar studies conducted in the United Kingdom (Wheeler and Giller, 1982), total SC, therefore, did not adequately explain the variation in species density in the East Harbor sites.

Perhaps, then, there exists a minimum threshold of total SC that is required to establish a significant negative correlation with species density. Total SC values for sites in East Harbor fell within the range of SC (400-1930 g/m²) obtained for managed and unmanaged, Phragmites wetlands (Wheeler and Giller, 1982). In fact, total SC for East Harbor sites was comparable to other studies conducted in Phragmites-dominated wetlands in Europe (Mason and Bryant, 1975; Haslam, 1971). Species density in the East Harbor sites also was within the range of values published for each of these studies. While these studies, including the East Harbor study, reported a negative relationship
between total SC and species density, SC only weakly explained the variation in species density data. A correlation, therefore, may exist between these variables, but evidence is lacking to establish strong inference.

Comparative data reported above refer to Phragmites wetlands in Europe and the United Kingdom where Phragmites populations are in decline. In North America, however, Phragmites is expanding its geographic range, particularly in the Northeast. Hence it is important to note that differences as yet undetermined exist between ecological conditions reported in this study and studies conducted in Europe. Nonetheless, values for total SC and species density were similar. Total SC, then, may not be the variable that best explains variation in species density in wetland plant communities. This may be true particularly for wetlands dominated by one or a few aggressive species such as Phragmites.

Lenssen et al. (2000) hypothesized that SC of the dominant species (Phragmites in their study) would account for a greater degree of the variation in species richness and composition than total SC. They observed a significant negative relationship between Phragmites SC and species density, but Phragmites SC accounted for only 22% of the variation in the data. Phragmites SC values in Sites R and A also were negatively correlated with species density, though Phragmites SC in Site B was not. Dominance in Site B was shared among several species not including Phragmites, however. Therefore, it is not surprising that Phragmites SC was unrelated to species density in Site B. In both Sites R and A the variation explained by Phragmites SC was greater than that for
total SC and also greater than the variation explained in the study conducted by Lenssen et al. (2000). Only in Site A, however, did *Phragmites* SC account for a substantial amount (59%) of the variation in species density. Species densities recorded in the East Harbor sites were comparable to those cited by Lenssen et al. (2000).

While *Phragmites* SC explained more variation in species density data than total SC, the value of this relationship was evident only at quantities of *Phragmites* SC comparable to those found in Site A (Table 3.2). These results indicate that *Phragmites* SC may have an effect on species density particularly in large quantities, but it is not a simple negative relationship attributable purely to the presence of *Phragmites*. Lenssen et al. (2000) also suggested that *Phragmites* SC was a good indicator of the plant's ability to compete with surrounding species for resources such as light and soil nutrients. But, there seems to be a threshold value of *Phragmites* SC at or below which other species may coexist with *Phragmites*. Growth and establishment of other plant species is inhibited when *Phragmites* SC exceeds this threshold. *Phragmites* SC in Site A may have exceeded this threshold value for the East Harbor wetland plant community, limiting species density beyond the constraints of other environmental filters.

Indeed, *Phragmites* SC in Site A (Table 3.2) exceeded values (mean ± SD) reported by Lenssen et al. (2000) for mixed vegetation samples (1007± 952 g/m²) and *Phragmites*-dominated samples (1459 ± 916 g/m²). But, the variation
in species density explained by *Phragmites* SC in Site R was equivalent to that reported by Lenssen et al. (2000) even though the amount of *Phragmites* SC in Site R was far less. Quantities of *Phragmites* SC may not have exceeded threshold values in the aforementioned sites. Alternatively, the relationship between *Phragmites* SC and species density may be site-specific.

Studies conducted in Europe may not be directly comparable to North American studies because of differences in *Phragmites* growth patterns. Unfortunately, comparative *Phragmites* SC data are lacking for freshwater, non-tidal marshes in North America. The most closely associated North American wetlands for which substantial data exist are tidal freshwater marshes. Mean *Phragmites* SC values in Sites R and B were less than values reported by Meyerson et al. (2000) for these wetlands. Data from Site A, however, were within the reported range (980-2642 g/m²). Standing crop values of non-*Phragmites* species in Sites R and A also were within the range reported (152-900 g/m²) by Meyerson et al. (2000). Site B data exceeded this range, owing to the abundance of woody species recorded on the site. Variation among sites and wetland types also exists within North America.

*Phragmites* SC was associated with reduced species richness in the literature reviewed by Meyerson et al. (2000). Species richness and species density for all three East Harbor sites exceeded published values for both *Phragmites*-dominated and non-*Phragmites*-dominated freshwater, non-tidal
marshes in North America, however. If *Phragmites* SC negatively affects species diversity as a rule, the results from this study suggest that there are limitations and exceptions to the rule.

This was most strikingly illustrated in that all diversity indices for Site R were much higher than those for Site A, supporting the proposition that *Phragmites* has a significant negative impact on species diversity at high values of SC. Simultaneously, species density in Site R was greater than that of Site B while all other indices were greater for Site B than for Site R. Both Sites A and B are hydrologically open to the harbor. But Site B was not dominated by *Phragmites* as in the case of Site A. The relative abundance of species, particularly dominant species, was more evenly distributed in Site B than it was in Site R or Site A. While *Phragmites* SC seems to influence the relative abundance of other species, especially at high values, it is incorrect to assume that all *Phragmites* invasions result in a loss of floristic diversity as is often implied (e.g.—Keller, 2000; Meyerson, et al., 2000). To the contrary, the results from this study show that species density did not differ between those quadrats where *Phragmites* was present and those quadrats where *Phragmites* was absent in any of the East Harbor sites. This suggests that factors in addition to the presence of *Phragmites* determine the limits of species density within a given site.

These results also suggest that the relationship between *Phragmites* SC and species density cannot be applied uniformly to all wetland sites. Because
SC is a complex variable, integrating both abiotic and biotic factors, it becomes difficult to discern if differences between sites is primarily a result of environmental gradients or a result of competitive interactions among species. It is possible that very different environmental variables dictate species richness and composition in different wetland sites dominated by the same species (Keddy, 2000). While the relationship between Phragmites SC and species density may reflect some of these differences, it may not account for all of them. This seems to be the case for the East Harbor wetland sites. A comparison of pertinent environmental variables should accompany future studies relating Phragmites SC and species density.

Elevated levels of Phragmites SC, for example, have been associated with nutrient-rich sites (Haslam, 1971). Soil nutrient parameters were not assessed in this study, but Welch (Chapter 4, 2001) found higher carbon and phosphorus concentrations in soil samples from Site A than were found in Site R. These results concur with hydrologic patterns governing each site. Site A is open to sediment exchange via East Harbor's hydrologic regime whereas Site R is virtually excluded from this interchange. This explanation would account for differences in Phragmites SC between Site R and Site A. It does not explain differences in the relationship of Phragmites SC to species density between these sites, however.

Both biotic and abiotic factors limit the distribution and abundance of plant species within wetland communities (Grime, 1979; Gaudet and Keddy, 1995;
Lenssen et al., 1999). While *Phragmites* is considered a competitive dominant in most plant communities it occupies (Haslam, 1971; Meyerson et al., 2000), results from this study support the argument that total SC and *Phragmites* SC are only weakly associated with species richness in freshwater marshes (Wheeler and Giller, 1982; Shipley et al., 1991; Lenssen et al., 1999). Some exception may be observed at high values of *Phragmites* SC (Table 3.2, Site A). Abiotic factors are typically more important in determining species richness in these communities even in the presence of *Phragmites* (Lenssen et al., 1999; Keddy, 2000).

Lenssen et al. (1999) found elevation to be a better predictor of species richness than SC. Contrary to the findings of Lenssen et al. (1999) there was no direct relationship between elevation and species density or elevation and *Phragmites* SC in the East Harbor sites when considered on a site-by-site basis. It is postulated, however, that the uniquely high values for species richness present in Site R are a result of heterogeneous topography (Welch, Chapter 4, 2001). This heterogeneity provides variable environmental conditions for a variety of species to exist simultaneously.

Lake Erie ecosystems also are unique in that they are located at the southern limit of many northern species and at the northern limit of many southern species (Moore, 1973). Additionally, Lake Erie is part of the larger
Great Lakes-St. Lawrence River basin and is an integral part of a major migratory bird route. All of these factors provide numerous opportunities for propagule dispersal and establishment uncommon to other regions of North America.

These hypotheses may account for the potential diversity these Lake Erie marshes have, but they do not explain completely the differences in species density observed among East Harbor sites. It is likely that in addition to variable micro-topography, soil nutrient (Grime, 1979; Gaudet and Keddy, 1995) and disturbance gradients (Keddy, 1982; Keddy and Reznicek, 1986; Day et al., 1988) all play important roles in determining species richness and composition of Lake Erie freshwater marshes. While *Phragmites* may be important as a biotic factor on a small scale, the competitive ability of *Phragmites* may be limited along various environmental gradients (Haslam, 1971; Gaudet and Keddy, 1995).

Welch (Chapter 4, 2001) explored the relationship between *Phragmites* and other plant species along nutrient and disturbance gradients within these marshes. He found species representative of each site were separated primarily along an elevation gradient. A gradient representing both *Phragmites* SC and soil fertility separated species along a second axis. These results further emphasize the importance of both biotic and abiotic factors in determining species richness and composition in *Phragmites*-dominated communities.

*Phragmites* is often associated with low species richness in wetland plant communities (Galatowitsch et al., 1999). While this may be the case in some systems, it not the case for all wetland ecosystems. The results from this study
indicate that *Phragmites* SC is only weakly correlated with species density if at all. Additionally, species density in East Harbor marshes far exceeds values for similar wetlands in other studies despite the presence of *Phragmites*. It is obvious from this study that other factors, most likely a combination of biotic factors and environmental gradients, determine species richness and composition within these wetlands.

While SC and, more specifically, *Phragmites* SC have been used to predict variation in species richness and composition across communities, there are limitations to using these variables as indicators. This study indicates that above a threshold value specific to a given wetland type *Phragmites* SC may serve as a useful predictor of species diversity. Below this threshold, however, factors such as topographic heterogeneity and nutrient availability may be better predictors. Further research is needed to identify environmental gradients that maximize species richness and may prove useful to wetland restoration and creation efforts designed to optimize plant species diversity. Gradients may also be identified that limit the growth and spread of *Phragmites* if that is the management objective. This information may prove valuable to wetland managers and restoration ecologists in the design, creation, and management of freshwater wetlands. Limited economic and human resources can be focused on preventative measures at the initiation of a project rather than being used ineffectively to combat a species that truly may not be having an impact on species diversity.

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List of references


81

Moore, D. L. 1973. Changes in the aquatic vascular plant flora of East Harbor State Park, Ottawa County, Ohio, since 1895. M. S. thesis. The Ohio State University, Columbus, OH.


Table 3.1: List of plant species recorded in June and August 2000 for each site in East Harbor State Park, Ohio. Wetland indicator status (WIS) follows USFWS (1996) for the Northeast Region and definitions for estimated probabilities of occurrence in wetland habitats, where OBL = obligate wetland (99%), FACW = facultative wetland (67-99%), FAC = facultative (34-66%), FACU = facultative upland (1-33%), UPL = obligate upland (0-1%), NL = no indicator, and NL = not listed. The "+" indicates a frequency towards the higher end of the category. The "-" indicates a frequency towards the lower end of the category.
Table 3.1: (continued)

<table>
<thead>
<tr>
<th>Species</th>
<th>Wetland Indicator Status</th>
<th>Site A</th>
<th>Site B</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Melissus officinalis</em></td>
<td>FACU</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td><em>Minuartia nigra</em></td>
<td>OBL</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td><em>Nepeta minor</em></td>
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<td>X</td>
<td></td>
</tr>
<tr>
<td><em>Nelumbo lutea</em></td>
<td>OBL</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td><em>Nympheae odorata</em></td>
<td>OBL</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td><em>Cannabis sativa</em></td>
<td>FACU</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td><em>Panicum capillare</em></td>
<td>FAC</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td><em>Panicum virgatum</em></td>
<td>NL</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td><em>Panicum virgatum</em></td>
<td>FAC</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td><em>Parnassia palustris</em></td>
<td>FACU</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td><em>Parnassia palustris</em></td>
<td>OBL</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td><em>Phlox subulata</em></td>
<td>FACW</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td><em>Phlox subulata</em></td>
<td>FACW</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td><em>Polypodium leucotrichum</em></td>
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<td></td>
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<td><em>Polypodium petersana</em></td>
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<td></td>
</tr>
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<td><em>Populus deltoids</em></td>
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<td>X</td>
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<td>X</td>
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<td><em>Rumex palustris</em></td>
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<td>X</td>
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<td>X</td>
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<td>X</td>
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<td>X</td>
<td></td>
</tr>
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<td>X</td>
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<td><em>Spergularia rubra</em></td>
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<tr>
<td><em>Vitis riparia</em></td>
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State-listed species: 10
Aggressive species: 7

Species that have been listed as threatened, rare, or endangered in Ohio.
Species considered non-native, invasive species in Ohio.
<table>
<thead>
<tr>
<th></th>
<th>Site R</th>
<th>Site A</th>
<th>Site B</th>
</tr>
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<tbody>
<tr>
<td>Total standing crop (g/m²)</td>
<td>741.13</td>
<td>1951.63</td>
<td>2098.33</td>
</tr>
<tr>
<td></td>
<td>(583.83-898.43)</td>
<td>(1658.02-2245.23)</td>
<td>(1566.84-2629.82)</td>
</tr>
<tr>
<td>Phragmites standing crop (g/m²)</td>
<td>475.47</td>
<td>1741.57</td>
<td>54.41</td>
</tr>
<tr>
<td></td>
<td>(312.21-638.74)</td>
<td>(1403.65-2079.49)</td>
<td>(-3.01-111.83)</td>
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<tr>
<td>Phragmites only standing crop (g/m²)</td>
<td>519.36</td>
<td>1919.28</td>
<td>240.96</td>
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<tr>
<td></td>
<td>(344.63-694.09)</td>
<td>(1586.26-2252.30)</td>
<td>(-3.91-485.83)</td>
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<td>Other species standing crop (g/m²)</td>
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<td>210.06</td>
<td>2043.92</td>
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<tr>
<td></td>
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<td>Species density (species/m²)</td>
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<td>3.46</td>
<td>5.81</td>
</tr>
<tr>
<td></td>
<td>(9.13-10.93)</td>
<td>(2.91-4.12)</td>
<td>(4.80-6.82)</td>
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<td>Elevation (m)</td>
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<td></td>
<td>(174.51-174.66)</td>
<td>(173.95-174.02)</td>
<td>(174.09-174.11)</td>
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</tbody>
</table>

Table 3.2: Mean (± 95% confidence interval) total standing crop, *Phragmites* standing crop, standing crop of other species, species density, and elevation within each study site. Data were collected in East Harbor State Park, Ohio in June and August 2000. *Phragmites* standing crop values include all quadrats within each site. *Phragmites* only standing crop excludes quadrats in which *Phragmites* was absent. Other species standing crop refers to the combined standing crop of all species except *Phragmites* within each site. Sites with different letters are significantly different from each other within each row (one-way ANOVA, using 95% confidence intervals).
Figure 3.1. Site locations within East Harbor State Park, Ohio.
Figure 3.2: Five most abundant plant species found in Site B, East Harbor State Park, Ohio. Values are based on the mean percentage of total standing crop represented by each species. Data were collected in June and August 2000. Species richness = 42 for these data.
Figure 3.3: Five most abundant plant species found in Site R, East Harbor State Park, Ohio. Values are based on the mean percentage of total standing crop represented by each species. Data were collected in June and August 2000. Species richness = 88 for this site.
Figure 3.4: Five most abundant plant species found in Site A, East Harbor State Park, Ohio. Values are based on the mean percentage of total standing crop represented by each species. Data were collected in June and August 2000. Species richness = 42 for these data.
Figure 3.5: Comparison of diversity indices calculated for each site in East Harbor State Park, Ohio, surveyed in June and August 2000. $H'$=Shannon-Wiener index, $N_1$=Hill's index of abundant species, $D$=Simpson's index, $N_2$=Hill's index of very abundant species, MHR=modified Hill's ratio (evenness), and SD=species density (with upper 95% confidence limit).
Figure 3.6: Relationship between the natural log of total standing crop and species density in Sites R, A, and B, respectively, in East Harbor State Park, Ohio. Represented are combined June and August 2000 data collected from 1 m² quadrats.
Figure 3.7: Relationship between the natural log of *Phragmites* abundance (standing crop) and species density in Sites R, A, and B, respectively, in East Harbor State Park, Ohio. Represented are combined June and August 2000 data collected from 1 m² quadrats.
Figure 3.8: Comparisons within each site of quadrats in which *Phragmites* was either present or absent. Data were collected in East Harbor State Park, Ohio in June and August 2000. Mean values (± 95% CI) for total SC (A), SC for other species (B), species density (C), and elevation (D) are depicted. Pairs for which error bars do not overlap are considered significantly different.
CHAPTER 4

DISTRIBUTION OF PLANT SPECIES IN EAST HARBOR STATE PARK, OHIO, IN RELATION TO ENVIRONMENTAL VARIABLES

Abstract

Models of stress and disturbance in freshwater wetland plant communities suggest that species diversity is maximized at intermediate positions along environmental gradients. Elevation, standing crop, and soil fertility often emerge as the dominant gradients determining both species richness and species composition. Few studies have investigated the effects of these gradients on species abundance (standing crop) and species composition in relation to aggressive stands of *Phragmites australis* in North America. This study explores the influence of key abiotic and biotic variables on species abundance and composition across Lake Erie wetland sites in East Harbor, Ohio. Standing crop was measured for 92 species in 95 1 x 1 m quadrats in three wetland sites that differed in hydrology and *Phragmites* abundance. Standard sediment analyses, wave exposure, distance to shoreline, elevation, light interference, species density, and *Phragmites* standing crop were measured for each quadrat. The
relationship between species data and environmental variables was investigated using canonical correspondence analysis (CCA). Two gradients combined to explain 62% of the variation in the species-environment relationships: an elevation gradient (35.7%) and a gradient of *Phragmites* standing crop-soil fertility (26.2%). Wave exposure was not a primary component of the first four canonical axes. Elevation was instrumental in describing species composition, separating wet meadow species from marsh species. The *Phragmites* standing crop-soil fertility gradient was more closely associated with species density. Species density was highest at low to moderate soil fertility for both wet meadow and marsh species. These findings support prevailing models describing the relationship between environmental gradients and species density and composition. Contrary to prevailing theories, however, species density was highest in the most sheltered sites. Additionally, a gradient of increasing wave exposure was associated with an increasing *Phragmites* standing crop-soil fertility gradient. The structural resistance of *Phragmites* stems to physical disturbance accounts, in part, for this positive relationship. Varying degrees of anthropogenic disturbance also may have differentially affected species density and composition in each wetland site. The presence of *Phragmites*, in particular, has been positively associated with anthropogenic disturbance. Variation among sites in topographic heterogeneity also is proposed as a factor contributing to the departure from prevailing theories.
Introduction

Stress and disturbance models (Connell, 1975; Grime, 1979; Huston, 1979) are often invoked to explain variation in plant species diversity and composition along environmental gradients in freshwater marsh ecosystems. Indeed, a growing literature base suggests that gradients of soil fertility (Day et al., 1988), wave exposure (Keddy, 1983, 1984), and water-depth fluctuation (Harris and Marshall, 1963; Hutchinson, 1975; Spence, 1982; Keddy and Reznicek, 1986; Wilcox and Meeker, 1991) strongly influence wetland plant species richness and composition. Accordingly, species richness peaks at some intermediate position along the dominant environmental gradient. Keddy and Reznicek (1986) cautioned that while it is believed that natural disturbance maintains diversity, the amplitude and frequency of change needed to maximize diversity is not known for most ecosystems.

Other studies, however, conducted along brackish shorelines (Ellison, 1987), in tropical forests (Hubbell et al, 1999), and along freshwater lakeshores (Grelsson and Nilsson, 1991) suggest that disturbance and stress are not the sole predictors of variation in plant communities. Keddy (1984) and van der Valk (1981) surmised that disturbance alone cannot explain local floristic diversity.

As a result, several studies conducted in dynamic freshwater wetlands have combined multiple environmental variables with measures of standing crop using multivariate analysis techniques to explain variation in plant diversity and composition (Day et al., 1988; Shipley et al., 1991; Gough et al., 1994; Toivonen...
and Huttunen, 1995). Standing crop has been used successfully to explain variation in species density (number of species/unit area) across some wetland communities (Wheeler and Giller, 1982; Day et al, 1988; Wisheu et al., 1990). Gough et al. (1994) found, however, that standing crop accounted for a very small percentage of the variation in species richness in coastal wetlands adjacent to the Gulf of Mexico. Lenssen et al. (2000) suggested using standing crop of the dominant species as a biotic predictor variable within communities. They found that standing crop of the dominant species explained 64% of the variation in species richness data when coupled with litter measurements. However, most of these studies concluded that elevation and soil fertility emerged as the primary gradients associated with species composition and richness in wetland plant communities.

Few, if any studies, have evaluated the influence of these gradients on plant species composition and diversity in freshwater marshes undergoing invasion by *Phragmites australis* (Cav.) Trin. ex Steud. (Meyerson et al., 2000). *Phragmites* is tolerant of a variety of environmental stresses (Haslam, 1972) and is resistant to physical disturbance (Coops et al., 1996). While numerous studies have investigated the autecology of *Phragmites* throughout the world, there is a poor understanding of the specific mechanisms responsible for its rapid expansion throughout the Great Lakes region of North America. Conflicting theories also exist pertaining to the response of *Phragmites* to wave exposure (Haslam, 1972; Weisner, 1987, 1991) and other environmental variables.
(Ostendorp, 1989). Furthermore, references to the relationship between
*Phragmites* and species diversity in freshwater, non-tidal marshes of North
America are limited and primarily anecdotal (Meyerson et al., 2000).

The freshwater marshes of East Harbor, Ohio, are among the most
floristically diverse wetlands on Lake Erie (Stuckey, 1988). In the past 30 years,
rapid expansion of *Phragmites* from historically isolated populations has
coincided with large-scale anthropogenic disturbances. These disturbances
separated portions of the marsh complex from hydrologic interaction with the
harbor. Stuckey (1988) suggested that altered hydrologic interactions were
responsible for *Phragmites* expansion throughout East Harbor and would result
in a loss of floristic diversity. Current restoration efforts will reconnect the
hydrology of the harbor and the diked wetland via a small opening in the dike
wall.

The objectives of this descriptive study are to:

1) characterize the key abiotic and biotic variables associated with plant
   species richness and composition across three East Harbor wetlands,
2) determine what role, if any, wave exposure plays in determining
   species richness and composition across these sites, and
3) identify maximum levels of species richness along the dominant stress
   and disturbance gradients.
Methods

Site descriptions

The three sites surveyed in this study are located in East Harbor State Park, Ottawa County, Ohio, along the northern edge of the Lake Erie Marblehead peninsula (41°33'N, 82°48'W) (Figure 4.1). The Park has a temperate climate with a growing season that extends from mid-April to early September.

East Harbor State Park includes two harbors, East Harbor and Middle Harbor, separated by a causeway built in 1945. The surface hydrology of the two harbors is linked via a 2 m diameter culvert. Both harbors are separated from Lake Erie by a sand bar formed by lateral shoreline drift. Dredge material removed from East Harbor in 1967 was deposited on the harbor-side of the East Harbor sand bar, tripling the width of the sand bar. Middle Harbor, however, is a wildlife sanctuary and has experienced minimal anthropogenic disturbance. East Harbor is connected directly to Lake Erie by a boat channel cut through the sand bar. Middle Harbor is connected to Lake Erie indirectly by its connection to East Harbor. As a result, East Harbor and Middle Harbor both are subject to wave exposure, seiche activity, and water level fluctuations characteristic of the Lake Erie disturbance regime. The average depth of both harbors is < 2 m.

Site R is a 6.4 ha rectangular, freshwater, non-tidal marsh created from dredge spoils taken from East Harbor in 1967 and placed along the interior of the sand bar (Moore, 1973). The dredge material is predominantly sand. The site is separated from the harbor's surficial hydrology by an earthen dike except for a
standing pipe at the northwest corner and a culvert at the southwest corner of the wetland. Both pipes are less than 1 m in diameter. At least a partial groundwater connection between the marsh and the harbor is suspected to exist (S. Mackey, ODNR Geologist, pers. comm.). Pockets of standing water exist in the marsh prior to July in low water years, and throughout the year in high water years. However, portions of the marsh are never inundated. The mean elevation of the site is 174.59 m (IGLD, 1985), ranging from 173.94 to 175.17 m. The substrate of 0.6 ha of Site R at the southern end was mechanically disturbed in 1992 by using a low ground pressure bulldozer. Sporadic mowing also has occurred throughout the site except in the disturbed substrate area. A nature trail runs parallel to and inside the dike wall. Although several aggressive species are present, *Phragmites* has formed monodominant stands throughout the marsh over the past 30 years. *Phragmites* stands are more robust in Site A than they are in Site R. Plans have been made to restore the natural hydrology to Site R in an attempt to reduce the abundance of *Phragmites* and enhance floristic diversity.

Site A is a 7.5 ha marsh located in East Harbor on the harbor-side of the sand bar and south of Site R. Site A is directly open to hydrologic interaction with East Harbor except for a pile of rip-rap 20 m long and 1 m wide on the southern lip of the opening to the marsh. The rip-rap is submersed during seiche and storm events and was covered by water during the 1998 field season. The mean elevation of the site is 173.90 m (IGLD, 1985), ranging from 173.59...
to 174.09 m. *Phragmites* has formed a vigorous, mature stand over the past 30 years across most of the site except for localized populations of *Typha angustifolia* L.

Site B is a 3 ha marsh located in the northwest corner of Middle Harbor. Site B is completely open to the harbor. The mean elevation of the site is 174.10 m (IGLD, 1985), ranging from 174.05 to 174.14 m. This site is fringed by sparse stands of *Phragmites* but was dominated by *Populus deltoides* Marshall., *Salix* spp. saplings, and mudflat annuals during this study. *Populus* and *Salix* species emerged following a drawdown event in 1999. Woody species and mudflat conditions have persisted for >2 years. Site B is completely inundated during high water years.

**Vegetation surveys**

Vegetation surveys were conducted at each site in early June and mid-August 2000. A baseline was established along the nature trail in Site R, whereas baselines were established along the shoreline in Sites A and B. Each baseline was divided into 50-m intervals and transects established perpendicular to this baseline. One transect was located randomly within each interval. One 1 x 1 m quadrat was placed every 25 m along each transect. The compass direction for each transect was recorded. Within each quadrat, plant species were identified, stems counted, percent cover estimated, and average stem height noted for all species. Taxonomic nomenclature follows Gleason and
Cronquist (1991). Percent cover was estimated to the nearest 5% increment. Cover estimates exceeded 100% in some cases because of the presence of multiple plant strata. Water depth and distance to the shoreline also were recorded. Elevations were measured using an auto-level and calibrated with both Lake Erie water levels and existing survey points established by the Ohio Department of Natural Resources, Geologic Survey Division. Elevation data were substituted for the inverse of water depth because water depth data were lacking from many quadrats as a result of declining Lake Erie water levels.

Five light meter readings were taken at ground level within each quadrat. Five control readings were taken at the beginning and the end of each transect, outside the influence of vegetation stands. Mean incident light (lux) at ground level was compared to the mean light measurement obtained outside the vegetation stand and subtracted from unity to calculate the fraction of light intercepted (shading) by the vegetation in each quadrat.

Estimates of standing crop

Ten stems of each species were harvested at ground level adjacent to five random quadrats within each site. This technique ensured that representative samples were collected while avoiding the harvest of samples from within quadrats. A minimum of 50 stems/species was harvested in both June and August surveys. A minimum of 100 stems/species was harvested for species <15 cm tall. In the case of rare, threatened, or endangered species, a species
with similar growth form and size was substituted. The height of each stem was recorded, and the specimens were bagged by species in paper sacks. Specimens were dried to a constant weight at 70°C and then weighed. Stem weights were classified by height for each species. A mean weight was calculated for each height class. The mean weight was divided by the height to obtain a height-adjusted weight (g/cm) for each species. The corresponding height-weight ratio was multiplied by the average height measured in the field for each species. Standing crop (SC) was estimated by multiplying this number by the stem density of each species within a given quadrat. This estimate was calculated for all species in all quadrats and is site-specific.

Soil samples

Three subsamples of the top 10 cm of soil were collected from each quadrat and combined. All quadrats in each site were sampled in one day. Samples were transported to Ohio State University where they were stored at 5°C until analysis. Samples were air dried at 25°C. A portion of each sample was ashed at 950°C to determine total carbon content. Another portion of each sample was sent to Ohio State University’s Service, Testing, and Research Laboratory for standard analyses, including pH, available Bray (no.1) phosphorus, cation exchange capacity, exchangeable potassium, calcium, and magnesium. Soil moisture content also was calculated for each sample.
Wave exposure

Wave exposure ($E_m$) was calculated according to Keddy (1982). Wind velocity, direction, and frequency data collected at Marblehead United States Coast Guard station were obtained from the National Oceanic and Atmospheric Administration's National Climatic Data Center for all available years (1976-1996). Fetch was measured along each of 16 compass directions (every 22.5°) for every quadrat using an aerial photograph. Wind direction was matched with directions of fetch. Exposure was then calculated for all 16 compass directions as

$$E_m = \sum \text{mean wind velocity}_{22.5^\circ} \times \text{percent frequency}_{22.5^\circ} \times \text{fetch}_{22.5^\circ}.$$

Data analysis

Canonical correspondence analysis (CCA; ter Braak, 1991) was used to explore the relationships between abiotic and biotic variables, and species composition and abundance across all three wetland sites in East Harbor. Standing crop data (Table 4.1) were arranged in a 92 species by 95 quadrat data matrix. Similarly, predictor variables (Table 4.2) were arranged in a 14 variable by 95 quadrat data matrix. *Phragmites* SC (Lenssen et al., 2000) and species density (Tilman et al., 1997) were used as biotic predictor variables because of their strong but indirect influences on species composition and ecosystem processes. *Phragmites* SC can alter soil properties, limit incident light, and alter
site elevation (Haslam, 1972), thereby affecting the types and number of species existing in an occupied wetland. Similarly, species density has been correlated with plant productivity, nitrogen uptake, and the amount of incident light reaching the soil (Tilman et al., 1997). These processes, in turn, affect species composition and abundance in a given plant community.

Potassium concentration, pH, cation exchange capacity, and magnesium concentration were eliminated from the CCA analysis because of a high degree of inter-correlation (r > 0.85), typical of these variables. Calcium concentration was used to represent these soil variables because initial analyses indicated that the variance inflation factor was lowest of the soil chemistry variables (see ter Braak, 1986 for details).

Standing crop data were transformed (ln (10x + 1)) to enhance normality. Species with infrequent occurrences were down-weighted so that species with few occurrences would not inequitably influence gradient analyses (ter Braak, 1991). A species-environment joint plot and a quadrat scatter plot were produced showing species with >5% fit and quadrats with >10% fit in relation to the first two canonical axes. No quadrats were discarded. The significance of the relationship between predictor variables and species data was tested using 99 permutations of a Monte Carlo permutation test (ter Braak, 1991).
Results

Canonical correspondence analysis

The first four canonical axes accounted for 82.3% of the variation in the weighted averages of the species with respect to each environmental variable (Table 4.3). Together Axes I and II accounted for the majority (61.9%) of this variation and explained 17.9% of the total inertia in the species data set. Axis I was associated with increasing soil calcium, soil moisture, and negatively associated with elevation (Table 4.4; Figure 4.2). Axis I, therefore, represented a gradient of decreasing elevation or, indirectly, a gradient of increasing water depth. Axis II was associated with increasing *Phragmites* SC, total carbon, and to a lesser degree, soil phosphorus (Table 4.4; Figure 4.2). Consequently, Axis II represented a combined *Phragmites* SC-soil fertility gradient.

Based on a Monte Carlo permutation test, the relationship between species scores and the environmental variables was significant ($n=99$, $F=3.43$, $p=0.01$). Species scores and environmental variables were strongly correlated for Axis I ($r=0.929$) and Axis II ($r=0.856$).

Axis III also produced a strong correlation between species scores and environmental variables but did not add appreciably to the variance explained (13%) in the species-environment relationship.

*Phragmites* SC, soil phosphorus, and soil carbon were nearly orthogonal to elevation (i.e.—there was no relationship between elevation and these three variables), suggesting that these gradients were appropriate descriptors for the
first two axes. Furthermore, all of the soil-related environmental variables were strongly and positively related (Figure 4.2). Distance, soil phosphorus, and shade did not influence the ordination axes as strongly as the other variables. (The relative length of the line for each environmental variable indicates its relative importance.) Species density was negatively correlated with all environmental variables except elevation and distance from shoreline. The relationship between exposure and species density was inversely related as was the relationship between soil moisture and species density.

Species scores

As expected, Phragmites had the highest number of effective occurrences (Hill's $N_2$) across all sample quadrats. As a result, the centroid representing Phragmites abundance was located near the center of Axis I and, to a lesser extent, near the center of Axis II. Ubiquitous species are often located near the center of ordination axes in CCA diagrams, indicating that they have a broad response curve associated with a given set of environmental variables (ter Braak, 1991). Despite its prevalence throughout the sample quadrats, Phragmites had only the second largest tolerance band, or niche width, given the environmental variables measured (Table 4.5). The tolerance for each species was calculated as a part of the CCA procedure and represents the root mean squared deviation
for each species for the set of environmental variables measured. *Lythrum salicaria* L. had the broadest niche width, although its effective number of occurrences was relatively low (Table 4.5).

Figure 4.2 illustrates the joint species-environment plot with respect to Axes I and II. Only species with > 5% fit to the environmental variables were depicted. Species to the right of the origin were associated with wet habitats (marsh species) while species to the left of the origin were associated with moist soil conditions (wet meadow species). Axis II sorted species along a gradient of increasing *Phragmites* SC and soil fertility. Because SC reflects the combined influence of several environmental variables on soil fertility (Wisseu et al., 1990; Gaudet and Keddy, 1995), Axis II was interpreted as a gradient of increasing soil fertility. Species plotted above the origin were associated with high levels of soil fertility while species below the origin were related to declining soil fertility.

Most of the species associated with moist soil conditions also were associated with moderate soil fertility (e.g.—*Agalinis tenuifolia* (M. Vahl.) Raf., *Eupatorium perfoliatum* L., and *Panicum virgatum* L.), whereas several of the wet soil species were associated with low soil fertility (e.g.—*Penthorum sedoides* L., *Cyperus strigosus* L., and *Hibiscus moscheutos* L.).

Few species were associated with high exposure values. *Rorippa palustris* (L.) Besser., *Typha angustifolia*, *Scirpus validus* Vahl., *Sagittaria calycina* Engelm., *Scirpus fluviatilis* (Torr.) A. Gray, and *Phragmites*, in descending rank, were at the upper end of the exposure gradient. Many of the
remaining wet soil species were ranked along moderate levels of exposure. Species associated with moist conditions were not exposed to wave action.

Both species associated with moist soil conditions and species associated with wet soil conditions were ranked at high levels of species density (Figure 4.2). Species ranked highest along the species density gradient also were ranked highest on the elevation gradient. While both wet meadow species and marsh species were associated with high species density, the highest levels of diversity were associated with wet meadow species.

Sample scores

Figure 4.3 is a scatter plot of sample scores on Axes I and II. I calculated species scores as weighted means of sample scores. Species scores ordinated in the species-environment joint plot (Figure 4.2) represent the centroid for each species as a function of all quadrats where that species occurred. Quadrats depicted in the sample scatter plot (Figure 4.3), then, are those samples most closely associated with species scores in the same multivariate space. Ecologically speaking, quadrats depicted in Figure 4.3 represent the central tendency of the species depicted in the same graphical location in Figure 4.2.

With only three exceptions, Plots A91, A102, and A103, all quadrats from Site A were associated with high soil fertility and Phragmites SC. Plots A91, A102, and A103 represented sheltered quadrats dominated by Typha instead of
Phragmites. Four quadrats from Site R also were associated with high Phragmites SC, but were ranked more moderately than most quadrats from Site A. These four quadrats from Site R were located in areas with standing water throughout much of the year, despite their high elevation. Elevation for these quadrats was relatively high compared to Sites A and B but low within Site R. Most quadrats in Site R were closely related to gradients of high elevation and moderate Phragmites SC-soil fertility. All quadrats from Site B were related to low elevation and low Phragmites SC-soil fertility.

Quadrats from Site A only were associated with an increasing exposure gradient. Quadrats in Site B were ranked at intermediate to low positions along the exposure gradient. Quadrats in Site R were not exposed to wave action. Table 4.2 lists summary statistics for theoretical exposure values that would result from removal of the dike at Site R. Hypothetical values for Site R exceeded maximum values calculated for Sites A and B. Based on the species-environment relationships depicted in Figure 4.2, only a few disturbance-tolerant species such as Phragmites and Typha could exist under such conditions.

At a broader scale, quadrats depicted in Figure 4.3 were separated into distinct groups by site. Clusters of quadrats were associated with corresponding clusters of species depicted in Figure 4.2. These results indicate that there was little overlap between the species present in each site along the gradients measured.
Discussion

Dominant gradients

Elevation was the dominant gradient that emerged from canonical correspondence analysis (CCA) conducted in this study. Elevation is a complex variable that mediates other physical factors such as water depth during flooding events, wave exposure, ice scour, sediment and litter deposition, soil chemistry, and soil moisture in drier sites and during drawdown events. All of these factors are integral components of the hydrologic regime specific to a given wetland. Likewise, all of these variables have been directly linked to floristic diversity and composition within wetlands (Hutchinson, 1975; van der Valk, 1981; Spence, 1982; Keddy and Reznicek, 1986). Day et al. (1988) and Gough et al. (1994) concluded that a similarly complex relationship between the primary gradient in their studies—elevation in both cases—and species richness existed. Elevation also played an important role in determining the dominant species along freshwater shorelines associated with *Phragmites* stands (Lenssen et al., 1999). The wetlands in East Harbor are no exception to these findings.

Soil calcium contributed strongly to Axis 1 and was inversely related to elevation. This relationship is consistent with the interpretation of Axis 1 as a gradient of inundation (as related to elevation). Lake Erie waters are influenced by calcareous geology and, hence, contain high concentrations of calcium and magnesium ions as well as sediment particles associated with complexes of these cations (Herdendorf, 1987). Quadrats at lower elevation, therefore, are
influenced directly by Lake Erie sediment and water chemistry. Not surprisingly, species were ordinated according to specific tolerances to water depth and soil moisture associated with the elevation gradient. Wet meadow species associated with infrequent flooding such as *Agalinis tenuifolia* and *Eupatorium perfoliatum* were related to high elevation, while marsh emergents associated with infrequent drawdowns such as *Typha* and *Scirpus fluviatilis* were related to lower elevations (Table 4.1; Figure 4.2). *Phragmites* and *Lythrum* had reasonably wide elevation tolerances and were, therefore, ordinated near the center of the elevation gradient. This is an artifact of CCA (ter Braak, 1991).

The second canonical axis (Axis II) was associated with *Phragmites* SC, total carbon, and to a lesser extent soil phosphorus. *Phragmites* SC, like elevation, is a complex variable integrating both biotic factors such as interspecific competition and abiotic factors that include physical disturbance, climatic conditions, and nutrient availability (Gaudet and Keddy, 1995). Studies conducted in riparian wetlands (Day et al., 1988), fen communities (Wheler and Giller, 1982), brackish marshes (Meyerson et al., 2000), and freshwater wetlands of the Great Lakes (Wisheu et al., 1990) have established strong relationships between standing crop, including *Phragmites* SC, and species diversity and composition.

Increasing standing crop is frequently associated with declining species density (species/unit area) and limited species composition (species identities). The mechanisms behind these relationships include light interception (Haslam,
1972; Gusewell and Edwards, 1999), germination inhibition (Cizkova et al., 1999), and accumulation of litter as a physical barrier to growth and reproduction (Nilsson and Grelsson, 1990). This latter mechanism is directly related to soil carbon, or soil organic matter (SOM). In this study, total carbon was measured rather than soil organic matter. Total carbon includes both organic and inorganic carbon components. Samples containing high percentages of total carbon, however, were obtained from quadrats with an appreciable Phragmites litter layer and yielded total carbon values in excess of inorganic carbon values typical of the resident soils (S. Frey, OSU Soil Scientist, pers. comm.). Any significant change in total carbon in this study, therefore, reflects a relative change in SOM. SOM is used, in turn, as an indicator of soil fertility (Wisheu et al., 1990) and has been directly related to standing crop. Soil phosphorus also is an indicator of soil fertility (Day et al., 1988). Standing crop, SOM, and soil phosphorus, then, are all correlated with soil fertility. Soil fertility has been shown to influence species density and species composition in a variety of studies. Species density reaches some optimal value at low to moderate levels of soil fertility (Grime, 1979; Day et al., 1988; Wisheu et al., 1990; Gaudet and Keddy, 1995; Lenssen et al., 2000).

Species most closely associated with high fertility and low elevation in this study were Rorippa palustris, Scirpus validus, Phragmites, Typha, and Sagittaria latifolia Willd. (Figure 4.2). Stress tolerant species (sensu Grime, 1979 and Day et al., 1988) such as Juncus alpinoarticulatus Chaix., Juncus torreyi Cov., Penthorum sedoides L., and Hibiscus moscheutos L. were associated with low
fertility and low elevation. Few species were associated with high fertility and high elevation aside from *Phragmites*. Only *Vitis riparia* Michx., found climbing *Phragmites* shoots, could be classified in this category. Most species found in elevated quadrats were associated with moderate levels of fertility. In fact, maximum species density was associated with low to intermediate levels of fertility in accordance with competitive dominance theory (Gaudet and Keddy, 1995). This was true for both wet meadow species and marsh species. Similar findings were obtained by Wilson and Keddy (1986), Day et al. (1988), and Wisheu et al. (1990) elsewhere in the Great Lakes and comply with models proposed by Grime (1979) and Huston (1979).

Species in this study were arranged along an elevation gradient based on reproductive and growth characteristics compatible with the prevailing hydrologic conditions (van der Valk, 1981). In terms of Axis II, fertility limited species density by controlling competitive interactions. From another perspective, light became the limiting factor in nutrient-rich sites such as Site A. This combination of environmental variables provided optimal conditions for *Phragmites*, a tall, rhizomatous species, to allocate resources toward improved growth and reproduction, allowing it to overshadow other, less vigorous species.

**Exposure gradient**

Exposure did not play a significant role in any of the first four canonical axes. Additionally, exposure was not inversely related to the fertility gradient as
suggested by Grime (1979), Huston (1979), and Day et al. (1988). According to these models, increasing exposure results in a loss of fertility via removal of fine sediment and litter (Keddy, 1984; Day et al., 1988). Similarly, these models suggest that intermediate levels of disturbance yield maximum levels of diversity.

I found that exposure was positively, though weakly, related to soil fertility. These gradients were by no means orthogonal. In fact, species associated with high fertility and low elevation also were associated with high levels of exposure. Additionally, species density was highest at low levels of exposure despite any relationship with the elevation gradient. The relationship between exposure and fertility gradients in this study does not conform to the models discussed above.

Several factors may account for this divergence from models of fertility and exposure. Exposure was effectively zero for all quadrats in Site R because a dike separated the site from East Harbor. This site, however, produced the highest values of species density in this study. Despite the lack of exposure, portions of Site R have experienced moderate substrate disturbance in the past and intermittent mowing events. It is possible that anthropogenic disturbances have been sufficient in frequency and intensity to limit competitive dominants and promote species diversity despite an apparent abundance of Phragmites. By conducting CCA on each site separately, future analyses may be able to isolate the differences between wave exposure and anthropogenic disturbance.

It is more likely, however, that topographic heterogeneity and nutrient limitations within Site R have limited the impact of Phragmites on the plant.
community (Chapter 3, Welch, 2001). Many areas with high elevation and low to moderate soil fertility exist throughout Site R providing sub-optimal conditions for *Phragmites* growth. As a result, other species tolerant of drier conditions occupy elevated areas. Still other species adapted to frequent inundation occupy deep-water areas—also sub-optimal conditions for *Phragmites* growth—as seed bank species or aquatic macrophytes. This explanation is consistent with the dominant gradients established in this study and is consistent with Keddy's (1990) definition of wet meadow communities.

Additionally, quadrats with the highest levels of exposure in this study were dominated by *Phragmites*. While this may seem counterintuitive, *Phragmites* is known to be structurally resistant to wave action (Weisner, 1987; Coops et al., 1996). *Phragmites* is also physiologically integrated (Hara et al., 1993), allowing ramets in stable environments to translocate resources to ramets in stressed environments. *Phragmites* roots and rhizomes form dense and deep networks that provide stability in shallow and deep waters (Weisner, 1987, 1991). Because of its physical structure, foundational stability, and high stem density, *Phragmites* can absorb and dissipate wave energy across the stand (Coops et al., 1996). This allows sediment to be deposited and litter to accumulate within a *Phragmites* stand under conditions that would normally flush organic matter from the system. Together these factors allow the leading edge of *Phragmites* stands to stabilize and expand (Weisner, 1991; Chambers et al., 1999) except under extreme levels of exposure (Coops et al., 1996). At very high levels of exposure,
though, few other herbaceous species would exist. In this way, soil fertility may increase along shorelines dominated by *Phragmites* even at high levels of exposure. *Phragmites*, then, provides one exception to the rule established by Grime (1979), Huston (1979), and Wilson and Keddy (1986) that competitive dominants are predictably found where stress and disturbance are minimal. As Yodzis (1985) emphasized, the effect of intermediate disturbance on species depends largely on the species within the community and how the disturbance differentially affects those species. *Phragmites* has too wide a tolerance to a variety of environmental conditions to be confined by generalities.

Sites associated with high species density and low elevation in this study were found predominantly in Site B. Site B, however, is experiencing its second year of mudflat conditions after a long period of inundation. This cycle is typical of most freshwater marshes. Exposure levels within the site were low to moderate while species density was moderate to high. *Phragmites* was limited to the fringe of this site. Coexistence of species in this site seems to be moderated by regular flooding punctuated by periodic drawdown events much the same as patterns recorded by Keddy and Reznicek (1986) and Wilcox and Meeker (1991) along freshwater shorelines of the Great Lakes region.

While a fluctuating hydrologic cycle may limit aggressive species such as *Phragmites* in Site B, it does not account for the differences in species density and species composition that exist between Site A and Site B. Similar water level fluctuations occur in both sites. Both sites are relatively uniform in
elevation, and, hence, fluctuating water levels function in the same way as the heterogeneous topography in Site R. Varying hydrology provides a heterogeneous environment in time rather than in space. Species present in Site B, however, are typical of species found in sheltered sites, deposited by both wind and water. Indeed, Grelsson and Nilsson (1991) found that species density was higher in sheltered sites along a lakeshore than in moderately or highly exposed sites. In exposed sites, propagules, like sediment and litter are flushed from the system. Propagules that are deposited in sites dominated by *Phragmites* such as Site A succumb to the growth-inhibiting properties associated with *Phragmites* litter. Both the source material and the appropriate conditions are required to establish diverse wetland communities (van der Valk, 1981).

Water level fluctuations also do not account for the difference in *Phragmites* abundance between Site A and Site B. The average elevation in Site A is lower than in Site B, so fluctuations in water level should be more limiting to *Phragmites* in Site A. Neither site is outside the water-depth tolerance for *Phragmites*. In this case, anthropogenic disturbance (deposition of dredge material in 1967) is most likely linked to the expansion of *Phragmites* in Site A and would explain the current lack of *Phragmites* in Site B which has not been disturbed to the extent that Site A has. *Phragmites*, like many emergent species, germinates in mudflat and shallow water conditions. The deposition of dredge
material in Site A would have provided ideal growing conditions for *Phragmites*. Over time, wave action and storm events will have scoured the site, reducing the elevation to its present level.

Together several factors have contributed to the departure of this study from the dominant models relating soil fertility to exposure. These include: anthropogenic disturbance, topographic heterogeneity, tolerance of *Phragmites* to exposure, its ability to influence sediment accretion, and variation in hydrology among sites. Barring this anomaly, results of this study are consistent with the prevailing stress and disturbance models proposed by Grime (1979), Huston (1979), Day et al. (1988), and Gough et al. (1994). Wave exposure did not play a significant role in determining species density and composition across wetland sites in East Harbor. Rather, elevation and soil fertility were the dominant gradients along which wetland plant species were associated. Also in concurrence with these models, species density generally declined with increasing fertility and *Phragmites* standing crop and reached maximum levels in both wet meadow and marsh environments. Wet meadow species were associated with high elevation and low to moderate soil fertility. Marsh species were associated with low elevations and a wider range of *Phragmites* SC-soil fertility than wet meadow species. Elevation in this study functioned well as a predictor of species composition because it represented hydrologic influences directly responsible for limiting species composition within wetlands. The wave exposure gradient separated species based on their structural tolerance to
physical disturbance. Water depth may be more of a limiting factor to aggressive species than exposure, however, particularly for species such as *Phragmites* that possess a high tolerance to wave exposure and a broad tolerance to many other environmental variables.

In conclusion, a series of complex variables with both biotic and abiotic components are associated with species density and composition in the East Harbor marsh complex. Exposure may play a minor role in this process, but results from this study suggest that elevation, *Phragmites* SC, and soil fertility account for a large percentage of the variation in the species-environment relationships examined. Low to intermediate positions along the *Phragmites* SC-soil fertility gradient were associated with increased species density. Elevations in both wet meadow and marsh habitats were associated with high species density depending on the position along the fertility gradient. Restoration efforts planned for Site R, therefore, should focus on strategies that optimize spatial heterogeneity and minimize soil fertility rather than focusing on enhancing physical disturbance associated with exposure. In general, increasing water depth will require decreasing soil fertility to limit *Phragmites* and foster species diversity.
List of references


<table>
<thead>
<tr>
<th>Species Abbreviation</th>
<th>Mean sU (g/m²)</th>
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(continued on the next page)

Table 4.1. List of species recorded in 2000 throughout the East Harbor State Park, Ohio, study sites. Abbreviations used in the canonical correspondence analysis are listed. Also listed are the mean, standard deviation (SD), and half of the 95% confidence interval (CI) for the standing crop (SC) of each species across all quadrats. The total number of occurrences of each species is also listed. N/A=frequency of occurrence did not allow for this calculation.
<table>
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<th>Species</th>
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<th>SD (gm²)</th>
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<td>Variable</td>
<td>Abbreviation</td>
<td>Units</td>
<td>Mean</td>
<td>SD</td>
<td>Half 95% CI</td>
</tr>
<tr>
<td>--------------------------</td>
<td>--------------</td>
<td>-------</td>
<td>------</td>
<td>-----</td>
<td>------------</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>SOILPHOS</td>
<td>ug/g</td>
<td>15.48</td>
<td>14.30</td>
<td>2.92</td>
</tr>
<tr>
<td>Calcium</td>
<td>SOILCALC</td>
<td>ug/g</td>
<td>3374.53</td>
<td>1691.49</td>
<td>340.14</td>
</tr>
<tr>
<td>Total Carbon</td>
<td>SOILCARB</td>
<td>%</td>
<td>8.09</td>
<td>7.20</td>
<td>1.45</td>
</tr>
<tr>
<td>Soil Moisture</td>
<td>SOILMOIS</td>
<td>%</td>
<td>41.66</td>
<td>21.58</td>
<td>4.34</td>
</tr>
<tr>
<td>Elevation</td>
<td>ELEVATIO</td>
<td>m</td>
<td>174.28</td>
<td>0.28</td>
<td>0.06</td>
</tr>
<tr>
<td>Distance to shoreline</td>
<td>DISTANCE</td>
<td>m</td>
<td>47.89</td>
<td>33.57</td>
<td>6.75</td>
</tr>
<tr>
<td>Exposure</td>
<td>EXPOSURE</td>
<td>Em</td>
<td>288.92</td>
<td>269.48</td>
<td>54.19</td>
</tr>
<tr>
<td>Hypothetical Exposure, Site R</td>
<td>EXPOSURE</td>
<td>Em</td>
<td>1239.31</td>
<td>160.56</td>
<td>60.00</td>
</tr>
<tr>
<td>Light Interception at 0 meters</td>
<td>SHADE 0M</td>
<td>%</td>
<td>89.96</td>
<td>12.55</td>
<td>2.52</td>
</tr>
<tr>
<td>Species Density</td>
<td>SPECdens</td>
<td>Species/m²</td>
<td>5.9</td>
<td>4.1</td>
<td>0.8</td>
</tr>
<tr>
<td>Phragmites Standing Crop</td>
<td>PHRAG SC</td>
<td>g/m²</td>
<td>1054.33</td>
<td>1197.95</td>
<td>240.89</td>
</tr>
</tbody>
</table>

Table 4.2. List of environmental variables used as part of the canonical correspondence analysis. The abbreviation, units of measure, mean, standard deviation (SD), half the 95% confidence interval (CI), minimum, and maximum values are also given for each variable measured in 2000. Additionally, summary data are given for hypothetical exposure values that would result if the harbor dike was removed from Site R, East Harbor State Park, Ohio.
Table 4.3. Cumulative variation explained in the species data set and in the weighted averages of the species with respect to each environmental variable. Canonical correspondence analysis (CCA) was performed using data obtained in 2000 from three wetlands in East Harbor State Park, Ohio. The total inertia detected in the species data based on a chi-square analysis is listed as is the total variation explained by CCA.
Table 4.4. Correlation between environmental variables examined and the first two canonical axes resulting from canonical correspondence analysis. Variables were measured in three East Harbor State Park wetlands in 2000. The species-environment correlation for Axis I was $r=0.9292$ and for Axis II $r=0.8562$.

<table>
<thead>
<tr>
<th>Environmental Variable</th>
<th>Canonical Axis I</th>
<th>Canonical Axis II</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOILPRES</td>
<td>0.1728</td>
<td>0.4141</td>
</tr>
<tr>
<td>SOILCALC</td>
<td>0.7778</td>
<td>0.1274</td>
</tr>
<tr>
<td>SOILCARB</td>
<td>0.3641</td>
<td>0.5144</td>
</tr>
<tr>
<td>SOILMOIS</td>
<td>0.6905</td>
<td>0.4891</td>
</tr>
<tr>
<td>ELEVATIO</td>
<td>-0.7772</td>
<td>0.0450</td>
</tr>
<tr>
<td>DISTANCE</td>
<td>-0.3577</td>
<td>0.1126</td>
</tr>
<tr>
<td>EXPOSURE</td>
<td>0.5789</td>
<td>0.3479</td>
</tr>
<tr>
<td>SHADE 0M</td>
<td>0.4574</td>
<td>0.2038</td>
</tr>
<tr>
<td>SPECDEN</td>
<td>-0.6218</td>
<td>-0.3484</td>
</tr>
<tr>
<td>PHRAG SC</td>
<td>0.1459</td>
<td>0.6772</td>
</tr>
</tbody>
</table>
Table 4.5. List of the five species with the broadest tolerances to the environmental conditions examined and the five species with the highest number of effective occurrences throughout the East Harbor study sites in 2000. Tolerance=root mean squared deviation for each species; \( N_2 = \text{Hill's } N_2 \), the effective number of occurrences of a species.

<table>
<thead>
<tr>
<th>Species</th>
<th>Tolerance</th>
<th>( N_2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carya ovata</td>
<td>89.27</td>
<td>1.92</td>
</tr>
<tr>
<td>Eupatorium perfoliatum</td>
<td>68.71</td>
<td>16.03</td>
</tr>
<tr>
<td>Lythrum salicaria</td>
<td>98.80</td>
<td>6.57</td>
</tr>
<tr>
<td>Phalaris arundinacea</td>
<td>83.83</td>
<td>24.50</td>
</tr>
<tr>
<td>Phragmites australis</td>
<td>96.40</td>
<td>73.47</td>
</tr>
<tr>
<td>Polygonum persicaria</td>
<td>90.98</td>
<td>2.00</td>
</tr>
<tr>
<td>Populus deltoides</td>
<td>76.29</td>
<td>14.02</td>
</tr>
<tr>
<td>Rorippa palustris</td>
<td>95.40</td>
<td>7.50</td>
</tr>
<tr>
<td>Typha angustifolia</td>
<td>82.36</td>
<td>15.71</td>
</tr>
</tbody>
</table>
Figure 4.1. Wetland site locations within East Harbor State Park, Ohio.
Figure 4.2. CCA ordination diagram depicting species and environmental variables in relation to the first two canonical axes. Data were obtained in 2000 from three wetlands in East Harbor State Park, Ohio. Axes I and II explained 35.7% and 26.2%, respectively, of the variation in species-environment relationships. Of the 92 species present in this study, only those species with >5% fit to the data (46 species) were included in the ordination. Graphic restrictions, however, excluded the names of nine species, though their symbols are present. These species are: AGALPURP, CHARFOET, CORNDRUM, EQUIARVE, MEUOFFI, PANICAPI, PANILANU, POPUDELT, and SCHISCOP. POPUDELT is centered near MIMURING whereas the other eight species are positioned to the left of the diagram among the other wet meadow species. Full species names and abbreviations are listed in Table 4.1. Environmental variables are listed in Table 4.2.
Figure 4.3. CCA quadrat scatter diagram plotted on the first two canonical axes. Data were obtained in 2000 from three wetlands located in East Harbor State Park, Ohio. Axis I (horizontal axis) is associated with a gradient of increasing elevation to the left and increasing soil calcium to the right. Axis II (vertical axis) is associated with an increasing gradient of Phragmites standing crop and soil fertility from bottom to top (see also Figure 4.2). Of the 95 quadrats examined, only those with >10% fit to the data (77 quadrats) are displayed. For each quadrat identifier, A=Site A, B=Site B, and R=Site R.
CHAPTER 5

CONCLUSIONS AND
IMPLICATIONS FOR MANAGEMENT

This study attempted to determine the relationship between Phragmites abundance and plant species diversity, both extant and potential, in a diked marsh located in East Harbor State Park, Ohio. I also explored the relationship between Phragmites standing crop and species diversity in this marsh and two proximate marshes open to hydrologic interaction. The relationship of these species to environmental and biotic variables also has been examined. While it is generally believed that expansion of Phragmites throughout Lake Erie wetlands has had a negative effect on species diversity, the results from this study indicate that the influence of Phragmites colonization on species diversity depends on the specific environmental and biotic factors existing in a given site. The most important factors emerging from this study were site elevation (as an indicator of hydrologic influence), Phragmites standing crop, and soil fertility. Seed bank diversity and topographic heterogeneity within a site played a
significant part in determining plant community structure following disturbance. These environmental and biotic gradients should be examined more closely as wetland restoration efforts continue within East Harbor.

Despite declining extant species richness and a relatively species-poor seed bank, the East Harbor disturbance site is a floristically-rich wetland compared to similar freshwater wetlands (Keddy and Reznicek, 1982; Thiet, 1998; Keller, 2000; Meyerson et al., 2000). Nonetheless, current management objectives intend to restore historic species diversity to all of Site R. Restoration measures include basin contouring via substrate disturbance and hydrologic connection of Site R to East Harbor via a water control structure. The existing seed bank is an integral part of this restoration effort. Restoration success will depend, in part, on maximum activation of seed bank reserves.

Basin contouring will entail the removal of soil in areas where elevation is to be reduced. This will remove seed stores and _Phragmites_ rhizome fragments along with the soil. A thin layer of the excavated material could be spread across the site after contouring is complete to utilize seed bank resources. But, this will also spread _Phragmites_ rhizome fragments throughout the site, encouraging the establishment of _Phragmites_ stands. To avoid the distribution of _Phragmites_ rhizome fragments throughout the site, excavated soil could be sieved through a large, mesh screen. The mesh would need to be large enough to allow course soil material and large seeds (e.g.—_Sparganium eurycarpum_) to pass, but small enough to screen rhizome fragments. Screens of this type are used by rock quarries to sort gravel, for example. (There is a limestone quarry on the
Marblehead Peninsula.) If material obtained from all parts of the site were mixed, this technique may be sufficient to capture isolated seed stores of rare and endangered plants. The resulting material could then be spread across the contoured basin. This process requires time, labor, and money that may be beyond the scope of this project. Volunteer resources, including the local military reserve units, may be a source of valuable assistance.

Alternatively, mature plants and seedlings could be planted in Site R following basin contouring. This technique avoids mortality associated with seed germination (Leek, 1989), increasing success rates of plant establishment (Primack, 1996). The success of planting efforts aimed at restoring diversity has been mixed (Pavlik, 1996). Some plants do not establish viable populations. Many plants succumb to herbivory from insects or waterfowl, particularly Canada geese (Primack, 1996). In some cases, however, plantings succeed, giving desired species a competitive edge over aggressive species.

Extant species richness exceeds seed bank species richness for Site R, however. Any excavation or substrate disturbance designed to activate the seed bank, then, may compromise subsequent species richness in the extant vegetation. With the existing vegetation eliminated, future vegetation components of the wetland will be more reliant on vegetative propagules and external seed sources because of the limited seed bank. Historic levels of species diversity may not be realized for years if at all.

Similarly, the results of this study indicate that restoration efforts planned for East Harbor will have little effect on aggressive species richness. The
number of aggressive species did not vary from year to year within the site despite previous disturbance events. Substrate disturbance will most likely result in a shift in species composition toward species adapted to wetter conditions. Since the most common species found throughout Site R are adapted to these conditions already, only those species associated with drier conditions will be negatively affected. In fact, it is quite possible that gaps formed throughout the site will provide ideal conditions for disturbance-tolerant species such as *Phragmites* and *Phalaris* as well as gap colonizers such as *Lythrum* (Day et al., 1988). Brown and Bedford (1997), for instance, noted that *Typha angustifolia* colonized gaps created by substrate disturbance in a restoration project in northern New York. Similarly, Havens et al. (1997) observed that *Phragmites* quickly colonized over 80% of the wetlands restored using substrate disturbance in Virginia. All of these aggressive species have been observed in and around Site R, particularly in wet areas.

Results from this study indicate that restoration activities will encourage the growth and reproduction of at least some state-listed species. Several state-listed species were consistently observed in the extant vegetation and the seed bank despite the re-colonization of the site by *Phragmites* and despite the presence of other aggressive species. These species include *Alisma triviale*, *Carex aurea*, *Eleocharis caribaea*, *Eleocharis palustris*, and *Hypericum majus* (Table 2.1).

It is not clear from this study, however, that an increase in the marsh-harbor hydrologic interaction will limit aggressive species, particularly
Phragmites, in the long-term. There was some indication that Phragmites stem density declined with declining water levels between 1999 and 2000. It is unclear if Phragmites abundance will increase, then, with increasing hydrologic activity. It may well depend on the degree and duration of inundation. Once established, Phragmites can tolerate all but the most extreme conditions typical of marsh ecosystems and, in fact, thrives in moderately inundated conditions (Haslam, 1972; Weisner and Graneli, 1989; Armstrong et al., 1999).

Based on the results of this study, however, the presence of Phragmites may not result in a loss of species diversity even if Phragmites re-colonizes Site R after substrate disturbance. Although Phragmites is often associated with low species richness in wetland plant communities, Phragmites standing crop was only weakly correlated with species diversity. Only at high values of Phragmites standing crop (1403-2079 g/m²) was species diversity strongly and adversely affected in this study. These values for Phragmites abundance exceeded values reported elsewhere in the literature for similar wetlands (Haslam, 1972; Keller, 2000; Meyerson et al., 2000). If restoration objectives are to limit the abundance of Phragmites while maximizing species diversity, restoration efforts must avoid establishing site conditions that will foster the most vigorous growth of Phragmites stands.

What factors should managers focus on to limit Phragmites and foster species diversity in the East Harbor restoration site? Based on the results obtained from three East Harbor wetlands (see Chapter 4), the key factors determining species density and composition were elevation, Phragmites
standing crop, and soil fertility. In this study, the elevation was used as an indicator of hydrologic influence. Wave exposure, thought to be a primary determinant of species diversity in freshwater marshes (Keddy, 1982; Weisner, 1987 and 1991), was found to be only of peripheral importance as an environmental variable in this study, however.

How do these environmental gradients relate to restoration plans scheduled for Site R? Restoration efforts in Site R will effectively lower the elevation of much of the site. Additionally, a control structure will provide hydrologic interaction between East Harbor and the restoration site. By lowering the elevation of the site, species composition will shift from wet meadow to marsh species. In other words, species better adapted to wetter conditions will dominate the extant vegetation (van der Valk, 1981).

The limited hydrologic connection established between the marsh and East Harbor will alter the current fertility gradient. Depending on the nature of the hydrologic interaction, fertility could decrease within the site, maintaining a relatively high level of species density with an altered species composition comparable to Site B. Future vegetation composition of the altered system would not only depend on the elevation and fertility gradients but would also be dictated by species present in the seed bank (van der Valk, 1981), active seed sources, flood frequency, and flood duration (Keddy, 1990).

It is also possible that species density will decrease and *Phragmites* abundance will increase beyond the current density that exists within Site R. With a reduction in elevation and a partial hydrologic connection, fertility could
increase rather than decrease. This would provide optimal hydrologic and nutrient conditions for *Phragmites* growth and reproduction. *Phragmites* rhizomes and seeds already exist in Site R. Site R is also oriented in the same direction as Site A and would, therefore, receive similar water-borne seed and nutrient inputs. Given these conditions, Site R could mimic Site A in species diversity and composition following project completion, Site A being much less diverse than Site R. Elevation gradients and hydrologic inputs need to be considered carefully if the reduction of *Phragmites* and the maintenance of species diversity are the primary management goals.

Consideration also must be given to the species composition that may result after the site is altered. Results from multivariate analyses suggest, for instance, that if the elevation within the site is reduced and the current level of soil fertility maintained or slightly reduced, *Lythrum*, *Typha*, and/or *Phalaris* may flourish in Site R instead of *Phragmites* (see Chapter 4; Figure 4.2). These species, considered aggressive and undesirable in Lake Erie marshes, could readily colonize gaps resulting from substrate disturbance during restoration activities. Each of these species has relatively wide environmental tolerances and produces copious amounts of seed.

Can *Phragmites* be prevented from dominating Site R following restoration? Several management options exist that may be worthy of investigation based on the results of this study and results obtained in other experiments (Marks et al., 1994). One option that has been suggested includes dismantling the dike that currently separates the wetland from East Harbor. It is
hypothesized that this would restore the hydrologic disturbance regime once typical of East Harbor. Once the dike has been removed, the East Harbor hydrologic regime hypothetically would promote disturbance-mediated coexistence of species, and foster plant species diversity throughout the site. If a dike wall did not exist between Site R and East Harbor, estimated wave exposure values would exceed the maximum exposure value calculated for all quadrats in the current study (see Chapter 4; Table 4.2). Exposure values would far exceed “intermediate” exposure in East Harbor. Species diversity was greatest, however, in quadrats with low to moderate wave exposure values. While this option may succeed in eliminating Phragmites, it most surely would eliminate all other species of interest.

Alternatively, the hydrologic connection between East Harbor and the wetland could be made large enough to allow for periodic purging of organic sediment and litter, limiting fertility and the negative effects associated with Phragmites litter within the site, and small enough to limit wave exposure. This free-flowing design still runs the risk of increasing fertility and encouraging Phragmites expansion in portions of Site R if not designed properly.

If the management goal is to enhance plant species diversity, the results from this study suggest that maintaining a heterogeneous basin morphology within the site may be the most productive method of achieving this goal. If properly designed, topographic variation would provide deep-water sites for emergent and floating aquatic species while preserving elevated areas that currently support wet meadow species. Both elevation extremes provide sub-
optimal conditions for Phragmites. Likewise, variation in elevation would contribute to variation in soil fertility throughout the site, further limiting Phragmites distribution throughout the restoration site.

Based on the above discussion, data from Chapter 2 (Figure 2.2), and results from Chapters 3 and 4, I recommend that the mean elevation of the restored marsh approximate the long-term mean Lake Erie water level for June (174.15 m). I suggest that one-third of the site be excavated to this elevation. I also suggest that another third of the site be excavated to elevations one meter below the long-term mean water level. Deeper sites also may be desirable for fish and waterfowl habitat. The last third would utilize existing areas within the marsh above the long-term mean water level. Variation in basin morphology should combine gradual transitions in elevation from high to low points with abrupt changes from deep pockets of standing water to elevated mounds. This degree of variation maximizes topographic heterogeneity in Site R, providing variable environmental conditions in high water and low water years. This variability in both time and space is necessary to foster plant species diversity throughout Site R while limiting the growth and establishment of Phragmites.

Excavation activities of this magnitude will require the shifting or transport of vast quantities of soil, however. Again, local military reserve units and other volunteer services may prove to be invaluable resources.

Spatial heterogeneity could be incorporated into any of the aforementioned hydrologic manipulation schemes to increase project effectiveness. In fact, it may be vital to the success of current management.
plans. If coupled with periodic inundation of the site to control *Phragmites*, these techniques could prove to be an effective means of promoting wetland plant diversity while limiting *Phragmites* abundance. The merits of manipulated inundation have been discussed elsewhere (Harris and Marshall, 1963).

Unfortunately for wetland managers, inundation as a *Phragmites*-control technique has met with varied success (Marks et al., 1994). *Phragmites* can persist at a wide variety of water depths depending on soil conditions, hydrologic regime, genetic diversity among *Phragmites* stands, and existing management practices (Haslam, 1972; Weisner, 1987, 1991, 1996; Squires and van der Valk, 1992; Marks et al., 1994; Gusewell et al., 2000). Future research efforts need to focus on defining the water-depth tolerances specific to *Phragmites* populations in Lake Erie wetlands. Experiments quantifying the response of *Phragmites* to flood duration, flood frequency, and water depth in combination with other environmental variables also need to be conducted. This information would assist wetland managers in their goal to control *Phragmites* populations in Lake Erie marshes.

Ultimately, the success of any restoration effort depends on clearly defined objectives and a firm understanding of the ecology of the system being modified. If current management objectives are to reduce *Phragmites* abundance throughout the East Harbor restoration marsh while increasing plant species richness and abundance, current restoration plans may produce undesirable results. Excavation and hydrologic alteration may increase *Phragmites* abundance and limit species richness. If the objective, however, is to
maintain the abundance of state-listed species throughout the marsh, plans may be successful. State-listed species are prevalent and relatively abundant components of the marsh seed bank.

Additionally, species richness in East Harbor marshes far exceeds values for similar wetlands in other studies despite the presence of *Phragmites*. It is obvious from this study that other factors, most likely a combination of biotic factors and environmental gradients, determine species richness and composition within these wetlands. Further research designed to identify environmental gradients that maximize species richness may prove useful to wetland restoration and creation efforts if the management objective is to optimize plant species diversity. Gradients may also be identified that limit the growth and spread of *Phragmites*. This information may prove valuable to wetland managers and restoration ecologists in the design, creation, and management of freshwater wetlands. Limited economic and human resources can be focused on preventive measures at the initiation of a project rather than being used ineffectively to combat a species that truly may not be having an impact on species diversity.


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IDENTIFICATION OF SYNTHETIC BENZOPYRANONES AS SELECTIVE AGENTS FOR MOLECULAR TARGETS IN BREAST CANCER

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
The Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By
Jennifer Lynn Whetstone, B.S. Biochemistry

The Ohio State University
2001

Dissertation Committee:
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Approved by
Robert W. Brueggemeier
Adviser
Pharmacy
ABSTRACT

Currently, one out of eight American women will develop breast cancer in her lifetime, making breast cancer the second highest cause of mortality of all female cancers. The majority of breast cancer cases (60-70%) are hormone-dependent, meaning that estrogens are needed for the growth of the tumor. Various antiestrogens, including tamoxifen, are widely used for the treatment of hormone-dependent breast cancer. However, the need for newer antiestrogens with greater specificity and reduced side effects exists. The hypothesis of this research is that the design, synthesis, and screening of substituted benzopyranone libraries would allow us to utilize the biological potential of these molecules and develop more selective therapeutic agents for molecular targets in breast cancer.

The benzopyranone ring system is the core structure found in a number of natural products such as the flavonoids and isoflavonoids. Substituted 4H-1-benzopyran-4-ones have shown activity as protein tyrosine kinase inhibitors, estrogen receptor agonists or antagonists, or inhibitors of steroidogenic enzymes. The prevalent literature methods for constructing benzopyranones were not ideally suited for making libraries as these methods suffer from harsh reaction conditions, poor substituent tolerance, and low yields. Initial synthetic chemistry produced a novel synthetic route utilizing readily
available salicylic acids and terminal alkynes as starting materials to construct the benzopyranone nucleus. This approach is characterized by mild and high yielding reactions with good functional group tolerance, and it is ideal for developing combinatorial libraries centered around the benzopyranone ring system.

The novel solution-phase chemistry developed to synthesize the benzopyranones can be accomplished in several steps. Retrosynthetically, it was envisioned to make the benzopyranones by the cyclization of alkynones. Substituted bis-TBDMS-salicylic acids underwent a one-pot acid chlorination-Sonogashira coupling resulting in the synthesis of the critical intermediate, alkynone, in excellent yields. To date, electronic and steric requirements for these coupling reactions have been determined. Substitutions at the 3-, 4-, and 5-position of salicylic acid, including halogens, aromatic, and methoxy functionalities, have been used in coupling and result in yields ranging from 40-96%. For the Sonogashira coupling, various terminal alkynes were used (aromatic, alkyl, acetal). The one-pot acid chlorination-Sonogashira coupling, key for introducing diversity, displays a wide substituent tolerance in both of the coupling partners.

Michael addition of the secondary amine to the alkynone, followed by a 6-endo-trig cyclization results in the formation of the six-membered benzopyranone with yields from 70-96%. By using a secondary amine addition to the alkynone, the synthetic strategy prevents the cyclization of the competing five-membered benzofuranone and thus resolves the regioselectivity problem encountered by previous efforts. Synthetic
approaches for diversifying the benzopyranone skeleton have also been pursued; substituents at the 3-position on the ring system would dramatically increase the diversity of our library. Evaluation of a library with more than forty synthetic benzopyranones in initial bioassays (cell proliferation, estrogen receptor binding, and aromatase) using human breast cancer cell lines has resulted in agents exhibiting enhanced and differential activities on breast cancer cell growth and on aromatase inhibition. Continued synthetic efforts will concentrate on development of more selective agents for molecular targets in breast cancer based upon the benzopyranone nucleus.
Dedicated to

Shawn

Mom, Dad, Beth, & Matt
ACKNOWLEDGMENTS

My graduate school career is a culmination of not only my individual accomplishments, but also the contributions of many individuals, both in my personal and professional life. I cannot begin to thank everyone for their support and guidance throughout this journey.

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PUBLICATIONS

Research Publications


FIELDS OF STUDY

Major Field: Pharmacy

Medicinal Chemistry
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CHAPTER 1

TARGETING BREAST CANCER WITH FLAVONOIDS

1.1 Breast Cancer Statistics

Cancer is the leading cause of death among women between the ages of 30 and 54. A woman living in the United States has a one in eight lifetime risk of developing breast cancer [1]. In 2001, the American Cancer Society estimates that 192,200 new invasive cases of breast cancer will be diagnosed among women in the United States, as well as nearly 47,100 additional cases of in situ breast cancer [1]. A total of 40,200 women are expected to die from this disease in 2001, making breast cancer the second highest cause of mortality of all female cancers. The reality is that many women, and their families, are affected by breast cancer.

With treatment options that exist today, the 5-year survival rate for localized breast cancer is 97%. If the breast cancer has spread regionally or with distant metastases, 5-year survival rates decrease dramatically to 77% and 21%, respectively [2]. Survival rates also continue to decline beyond five years and the need for newer therapies with greater specificity and reduced side effects still exists.
1.1.1 Risk Factors for Breast Cancer

Various factors that increase the relative risk for breast cancer in women are presented in Table 1.1. Risk factors for breast cancer can be grouped into several categories: hormonal, genetic, geographical, and environmental factors. The primary risk factors for developing breast cancer are gender and age. Women with a family history of breast cancer (first-degree relative like mother, sister, or daughter) are at higher risk due to genetic predisposition. Approximately 5% to 10% of breast cancer cases are a result of inherited mutations in breast cancer susceptibility genes (BRCA1 and BRCA2) [1]. Although the majority of risk factors are not modifiable (age, family history, age at first birth, early menarche, late menopause), other factors such as alcohol consumption, use of postmenopausal hormones, and obesity after menopause are modifiable. Because the exact cause of breast cancer is unknown and it is impossible to eliminate all risks of breast cancer, prevention and early detection through mammography and breast self-examinations are the best strategy for women to fight this disease.

1.2 Estrogens and Breast Cancer

The regulation of normal breast development, initiation of breast carcinogenesis, and growth and progression of breast cancer are dependent on hormonal factors. The majority of breast cancer cases (60-70%) are hormone-dependent, meaning that estrogen receptors are present and estrogens are needed for the growth of the tumor. 17β-Estradiol is the most potent endogenous estrogen (Figure 1.1). At the molecular level, estrogens bind to the estrogen receptor (ER) and cause the expression of various
<table>
<thead>
<tr>
<th>Relative Risk</th>
<th>Factor</th>
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<tr>
<td>&gt; 4.0</td>
<td>Certain inherited genetic mutations for breast cancer</td>
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<tr>
<td></td>
<td>Two or more first-degree relatives with breast cancer diagnosed at an early age</td>
</tr>
<tr>
<td></td>
<td>Personal history of breast cancer</td>
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<td></td>
<td>Age (65+ vs. &lt; 65 years, although risk increases across all ages until age 80)</td>
</tr>
<tr>
<td>2.1 - 4.0</td>
<td>One first-degree relative with breast cancer</td>
</tr>
<tr>
<td></td>
<td>Nodular densities on mammogram (&gt; 75% of breast volume)</td>
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<td></td>
<td>Atypical hyperplasia</td>
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<td>High-dose ionizing radiation to the chest</td>
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<td></td>
<td>Ovaries not surgically removed &lt; age 40</td>
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<tr>
<td>1.1 - 2.0</td>
<td>High socioeconomic status</td>
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<tr>
<td></td>
<td>Urban residence</td>
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<td></td>
<td>Northern US residence</td>
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<tr>
<td>Reproductive Factors</td>
<td>Early menarche (&lt; 12 years)</td>
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<tr>
<td></td>
<td>Late menopause (≥ 55 years)</td>
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<tr>
<td></td>
<td>No full-term pregnancies (for breast cancer diagnosed at age 40+ years)</td>
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<tr>
<td></td>
<td>Late age at first full-term pregnancy (≥ 30 years)</td>
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<tr>
<td>Other factors that affect circulating hormones or genetic susceptibility</td>
<td>Postmenopausal obesity</td>
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<tr>
<td></td>
<td>Alcohol consumption</td>
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<td></td>
<td>Recent hormone replacement therapy</td>
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<td></td>
<td>Recent oral contraceptive use</td>
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<tr>
<td></td>
<td>Tall</td>
</tr>
<tr>
<td></td>
<td>Personal history of cancer of endometrium, ovary, or colon</td>
</tr>
<tr>
<td></td>
<td>Jewish heritage</td>
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</table>

**Table 1.1**: Factors that increase the relative risk for breast cancer in women [1].
genes important for growth, i.e. growth factors, protein tyrosine kinases, and cyclin
dependent kinases. These proteins act by endocrine, paracrine, autocrine, and intracrine
interactions to promote the growth of breast cancer cells.

\[ \text{Figure 1.1: 17β-Estradiol, estrone, and estriol.} \]

1.2.1 ESTROGEN RECEPTOR α AND ESTROGEN RECEPTOR β

Estrogens not only exert their effects on the growth, differentiation, and functioning of
reproductive tissues, but also have important actions on other tissues including bone,
liver, cardiovascular system, and central nervous system. The majority of actions
influenced by estrogen are through two estrogen receptor (ER) subtypes, estrogen
receptor α (ERα) and the more recently discovered estrogen receptor β (ERβ) [3,4].
The tissue distribution and relative ligand binding affinity for ERα and ERβ are
different and could contribute to the selective action of ER agonists and antagonists in
different tissues [5].
ER is a member of the steroid hormone receptor superfamily which includes receptors for steroids (progesterone, cortisol, aldosterone, and testosterone), thyroid hormone, vitamin D, and retinoic acids. In humans, a single gene encodes for ERα (595 amino acids) and ERβ (530 amino acids) protein and is found on chromosomes 6 and 14, respectively [6]. ERα and ERβ proteins are similar in architecture to other steroid receptors; each is composed of five independent but interacting functional domains: A/B, C, D, E, and F domains (Figure 1.2). The N-terminal A/B domain encodes the ligand-independent activation function (AF-1) and is involved in protein-protein interactions and the transcriptional activation of gene expression. ERα and ERβ differ the most in the A/B domain, having only 18% amino acid homology [7]. The most conserved domain among nuclear hormone receptors is the C domain. It is the DNA binding domain and contains two zinc finger structures, which allow for DNA sequence-specific receptor binding and dimerization. Domain D is known as the hinge region and allows for ligand mediated conformational changes. The ligand-binding domain (LBD), domain E, of ERα and ERβ shows 56% amino acid homology. It is responsible for ligand binding, receptor dimerization, nuclear translocation, and ligand-dependent transaction (AF-2) of target genes. When a ligand is not present, a heat-shock protein Hsp90 binds and stabilizes the ER, and prevents dimerization and/or recognition of the estrogen responsive elements (ERE).
Figure 1.2: Schematics of human ERα and ERβ. The structural domains (A/B, C, D, E, and F) as well as the hormone binding, DNA binding and transactivation (AF-1, AF-2) functional domains are shown [7].

1.2.2 MECHANISM OF ESTROGEN RECEPTOR ACTIVATION

Steroids, like estrogens, are lipophilic molecules that are bound to steroid binding proteins in the blood stream to aid in transport as well as protection from metabolism. Upon arrival at a target tissue, an equilibrium exists between bound and unbound estrogen. Due to its lipophilic nature, estrogens can diffuse into the plasma membrane of the target tissue and translocate across the nuclear membrane to the nucleus. In the nucleus, estrogens will bind to an ER, displace the stabilizing heat-shock protein, and induce a conformational change (Figure 1.3). The conformational change of the estrogen-ER complex allows for homodimerization with another estrogen-ER complex via a zinc finger in the LBD. The homodimerized estrogen-ER complex recognizes specific sequences in the promoter regions of DNA called estrogen responsive elements (ERE). The DNA ERE is a palindromic consensus sequence of 12 base pairs separated by three-nucleotides (AGGTCAnnnTGACCT) [8]. Upon binding to the ERE, transcriptional machinery is recruited and results in the transcription of the gene. The
mRNA formed undergoes processing and translocation to the cytoplasm, followed by translation to form a protein which can alter cellular function by endocrine, paracrine, or autocrine interactions.

Figure 1.3: Mechanism of steroid hormone action.

The action of estrogen receptors is tripartite, involving the receptor (ERα or ERβ), its ligands, and the coregulatory proteins needed for transcription [7]. If the structural requirements of ligands needed to bind specifically to ERα and ERβ can be determined, the cell-specific and promoter-specific activities of estrogens in target cells could enable researchers to have an understanding of the exact role of estrogens in breast cancer cells and provide new insight into treatment. The role of ERβ in the breast remains to be fully defined; however, it may have a protective role in breast tissue [9]. Structural
information from x-ray crystallography is currently available for the ERα-LBD bound to 17β-estradiol [10,11], raloxifene [10], diethylstilbestrol [12], and 4-hydroxy-tamoxifen [12] and ERβ-LBD bound to raloxifene and genistein [13].

![Diagram of interactions between ligands, receptor, and effectors](image)

**Figure 1.4:** Tripartite nature of nuclear hormone receptors involving the interactions between ligands, receptor, and effectors [7].

### 1.3 Current Chemotherapy of Breast Cancer

The optimal treatment of breast cancer primarily depends on the stage of the breast cancer, the patient’s age and preferences, and the risks and benefits ascribed to each treatment. Surgery to remove the tumor is often combined with other treatments such as radiation therapy, chemotherapy, hormone therapy, and/or monoclonal antibody therapy [1]. The majority of human breast cancers are initially hormone-dependent and regress upon deprivation of the supporting hormone. The status of hormone dependence is usually determined by testing positive for the presence of estrogen or progesterone receptors. Because of the role of endogenous estrogens in the
development and maintenance of breast cancer, two main approaches have been developed to block or antagonize the action of these hormones. The first-line therapy for metastatic breast cancer is treatment that blocks the action of 17β-estradiol at the ER by means of antagonists, of which various antiestrogens including tamoxifen (Novaldex®) are used (Figure 1.5). About two-thirds of patients with ERα-positive breast tumors will respond favorably to tamoxifen treatment or other endocrine manipulations. Unfortunately, patients on tamoxifen therapy can relapse or acquire tamoxifen-resistant tumors as well as suffer from side effects such as increased incidence of uterine cancer, deep vein thrombosis and hot flushes [14]. The second approach is to inhibit the biosynthesis of 17β-estradiol locally within breast tissue by inhibiting aromatase, the cytochrome P450 enzyme that catalyzes the conversion of androgens into estrogens [15]. Aromatase inhibitors are the second-line defense for patients with ERα-positive breast tumors who fail tamoxifen treatment.

Various combinations of chemotherapy drugs are used in breast cancer, including cyclophosphamide, methotrexate, fluorouracil, doxorubicin (adriamycin), epirubicin, and paclitaxel (taxol). A recent clinical trial has demonstrated that tamoxifen can be used to reduce the risk of breast cancer in women at increased risk for developing the disease. A 47% reduction in the risk of invasive breast cancer and a 50% reduction in the risk of noninvasive breast cancer were observed in women taking tamoxifen as a preventative agent [14]. Table 1.2 provides a list of new anticancer treatments under development in breast cancer [16].
### New Anticancer Treatments under Development

- Cytotoxic drugs
  - New analogs
  - New molecular/mechanistic classes
- Modulators of drug resistance
- Immunologic approaches
  - Antibodies: monoclonal or polyclonal
  - Immunoconjugates:
    - With cytotoxic drugs
    - With radioactive substances
    - With toxins
- ADEPT systems
- Fusion proteins
- Vaccines
- Growth factor or growth factor receptor directed
  - HER-2/neu
  - EGFR
  - IGF-I
- Steroid hormones and their receptors
- Osteoclast activating factors
- Mammastatin
- Signal transduction inhibitors
  - Tyrosine kinase inhibitors
  - Farnesyl protein transferase inhibitors
  - Grb2 inhibitors
- Angiogenesis inhibitors
  - Anti-VEGF agents
  - Inhibitors of endothelial proliferation
- Inhibitors of tissue invasion and metastasis
  - Inhibitors of adhesion molecules, integrins
  - Matrix metalloprotease inhibitors
- Modulators of apoptosis
- Telomerase inhibitors

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**Table 1.2:** New anticancer treatments under development in breast cancer [16].
1.3.1 TARGETING THE ER WITH SERMS AND OTHER ER ANTAGONISTS

The structure activity requirements (SAR) of 17β-estradiol binding to ERα has been extensively researched [17]. The SAR of 17β-estradiol has been described as a planar hydrophobic structure which contains a phenol that acts as a H-bond donor (A-ring) separated from a second hydroxyl group that acts as a H-bond acceptor (D-ring) (Figure 1.5). Larger hydrophobic substituents are tolerated at positions 7α, 11β, and 17α. Nonsteroidal estrogens that possess this pharmacophore and bind to the ER exist and include synthetic potent estrogens (diethylstilbestrol, DES) and phytoestrogens (Figure 1.5). Phytoestrogens are weakly estrogenic compounds that have been isolated from plants. A few examples include the isoflavonoid genistein, coumestrol (found in clover), equol (plant metabolite), and the fungal metabolite zearalenone (Figure 1.5). These hormonal mimics, or agonists, bind to the ER and elicit a hormonal response.

Antiestrogens bind to the ER but due to structural differences prevent the expression of the genes that lead to cancerous growths. Antiestrogens are antagonists of the ER and includes derivatives of various backbones such as triphenylethylene, benzothiophene, steroidal, indole [18,19], napthalene [20,21], benzopyran [22], arylcoumarin [23], constrained tetracyclic cores [24], and other heterocyclic compounds (Figure 1.5-1.6) [25-27]. Although estrogens have a deleterious effect in breast tumors, they have protective effects in other tissues. Selective estrogen receptor modulators (SERMs) are a relatively diverse set of compounds that demonstrate both ER agonist and antagonist activity dependent upon the cell type and gene promoter targeted.
Tamoxifen, a triphenylethylene derivative, was among the first reported classes of SERMs and has been widely used for decades as treatment for breast cancer and more recently as a preventative agent for breast cancer. Tamoxifen functions as an antiestrogen in breast tissue, but has estrogenic activity in the uterus, liver, and bone. Tamoxifen has a triphenylethylene ring system with a basic amine side chain projecting off an aromatic ring and is hydroxylated in vivo to produce the active metabolite, 4-hydroxytamoxifen. Because tamoxifen produces an increased incidence of uterine cancer, efforts to modify this structure and reduce the effects in the uterus have been performed without much improvement (Figure 1.5). Toremifene is marketed for treatment of breast cancer with the same side effects in the uterus. Droloxifene and idoxifene were recently terminated from late stage clinical trials [28,29].

The majority of non-steroidal antiestrogens contain a common pharmacophore that includes two aryl groups separated by two atoms, often in a stilbene type arrangement. Typically one of these aryl groups contains a hydroxyl group and mimics the A-ring of 17β-estradiol. A third aryl group bearing a basic amine side chain is present and corresponds to the 11β-position of 17β-estradiol. This bulky basic amine side chain confers antiestrogenic activity by projecting into the estrogen receptor and displacing helix 12, which prevents interaction of the receptor with certain nuclear coactivator proteins and thus interferes with cellular transcription [10].
Raloxifene (Evista®) is a benzothiophene derivative that was approved in December 1997 for the treatment and prevention of osteoporosis in postmenopausal women. Raloxifene, like tamoxifen, antagonizes the effects of estrogen on breast tissue and mimics estrogens actions on bone; however, raloxifene does not induce significant uterine stimulation [30]. The orientation of the basic amine side chain has been determined to be critical in the selectivity of antiestrogens. The carbonyl hinge connecting the basic amine side chain to the benzothiophene ring system of raloxifene induces an orthogonal orientation [30]. The basic amine side chain is coplanar to the stilbene ring system of tamoxifen. Basic amine side chains have been appended to a variety of phytoestrogens and heterocyclic ring systems in appropriate positions in attempts to develop additional SERM-like molecules (Figure 1.5-1.6).
Figure 1.5: SAR of 17β-estradiol. Nonsteroidal, phytoestrogen, triphenylethylene, and benzothiophene derivatives of estrogen antagonists.
Figure 1.6: Steroid, indole, napthalene, benzopyran, and arylcoumarin derivatives of estrogen antagonists.
1.4 Estrogen Biosynthesis

The biosynthesis of estrogens, including 17β-estradiol, estrone, and estriol, occurs primarily in the ovaries in mature premenopausal women and is under the hormonal regulation of the hypothalamus-pituitary axis. The hypothalamus releases gonadotropin releasing hormone (GnRH) which acts on the pituitary to release follicle stimulating hormone (FSH) and leutinizing hormone (LH). FSH and LH bind to cell membrane receptors on granulosa and theca cells of the ovary, and through cAMP dependent cascade of events increases the amount of cholesterol and cytochrome P450 aromatase. In the theca cells of the ovary, cholesterol, a C27 precursor to the steroid hormones, is converted to pregnenolone (C21) in the rate determining step in steroid biosynthesis by the cytochrome P450 cholesterol side chain cleavage enzyme. Through a series of enzymatic steps, pregnenolone is converted to the androgen androstenedione (C19), which is transferred to the granulosa cells. In the granulosa cells of the ovary, androgens are converted to estrogens (C18) by the cytochrome P450 enzyme aromatase (estrogen synthase) (Figure 1.7).

Aromatase is encoded by the CYP19 located on chromosome 15q21.1 as a single gene. There are nine exons that encode to make aromatase, a 503 amino acid, 55 kDa NADPH-dependent cytochrome P450 reductase, which contains an iron-porphyrin prosthetic group. The regulation and expression of aromatase in different tissues is due to promoter switching of the CYP19 gene [31,32].
Estrogen biosynthesis does not exclusively occur in the ovaries. Other tissues that produce estrogens are the placenta, muscle, adipose tissue, brain, skin, and normal breast stromal cells. In addition, the biosynthesis of 17β-estradiol at low levels occurs locally with breast tumor tissue and is an important source of estrogen in postmenopausal women [33-39]. Because aromatase catalyzes the rate-limiting step in estrogen biosynthesis and is present locally within breast tumors, it is a potential treatment strategy in hormone dependent breast cancer.

Figure 1.7: Biosynthesis of estrogens from androgens via aromatase.

1.4.1 AROMATASE INHIBITORS

Aromatase inhibitors are primarily used as a second-line treatment for ER-positive breast cancer patients who fail tamoxifen treatment. Two classes of aromatase inhibitors, steroidal and nonsteroidal, are currently in use (Figure 1.8).
4-Hydroxyandrostenedione (Lentaron®) is a steroidal competitive inhibitor of aromatase resulting in either covalent or very tight binding of the inhibitor to the enzyme causing the inactivation of aromatase [40]. 7α-Thiosubstituted androstenedione inhibitors were synthesized by this laboratory as potent competitive (7α-APTA) or enzyme-activated irreversible inhibitors (7α-APTADD) [41-45]. The nonsteroidal aromatase inhibitors possess a heteroatom, usually a nitrogen-containing heterocycle that interferes with steroidal hydroxylation by coordinating with the iron of the heme group at the active site of aromatase and thus competing with androstenedione for the

**Figure 1.8**: Steroidal and non-steroidal aromatase inhibitors.
steroid binding site. Arimidex® and Femara® are highly potent, competitive, reversible aromatase inhibitors and are approved for the treatment of postmenopausal breast cancer [40].

1.5 FLAVONOIDs AS TARGETS IN BREAST CANCER

Found predominantly in higher plants, flavonoids are a class of natural products that encompasses the flavones, isoflavones, flavanones, and flavonols, each possessing the 4H-1-benzopyran-4-one ring system as the common chemical scaffold (Figure 1.9). Present in many food sources including fruits, vegetables, legumes, and whole grains, flavonoids have attracted considerable interest as dietary factors. Flavonoids may be responsible for the lower incidence of breast cancer in women from certain regions of the world, e.g., Japan and Finland [46,47]. A four to six-fold lower risk of breast cancer development exists in Asian women and has been linked to a traditional low-fat high-soy diet; flavonoids being present in soy [48,49]. The chemopreventive effects of vegetable- and fruit-rich diets may also be attributable to flavonoids.

![Figure 1.9: Flavonoids: a class of natural products.](image-url)
1.5.1 Biological Activities of Flavonoids

Over 4000 chemically unique flavones have been isolated from plants and exhibit a wide range of biological activities because of their ability to interact with various enzymes and receptor systems of pharmacological significance (Figure 1.10). Flavonoids demonstrate antiviral, anti-inflammatory, anti-allergic, antimutagenic, antioxidant, and anticarcinogenic activities [50-56]. Sharing structural similarities to the endogenous hormone 17β-estradiol, flavones and isoflavones possess many of the features (two phenols separated by approximately 11-12Å with a planar conformation) needed to satisfy the estrogen pharmacophore. It is not surprising that they possess estrogenic or antiestrogenic activities [48,51,53,57,58,59-60], bind to both ERα and ERβ [5,61], inhibit aromatase [62-64], and inhibit 17β-hydroxysteroid oxidoreductases type 1 [65,66]. Flavonoids have also shown activity as protein tyrosine kinase inhibitors [67-69], topoisomerase inhibitors [70], osteoclast inhibitors [71,72], angiogenesis inhibitors [73,74], and inhibit tubulin polymerization [75]. Because the 4H-1-benzopyran-4-one ring system is found in many different compounds that possess biological activity, we believe that modifications to this core skeleton can result in more selective therapeutic agents for molecular targets in breast cancer.
**Figure 1.10:** Biologically active flavonoids.

### 1.5.2 Phytoestrogens

A phytoestrogen has been loosely defined as any plant-derived compound that can regulate gene expression mediated by an ERE, in a manner either comparable or apparently antagonistic to 17β-estradiol, as a result of direct binding to ER [76]. There are several groups of phytoestrogenic compounds which includes the lignans, isoflavones (genistein), flavones (apigenin), chalcones (phloretin), flavanones (naringenin) and mycotoxins (zearalenone) (Figure 1.5, 1.11). Isoflavonoids may reduce the risk in developing breast cancer [77] and have been shown to inhibit the *in vitro* growth of MCF-7, T47D, MD-MBA-231, SKBR3, and ZR-75-1 human breast cancer cells [78-81]. Genistein, a principle isoflavone present in soy, has weak estrogenic activity [48], exhibits differential affinities for ERα and ERβ [5], inhibits protein tyrosine kinases (PTK) [67,68], regulates specific phases of the cell cycle,
inhibits DNA topoisomerase II activity, induces cellular differentiation, inhibits production of reactive oxygen species, and may have dietary chemopreventive benefits [46,47,82]. The SAR for genistein, as well as other flavonoids, have been determined with respect to genistein's estrogenic and PTK inhibitory activity [58,68]. For maximal estrogenic activity, the diaryl ring structure and hydroxyl substituents at the 4'- and 7-positions are necessary, and a hydroxyl group at the 5-position increases the estrogenicity of genistein [58]. The hydroxy group at the 5-position is essential for PTK activity and the hydroxyl groups at the 4'- and 7-position are necessary for full expression of PTK activity [68]. The dietary exposure of phytoestrogenic compounds and their risk/benefit to various cancers, including breast cancer, remains to be fully elucidated.

Figure 1.11: Phytoestrogenic compounds.

1.5.3 Flavonoids that Inhibit Aromatase

Various flavonoids have shown activity as aromatase inhibitors in vitro (Figure 1.12) [62,63,83-85]. It has also been shown that apigenin, naringenin, and chrysin did not significantly reduce androstenedione-induced uterine growth, indicating a lack of aromatase-inhibiting effect in vivo [64]. The SAR for various flavonoids has been
determined with respect to aromatase inhibition. Kao et al. suggested from site-directed mutagenesis studies that flavonoids bind to the active site in an orientation such that their A and C rings mimic rings D and C of the steroid, respectively [86]. As seen in the SAR of genistein for ER binding and PTK activity, the number and placement of hydroxyl groups are also important for aromatase inhibition [63,64,86]. For maximal aromatase inhibition, a 7-hydroxyl on a flavone skeleton is necessary. It was shown that reduction of the C-2, C-3 double bond or methylation of the 7-hydroxyl reduces the inhibitory effect. The farther the hydroxyl group was from the C-4 carbonyl, the higher the inhibition. Dihydroxyl groups at the 3'- and 4'-position or hydroxyl groups at the 4'- or 3-positions decrease the inhibitory capacity of the flavones.

Recanatini et al. recently designed a new class of nonsteroidal aromatase inhibitors based upon the chromone and xanthone skeletons with either an imidazole or 1,3,4-triazole linked to the aromatic moiety (Figure 1.13) [87]. The xanthone derivatives were able to potently and selectively inhibit cytochrome P450 aromatase (IC$_{50}$ = 43 and 40 nM) while showing minimal inhibition of 17α-hydroxylase/C17,20-lyase (P450$_{17}$).
Figure 1.12: Flavonoids as inhibitors of aromatase. For reference, the $K_m$ for the substrate androstenedione is approximately 20 nM. The current clinical drugs of anastrozole and letrozole have IC$_{50}$ values of 15 nM and 11.5 nM, respectively.

Figure 1.13: Xanthone and chromone nucleus as aromatase and 17α-hydroxylase inhibitors [87].
1.6 TARGETING BREAST CANCER WITH FLAVONOIDS

The 4H-1-benzopyran-4-one skeleton is present in the class of natural products known as the flavonoids. It has been shown that minor changes in flavonoid structure have resulted in major changes of biological activity. Structure activity requirements of flavonoids for ER binding, PTK, aromatase, and 17β-hydroxysteroid oxidoreductase, type 1 have been described [64]. The design, synthesis, and screening of a novel substituted 4H-1-benzopyran-4-ones solution phase library will allow us to harvest the biological potential of these molecules and develop selective agents for molecular targets in breast cancer.
CHAPTER 2

HYPOTHESIS

2.1 THE 4H-1-BENZOPYRAN-4-ONE RING SYSTEM AS A POTENTIAL TARGET

The 4H-1-benzopyran-4-one ring system is the core structure found in a number of natural products termed flavonoids which includes such classes as the flavones and isoflavones. Flavonoids exhibit numerous biological properties and interact with various enzymes and receptor systems of pharmacological significance. These natural products have shown antiviral, anti-inflammatory, anti-allergic, antifungal, and anticarcinogenic activities [50-53,55,88]. In fact, flavones and isoflavones share structural similarities to the endogenous 17β-estradiol, and substituted 4H-1-benzopyran-4-ones have shown activity as protein tyrosine kinase inhibitors, estrogen receptor agonists/antagonists, or inhibitors of steroidogenic enzymes including P450 aromatase [5,57-59,62,63,67,68]. Because the benzopyranone ring system is found in many different compounds that possess biological activity, we believe that modifications to this core skeleton can result in more selective therapeutic agents for molecular targets in breast cancer (Figure 2.1). Modifications to this skeleton could include alkyl, hydroxyl, ether, ester, amine, halogen, aromatic, heteroaromatic, or cycloalkyl substituents.
The 4H-1-benzopyran-4-one skeleton is the core structure present in many compounds including estrogenic flavonoids.

Figure 2.1: The 4H-1-benzopyran-4-one skeleton as a potential target.

2.1.1 INHIBITOR DESIGN

Although nature has provided many examples of natural products containing the benzopyranone skeleton, we believe that the opportunity still exists to make novel compounds targeted to breast cancer. With the advent of combinatorial chemistry, chemists are employing solid-phase techniques to quickly make libraries of compounds. The first example of 2,3-disubstituted benzopyran-4-ones has just recently been synthesized using a solid-phase diisopropylsilyloxy traceless linker yielding only a nine-membered library with limited side chain diversity [89] (Figure 2.2). Thus, our objective was to develop a novel solution-phase methodology that would not only be amenable to solid-phase but also have the potential to introduce multiple sites of chemical diversity with diverse side chains not found readily in nature (Figure 2.1). Existing synthetic methods for constructing benzopyranones are not ideally suited for making solid phase combinatorial chemistry libraries; they suffer from harsh reaction conditions, low yields, and poor substituent tolerance. Thus, we envisioned making benzopyranones retrosynthetically by cyclization of alkynones; which can in turn be made from salicylic acids and terminal alkynes (Figure 2.3). A synthetic chemistry
approach has been accomplished using these starting materials and is characterized by mild, high yielding reactions with good functional group tolerance; ideal for developing combinatorial libraries centered around the benzopyranone ring system.

**Figure 2.2:** Retrosynthetically, 2,3-disubstituted-benzopyran-4-ones were derived from salicyl aldehydes, amide acetals, and organometallic reagents [89].

**Figure 2.3:** Retrosynthetic approach of substituted-4H-1-benzopyran-4-ones.
2.2 Classical Methods for Flavone Synthesis

The synthetic methods in the literature that have been used to synthesize flavones can be grouped into 3 categories: (I) the heterocyclic ring is formed during the synthesis; (II) the heterocyclic ring is found in the starting material but in a different oxidation state or different ring size; and (III) alteration of another flavone. By far, the majority of the synthetic methodologies rely on the synthesis of the 4\(H\)-1-benzopyran-4-one ring system. Because of our interest in the synthesis of a diverse library of benzopyranones, alteration of another flavone is not applicable and therefore will not be discussed.

2.2.1 Forming the 2-Phenyl-4\(H\)-1-Benzopyran-4-one Ring System

The majority of synthetic methodologies for synthesis of the 4\(H\)-1-benzopyran-4-one involve the synthesis of the heterocyclic ring system. Four possible disconnection approaches (A-D) that are predominantly found in the literature for the synthesis of the benzopyranone skeleton can be seen in Figure 2.4 [90]. The carbon skeleton is usually formed by the reaction of two aromatic compounds. The primary approaches that have gained importance for the laboratory synthesis of flavones are approaches A and B: (A) acylation of phenols with a cinnamic acid derivative which corresponds to the biosynthetic pathway; and (B) condensation of a 2-hydroxyacetophenone with either an aromatic aldehyde or an aromatic carboxylic ester [91]. Approach C is the condensation of an \(o\)-alkoxycarboxylic ester with an acetophenone. No method based
upon approach D is known. Approach B is the most widely used method for the synthesis of flavonoids with the majority using an o-hydroxyaryl alkyl ketone as the starting material.

![Figure 2.4: Four possible disconnection approaches (A-D) that are predominantly found in the literature for the synthesis of the benzopyranone skeleton.](image)

2.3 SYNTHESIS OF FLAVONOIDS FROM AN O-HYDROXYARYL ALKYL KETONE

2.3.1 CLAISEN CONDENSATION

The Claisen condensation of o-hydroxyaryl alkyl ketones with a carboxylic ester is one of the most frequently used preparative methods of the 4H-1-benzopyran-4-one skeleton (Figure 2.5) [91]. It involves the condensation of 2-hydroxyacetophenone in the presence of a strong base to form a 1,3-diketone intermediate, which is cyclized upon heating in acidic medium to give the benzopyranone. Substituents on the aromatic ring
of the acetophenone have minimal effects on the condensation and both electron-donating and electron-withdrawing groups are compatible. The variation of the $R_2$ acetyl group is less because of the reactivity of some substituents towards nucleophiles. Because substituents on the acetyl group have a direct effect on the condensation, an alternative method for 3-substituted benzopyranones involves alkylation of the intermediate 1,3-diketone. The C-2 substituent arises from the ester component, which is commonly an alkyl or ethoxycarbonyl. This is a major limitation in forming flavones which have an aromatic group at $R_3$. The Claisen condensation requires a strong base for the condensation and acidic conditions for the cyclization, and therefore is not ideal for our synthetic pursuits.

![Figure 2.5: Claisen condensation](image)

**2.3.2 Baker-Venkataraman Rearrangement [92]**

Another source of the 1,3-diketone intermediate involves the $O$-acylation of 2-hydroxy-acetophenone to form an acyloxyacylbenzene intermediate followed by a Baker-Venkataraman rearrangement. Treatment of the acyloxyacylbenzene intermediate with base in pyridine initiates an intramolecular rearrangement in which the acyl moiety migrates from the oxygen to the carbon atom $\alpha$ to the carbonyl of the other acyl group.
This intramolecular rearrangement is known as the Baker-Venkataraman rearrangement. An advantage to this method is that the migrating acyl group may be aliphatic or aryl in nature and thus lead to the synthesis of flavones. The rearrangement occurs under a variety of basic catalysts including potassium carbonate, potassium hydroxide, sodium hydroxide, sodium metal, and sodium hydride. It has been suggested that the stronger basic species are more effective than potassium carbonate.

The proposed mechanism (Figure 2.6) is probably a base-catalyzed intramolecular Claisen condensation followed by ring opening to the diketone. The subsequent cyclization can be effected not only in sulfuric acid in ethanol, but also with glacial acetic acid and sodium acetate, or by heating the diketone in a vacuum.

\[ \text{Mechanism:} \]

**Figure 2.6: Baker-Venkataraman Rearrangement**
1,3-Diketone is a common intermediate for the synthesis of flavones and construction of the 4H-1-benzopyran-4-one skeleton. Fougerousse et al. have recently reported a new approach to synthesize flavonols using a Baker-Venkataraman rearrangement to form a 1,3-diketone intermediate (Figure 2.7) [93]. Other methodologies exist for the synthesis of the 1,3-diketone: (1) the Claisen reaction between salicylic acid derivatives like methyl 2-methoxybenzoate and acetone; (2) the direct acylation of lithium enolates of acyl phenols; and (3) the DBU catalyzed reaction of acetophenones with aryl or alkanoyl chlorides (Figure 2.8) [69,92,94]. Methyl salicylates have also been condensed with dilitiated β-diketones for the preparation of 2-phenacyl-4H-1-benzopyran-4-ones via a triketone intermediate) [95] or bromocrotononitrile for the preparation of 2-cyano-methylthiomethyl-4H-1-benzopyran-4-ones (Figure 2.9) [96]. These routes provide new classes of 2-substituted benzopyranones that were not easily obtainable by previous synthetic efforts.
Figure 2.8: Other sources of 1,3-diketones

Figure 2.9: Condensation and cyclization of methyl salicylates with dilithiated \( \beta \)-diketones [95] or bromocrotononitrile [96].
2.3.3 Allan-Robinson Condensation

The Allan-Robinson condensation is a one step condensation of an \( \alpha \)-hydroxy-acetophenone with the anhydride of an aromatic acid in the presence of the salt of the same acid, followed by alkaline hydrolysis (Figure 2.10). This reaction is performed at an oil bath temperature with either trimethylamine or pyridine as catalyst. The best results of this method occur when the ketone has oxygen in the \( \alpha \)-position (either as an alkoxy or aroyloxy group) and thus is suitable for the synthesis of 3-hydroxy- and 3-alkoxyflavones [90]. This method has been primarily used for the synthesis of 3-methoxyflavones. The first step of the Allan-Robinson reaction is evidently the formation of an \( \alpha \)-aryloxyacetophenone, which undergoes a Baker-Venkataraman rearrangement. Upon loss of water, the cyclized flavone is formed.

Figure 2.10: Allan-Robinson Condensation.

2.4 Synthesis of the 4H-1-Benzopyran-4-one Ring from Chalcones

The second method for synthesis of the 4H-1-benzopyran-4-one ring system is one in which the heterocyclic ring is found in the starting material but in a different oxidation state or different ring size. Reactions belonging to this class include the conversion of other flavonoids, such as flavanones or the chalcones, into the corresponding flavones.
2.4.1 ALGAR, FLYNN, AND OYAMADA (AFO) REACTION

The Algar, Flynn, and Oyamada (AFO) reaction of 1934 is one of the most frequently used synthetic methods for the oxidative conversion of 2'-hydroxychalcones into flavonols. This reaction involves the one step oxidation of 2'-hydroxychalcone to the flavonol (20-40% yield) with hydrogen peroxide in alkaline medium (Figure 2.11). Limitations for the AFO method exist. If the chalcone has a substituent in the 6'-position, the resulting yield of the flavonol is low due to the opening of the intermediate epoxide and results in the formation of aurones [93].

![Figure 2.11: Algar, Flynn, and Oyamada (AFO) Reaction](image)

Other methodologies exist for the cyclization of 2'-hydroxychalcones into flavones including the addition of bromine, refluxing with selenium dioxide, heating with DMF or disulfides, or stirring with palladium. Many of these methods are not successful for the general synthesis of flavones due to low yields and the formation of different products (flavone, aurones, flavanols, and flavanones) depending on the reaction conditions.
2.5 Heteroannulation Reactions

Substituted 4H-1-benzopyran-4-ones have classically been synthesized via a Claisen ester condensation followed by oxidative cyclization or a Baker-Venkataraman rearrangement. Besides requiring harsh acidic conditions for the final condensation step, these approaches also suffer from poor substituent tolerance and low yields. An alternative approach for the synthesis of benzopyranones involves heteroannulation reactions in which o-iodophenols react with terminal acetylenes in the presence of a base and palladium catalyst to undergo a carbonylative cyclization (Figure 2.12). A drawback to this chemistry in the past has been the requirement of high CO pressures and the formation of a mixture of benzopyranones and benzofuranones [97-99]. Recently, by using a new palladium complex as a catalyst (PdCl₂(Ph₃P)₂-thiourea-dppp), a highly efficient carbonylative cyclization of o-acetoxyiodobenzenes with aryl acetylenes to construct the corresponding flavones under mild conditions has been developed [100]. In developing our synthetic strategy, we wanted mild, high yielding reactions that would tolerate various functional groups to give exclusively the benzopyranone ring system, and still be amenable to solid-phase chemistry.
2.6 SYNTHETIC RATIONALE

Many investigators have researched the palladium-catalyzed carbonylative coupling of o-iodophenols with terminal alkynes and subsequent cyclization to form a mixture of 4H-1-benzopyran-4-ones and 3-(2H)-benzofuranones. A major drawback of this chemistry is the requirement for high carbon monoxide (CO) pressures and elevated temperatures to afford the alkynone intermediate, which upon cyclization produces a mixture of products. Because of our exclusive interests in the synthesis of benzopyranones and using chemistry applicable to solid phase, this chemistry at first glance was not ideally suited to our synthetic needs. However, upon closer inspection, this chemistry was more tolerant of various functional groups and provided higher yields than other existing protocols. If these problems could be resolved (i.e.; high CO pressure, high temperature, and non-regiospecific cyclization), a novel route for the regioselective formation of 4H-1-benzopyran-4-ones could possibly be developed.

Figure 2.12: Prevalent literature methods for the synthesis of benzopyranones include heteroannulation reactions.
Our solution phase chemistry studies started with the approach to synthesize 2-substituted-4H-1-benzopyran-4-ones from the palladium-catalyzed oxidative coupling of salicylic acids with terminal alkynes. By beginning with salicylic acids, instead of o-iodophenols, we could alleviate the high CO pressures and high temperatures need for the CO insertion to make the alkynone intermediate. Not to mention, the use of inexpensive, commercially available starting materials like salicylic acids and terminal alkynes would be ideal. The carboxylic acid of salicylic acids is not able to directly couple with a terminal alkyne. However, the conversion of the carboxylic acid on the salicylic acid to an acid chloride would enable the palladium-catalyzed Sonogashira coupling. The scope of this one-step Sonogashira coupling for the preparation of 1-alkynyl-ketones (alkynones) by coupling acyl chlorides with terminal alkynes can be accomplished in good yields [101]. Thus, by making an acyl-chloride from salicylic acids, and coupling it with terminal alkynes under Sonogashira conditions, we could make our key carbon-carbon bond.

The second major problem to resolve was the non-regiospecific cyclization of the alkynone intermediate. As seen in the literature, a free phenolic hydroxyl can effect either a 6-endo-dig or 5-exo-dig cyclization resulting in the nonselective formation of benzopyranones and benzofuranones. We rationalized that if the phenol was protected via protecting group like tert-butyldimethylsilyl (TBS); cyclization would be prevented until its removal. Michael addition of a secondary amine to the alkynone would result in the formation of enaminoketones (β-aminovinyl ketones) [102]. We reasoned that if
the alkynones were first converted to enaminoketones and then subjected to TBS deprotection, the system would be prone to undergo a 6-endo-\textit{trig} cyclization resulting in the regioselective formation of the 4\textit{H}-1-benzopyran-4-ones. By using a secondary amine addition to the alkynone, the synthetic strategy prevents the cyclization of the competing five-membered benzofuranone and thus resolves the regioselectivity problem encountered by previous efforts.

![Image of synthetic methodology]

\textbf{Figure 2.13:} Synthetic methodology for synthesis of substituted-4\textit{H}-1-benzopyran-4-ones.

Depending upon the variety of salicylic acids and terminal alkynes chosen, a diverse library of 2-substituted-4\textit{H}-1-benzopyran-4-ones can be synthesized. Synthetic approaches for diversifying the benzopyranone skeleton have also been pursued; substituents at the 3-position on the ring system would dramatically increase the diversity of our library. Evaluation of the synthetic benzopyranone library in initial bioassays (cell proliferation, aromatase inhibition, and estrogen receptor binding) using
human breast cancer cell lines has resulted in agents exhibiting enhanced and differential activities on breast cancer cell growth and aromatase inhibition. Continued synthetic efforts will concentrate on development of more selective agents for molecular targets in breast cancer based upon the benzopyranone nucleus.
CHAPTER 3

FORMATION OF ALKYNONE INTERMEDIATES VIA ACID CHLORINATION AND SONOGASHIRA COUPLINGS

3.1 ACID CHLORINATION

Various methods of acid chloride formation from carboxylic acids are reported in the literature. Table 3.1 compares the features of various reagents including thionyl chloride, oxalyl chloride, phosphorus compounds, and benzoyl chlorides for the formation of acid chlorides from carboxylic acids. The volatile reagents thionyl chloride and oxalyl chloride are often used for the formation of acid chlorides from carboxylic acids not only because they may be used in excess but evaporation of excess reagent affords the acid chloride product with good levels of purity [103]. An additional advantage for these reactions is the formation of gaseous by-products, both sulfur dioxide and HCl gas with thionyl chloride and carbon monoxide, carbon dioxide, and HCl gas with oxalyl chloride. Because this conversion involves acidic conditions, its application is limited to starting materials that lack acid-sensitive functionalities. The end point of this reaction is often judged once the evolution of gas has stopped. DMF (dimethylformamide) is often added as a catalyst to accelerate the reaction;
allowing the reaction to be conducted at room temperature or for shorter reaction times [103]. The amount of DMF added is typically catalytic, such as one or two drops of DMF are added.

<table>
<thead>
<tr>
<th></th>
<th>$\text{SOCl}_2$</th>
<th>$(\text{COCl})_2$</th>
<th>$\text{PPh}_3\text{Cl}_2$</th>
<th>$\text{PhCOCl}$</th>
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<tr>
<td><strong>Boiling point (°C)</strong></td>
<td>78-79</td>
<td>63-64</td>
<td>198-199</td>
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<td><strong>Molecular mass</strong></td>
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<td>126.93</td>
<td>140.57</td>
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<td><strong>Relative cost</strong></td>
<td>1</td>
<td>15</td>
<td>1</td>
<td>3</td>
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<td><strong>By-products</strong></td>
<td>$\text{SO}_2$, HCl</td>
<td>$\text{CO}_2$, HCl</td>
<td>$\text{Ph}_3\text{CO}$, $\text{HCCl}_3$</td>
<td>$\text{PhCO}_2\text{H}$</td>
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<tr>
<td><strong>Advantages</strong></td>
<td>Low cost</td>
<td>Simple workup</td>
<td>DMF catalyzed</td>
<td>Also polymer</td>
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<td></td>
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<tr>
<td><strong>Disadvantages</strong></td>
<td>Harsh conditions</td>
<td>Pyridine to remove</td>
<td>Removal of $\text{Ph}_3\text{PO}$</td>
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<td>Not compatible</td>
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Table 3.1: Comparison of reagents for the formation of acid chlorides [103].

3.2 Using Oxalyl Chloride

Adams and Ulich first introduced oxalyl chloride as a reagent for the conversion of carboxylic acids to the corresponding acid chlorides in 1920 [103]. The general reaction scheme is shown in Figure 3.1. Oxalyl chloride reacts with a carboxylic acid forming the desired acid chloride as well as carbon monoxide, carbon dioxide, and hydrogen chloride gas. In most cases, this reaction is performed at room temperature in an inert solvent such as benzene or dichloromethane in the presence of catalytic
amounts of DMF. Typically, the reaction is complete after 12h; however, the reaction is often substrate dependent. This reaction can often be judged complete when gas evolution ceases.

\[
\begin{align*}
\text{Cl}_2\text{C}=\text{O}\text{Cl} + \text{R} \text{CH}_2\text{OH} & \rightarrow \text{R} \text{Cl} + \text{HCl} + \text{CO} + \text{CO}_2 \\
\end{align*}
\]

**Figure 3.1:** General reaction scheme for acid chlorination of carboxylic acids using oxalyl chloride to produce acid chloride with carbon monoxide, carbon dioxide, and hydrogen chloride.

Until 1978, most methods accomplished the conversion of carboxylic acids into acid chlorides under acidic conditions. At that time, Wissner and Grudzinskas reported a methodology for forming carboxylic acid chlorides under neutral conditions [104]. In this communication, it was demonstrated that the reaction of tert-butyldimethylsilyl (TBS) esters with oxalyl chloride in the presence of catalytic amounts of DMF was an effective way to generate acid chlorides under neutral conditions [104]. In this reaction, TBS esters react with oxalyl chloride with catalytic amounts of DMF to generate the acid chloride, carbon dioxide, carbon monoxide, and TBS chloride in excellent yields (87-92%) (Figure 3.2). The proposed mechanism for the acid chlorination is shown in Figure 3.3 and involves the formation of dimethylformiminium chloride as the reactive intermediate. From the mechanism, it can be seen that tert-butyldimethylchlorosilane is generated instead of HCl, thus occurring under neutral conditions. Wissner et al. also reported that TBS ethers are stable to oxalyl chloride-DMF in the presence of TBS.
Figure 3.2: Preparation of acid chlorides under neutral conditions using tert-butyldimethylsilyl esters with oxalyl chloride and DMF [104].

Figure 3.3: Proposed mechanism of the acid chlorination of tert-butyldimethylsilyl esters with oxalyl chloride in the presence of DMF implicating dimethylformimininium chloride as the reactive intermediate.

esters under these reaction conditions [104]. In fact, this method was shown to be particularly useful for the preparation of acid chlorides derived from hydroxy-benzoic acids. The hydroxy-benzoic acid can be bis-silylated in a single step, converted to the corresponding acid chloride and then to the ethyl ester in yields of 98% [104] (Figure 3.4).
The key alkynone intermediate is synthesized by coupling acyl chlorides with terminal alkynes via a Sonogashira coupling. Acid chloride generation using oxalyl chloride produces volatile by-products that simply can be removed in vacuo. Therefore, we believed that the acid chloride generation could be followed directly by the Sonogashira coupling without intermediate purification resulting in a one-pot acid chlorination/Sonogashira coupling. To demonstrate the feasibility of a one-pot acid chlorination/Sonogashira coupling, 4-methoxy-bis-TBS-salicylic acid was used to determine if the acid chloride was generated cleanly and in high yield. To synthesize the acid chloride, oxalyl chloride (1.2 mmol) was added to a cold solution (0°C) of bis-TBS-salicylic acid in dichloromethane with 3 drops of DMF [104]. The resulting solution was stirred at 0°C for two hours. The reaction was then warmed to room temperature and stirred overnight. When the reaction was quenched with an ethanol in pyridine/ether (1:1:1) mixture, the ethyl ester was isolated in 77-91%, indicative of the clean formation of the acid chloride. Figure 3.5 illustrates the yields of the conversion of the TBS esters to the ethyl ester with various methoxy substituents.
Figure 3.5: Acid chloride generation followed by quenching with a mixture of ethanol:ether:pyridine (1:1:1) resulting in the isolation of the ethyl ester.

3.3 PROTECTIONS OF SALICYLIC ACIDS

Our synthetic rationale of using substituted salicylic acids as starting materials to synthesize the benzopyranone ring system is a direct application of Wissner and Grudzinskas communication. Thus, commercially available salicylic acid was used to optimize tert-butyldimethylsilyl (TBS) protection procedures. Both the carboxylic acid and phenolic hydroxyl of salicylic acid can be silylated in a single step. Initial protection procedures using t-BuMe2SiCl, imidazole, and DMAP in DMF were not always successful. Protection of 2,4-dihydroxybenzoic acid or 5-bromo-salicylic acid with tert-butyldimethylchlorosilane, imidazole, 4-dimethylaminopyridine (DMAP) in DMF provided the tri-silated derivative in 66% yield and the bis-silated in 43-75% yield [105]. Although these conditions provided the silylated salicylic acids in good yields, these conditions did not always work in all cases at all times. Often, the steric bulk of the tert-butyl group significantly diminishes the rate of silylation. Even when using the
basic activators, imidazole and DMAP, and performing the reaction in the dipolar aprotic solvent DMF, silylation was slow and inconsistent [106]. Salicylic acid was treated with 2.2 equiv of t-BuMe₂SiCl and triethylamine in CH₂Cl₂ to generate the bis-TBS protected salicylic acid in quantitative yield [107]. Salicylic acid could be protected in most cases in quantitative yields using tert-butylidemethylchlorosilane with Et₃N in dichloromethane regardless of the substitution pattern as illustrated in Figure 3.6.

![Figure 3.6: Protection of salicylic acid using tert-butylidemethylsilyl chloride and triethylamine in dichloromethane occurs in quantitative yield [107].](image)

**3.4 SUBSTITUTED SALICYLIC ACIDS USED**

This methodology was used to protect a variety of substituted salicylic acids including halogen, aromatic, methoxy, amine, and alkyl functionalities at the three, four, five, and six positions. The salicylic acids that were formed following the TBS protection in yields ranging from 88-100% can be seen in Figure 3.7.

Many salicylic acids are commercially available or can be readily synthesized. 4-Methoxysalicylic acid can also be prepared in 50% yield by selective methylation of the 4-hydroxyl group of 2,4-dihydroxybenzoic acid using dimethylsulfate in a 20%
solution of sodium hydroxide (Figure 3.8) [108]. 5-Phenylsalicylic acid was prepared in 91% yield by an aqueous Suzuki coupling of 5-bromosalicylic acid and phenylboronic acid in the presence of Pd(OAc)$_2$ as the base [109]. 5-(tert-Butoxycarbonyl)-amino salicylic acid was synthesized via the Boc protection of 5-aminosalicylic acid with di-tert-butylcarbonate in dioxane-H$_2$O and Et$_3$N [110].

![Chemical Structures](attachment:image1.png)

**Figure 3.7:** Substituted salicylic acids and the yields resulting from the protection with TBS-Cl.

![Chemical Structures](attachment:image2.png)

**Figure 3.8:** Preparation of 4-methoxysalicylic acid by selective methylation of 2,4-dihydroxybenzoic acid using dimethylsulfate ((CH$_3$O)$_2$SO$_2$) in a 20% solution of sodium hydroxide [108].
Figure 3.9: Preparation of 5-phenylsalicylic acid and NHBoc-salicylic acid. 5-Phenylsalicylic acid was prepared by an aqueous Suzuki coupling of 5-bromosalicylic acid and phenylboronic acid in the presence of Pd(OAc)$_2$ with Na$_2$CO$_3$ as the base [109]. 5-(tert-Butoxycarbonyl)aminosalicylic acid was synthesized via the Boc protection of 5-aminosalicylic acid with di-tert-butylcarbonate in dioxane-H$_2$O and Et$_3$N [110].

3.5 THE SONOGASHIRA COUPLING

Carbon-carbon bond forming reactions, especially those that are simple, efficient, high yielding, and tolerant of a wide range of functional groups, are important in organic synthesis. One such reaction, developed in 1975 by Sonogashira, typically involves the coupling of terminal alkynes with aryl or alkenyl halides in the presence of catalytic amounts of palladium (II) and copper (I) iodide in an amine solvent [111]. The general forms of this reaction are shown in Figure 3.10.
Figure 3.10: A. General forms of the Sonogashira coupling [111]; B. Sonogashira coupling extended to include preparation of 1-alkynyl ketones [112]

Kenkichi Sonogashira developed this reaction at the same time as both Heck and Cassar reported a similar process. However, their methods for making a carbon-carbon bond lacked the involvement of copper as a catalyst and required more forcing conditions.

The most common derivation of the Sonogashira coupling is the coupling of an aromatic iodide with a terminal alkyne at room temperature. Typically, this reaction is complete in a few hours, although often left overnight. It has been noted that dramatic color changes occur during the time course of the reaction but not in a predictive manner.
Sonogashira originally used palladium (II) (i.e., PdCl₂(PPh₃)₂) and copper (I) iodide as co-catalysts in this reaction. However, other palladium (I) and (II) catalysts can be found in the literature to catalyze the reaction (i.e. Pd(PPh₃)₄, Pd(OAc)₂-PPh₃, and polymer-bound palladium-phoshine) [113]. Copper (I) iodide has been found to be essential for the Sonogashira reaction to proceed at room temperature, which agrees with synthetic efforts in this laboratory. In our laboratory, Sonogashira couplings in the presence of PdCl₂(PPh₃)₂ without copper iodide were not successful.

More recent reports challenge the use of palladium as a co-catalyst. Chowdhury et al. report that acylation of terminal alkynes can be effected in the presence of 5 mol% copper (I) iodide without the need for a palladium catalyst to synthesize α,β-acetylenic ketones with yields ranging from 48-83% (Figure 3.11) [114,115]. This reaction has been shown to be applicable to aryl, heteroaryl, or alkyl terminal alkynes and aryl or branched acid chlorides [114]. Under these conditions, the acid chloride had to be aromatic; straight chain aliphatic acid chlorides did not work. Mechanistically, it is proposed that the acid chloride reacts with a copper acetylide reactive intermediate leading to the formation of the acetylenic ketone. This process eliminates the possibility of the competing oxidative homocoupling of alkynes, known as the Glaser coupling which are usually formed in the presence of palladium [115]. This methodology was used to prepare a uracil derivative with an acetylenic ketone functionality substituted at the C-5 position from the corresponding 5-ethynyl-2,4-dimethoxy pyrimidine and p-toluoyl chloride [115].
Figure 3.11: A facile method for the synthesis of \( \alpha,\beta \)-acetylenic ketones using terminal alkynes and acid chlorides in the presence of copper(I) iodide catalyst in triethylamine [114, 115].

The scope of this one-step Sonogashira coupling was extended to include the preparation of 1-alkynyl ketones (Figure 3.10). Aliphatic and acyl chlorides can be coupled with terminal alkynes in triethylamine with Pd(PPh\(_3\))\(_2\)Cl\(_2\) and CuI as seen in Figure 3.10 to afford the desired ketones in good yields [112]. Under these conditions, the highest yield was observed for coupling an acyl chloride with phenylacetylene. Yields decreased when either coupling partner is aliphatic.

Thorand et al. have shown that by coupling \( p \)-substituted-aryl bromides with terminal alkynes with catalytic amounts of Pd(PPh\(_3\))\(_2\)Cl\(_2\) and CuI in triethylamine and THF as solvent at room temperature produce arylalkynes in excellent yields (>82%) [116]. Their results suggest an increase of reactivity when the coupling is performed in THF instead of an amine as solvent. This is a marked improvement to literature methods for aryl bromide couplings, which due to their rather low reactivity require harsh conditions.
(i.e., high temperatures). The Glaser coupling was seen only in trace amounts (<5%). This side reaction is believed to be minimized due to the slow addition of the alkyne which keeps its concentration in the reaction mixture low [116].

Fu et al. reports the use of Pd(PhCN)2Cl2/P(t-Bu)3 as an efficient catalyst for room-temperature Sonogashira reactions of aryl bromides, which are the least reactive of the commonly employed organic halides (vinyl iodide, vinyl bromide > aryl iodide > vinyl chloride >> aryl bromide) [117].

The exact mechanism of the Sonogashira coupling is not known; however, the proposed mechanism is shown in Figure 3.12 [118]. It follows the normal oxidative addition-reductive elimination process common to palladium-catalyzed carbon-carbon bond forming reactions. The process involves generating the active palladium (0) species 1 which inserts via oxidative addition into the acyl chloride bond to give the acylpalladium (II) intermediate 2. Subsequent transmetallation of the acylpalladium intermediate 2 with the terminal alkyne, possibly via a transient copper acetylide species, leads to the alkynylpalladium (II) derivative 3. Derivative 3 can collapse via a reductive elimination to form the product and regeneration of the Pd(PPh3)2Cl2 catalyst. The rate determining step of the reaction is the oxidative addition (step i) of the aryl/acyl halide to the palladium (0) species. The reaction can be carried out without the presence of the copper (I) iodide with more stringent forcing conditions and more active substrates [118].
Figure 3.12: Proposed mechanism of the Sonogashira coupling which involves (i) oxidative addition, (ii) transmetallation, and (iii) reductive elimination steps [118,119].

The proposed mechanism, as shown in Figure 3.12 in one catalytic cycle, is more accurately believed to be a combination of two catalytic cycles [119]. Figure 3.13 shows the second catalytic cycle, which involves the formation of the unsaturated 14-electron complex Pd(0)(PPh₃)₂. The complex Pd(0)(PPh₃)₂ is produced by the reductive elimination of a Pd-acetylide complex generated by the transmetallation of the palladium (II) catalyst, PdCl₂(PPh₃)₂ with a terminal acetylene.
Various reaction conditions have been used for the Sonogashira coupling depending upon the reactivity profile of the starting materials. The reactivity of the organic halides for coupling decreases in the order vinyl iodide ≈ vinyl bromide > aryl iodide > vinyl chloride >> aryl bromide [119]. Aryl halides that have electron-withdrawing groups ortho or para to the halide react more readily [118]. This can be correlated to the mechanism in that the more electron-deficient the aryl halide, the more rapidly the Pd(0) species can insert, or rather undergo oxidative addition to the aryl-halide [118]. Various amines have been used as solvent and play a critical role in the coupling. The rate of the reaction depends upon the type of amine used and decreases in the order n-BuNH₂ > Et₃N > i-Pr₂NH > Et₂NH > K₂CO₃ [119].
3.6 SYNTHESIS OF THE PALLADIUM CATALYST

The palladium (II) catalyst dichlorobis(triphenylphosphine)palladium (Pd(PPh₃)₂Cl₂) is commercially available. It also can be readily synthesized following the literature procedure of Herrman & Salzer in which sodium tetrachloropalladate (II) (0.5 g, 1.7 mmol) and triphenylphosphine (0.95 g, 3.6 mmol) are suspended in EtOH (50 mL) under argon and stirred at room temperature for 24 h [120]. Upon addition of triphenylphosphine to sodium tetrachloropalladate solution, color change from brownish red to yellow. The resulting reaction mixture was filtered by gravity to give a yellow precipitate, which was further washed with H₂O, EtOH, and Et₂O. Solid was recrystallized from CHCl₃/petroleum ether to yield 1.1 g (90%) of the title compound as a yellow powder. The Pd catalyst is stable for long term storage.

3.7 GLASER COUPLING

In 1869, the synthesis of the symmetrical diphenylbutadiyne from phenylacetylene and air was performed by Glaser [121]. Glaser’s procedure involved using preformed copper acetylides that undergo an oxidative homocoupling according to the equation in Figure 3.14. Variations of this chemistry have since been used to produce a large variety of acetylenic compounds that have been in essence dimerized to form diynes. The amount of copper needed is usually stoichiometric. The Glaser coupling of terminal alkynes is an important side reaction that sometimes occurs during the Sonogashira coupling. Hence, reaction conditions need to minimize the Glaser homocoupling in the presence of the Sonogashira coupling.
2 R\equiv + \frac{1}{2} O_2 \rightarrow R\equiv \equiv R + H_2O

**Figure 3.14:** The overall equation of the Glaser homocoupling of terminal alkynes in the presence of copper [121].

### 3.8 Functional Group Compatibility of the Sonogashira Coupling

#### 3.8.1 Initial Couplings with Phenylacetylene

Efforts to synthesize the alkynyl ketones via the Sonogashira coupling were initially investigated using salicylic acid and phenylacetylene. The scope of the Sonogashira coupling includes the condensation of copper(I) salts of alkynes with acylhalides to provide a useful synthesis of 1-alkynyl ketones. As seen in Figure 3.15, acyl chloride can be coupled with phenylacetylene in Et$_3$N in presence of CuI and Pd(PPh$_3$)$_2$Cl$_2$ as catalysts to provide the alkynyl ketone in a yield of 96% [112]. This method seemed advantageous to synthesize the alkynyl ketones that we were interested in because it is a one step condensation reaction, employs mild reaction conditions, and is amenable to combinatorial applications.

\[
\begin{align*}
\text{Cl} & \quad \text{Pd(PPh$_3$)$_2$Cl$_2$} \\
\text{C$_6$H$_5$} & \quad \text{C$_6$H$_5$} \\
\text{Cu, Et$_3$N, rt, 18h} & \quad \text{Et$_3$NH}^{+}\text{Cl}^{-}
\end{align*}
\]

**Figure 3.15:** An acyl chloride can be coupled with phenylacetylene under Sonogashira conditions (Et$_3$N in the presence of CuI and Pd(PPh$_3$)$_2$Cl$_2$ as co-catalysts) to provide the alkynyl ketone in a yield of 96% [112].
Figure 3.16 shows one example from the literature where a salicyloyl chloride was coupled with phenylacetylene. In this example, salicyloyl chloride was coupled with phenylacetylene in trioctylamine at 50°C for six hours with Pd$_2$(dba)$_3$ catalyst resulting in the formation of the alkynyl ketone in 56% yield, the benzopyranone in 14% yield, and the benzofuranone in 19% yield [122]. The outcome and product distribution of this reaction suggests that the free phenol in the ortho position influences the reaction.

![Chemical Reaction](image)

**Figure 3.16:** An and Chiusoli et al. coupled salicyloyl chloride with phenylacetylene resulting in the formation of the alkynone (56%), benzopyranone (14%) and benzofuranone (19%) [122].

Previous efforts in our laboratory attempting to synthesize the alkynone from the salicyloyl chloride and phenylacetylene failed using various conditions (Figure 3.17). A Stille reaction with allyltributyltin also failed to give any coupled product (Figure 3.17) [105]. These coupling experiments emphasized the need for protecting the phenol during the coupling reaction. It was our hypothesis that, as a result of protecting the latent hydroxy group as a TBS ether, the cyclization forming both the benzopyranone and benzofuranone would be prevented and the alkynone could be isolated in high yield. Initial attempts of a Stille coupling of TBS-salicyloyl chloride with allyltributyltin also failed (Figure 3.18).
Our laboratory initially examined the coupling reactions between phenylacetylene and the acid chlorides generated from bis-TBS-salicylic acid under Sonogashira coupling conditions. The acid chloride (1 mmol) in 4 mL of $\text{Et}_3\text{N}$ was treated with of phenylacetylene (1 equiv), 1 mg of Cul, and 1 mg of Pd($\text{PPh}_3)_2\text{Cl}_2$. The reaction was stirred for 15 hours under argon and after workup and flash chromatography provided the alkynyl ketone in >80% yield. This reaction was unsuccessful when attempted with only the copper salts (Cul or Cud) in absence of the Pd catalyst. In addition, other
palladium catalysts were evaluated including Pd(PPh\textsubscript{3})\textsubscript{4} and PhCH\textsubscript{2}Pd(PPh\textsubscript{3})Cl\textsubscript{2}, however, complex mixtures of unidentifiable products were obtained.

The usefulness of the Sonogashira coupling as it relates to electronic and steric requirements of the terminal alkyne was determined. When using aliphatic and aromatic alkynes in conjunction with the Pd(PPh\textsubscript{3})\textsubscript{2}Cl\textsubscript{2} and Cul catalysts in Et\textsubscript{3}N, it was determined that using an excess of alkyne was optimal. Previous experimentation in our laboratory established that using 7 equivalents of aliphatic or 4 equivalents of aromatic alkynes was often necessary [105,123]. Because the homocoupling of alkynes is catalyzed in the presence of molecular oxygen, the reaction mixtures were deoxygenated by bubbling argon directly into the reaction mixture thereby reducing the formation of dieynes [116]. In order to determine the generality of the one-pot acid chlorination/Sonogashira coupling, various terminal alkynes including 4-ethynyltoluene, 5-chloro-1-pentyne, methyl propargyl ether, propiolaldehyde diethyl acetal, 2-methyl-3-butyn-2-ol, and 1-ethynyl-1-cyclohexene were coupled to salicylic acid under these procedures resulting in alkynone formation in moderate to excellent yields (74-96\%) (Figure 3.19) [105,123]. Initial investigations of Sonogashira coupling illustrated the usefulness for the formation of alkynones from silylated salicylic acids.
Figure 3.19: Various terminal alkynes including 4-ethynyltoluene, 5-chloro-1-pentyne, methyl propargyl ether, propiolaldehyde diethyl acetal, 2-methyl-3-butyn-2-ol, and 1-ethynyl-1-cyclohexene were coupled to bis-TBS-salicylic acid via a one-pot acid chlorination/Sonogashira coupling resulting in alkynone formation in moderate to excellent yields (74-96%) [105,123].

3.8.2 Sonogashira Coupling of Phenylacetylene with Substituted Salicylic Acids

Substituted bis-TBS-salicylic acids were coupled with phenylacetylene to evaluate the effect of substitutions of the salicylic acid component on the coupling reaction. The bis-TBS-salicylic acids used were 4-methoxy-bis-TBS-salicylic acid, 4-chloro-bis-TBS-salicylic acid, 5-phenyl-bis-TBS-salicylic acid, and 5-NHBoc-bis-TBS-salicylic acid. All of the coupling reactions gave the desired alkynones in excellent yields (78-92%).
The acid sensitive NH-Boc functionality is successfully carried through the acid chlorination step, emphasizing the mild nature of the reaction conditions. The synthesis of 5-phenylsalicylic acid and 5-NHBoc-salicylic acid is depicted in Figure 3.9.

![Chemical structure: 5-Ph, 4-OCH3, 4-Cl, 5-NHBoc](image)

<table>
<thead>
<tr>
<th>R</th>
<th>% Yield of Alkynone</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-Ph</td>
<td>90%</td>
</tr>
<tr>
<td>4-OCH3</td>
<td>92%</td>
</tr>
<tr>
<td>4-Cl</td>
<td>83%</td>
</tr>
<tr>
<td>5-NHBoc</td>
<td>78%</td>
</tr>
</tbody>
</table>

**Figure 3.20:** Substituted bis-TBS-salicylic acids (i.e.; 4-methoxy-bis-TBS-salicylic acid, 4-chloro-bis-TBS-salicylic acid, 5-phenyl-bis-TBS-salicylic acid, and 5-NHBoc-bis-TBS-salicylic acid) undergo a one-pot acid chlorination/Sonogashira coupling with phenylacetylene to provide the corresponding alkynones [105,123].

### 3.9 Examining the Scope of the Sonogashira Coupling

#### 3.9.1 Sonogashira Coupling Variations Used

Various conditions (scale of reaction, equivalents of alkyne, time, and coupling procedure) used for the Sonogashira coupling of bis-TBS-salicylic acid, 3-methyl-bis-TBS-salicylic acid, 3-methoxy-bis-TBS-salicylic acid, 4-methoxy-bis-TBS-salicylic acid, 4-methyl-bis-TBS-salicylic acid, 4-chloro-bis-TBS-salicylic acid, 5-chloro-bis-TBS-salicylic acid, and 5-phenyl-bis-TBS-salicylic acid are illustrated in Table 3.2 and Table 3.3. There were four different procedures used for the couplings and include:
**Coupling A**: Et$_3$N added to acid chloride followed by deoxygenation. Alkyne, Pd(PPh$_3$)$_2$Cl$_2$, and CuI added followed by deoxygenation. **Coupling A**: This is the same procedure as A but without deoxygenation. **Coupling B**: Et$_3$N added to acid chloride and deoxygenated. Alkyne and CuI added and deoxygenated. Pd(PPh$_3$)$_2$Cl$_2$ added and deoxygenated. **Coupling C**: Et$_3$N, alkyne, and CuI are stirred under argon for 15 min, followed by cannulation into acid chloride and finally addition of Pd(PPh$_3$)$_2$Cl$_2$. Deoxygenation is defined as bubbling argon directly into the reaction to remove any trace amounts of oxygen. **Coupling D**: Et$_3$N added to acid chloride followed by deoxygenation. Pd(PPh$_3$)$_2$Cl$_2$ and CuI added and deoxygenated. Alkyne (1.2 equiv) is added followed by an additional 1.2 equiv of alkyne added 4 h later. It is believed that the slow addition of alkyne keeps its concentration low in the reaction, thus reducing the Glaser coupling [116].

**3.9.2 Sonogashira Couplings with 4-Methoxysalicylic Acid**

Because the one-pot acid chlorination/Sonogashira coupling reaction is key for introducing diversity to our solution phase library of benzopyranones, the scope of this reaction was examined in closer detail. 4-Methoxysalicylic acid was initially chosen to examine more closely because of the placement of the methoxy group. Many active flavonoids have hydroxyl groups at this position; therefore it would be advantageous to make 7-hydroxy or 7-methoxybenzopyranones for further biological evaluation.
4-Methoxy-bis-TBS-salicylic acid coupled with phenylacetylene in high yield (92%) and the effect of an electron-donating group para to the acid chloride could also be determined.

![Chemical reaction diagram](image)

**Figure 3.21:** % Yields of one-pot acid chlorination and Sonogashira coupling of various terminal alkynes with 4-methoxysalicylic acid. The alkynes used were phenylacetylene, 4-ethynyltoluene, 1-ethynyl-1-cyclohexene, propionaldehyde diethyl acetal, 5-chloro-1-pentyne, and 1-pentyne.

4-Methoxysalicylic acid was treated with TBS-Cl (2.2 equiv) and Et$_3$N in CH$_2$Cl$_2$ to generate 4-methoxy-bis-TBS-salicylic acid in quantitative yield (Figure 3.7) [107]. The protected salicylic acid was reacted with 1.2 equivalents of oxalyl chloride in the presence of catalytic amounts of DMF in CH$_2$Cl$_2$ to provide the acid chloride under neutral conditions [104]. Previous experiments demonstrated the clean formation of the acid chloride in high yields (Figure 3.5). The acid chloride was concentrated *in vacuo* and followed directly by the Sonogashira coupling. Following *Coupling A* for the
Sonogashira coupling, the acid chloride in triethylamine was reacted with phenylacetylene, in the presence of catalytic amounts of Pd(PPh₃)₂Cl₂ and CuI to give the desired alkynone in 92% yield (Figure 3.21, Table 3.2) [112]. As seen in Table 3.2, the effect of varying the equivalents of phenylacetylene and reaction time were examined for this coupling. Shorter reaction times (3-5 h) gave higher yields (92-96%), in both small scale (0.7 mmol) and large scale (5.1 mmol) reactions. In the majority of experiments, an excess (4-5 mol excess) of phenylacetylene was used. The % yield of alkynone decreased to 54% when DMF was not used in the acid chlorination reaction.

To determine the generality of the one-pot acid chlorination/Sonogashira coupling, various terminal alkynes (aromatic, alkyl, acetal) were coupled to 4-methoxysalicylic acid under the same procedures (Figure 3.21) resulting in alkynone formation in moderate to excellent yields (42-90%). Based on further experimentation (Table 3.2), optimal yields of alkynone can be achieved on a case by case basis. For example, a yield of 96% resulted when phenylacetylene (4 equiv) was coupled for 4 hours with Coupling C; a yield of 57% resulted with 5-chloro-1-pentyne (10 equiv) was coupled for 5 hours with Coupling B; and finally a yield of 84% resulted when propiolaldehyde diethyl acetal (5 equiv) was coupled for 19.5 hours with Coupling A.
<table>
<thead>
<tr>
<th>Notebook #</th>
<th>Salicylic acid</th>
<th>Alkyne</th>
<th>Scale (mmol)</th>
<th>Alkyne (equiv)</th>
<th>Time (h)</th>
<th>Procedure</th>
<th>% Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>I-108</td>
<td>4-OCH₃ phenylacetylene</td>
<td>1</td>
<td>3</td>
<td>22</td>
<td>A</td>
<td>55%</td>
<td></td>
</tr>
<tr>
<td>I-123</td>
<td>4-OCH₃ phenylacetylene</td>
<td>1</td>
<td>5</td>
<td>24</td>
<td>A</td>
<td>53%</td>
<td></td>
</tr>
<tr>
<td>I-141</td>
<td>4-OCH₃ phenylacetylene</td>
<td>0.7</td>
<td>5</td>
<td>15</td>
<td>A</td>
<td>91%</td>
<td></td>
</tr>
<tr>
<td>I-126</td>
<td>4-OCH₃ phenylacetylene</td>
<td>1</td>
<td>5</td>
<td>3.5</td>
<td>A</td>
<td>92-95%</td>
<td></td>
</tr>
<tr>
<td>I-111</td>
<td>4-OCH₃ phenylacetylene</td>
<td>1.5</td>
<td>5</td>
<td>24</td>
<td>A</td>
<td>84%</td>
<td></td>
</tr>
<tr>
<td>I-185</td>
<td>4-OCH₃ phenylacetylene</td>
<td>4.3</td>
<td>4</td>
<td>20</td>
<td>A</td>
<td>81%</td>
<td></td>
</tr>
<tr>
<td>II-078</td>
<td>4-OCH₃ phenylacetylene</td>
<td>5.1</td>
<td>4</td>
<td>4</td>
<td>A</td>
<td>94%</td>
<td></td>
</tr>
<tr>
<td>II-138</td>
<td>4-OCH₃ phenylacetylene</td>
<td>1</td>
<td>5</td>
<td>80</td>
<td>A</td>
<td>54% No DMF used</td>
<td></td>
</tr>
<tr>
<td>I-196</td>
<td>4-OCH₃ phenylacetylene</td>
<td>1</td>
<td>1.2</td>
<td>40</td>
<td>A**</td>
<td>no reaction</td>
<td></td>
</tr>
<tr>
<td>I-210</td>
<td>4-OCH₃ phenylacetylene</td>
<td>1</td>
<td>4</td>
<td>4</td>
<td>C</td>
<td>81%</td>
<td></td>
</tr>
<tr>
<td>I-212</td>
<td>4-OCH₃ phenylacetylene</td>
<td>1</td>
<td>4</td>
<td>4</td>
<td>C</td>
<td>96%</td>
<td></td>
</tr>
<tr>
<td>I-213</td>
<td>4-OCH₃ phenylacetylene</td>
<td>1</td>
<td>2</td>
<td>4</td>
<td>C</td>
<td>76%</td>
<td></td>
</tr>
<tr>
<td>I-114</td>
<td>4-OCH₃ 4-ethyltoluene</td>
<td>1.5</td>
<td>5</td>
<td>17</td>
<td>A</td>
<td>70%</td>
<td></td>
</tr>
<tr>
<td>II-039</td>
<td>4-OCH₃ 4-ethyltoluene</td>
<td>2.7</td>
<td>3</td>
<td>4</td>
<td>A</td>
<td>no reaction; NMR indicates Me ester</td>
<td></td>
</tr>
<tr>
<td>I-116</td>
<td>4-OCH₃ 1-ethyl-1-cyclohexene</td>
<td>1</td>
<td>8.5</td>
<td>15.5</td>
<td>A</td>
<td>65%</td>
<td></td>
</tr>
<tr>
<td>I-192</td>
<td>4-OCH₃ 1-ethyl-1-cyclohexene</td>
<td>1.1</td>
<td>4</td>
<td>25</td>
<td>A</td>
<td>50%</td>
<td></td>
</tr>
<tr>
<td>II-043</td>
<td>4-OCH₃ 1-ethyl-1-cyclohexene</td>
<td>2.7</td>
<td>4</td>
<td>9</td>
<td>A</td>
<td>89%</td>
<td></td>
</tr>
<tr>
<td>I-110</td>
<td>4-OCH₃ 5-chloro-1-pentyne</td>
<td>1.1</td>
<td>7</td>
<td>21</td>
<td>A</td>
<td>23%</td>
<td></td>
</tr>
<tr>
<td>I-130</td>
<td>4-OCH₃ 5-chloro-1-pentyne</td>
<td>1</td>
<td>10</td>
<td>2.5</td>
<td>A</td>
<td>42%</td>
<td></td>
</tr>
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<td>I-201</td>
<td>4-OCH₃ 5-chloro-1-pentyne</td>
<td>1.1</td>
<td>10</td>
<td>5</td>
<td>B</td>
<td>57%</td>
<td></td>
</tr>
<tr>
<td>II-024</td>
<td>4-OCH₃ 1-pentyne</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>A</td>
<td>99%</td>
<td></td>
</tr>
<tr>
<td>II-032</td>
<td>4-OCH₃ 1-pentyne</td>
<td>2.5</td>
<td>3</td>
<td>4.5</td>
<td>A</td>
<td>89%</td>
<td></td>
</tr>
<tr>
<td>I-115</td>
<td>4-OCH₃ propiolaldehyde diethyl acetal</td>
<td>1</td>
<td>5</td>
<td>19.5</td>
<td>A</td>
<td>94%</td>
<td></td>
</tr>
<tr>
<td>I-135</td>
<td>4-OCH₃ propiolaldehyde diethyl acetal</td>
<td>0.53</td>
<td>5</td>
<td>20.5</td>
<td>A</td>
<td>66%</td>
<td></td>
</tr>
<tr>
<td>I-195</td>
<td>4-OCH₃ propiolaldehyde diethyl acetal</td>
<td>1</td>
<td>2.4</td>
<td>25</td>
<td>D</td>
<td>29% benzofuranone</td>
<td></td>
</tr>
<tr>
<td>I-219</td>
<td>4-OCH₃ propiolaldehyde diethyl acetal</td>
<td>1</td>
<td>5</td>
<td>19</td>
<td>C</td>
<td>24%</td>
<td></td>
</tr>
</tbody>
</table>

A El₃N added to acid chloride followed by deoxygenation. Alkyne, Pd(PPh₃)₃Cl₂, and Cul added followed by deoxygenation.
B El₃N added to acid chloride followed by deoxygenation. Alkyne and Cul added followed by deoxygenation. Pd(PPh₃)₃Cl₂ added followed by deoxygenation.
C El₃N, alkyne, and Cul are cannulated into acid chloride after being stirred under argon for 15 min, followed by cannulation into acid chloride and addition of Pd(PPh₃)₃Cl₂.
D El₃N added to acid chloride followed by deoxygenation. Pd(PPh₃)₃Cl₂ and Cul added and deoxygenated. Alkyne (1.2 eq) is added, another 1.2 eq added 4 h later.
** No Pd(PPh₃)₃Cl₂ added

Table 3.2: Effects of various procedures on the % yield of alkynone formation for the Sonogashira coupling of 2-[[1,1-dimethylethyl]dimethylsilyl]oxy]-4-methoxy-benzoic acid, (1,1-dimethylethyl)dimethylsilyl ester with terminal alkynes (phenylacetylene, 4-ethynyltoluene, 1-ethyl-1-cyclohexene, 5-chloro-1-pentyne, 1-pentyne, propiolaldehyde diethyl acetal). The effects due to changing the scale of the reaction (mmol), the reaction time (h), and amount of alkyne (equivalents) can be evaluated (Notebook # refers to either JLW-I-xxx or JLW-II-xxx).
The electronics and steric of the alkyne as well as the salicylic acid need to be considered when determining the optimal coupling conditions. The lowest yields (23-57%) were found when coupling 5-chloro-1-pentyne to 4-methoxy-bis-TBS-salicylic acid. To determine if this effect was due to an alkyl alkyne, 1-pentyne was coupled with 4-methoxy-bis-TBS-salicylic acid and resulted in yields of 89-99%. A dramatic difference in yield exists when comparing 5-chloro-1-pentyne and 1-pentyne. One suggestion is that the base Et$_3$N could be displacing the chloro group; however, no evidence of this has been found. Another possibility is that the Pd catalyst could be complexing with the chloro group on the alkyne forming an alternative palladium intermediate and thus not available for the coupling reaction. Yields are improved when a large excess (10 equiv) of 5-chloro-1-pentyne is used, suggesting another possibility that a higher amount of alkyne homocoupling exists which may explain thin-layer chromatography results.

3.9.3 Sonogashira Couplings with 3-Methoxy, 5-Methoxy, and 6-Methoxy-salicylic Acid

The effect of a methoxy substituent at the 3-, 4-, 5-, and 6-position of bis-silylated salicylic acid on the Sonogashira coupling was determined. A comparison of the yields of the one-pot acid chlorination and Sonogashira coupling for the various methoxy substitutions is illustrated in Figure 3.22. Details of the conditions of the reactions can be found in Table 3.3. Coupling 3-methoxy- and 5-methoxy-bis-TBS-salicylic acid with phenylacetylene resulted in similar coupling yields, i.e. 70% and 75% respectively. In both these cases, the methoxy was meta to the acid chloride. When the methoxy was
para to the acid chloride, as with 4-methoxy-bis-TBS-salicylic acid, a higher coupling yield was found only when coupling phenylacetylene. 6-Methoxy-bis-TBS-salicylic acid did not couple with phenylacetylene under the conditions used. As seen in Figure 3.5, the acid chloride was generated with 6-methoxy-bis-TBS-salicylic acid in good yield. Further studies investigating the effects of substituents at the 6-position (ortho) would be useful to determine if this is a general limitation of the Sonogashira coupling of acyl chlorides with terminal alkynes.

<table>
<thead>
<tr>
<th>Methoxy salicylic acid</th>
<th>Terminal Alkynes</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="ChemicalStructure.png" alt="Chemical Structure" /></td>
<td><img src="ChemicalStructure.png" alt="Chemical Structure" /> <img src="ChemicalStructure.png" alt="Chemical Structure" /> <img src="ChemicalStructure.png" alt="Chemical Structure" /></td>
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</tr>
<tr>
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<td><img src="ChemicalStructure.png" alt="Chemical Structure" /> <img src="ChemicalStructure.png" alt="Chemical Structure" /> <img src="ChemicalStructure.png" alt="Chemical Structure" /></td>
</tr>
</tbody>
</table>

**Figure 3.22:** Comparison of % yields of one-pot acid chlorination and Sonogashira coupling of various terminal alkynes with 4-methoxy, 3-methoxy, 5-methoxy, and 6-methoxy-bis-TBS-salicylic acid. The alkynes used were phenylacetylene, 4-ethynyltoluene, 1-ethynyl-1-cyclohexene, and 5-chloro-1-pentyne. ** The overall yield for coupling and cyclization is 22%.**
### Table 3.3: Effects of various procedures on the % yield of alkyne formation for the Sonogashira coupling (Notebook # refers to either JLW-I-xxx, JLW-II-xxx, or ASB [105]).

<table>
<thead>
<tr>
<th>Notebook #</th>
<th>Salicylic acid</th>
<th>Alkyne</th>
<th>Scale (mmol)</th>
<th>Coupling Procedure</th>
<th>Alkyne % Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>I-51</td>
<td>allyltributyltin</td>
<td>1.4</td>
<td>15</td>
<td>19</td>
<td>no reaction</td>
</tr>
<tr>
<td>I-57</td>
<td>allyltributyltin</td>
<td>1.4</td>
<td>11</td>
<td>overnight</td>
<td>no reaction</td>
</tr>
<tr>
<td>I-72</td>
<td>phenylacetylene</td>
<td>1.4</td>
<td>1</td>
<td>23</td>
<td>A*</td>
</tr>
<tr>
<td>I-85</td>
<td>phenylacetylene</td>
<td>1</td>
<td>1</td>
<td>18.5</td>
<td>A*</td>
</tr>
<tr>
<td>I-94</td>
<td>phenylacetylene</td>
<td>1</td>
<td>1</td>
<td>18.5</td>
<td>A*</td>
</tr>
<tr>
<td>I-165</td>
<td>phenylacetylene</td>
<td>1</td>
<td>5</td>
<td>21</td>
<td>A</td>
</tr>
<tr>
<td>I-184</td>
<td>phenylacetylene</td>
<td>4.6</td>
<td>4</td>
<td>20</td>
<td>A</td>
</tr>
<tr>
<td>I-208</td>
<td>phenylacetylene</td>
<td>1.1</td>
<td>4</td>
<td>6</td>
<td>B</td>
</tr>
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<tr>
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<td>3</td>
<td>overnight</td>
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<tr>
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<td>A 81%</td>
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</tbody>
</table>

A  Et₃N added to acid chloride followed by deoxygenation. Alkyne, Pd(PPh₃)₂Cl₂, and Cul added followed by deoxygenation.
A* Et₃N, alkyne, Pd(PPh₃)₂Cl₂, and Cul added to acid chloride sequentially without deoxygenation.
B Et₃N added to acid chloride followed by deoxygenation. Alkyne and Cul added followed by deoxygenation. Pd(PPh₃)₂Cl₂, added followed by deoxygenation.
C Et₃N, alkyne, and Cul are stirred under argon for 15 min, followed by cannulation.
3.9.4 Sonogashira Couplings with 3-Methylsalicylic Acid

3-Methyl-bis-TBS-salicylic acid was coupled with phenylacetylene resulting in the formation of the alkynone in 88-92% yield following Coupling A (Table 3.3). Because the yield of this reaction was excellent, further examples of coupling 3-methyl-bis-TBS-salicylic acid with alkynes followed Coupling C and were immediately cyclized without isolation of the alkynone.

3.9.5 Sonogashira Couplings with 4-Chlorosalicylic Acid

4-Chloro-bis-TBS-salicylic acid was coupled with 4-ethynyltoluene (3 equiv) following Coupling B (allowing preformation of the copper acetylide species). The major product synthesized was the corresponding alkynone, isolated in 62% yield (Table 3.3). After flash chromatography, a minor amount of the cyclized benzopyranone (15 mg, 5% yield) and benzofuranone (57 mg, 21% yield) was evident by $^1$H NMR. Previous coupling attempts with 4-ethynyltoluene and 4-chloro-bis-TBS-salicylic acid resulted in the formation of a 40% mixture of two alkynones: one in which the alkyne not only coupled with the acid chloride, but also the aryl chloride at the 4-position (Figure 3.23).

Figure 3.23: Sonogashira coupling of 4-chloro-bis-TBS-salicylic acid with 4-ethynyltoluene produces a mixture of products in 40% yield.
Other couplings were examined with 4-chloro-\textit{bis}-TBS-salicylic acid, in each case following \textit{Coupling B}. When coupling 4-chloro-\textit{bis}-TBS-salicylic acid with phenylacetylene (3 equiv), the alkynone was isolated in 84% yield, while a small amount of the corresponding benzopyranone was also isolated (~3% yield). When coupling with 1-ethynyl-1-cyclohexene (3 equiv), the coupling worked very well (~96%), however, cyclization resulted in an overall yield from the protected salicylic acid of 47% (previous efforts 70%). 4-Ethynyltoluene (3 equiv) was coupled with 4-chloro-\textit{bis}-TBS-salicylic acid at room temperature overnight under these conditions to yield 62% of the alkynone, 21% benzo[\textit{f}]uranone, and 5% benzopyranone. In each of these experiments, the coupling yields were moderate to high (62-96%) and yielded a small amount of either benzopyranone and/or benzo[\textit{f}]uranone. Coupling efforts with 5-chloro-1-pentyne (5 equiv) for 30 hours resulted in direct formation of the benzopyranone in 42% yield and no isolation of alkynone.

From experimental data collected, one can compare the effect of a methoxy and chloro substituent at the 4-position of silylated salicylic acids. In general, Sonogashira coupling yields are slightly lower with the 4-chloro-\textit{bis}-TBS-salicylic acid. However, it can also be said that lower yields are seen when the 4-chloro is coupled with aromatic alkynes. This is exemplified by the example shown in Figure 3.23 where 4-ethynyltoluene in fact coupled with the aryl chloride. Possibly, the chloro group when \textit{para} to the acid chloride can become activated for coupling with a fairly electron rich
alkyne like 4-ethynyltoluene. This is noteworthy because in most cases the reaction of aryl chlorides under Sonogashira conditions only occurs when electron-withdrawing groups, particularly nitro, are located at the *ortho* or *para* position [118].

### 3.9.6 Sonogashira Couplings with 5-Chlorosalicylic Acid

5-Chloro-*bis*-TBS-salicylic acid was reacted with phenylacetylene, 4-ethynyltoluene, 1-ethynyl-1-cyclohexene, or propiolaldehyde diethyl acetal following *Coupling A* resulting in the corresponding alkynes being isolated in yields ranging from 62-84% (Table 3.3). When 5-chloro-1-pentyne was coupled overnight with 5-chloro-*bis*-TBS-salicylic acid, 6-chloro-2-(3-chloropropyl)-4H-1-benzopyran-4-one was isolated in 14-29% instead of the alkynone.

### 3.9.7 Sonogashira Coupling Variations with 5-Phenylsalicylic Acid

To determine how to increase yields, variations of the standard Sonogashira coupling procedure were examined with 5-phenyl-*bis*-TBS-salicylic acid. As discussed earlier, the exact mechanism of the Sonogashira coupling and the role of the copper catalyst are not known. One possible mechanism involves a transient copper acetylide species reacting with an arylpalladium (II). If a copper acetylide species is allowed to form first without interference from other reactants, yields may be increased. In order to test this hypothesis, *Coupling C* was followed in which copper iodide and 3 equiv of phenylacetylene were preincubated in triethylamine for 10 min. This was then
cannulated into the acid chloride followed by the addition of Pd(PPh₃)₂Cl₂ catalyst. The result of this coupling was isolation of the alkynone in 76% yield, lower than previous coupling results of 90%.

In attempting Coupling B, triethylamine was added to the acid chloride, followed by addition of copper iodide and alkyne. This was allowed to stir at room temperature while bubbling argon directly into the reaction for 5 min before the addition of the palladium catalyst. This method was used to couple 4-ethynyl-toluene, 1-ethynyl-1-cyclohexene, and 5-chloropentyne and resulted in formation of alkynones. The alkynones were then cyclized resulting in overall yields over the 3 steps of 77%, 68% and 39% of the corresponding benzopyranones, respectively. The overall yields when a phenyl substituent is located at the five position were excellent. There was no evidence of a marked improvement in coupling yields when the copper iodide was preincubated with the alkyne for 5-phenylsalicylic acids.

3.10 Conclusions About One-Pot Acid Chlorination-Sonogashira Coupling Reaction

The highest yield for the alkynone formation from the one-pot acid chlorination/Sonogashira coupling for the nine substituted bis-silylated-salicylic acids and the six terminal alkynes used is presented in Figure 3.24.
Terminal Alkynes

% Yield of Alkynone

<table>
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<th>Terminal Alkynes</th>
<th>OTBS CH₂</th>
<th>CH₃O' OTBS</th>
</tr>
</thead>
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<tr>
<td>92%</td>
<td>95%</td>
<td>96%</td>
</tr>
<tr>
<td>70%</td>
<td>72%</td>
<td>68%</td>
</tr>
<tr>
<td>92%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>96%</td>
<td>70%</td>
<td>89%</td>
</tr>
<tr>
<td>83%</td>
<td>40%</td>
<td>82%</td>
</tr>
<tr>
<td>84%</td>
<td>77%</td>
<td>74%</td>
</tr>
<tr>
<td>90%</td>
<td>84%</td>
<td>73%</td>
</tr>
<tr>
<td>75%</td>
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<td>No rxn</td>
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</tbody>
</table>

Figure 3.24: Highest % yield of alkynone formation.
CHAPTER 4

CYCLIZATIONS OF ALKYNONE TO FORM 4H-1-BENZOPYRAN-4-ONES

4.1 Competing Cyclization of Benzopyranones versus Benzofuranones, Mechanism of Cyclization

The palladium catalyzed carbonylative cyclization of o-iodophenols and terminal acetylenes in the presence of base (usually Et₂NH or Et₃N) is known to produce a mixture of six-membered benzopyranones and five-membered benzofuranones (Figure 4.1) [97-99]. The mechanistic rationale for the formation of benzopyranones versus benzofuranones is shown in Figure 4.2 [100]. In this catalytic cycle, the o-iodophenol undergoes oxidative addition by the Pd[0], CO insertion, and complexation with the alkyne to form intermediate D, an alkynone. It is hypothesized that intermediate D can then undergo three different routes to form either the benzopyranones or the benzofuranones. The alkynone can form benzopyranones by two possible routes: (1) a 6-endo-dig cyclization of the alkynone or (2) a 6-endo-trig cyclization of the enaminoketone E formed by the Michael addition of a secondary amine to the alkynone D. Benzofuranone formation could arise by the complexation of alkynone D with palladium (0) to form complex G. Intermediate G following a rearrangement and reductive elimination could give rise to the benzofuranones.
Figure 4.1: Palladium catalyzed carbonylative cyclization of \( o \)-iodophenols and acetylenes produces a mixture of benzopyranones and benzofuranones.

Figure 4.2: The mechanistic rationale for the formation of benzopyranones versus benzofuranones [100].
Evidence by Korshunov et al. supports the idea of the alkynone being a critical intermediate for benzopyranone and benzofuranone formation. Korshunov et al. examined the role of the hydroxyl group in the intramolecular cyclization of 3-aryl-1-(4-hydroxyaryl)-2-propyn-1-ones (an alkynone) with various primary, secondary, and tertiary amines (Figure 4.3) [102]. They found that the product formed from the reaction of the alkynone with an amine was controlled by the nature of the amine. The reaction of the alkynone with a primary, secondary, or tertiary amine resulted in the formation of a β-aminovinyl ketone (enaminoketone). Depending on the amine used, the enaminoketone could further undergo an intramolecular cyclization with the participation of the ortho hydroxyl group. The end result was that a primary amine led only to enaminoketone formation, a secondary amine led to benzopyranone formation, and a tertiary amine led to benzofuranone formation [102]. Their results suggest that product formed by the intramolecular cyclization of an alkynone can be controlled by the nature of the amine. Depending on reaction conditions, 3-aryl-1-(4-hydroxyaryl)-2-propyn-1-ones can also be cyclized in basic media (potassium carbonate in acetone or sodium ethoxide in EtOH) to afford a mixture of benzopyranones or benzofuranones [124].
Figure 4.3: The reaction of 3-aryl-1-(2-hydroxylaryl)-2-propyn-1-ones with a primary, secondary, or tertiary amine resulting in the formation of β-aminovinylketone (enaminoketone), benzopyranone, or benzofuranone, respectively [102].

The factors controlling the regioselectivity for benzopyranone and benzofuranone formation have been examined in the reaction of substituted o-iodophenols with substituted phenylacetylene [98,99]. Ciattini et al. carried out this reaction in DMF at 60°C under 1 atm CO pressure using DBU as the base and Pd(OAc)$_2$(DPPF)$_2$ as the catalyst to afford a mixture of benzopyranones and benzofuranones (Figure 4.4). In this experiment, the carbonylative coupling was accomplished using milder conditions and ambient CO pressure than previous experimental attempts. The regioselectivity of the palladium-catalyzed cyclization could be controlled depending on the base used. It should be noted that only the more stable Z-isomer of the benzofuranone was isolated.
Figure 4.4: o-Iodophenol was reacted under atmospheric CO pressure with phenylacetylene in DMF at 60°C using 1,8-diababicyclo[5.4.0]undec-7-ene (DBU) as base and the catalyst generated in situ from 3 mol% of Pd(OAc)$_2$ and 6 mol% 1,1’=bis(diphenylphosphino)ferrocene (DPPF) [98].

The synthesis of benzopyranones and quinolones via the palladium-catalyzed carbonylation of o-iodophenols and o-iodoanilines in the presence of acetylenes via a one-pot reaction has been performed (Figure 4.5) [97,99]. Torii et al. accomplished a one-pot coupling and cyclization of alkynones using an autoclave and an excess of diethylamine for use as base and solvent, 5 mol% Pd(PPh$_3$)$_2$Cl$_2$, high CO pressures (20 kg/cm$^2$) at 120°C for 6 h to give exclusively the 6-membered benzopyranones or quinolones. By using an excess of diethylamine as base and solvent, the enamino ketone intermediate was generated thus resolving regioselectivity problems encountered by previous efforts. Benzofuranones or the indoxyl derivatives were not detected when secondary amines were used. This reaction is general for secondary amines; diethylamine, morpholine and piperidine worked; however, the basicity and sterics of the amine were found to influence the reaction. Because of the high temperatures and pressures used, this reaction is not ideally suited for combinatorial synthesis of flavonoid libraries.
Figure 4.5: Regioselective synthesis of benzopyranones and quinolones via a one-pot palladium catalyzed carbonylation of o-iodophenols and o-iodoanilines in the presence of acetylenes [97,99].

4.2 CYCLIZATION OF ALKYNONES USING DIETHYLAMINE AND ETHOH

As seen in the literature, a free phenolic hydroxyl can effect either a 6-endo-dig or 5-exo-dig cyclization resulting in the nonselective formation of benzopyranones and benzofuranones, respectively. Because of our exclusive interest in the benzopyranone ring system, we were interested in synthetic methodology that would allow us to regioselectively synthesize the benzopyranones. We reasoned that if the latent ortho hydroxyl group is protected as a TBS ether, the cyclization of the alkynones could not occur until the protecting group was removed. If the alkynones were first converted to enaminoketones and then subjected to TBS deprotection, the system would be prone to undergo Michael addition followed by elimination of secondary amine to yield the desired benzopyranones as a 6-endo-trig cyclization (Figure 4.6). The potential for benzofuranone formation by the 5-exo-dig cyclization option would thus be eliminated. The enaminoketone intermediate could also be used to introduce various electrophiles at the three-position [125].
Figure 4.6: Alkynone is reacted with diethylamine to form the enaminoketone intermediate. The enaminoketone undergoes TBS deprotection, allowing the hydroxy group to attack the sp² carbon resulting in a 6-endo-trig cyclization to form exclusively the benzopyranones [123].

4.2.1 INITIAL STUDIES OF CYCLIZATIONS

In order to determine if this synthetic rationale would work, bis-TBS-salicylic acid was first coupled with phenylacetylene to form the alkynone by methodology previously discussed. To an ethanolic solution of alkynone, diethylamine (1.2 equiv) was added and stirred at room temperature under argon for 4 hours to afford the protected enaminoketone intermediate in 72% yield (Table 4.1, entry I-102). When an excess of diethylamine (10 equiv) was added to the alkynone in EtOH, the reaction yielded 51% of the protected enaminoketone, 16% of the deprotected enaminoketone, and 6% of the benzopyranone (Table 4.1, entry I-156). This led us to investigate the direct conversion of the alkynones to the benzopyranones in a single step.

To our surprise, it was discovered that the conversion of the alkynones to enaminoketones and subsequent cyclization could be effected in a single step. Thus, an ethanolic solution of an alkynone and 10 equiv of diethylamine could afford the TBS-protected-enaminoketone in less than 2 hours. Upon rotary evaporation of diethylamine and ethanol, the TBS-protected-enaminoketone was resuspended in ethanol and
refluxed for 18 hours to provide the benzopyranone in yields of 96% (Figure 4.7). After conditions for the cyclization were worked out, the protocol was applied to a diverse set of alkynones as seen in Figure 4.7 to provide 2-substituted-benzopyranones in yields ranging from 54-96% [105,123]. Similar cyclization results were obtained by using other secondary amines such as dimethylamine (2M solution in THF), pyrrolidine, and N-benzyl-ethylamine. Diisopropylamine reacted sluggishly with an ethanolic solution of 1-[2-[[1,1-dimethylth)dimethylsilyl]oxy]-phenyl]3-phenyl-2-propyn-1-one, so the reaction mixture was refluxed for 24 hours and after workup, the enaminoketone was isolated in quantitative yield [105].
Figure 4.7: Cyclization of alkynone with excess of diethylamine in EtOH to provide the enaminoketone intermediate, which can undergo 6-endo-*trig* cyclization when refluxed in EtOH [105,123].
<table>
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<th>Cyclization Procedure</th>
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<th>Product #</th>
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<td></td>
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</tr>
<tr>
<td>ASB</td>
<td>H phenylacetylene</td>
<td>C</td>
<td>96%</td>
<td>10</td>
<td>2-Phenyl-4H-1-benzopyran-4-one</td>
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<tr>
<td>I-246</td>
<td>H phenylacetylene</td>
<td>F</td>
<td>73%</td>
<td>10</td>
<td>2-Phenyl-4H-1-benzopyran-4-one</td>
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<tr>
<td>I-240</td>
<td>H phenylacetylene</td>
<td>F</td>
<td>50%</td>
<td>10</td>
<td>2-Phenyl-4H-1-benzopyran-4-one</td>
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<tr>
<td>ASB</td>
<td>H 4-ethynyltoluene</td>
<td>C</td>
<td>87%</td>
<td>11</td>
<td>2-(4'-Methylphenyl)-4H-1-benzopyran-4-one</td>
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<tr>
<td>I-247</td>
<td>H 4-ethynyltoluene</td>
<td>F</td>
<td>48%</td>
<td>11</td>
<td>2-(4'-Methylphenyl)-4H-1-benzopyran-4-one</td>
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<tr>
<td>ASB</td>
<td>H 1-ethynyl-1-cyclohexene</td>
<td>C</td>
<td>82%</td>
<td>12</td>
<td>2-(1-Cyclohexen-1-yl)-4H-1-benzopyran-4-one</td>
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<tr>
<td>I-248</td>
<td>H 1-ethynyl-1-cyclohexene</td>
<td>F</td>
<td>71%</td>
<td>12</td>
<td>2-(1-Cyclohexen-1-yl)-4H-1-benzopyran-4-one</td>
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<tr>
<td>ASB</td>
<td>H 5-chloro-1-pentyne</td>
<td>C</td>
<td>73%</td>
<td>13</td>
<td>2-(3-Chloropropyl)-4H-1-benzopyran-4-one</td>
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<td>I-249</td>
<td>H 5-chloro-1-pentyne</td>
<td>F</td>
<td>51%</td>
<td>13</td>
<td>2-(3-Chloropropyl)-4H-1-benzopyran-4-one</td>
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<td>ASB</td>
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<td>C</td>
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<td>I-250</td>
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<td>F</td>
<td>21%</td>
<td>14</td>
<td>2-(Diethoxymethyl)-4H-1-benzopyran-4-one</td>
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<tr>
<td>I-171</td>
<td>3-OCH3 phenylacetylene</td>
<td>C</td>
<td>88%</td>
<td>20</td>
<td>8-Methoxy-2-phenyl-4H-1-benzopyran-4-one</td>
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<tr>
<td>I-224</td>
<td>3-OCH3 phenylacetylene</td>
<td>F</td>
<td>95%</td>
<td>41%</td>
<td>2-(Diethoxymethyl)-4H-1-benzopyran-4-one</td>
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<tr>
<td>I-227</td>
<td>3-OCH3 phenylacetylene</td>
<td>F</td>
<td>89%</td>
<td>20</td>
<td>8-Methoxy-2-phenyl-4H-1-benzopyran-4-one</td>
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<tr>
<td>I-42</td>
<td>3-OCH3 4-ethynyltoluene</td>
<td>C</td>
<td>82%</td>
<td>59%</td>
<td>2-(4'-Methylphenyl)-4H-1-benzopyran-4-one</td>
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<tr>
<td>I-228</td>
<td>3-OCH3 4-ethynyltoluene</td>
<td>F</td>
<td>88%</td>
<td>21</td>
<td>8-Methoxy-2-(4'-methylphenyl)-4H-1-benzopyran-4-one</td>
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<tr>
<td>I-48</td>
<td>3-OCH3 1-ethynyl-1-cyclohexene</td>
<td>C</td>
<td>72%</td>
<td>49%</td>
<td>2-(1-Cyclohexen-1-yl)-8-methoxy-4H-1-benzopyran-4-one</td>
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<tr>
<td>I-225</td>
<td>3-OCH3 1-ethynyl-1-cyclohexene</td>
<td>F</td>
<td>65%</td>
<td>44%</td>
<td>2-(1-Cyclohexen-1-yl)-8-methoxy-4H-1-benzopyran-4-one</td>
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<tr>
<td>I-253</td>
<td>3-OCH3 5-chloro-1-pentyne</td>
<td>F</td>
<td>22%</td>
<td>23</td>
<td>8-Methoxy-2-(3-Chloropropyl)-4H-1-benzopyran-4-one</td>
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<tr>
<td>I-230</td>
<td>3-OCH3 propiolaldehyde diethyl acetal</td>
<td>C</td>
<td>53%</td>
<td>24</td>
<td>2-(Diethoxymethyl)-8-methoxy-4H-1-benzopyran-4-one</td>
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<tr>
<td>I-172</td>
<td>3-CH3 phenylacetylene</td>
<td>C</td>
<td>87%</td>
<td>40</td>
<td>8-Methyl-2-phenyl-4H-1-benzopyran-4-one</td>
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<tr>
<td>I-177</td>
<td>3-CH3 phenylacetylene</td>
<td>C</td>
<td>82%</td>
<td>40</td>
<td>8-Methyl-2-phenyl-4H-1-benzopyran-4-one</td>
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<tr>
<td>I-252</td>
<td>3-CH3 phenylacetylene</td>
<td>F</td>
<td>84%</td>
<td>40</td>
<td>8-Methyl-2-phenyl-4H-1-benzopyran-4-one</td>
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<td>I-232</td>
<td>3-CH3 4-ethynyltoluene</td>
<td>F</td>
<td>81%</td>
<td>41</td>
<td>8-Methyl-2-(4'-methylphenyl)-4H-1-benzopyran-4-one</td>
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<tr>
<td>I-233</td>
<td>3-CH3 1-ethynyl-1-cyclohexene</td>
<td>F</td>
<td>83%</td>
<td>42</td>
<td>2-(1-Cyclohexen-1-yl)-8-methyl-4H-1-benzopyran-4-one</td>
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</tr>
<tr>
<td>I-251</td>
<td>3-CH3 5-chloro-1-pentyne</td>
<td>F</td>
<td>40%</td>
<td>43</td>
<td>2-(3-Chloropropyl)-8-methyl-4H-1-benzopyran-4-one</td>
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<tr>
<td>II-38</td>
<td>4-CH3 phenylacetylene</td>
<td>C</td>
<td>80%</td>
<td>65%</td>
<td>7-Methyl-2-phenyl-4H-1-benzopyran-4-one</td>
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</table>

Table 4.1: % Yield of substituted-4H-1-benzopyran-4-one resulting from the appropriate cyclization procedure (Notebook # refers to either JLW-I-xxx, JLW-II-xxx, or ASB [105]).
Table 4.1, continued

<table>
<thead>
<tr>
<th>#</th>
<th>Salicylic acid</th>
<th>Alkyne</th>
<th>Cyclization Procedure</th>
<th>% Yield</th>
<th>Product</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>I-144</td>
<td>4-OCH3 phenylacetylene</td>
<td>B</td>
<td>78%</td>
<td>Protected enaminoacetone after 45 min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I-145</td>
<td>4-OCH3 phenylacetylene</td>
<td>B</td>
<td>65%</td>
<td>Protected enaminoacetone after 5 min</td>
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<td></td>
</tr>
<tr>
<td>I-146</td>
<td>4-OCH3 phenylacetylene</td>
<td>B, H</td>
<td>70%</td>
<td>Deprotected enaminoacetone</td>
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<td></td>
</tr>
<tr>
<td>I-120</td>
<td>4-OCH3 phenylacetylene</td>
<td>C</td>
<td>90%</td>
<td>7-Methoxy-2-phenyl-4H-1-benzopyran-4-one</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I-139</td>
<td>4-OCH3 phenylacetylene</td>
<td>C</td>
<td>92%</td>
<td>7-Methoxy-2-phenyl-4H-1-benzopyran-4-one</td>
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<tr>
<td>I-150</td>
<td>4-OCH3 phenylacetylene</td>
<td>A</td>
<td>69%</td>
<td>7-Methoxy-2-phenyl-4H-1-benzopyran-4-one</td>
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<tr>
<td>I-188</td>
<td>4-OCH3 phenylacetylene</td>
<td>C</td>
<td>55%</td>
<td>7-Methoxy-2-phenyl-4H-1-benzopyran-4-one</td>
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<tr>
<td>I-211</td>
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<td>C</td>
<td>90%</td>
<td>7-Methoxy-2-phenyl-4H-1-benzopyran-4-one</td>
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<tr>
<td>I-214</td>
<td>4-OCH3 phenylacetylene</td>
<td>C</td>
<td>85%</td>
<td>7-Methoxy-2-phenyl-4H-1-benzopyran-4-one</td>
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</tr>
<tr>
<td>I-219</td>
<td>4-OCH3 phenylacetylene</td>
<td>A</td>
<td>77%</td>
<td>7-Methoxy-2-phenyl-4H-1-benzopyran-4-one</td>
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<tr>
<td>II-201</td>
<td>4-OCH3 phenylacetylene</td>
<td>D</td>
<td>59%</td>
<td>7-Methoxy-2-phenyl-4H-1-benzopyran-4-one</td>
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<tr>
<td>II-31</td>
<td>4-OCH3 phenylacetylene</td>
<td>C</td>
<td>71%</td>
<td>7-Methoxy-2-phenyl-4H-1-benzopyran-4-one</td>
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<tr>
<td>I-274</td>
<td>4-OCH3 phenylacetylene</td>
<td>F</td>
<td>77%</td>
<td>7-Methoxy-2-phenyl-4H-1-benzopyran-4-one</td>
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<tr>
<td>I-242</td>
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<td>F</td>
<td>70%</td>
<td>7-Methoxy-2-phenyl-4H-1-benzopyran-4-one</td>
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<tr>
<td>II-78</td>
<td>4-OCH3 phenylacetylene</td>
<td>C</td>
<td>90%</td>
<td>7-Methoxy-2-phenyl-4H-1-benzopyran-4-one</td>
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<tr>
<td>I-120</td>
<td>4-ethynyltoluene</td>
<td>H</td>
<td>64%</td>
<td>7-Methoxy-2-(4'-methylphenyl)-4H-1-benzopyran-4-one</td>
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<tr>
<td>I-134</td>
<td>4-ethynyltoluene</td>
<td>C</td>
<td>80%</td>
<td>7-Methoxy-2-(4'-methylphenyl)-4H-1-benzopyran-4-one</td>
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<tr>
<td>II-45</td>
<td>4-ethynyltoluene</td>
<td>C</td>
<td>29%*</td>
<td>7-Methoxy-2-(4'-methylphenyl)-4H-1-benzopyran-4-one</td>
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<tr>
<td>I-287</td>
<td>4-ethynyltoluene</td>
<td>G</td>
<td>25%</td>
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<tr>
<td>I-133</td>
<td>1-ethyl-1-cyclohexene</td>
<td>C</td>
<td>73%</td>
<td>2-(1-Cyclohexen-1-yl)-7-methoxy-4H-1-benzopyran-4-one</td>
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<tr>
<td>I-203</td>
<td>1-ethyl-1-cyclohexene</td>
<td>C</td>
<td>44%</td>
<td>2-(1-Cyclohexen-1-yl)-7-methoxy-4H-1-benzopyran-4-one</td>
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<td>I-46</td>
<td>1-ethyl-1-cyclohexene</td>
<td>C</td>
<td>64%</td>
<td>50%</td>
<td>7-Methoxy-2-(1-Cyclohexen-1-yl)-7-methoxy-4H-1-benzopyran-4-one</td>
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<tr>
<td>II-292</td>
<td>1-ethyl-1-cyclohexene</td>
<td>G</td>
<td>48%</td>
<td>2-(1-Cyclohexen-1-yl)-7-methoxy-4H-1-benzopyran-4-one</td>
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<tr>
<td>I-132</td>
<td>5-chloro-1-pentene</td>
<td>C</td>
<td>60%</td>
<td>2-(3-Chloropropyl)-7-methoxy-4H-1-benzopyran-4-one</td>
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<tr>
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<td>5-chloro-1-pentene</td>
<td>C</td>
<td>55%</td>
<td>2-(3-Chloropropyl)-7-methoxy-4H-1-benzopyran-4-one</td>
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<td>II-25</td>
<td>1-pentene</td>
<td>E</td>
<td>80%</td>
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<td>I-33</td>
<td>1-pentene</td>
<td>C</td>
<td>85%</td>
<td>7-Methoxy-2-propyl-4H-1-benzopyran-4-one</td>
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<tr>
<td>I-130</td>
<td>propionaldehyde diethyl acetal</td>
<td>C</td>
<td>85%</td>
<td>2-(Diethoxymethyl)-7-methoxy-4H-1-benzopyran-4-one</td>
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<tr>
<td>I-199</td>
<td>propionaldehyde diethyl acetal</td>
<td>C</td>
<td>85%</td>
<td>2-(Diethoxymethyl)-7-methoxy-4H-1-benzopyran-4-one</td>
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<tr>
<td>ASB</td>
<td>phenylacetylene</td>
<td>C</td>
<td>85%</td>
<td>7-Chloro-2-phenyl-4H-1-benzopyran-4-one</td>
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<tr>
<td>I-273</td>
<td>phenylacetylene</td>
<td>C</td>
<td>49%</td>
<td>7-Chloro-2-phenyl-4H-1-benzopyran-4-one</td>
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<tr>
<td>I-274</td>
<td>4-ethynyltoluene</td>
<td>C</td>
<td>68%</td>
<td>7-Chloro-2-(4'-methylphenyl)-4H-1-benzopyran-4-one</td>
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<tr>
<td>I-278</td>
<td>1-ethyl-1-cyclohexene</td>
<td>C</td>
<td>47%</td>
<td>7-Chloro-2-(1-cyclohexen-1-yl)-4H-1-benzopyran-4-one</td>
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</table>

continued
**Table 4.1, continued**

<table>
<thead>
<tr>
<th>Notebook</th>
<th>Salicyclic acid</th>
<th>Alkyne</th>
<th>Cyclization Procedure</th>
<th>% Yield</th>
<th>Product</th>
<th>Product</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASB 5-Cl</td>
<td>phenylacetylene</td>
<td>C</td>
<td>83%</td>
<td>6-Chloro-2-phenyl-4H-1-benzopyran-4-one</td>
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<tr>
<td>I-260 5-Cl</td>
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<tr>
<td>I-235 5-Cl</td>
<td>phenylacetylene</td>
<td>F</td>
<td>60%</td>
<td>6-Chloro-2-phenyl-4H-1-benzopyran-4-one</td>
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<tr>
<td>I-236 5-Cl</td>
<td>4-ethynyltoluene</td>
<td>F</td>
<td>6%</td>
<td>6-Chloro-2-(4'-methylphenyl)-4H-1-benzopyran-4-one</td>
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<td>63%</td>
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<td>4-ethynyltoluene</td>
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<td>7%</td>
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<td>II-289 5-Cl</td>
<td>1-ethynyl-1-cyclohexene</td>
<td>G</td>
<td>23%</td>
<td>6-Chloro-2-(1-cyclohexen-1-yl)-4H-1-benzopyran-4-one</td>
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<tr>
<td>ASB 5-Ph</td>
<td>phenylacetylene</td>
<td>C</td>
<td>94%</td>
<td>2,6-Diphenyl-4H-1-benzopyran-4-one</td>
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</tr>
<tr>
<td>I-264 5-Ph</td>
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<td>C</td>
<td>61%</td>
<td>2,6-Diphenyl-4H-1-benzopyran-4-one</td>
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</tr>
<tr>
<td>I-267 5-Ph</td>
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<td>77%</td>
<td>2-(4'-Methylphenyl)-6-phenyl-4H-1-benzopyran-4-one</td>
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<tr>
<td>ASB 5-Ph</td>
<td>1-ethynyl-1-cyclohexene</td>
<td>C</td>
<td>92%</td>
<td>2-(1-Cyclohexen-1-yl)-6-phenyl-4H-1-benzopyran-4-one</td>
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<tr>
<td>I-268 5-Ph</td>
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<td>C</td>
<td>68%</td>
<td>2-(1-Cyclohexen-1-yl)-6-phenyl-4H-1-benzopyran-4-one</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I-277 5-Ph</td>
<td>5-chloro-1-pentyne</td>
<td>C</td>
<td>53%</td>
<td>2-(3-Chloropropyl)-6-phenyl-4H-1-benzopyran-4-one</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>II-65 5-OCH$_3$</td>
<td>phenylacetylene</td>
<td>C</td>
<td>71%</td>
<td>6-Methoxy-2-phenyl-4H-1-benzopyran-4-one</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(A) Et$_3$NH, neat; (B) Et$_3$NH, EtOH; (C) 1. Et$_3$NH, EtOH, 2. EtOH, reflux; (D) Pyrrolidine, 2. EtOH, reflux; (E) Pyrrolidine; (F) Et$_3$N, alkyne and Cul are cannulated into acid chloride after being stirred under argon for 15 min, followed by cannulation into acid chloride and addition of Pd(PPh$_3$)$_4$Cl$_2$.

Intermediate alkyne is filtered thru pad of celite and concentrated. 1. Et$_3$NH & EtOH; 2. EtOH, reflux; (G) 1. Pyrrolidine, EtOH, 2. EtOH, reflux; (H) KF, 18-crown-6, DMF; (I) pyridinium p-toluensulfonate, MeOH.

$^1$51% enaminoketone and 15% of deprotected enaminoketone formed in reaction. $^2$12% benzofuranone formed in coupling step. $^3$Methyl-ester formed in coupling step; $^4$majority of product is deprotected alkyne.
4.2.2 Cyclizations with the Alkynone 1-[2-[(1,1-Dimethylethyl)-dimethylsilyl]oxy]-4-methoxynaphthal-2-propyn-1-one

The next step was to examine the effect that a 4-methoxy substituent on the alkynone has on the cyclization yield. Diethylamine (10 equiv) and EtOH were added to the alkynone isolated from the coupling of 4-methoxynaphthalic acid and phenylacetylene (Figure 4.8). After stirring under argon for 45 minutes, the protected enaminoketone was isolated in 78% yield. When the secondary amine pyrrolidine was added to an ethanolic solution of the alkynone, the alkynone disappeared by TLC in less than 5 minutes and led to isolation of the protected enaminoketone in 69% yield (Figure 4.8, Table 4.1). Although the pyrrolidine underwent a faster 1,4 addition, the Et₂NH provided the enaminoketone in higher yield. The 4-methoxy did not seem to affect the formation of the enaminoketone.

![Enaminoketone generation using different secondary amines (i.e. diethylamine and pyrrolidine).](image)

**Figure 4.8:** Enaminoketone generation using different secondary amines (i.e. diethylamine and pyrrolidine).
The one-pot acid chlorination/Sonogashira coupling of 4-methoxysalicylic acid with various terminal alkynes generated various methoxyalkynones (Table 3.2). The alkynones were reacted with diethylamine in EtOH to first generate the enaminoketone intermediates. The enaminoketones were resuspended in EtOH and refluxed to provide the 2-substituted-7-methoxy-4H-1-benzopyran-4-ones in yields ranging from 73-92% as seen in Figure 4.9. The cyclization to provide 2-(1-cyclohexen-1-yl)-7-methoxy-4H-1-benzopyran-4-one resulted in the lowest yield (73%). The lower yield may be due to the ability of the nitrogen nucleophile of the amine to do a 1,6-Michael addition to the double bond of the cyclohexene. However, the product from the 1,6 addition has not been detected.

![Chemical structure](image)

**Table 3.2**

<table>
<thead>
<tr>
<th>4-Methoxysalicylic acid</th>
<th>Terminal Alkynes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Coupling Yields</strong></td>
<td><strong>Cyclization Yields</strong></td>
</tr>
<tr>
<td>92%</td>
<td>92%</td>
</tr>
<tr>
<td>70%</td>
<td>88%</td>
</tr>
<tr>
<td>65%</td>
<td>73%</td>
</tr>
<tr>
<td>84%</td>
<td>89%</td>
</tr>
<tr>
<td>42%</td>
<td>80%</td>
</tr>
<tr>
<td>90%</td>
<td>85%</td>
</tr>
</tbody>
</table>

**Figure 4.9**: Coupling yields of 4-methoxysalicylic acids with various alkynes followed by their cyclization with diethylamine and EtOH to produce 2-substituted-7-methoxy-4H-1-benzopyran-4-ones.

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4.2.3 CYCLIZATION YIELDS OF THE ALKYNONES

All of the alkynones made from the Sonogashira couplings were cyclized to the benzopyranone. The highest yields of these cyclizations can be seen in Figure 4.10 and Table 4.1.

<table>
<thead>
<tr>
<th>% Yield of Benzopyranone</th>
<th>Terminal Alkynes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CH₃</td>
</tr>
<tr>
<td></td>
<td>96% 87% 82% 73% 75%</td>
</tr>
<tr>
<td></td>
<td>OTBS</td>
</tr>
<tr>
<td></td>
<td>95% 82% 72%</td>
</tr>
<tr>
<td></td>
<td>OCH₃</td>
</tr>
<tr>
<td></td>
<td>87%</td>
</tr>
<tr>
<td>Salicylic Acids</td>
<td>CH₃</td>
</tr>
<tr>
<td></td>
<td>92% 88% 73% 80% 89% 85%</td>
</tr>
<tr>
<td></td>
<td>CH₃</td>
</tr>
<tr>
<td></td>
<td>80%</td>
</tr>
<tr>
<td></td>
<td>CH₃</td>
</tr>
<tr>
<td></td>
<td>85% 68%</td>
</tr>
<tr>
<td></td>
<td>CH₃</td>
</tr>
<tr>
<td></td>
<td>83% 63%</td>
</tr>
<tr>
<td></td>
<td>CH₃</td>
</tr>
<tr>
<td></td>
<td>94% 92% 53%</td>
</tr>
<tr>
<td></td>
<td>CH₃</td>
</tr>
<tr>
<td></td>
<td>71%</td>
</tr>
</tbody>
</table>

Figure 4.10: Cyclization yields of the alkynones derived from the coupling of the salicylic acids and terminal alkynes via methodology in Table 4.1.
4.2.4 Protecting the Hydroxyl Group to Prevent Cyclization

Since our initial publication of this chemistry, Miao and Yang have developed a highly efficient synthetic technology for carbonylative cyclization of o-acetoxyiodobenzenes with arylacetylenes to construct the corresponding benzopyranones under mild conditions (40°C under a balloon pressure of CO) using \( \text{PdCl}_2(\text{Ph}_3\text{P})_2\)-thiourea-dppp (1:1:1) complex as the catalyst (Figure 4.11) [100]. They also reasoned that if the latent ortho-hydroxy group was protected, in this case as an acetoxy, they could prevent the formation of aurones. By protecting the hydroxyl, they determined that the reaction rate of the Pd-catalyzed coupling was also increased. This possibly could be due to the electron-withdrawing effect of the acetoxy, since it reduces the electron density of the aromatic ring, thus increasing the oxidative addition of Pd. Using these conditions, the benzopyranones were formed in a one-pot reaction with overall yields ranging from 68-92% and the five-membered benzofuranone formation was prevented.

![Chemical Reaction]

**Figure 4.11:** Regiospecific carbonylative annulation of iodophenol acetates and arylacetylenes to construct benzopyranones by a new catalyst of palladium-thiourea-1,3-bis(diphenylphosphino)propane (dppp) complex [100].

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4.3 Other Cyclization Protocols Attempted

Various cyclization protocols of alkynones have been attempted in the literature. It has been shown that the reaction of various secondary amines with alkynones results in the formation of the enaminketone intermediate, which can then undergo cyclization to the benzopyranone.

Theoretical calculations on the cyclization of o-hydroxyphenyl ethynyl ketones (alkynones) have shown that both the 6-endo-dig and 5-endo-dig cyclizations are endothermic and reversible in aprotic media, and the irreversible protonation of the resulting anions is critical for the distribution of products [126]. To determine the distribution of products in aprotic media, Nakatani et al. desilyated o-silyloxyphenyl ethynyl ketones using sources of fluoride ion to generate a phenoxide ion which cyclized to give either benzopyranones or benzofuranones. They showed that KF in aprotic media allows the irreversible protonation of the resulting vinyl anion from a 6-endo-dig cyclization to give rise to the benzopyranone with high selectivity. The basis for this selectivity is unknown. The theoretical potential energy diagram is Figure 4.12 suggests that the transition states are very close to each other; however, the benzopyranone vinyl anion is more stable than the benzofuranone vinyl anion. The irreversible protonation of the benzopyranone vinyl anion results in the formation of the benzopyranone over the benzofuranone. Application of this methodology (i.e., KF,
18-crown-6, DMF) to our alkynones resulted in the formation of the benzopyranones in less than 4 hours in excellent yields (>95%) (Figure 4.13). This methodology did not work as well when the substitution of the alkynone was changed.

A.

\[
\begin{align*}
\text{OTBS} & \quad \text{OTHP} \\
\text{KF, 18-crown-6} & \quad \text{DMF} \\
\text{97% yield} & \quad \text{OTHP}
\end{align*}
\]

B.

\[
\begin{align*}
\text{OTBS} & \quad \text{OTHP} \\
\text{TBAF, THF} & \quad \text{90% yield} \\
\text{Benzopyranone: Benzofuranone (47:53)}
\end{align*}
\]

Figure 4.12: A. Alkynones can be cyclized under basic conditions using potassium fluoride, 18-crown-6, and DMF via a 6-endo or 5-exo-digonal cyclization resulting in the selective formation of benzopyranones. The reaction of an alkynone with a solution of TBAF in THF produced a mixture of benzopyranone and benzofuranone in 90% yield with low selectivity (47:53) [126]. B. The theoretical potential energy diagram for this selectivity.
Figure 4.13: Alkynones can be cyclized using potassium fluoride, 18-crown-6, and DMF or pyridinium p-toluenesulfonate (PPTS) in MeOH via a 6-endo-\textit{dig} cyclization resulting in the selective formation of benzopyranones.

4.4 \textbf{Benzofuranone Formation during Sonogashira Couplings}

The chemistry that we have developed has allowed us to regioselectively synthesize the benzopyranones. The competing 5-exo-\textit{dig} cyclization yielding benzofuranones has not been observed following our cyclization protocols. As seen in Figures 4.2 and 4.14, there are 3 possible mechanisms of cyclization of alkynones. Alkynones could undergo a 5-exo-\textit{dig} cyclization to form either the Z or E isomer of a benzofuranone. Both a 6-endo-\textit{dig} cyclization of the alkynone or a 6-endo-\textit{trig} cyclization of the enaminoketone would result in the formation of benzopyranones. An ethanolic solution of a secondary amine, predominantly diethylamine, has been used to cyclize the alkynone intermediate, without any evidence of benzofuranone formation. However, in six cases, the Z-benzofuranone has been isolated in low yields during the Sonogashira coupling of acid chlorides and terminal alkynes which uses triethylamine as solvent and

<table>
<thead>
<tr>
<th>Method</th>
<th>R_1</th>
<th>R_2</th>
<th>% Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>H</td>
<td>H</td>
<td>&gt;95%</td>
</tr>
<tr>
<td>B</td>
<td>H</td>
<td>H</td>
<td>45%</td>
</tr>
<tr>
<td>A</td>
<td>4-OCH₃</td>
<td>CH₃</td>
<td>64%</td>
</tr>
</tbody>
</table>
Korshunov et al. demonstrated that diarylpropynones containing a hydroxyl group ortho to the alkyne in triethylamine led to the formation of only benzofuranones [102]. If the TBS protecting group was removed during the reaction, the nucleophilic attack of the unmasked phenolic hydroxyl on the alkyne could proceed via two routes: a 5-exo-dig pathway to provide the benzofuranone or the 6-endo-dig pathway to provide the benzopyranones. According to Baldwin’s cyclization rules, both of these modes are favored [127].

**Figure 4.14:** Mechanism of cyclization of the alkynone to the benzopyranone and benzofuranone.
The Z-benzofuranone and benzopyranone have been isolated in yields of 3-29% and 3-42%, respectively, during the Sonogashira coupling of various salicylic acids and terminal alkynes (Figure 4.15, Table 3.3). During the course of our experimentation, the coupling of salicylic acid and phenylacetylene has been performed numerous times and under a variety of conditions. Only in one case has the benzofuranone been detected in 12% yield (Table 3.3). The largest yield (29%) of benzofuranone isolated was formed in the coupling of 4-methoxysalicylic acid and propiolaldehyde diethyl acetal. In this case, the Et$_3$N used in the Sonogashira coupling was distilled from KOH instead of the usual distillation procedure from phthalic anhydride and calcium hydride. In this instance, no alkynone was even detected. When this coupling was repeated with Et$_3$N distilled from CaH$_2$, the alkynone coupling yield ranged from 24-84%, without detection of the benzofuranone. Each time 5-chloro-bis-TBS-salicylic acid was coupled to 5-chloro-1-pentyne, only the benzopyranone was isolated (14-29%); the alkynone was not detected each time. This is the only example when a specific alkynone could not be isolated after a Sonogashira coupling.
Figure 4.15: Formation of Z-benzofuranones during the Sonogashira coupling of salicylic acids and terminal alkynes.

4.5 NAMING OF THE FLAVONOIDs

All flavonoids contain fifteen carbon atoms in the parent skeleton and feature two phenyl rings linked by a three-carbon chain. There are countless examples of flavonoids in the literature since they represent one of the most diverse and widespread groups of natural products. Many flavonoids bear numerous hydroxy or methoxy groups and often exist as O-glycosides or as dimers. The class of flavonoids includes the flavones, flavonols, isoflavones, flavanones, dihydroflavonols, aurones, and finally chalcones. The skeletons and numbering schemes commonly found in the literature for the flavonoids can be seen in Figure 4.16 [128,129].
Many of the flavonoids have a fused benzene and pyran ring and are thus called benzopyrans. There are two classes of benzopyrans which depend on the placement of the oxygen, 1-benzopyran and 2-benzopyran (Figure 4.17). Note that the placement of the double bond in the 1-benzopyrans can be in the 2,3 or the 3,4 position. When trying to search for the flavonoids in the literature, one also has to be aware of the nomenclature of the 1-benzopyran classes, which includes the chroman, 2\(H\)-chromene, 4\(H\)-chromene, 3-chromanone, 4-chromanone, 2,4-chromandione, and chromone (Figure 4.18).

**Figure 4.16:** Skeletons and numbering schemes commonly employed in the literature for the class of flavonoids.

**Figure 4.17:** Types of benzopyrans.
A variety of names have been used for the chromone ring system including benzo-γ-pyrone, γ-benzopyrone, pheno-γ-pyrone, 2,3-benzopyrone-(4), 4-oxo[1,4-chromen], 4-oxochromen, and 4H-1-benzopyran-4-one. In 1972, Chemical Abstracts replaced the trivial name of chromone with the systematic name of 4H-1-benzopyran-4-one [130]. Thus, the benzopyranones and the benzofuranones shown in Figure 4.19 have the systematic names of 2-phenyl-4H-1-benzopyran-4-one and 2-phenyl-(2Z)-3(2H)-benzofuranone, respectively, and belong to the larger class of flavonoids. Other names for the benzofuranone ring system include β-coumaranone, 2,3-dihydro-1-benzofuran-3-one, 2,3-dihydro-3-oxobenzofuran, benzo[b]furan-3(2H)-one, coumaran-3-one, and coumaranone.
4.6 STRUCTURE ELUCIDATION

Although often taken for granted, structure elucidation of the benzopyranones and the benzofuranones should not be overlooked and is not a trivial task. All products synthesized in our laboratory have been fully characterized by $^1$H and $^{13}$C Nuclear Magnetic Resonance Spectroscopy (NMR), Infrared Spectroscopy (IR), Ultraviolet-visible Spectroscopy (UV-Vis) and High Resolution Mass Spectrometry (HRMS). Figure 4.19 reveals the general structures of unsubstituted benzopyranones and benzofuranones. The olefin bond of the benzofuranones introduces two possible geometric isomers, most of the naturally occurring aurones possess Z-olefinic configuration and are named Z-aurones or Z-benzofuranones [129].

There are numerous sources of information for isolation, purification, and identification of the flavonoids. In particular, much work has been accumulated on the physical properties and spectra (including UV, $^1$H, and $^{13}$C NMR) of various flavonoids [128-132].
4.6.1 $^1$H NMR Spectroscopy

The distinguishing characteristic between these two classes of compounds begins with the distinction of the H-3 of benzopyranones and the benzylic methine of benzofuranones [129]. The chemical shifts of these protons both would appear as singlets in the $^1$H NMR spectra, each having a chemical shift from 6.0-8.0 ppm. Figure 4.20 shows that the $^1$H NMR chemical shifts for the H-3 of 6-methoxy-2-phenyl-4H-1-benzopyran-4-one and the benzylic proton of 6-methoxy-2-phenyl-3(2H)-benzofuranone both appear as singlets at 6.80 and 6.85 ppm, respectively. $^1$H NMR data by itself is not adequate to differentiate the structures from each other and other experimental proof like $^{13}$C NMR and UV are needed to confirm these structures.

![Figure 4.20: $^1$H NMR chemical shifts for the H-3 of 6-methoxy-2-phenyl-4H-1-benzopyran-4-one and the benzylic proton of 6-methoxy-2-phenyl-3(2H)-benzofuranone from data collected in CDCl$_3$.](image)

For benzopyranones with non-aromatic substituents at the C-2 position, like 2-(diethoxymethyl)-7-methoxy-4H-1-benzopyran-4-one in Figure 4.21, chemical shift and coupling constants from $^1$H NMR data is adequate to determine the structure. For
the benzopyranone, H-3 appears as a singlet at 6.49 ppm and the allylic proton appears as a singlet at 5.26 ppm. These two protons are not coupled to each other because they are four bonds away. However, the benzofuranone H-2 and the allylic proton are three bonds apart and are coupled. They both appear as doublets with $^3J = 7.5$ Hz.

![Chemical Structures](image)

**Figure 4.21:** $^1$H NMR chemical shifts for the H-3 of 2-(diethoxymethyl)-7-methoxy-4H-1-benzopyran-4-one and the H-2 of 7-methoxy-2-(diethoxymethyl)-3(2H)-benzofuranone from data collected in CDCl$_3$.

4.6.2 $^{13}$C NMR Spectroscopy

$^{13}$C NMR spectroscopy has been widely used for the characterization of various categories of flavonoids. From $^{13}$C NMR data, one can not only determine if the compound is a benzopyranone or benzofuranone, but also determine the geometry of the olefin bond of the benzofuranones.

Figure 4.22 presents the $^{13}$C NMR chemical shifts of 2-phenyl-4H-1-benzopyran-4-one and 2-phenyl-3(2H)-benzofuranone in CDCl$_3$ [129]. As seen in Figure 4.22, the carbon resonances from the aromatic A and B rings for the benzopyranone and benzofuranone...
overlap and cannot be readily distinguished from each other. $^{13}$C NMR can be used to differentiate the sp$^2$ hybridized olefin bond (C-2 and C-3) of benzopyranones and benzofuranones. The C-3 resonance of flavonoids typically appears from 102.3-113.7, depending on substitution patterns found on the ring system; however, for the majority of flavonoids, the C-2 appears at ~107 ppm \[129\]. The resonance at ~107 ppm is unique and distinct from resonances from aurones. The exocyclic olefin methine (C-2) of unsubstituted Z-aurones typically absorbs at 108.1-112.8 ppm, while the methine C-2 of E-aurones is deshielded and appears at 119.9-122.2 ppm. If the Z-aurone has a 2'-oxy substituent, the C-2 is shifted upfield to 104.0-105.9 ppm. As can be seen in Figure 4.22, the C-3 resonance of the flavonoids appears at 107.6 ppm and the closest resonance to this in aurones appears at 112.8 ppm (C-2 and C-8 of Z-aurone) or 112.1 ppm (C-8 of E-aurone).
<table>
<thead>
<tr>
<th>Carbon #</th>
<th>Benzofuranone</th>
<th>Z</th>
<th>E</th>
<th>Benzopyranone</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-2</td>
<td>112.8</td>
<td>122.2</td>
<td>163.2</td>
<td></td>
</tr>
<tr>
<td>C-3</td>
<td>146.8</td>
<td>148.5</td>
<td>107.6</td>
<td></td>
</tr>
<tr>
<td>C-4</td>
<td>184.5</td>
<td>182.8</td>
<td>178.4</td>
<td></td>
</tr>
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</tr>
<tr>
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<td>132.4</td>
<td>125.2</td>
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</tr>
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<td></td>
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<tr>
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<td>123.3</td>
<td>124.0</td>
<td></td>
</tr>
<tr>
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<td>131.9</td>
<td>131.8</td>
<td></td>
</tr>
<tr>
<td>C-2'</td>
<td>131.5</td>
<td>130.8</td>
<td>126.3</td>
<td></td>
</tr>
<tr>
<td>C-3'</td>
<td>128.8</td>
<td>128.4</td>
<td>129.0</td>
<td></td>
</tr>
<tr>
<td>C-4'</td>
<td>123.8</td>
<td>130.2</td>
<td>131.6</td>
<td></td>
</tr>
<tr>
<td>C-5'</td>
<td>128.8</td>
<td>128.4</td>
<td>129.0</td>
<td></td>
</tr>
<tr>
<td>C-6'</td>
<td>131.4</td>
<td>130.8</td>
<td>126.3</td>
<td></td>
</tr>
</tbody>
</table>

Figure 4.22: $^{13}$C NMR chemical shifts of 2-phenyl-4H-1-benzopyran-4-one and 2-phenyl-3(2H)-benzofuranone in CDCl$_3$ [129,133].

4.6.3 Two-dimensional $^{13}$C NMR Spectroscopy

2D NMR experiments have been used to fully assign the proton and carbon skeleton of a few model systems. Heteronuclear shift correlation (XHCORR) spectroscopy is a very useful 2D technique used to determine which protons are directly bonded to which $^{13}$C nuclei. In this technique, only $^{13}$C's directly attached to $^1$H's are detected, all quaternary carbons are missing. A COLOC (Correlation Spectroscopy via Long Range Coupling) experiment is a 2D heteronuclear correlation technique that can be used to
determine which $^1$H's are bonded to which $^{13}$C nuclei via small long-range couplings ($^{n}$/$^{1}$CH, n>1). Thus, in this experiment, all $^{13}$C resonances are detected, not just those bonded directly to protons. Figures 4.23 to 4.26 present various 2D experiments used to establish either the benzopyranone or benzofuranone skeleton. In Figure 4.23, a cross peak exists for the H-3 and the carbon at 106.7 ppm, thus indicating that the compound is a benzopyranone.

Benzofuranones can be readily distinguished by the chemical shift of the exocyclic olefinic methine (C-2). The exocyclic olefinic methine carbon appears at 104.0-112.8 ppm in most Z-benzofuranones. However, the methine C-2 is deshielded by 10 ppm in E-isomers and absorbs at 119.9-122.2 ppm [129,133,134]. It should be noted that according to past $^{13}$C NMR experiments in the literature, the E and Z-isomers cannot be distinguished on basis of the chemical shifts of the C-3 olefinic quaternary carbon or the C-4 carbonyl carbon because they absorb almost at the same position (Figure 4.27) [134]. The olefinic quaternary carbon C-3 absorbs in the range of 147.0-148.5 ppm while the C-4 carbonyl carbon absorbs in the range of 178.9-185.8 ppm for both isomers.
Figure 4.23: Heteronuclear shift correlation experiment of 7-hydroxy-2-phenyl-4H-1-benzopyran-4-one (60).
Figure 4.24: Heteronuclear shift correlation experiment (upper) and COLOC (Correlation Spectroscopy via Long Range Coupling) (lower) of 7-hydroxyl-2-(4'-methylphenyl)-4H-1-benzopyran-4-one (61).
Figure 4.25: Heteronuclear shift correlation experiment of 7-chloro-2-phenyl-3(2H)-benzofuranone. A cross peak exists for the H-2 and the carbon at 114.1 ppm, thus indicating that the compound is a benzofuranone.
Figure 4.26: Heteronuclear shift correlation experiment of 6-methoxy-2-phenyl-3(2H)-benzofuranone. A cross peak exists for the H-2 and the carbon at 113.1 ppm, thus indicating that the compound is a benzofuranone.
Figure 4.27: Mean difference in chemical shift of $^{13}$C NMR resonances between Z- and E-isomers of 2-phenyl-3-(2H)-benzofuranone [134].

4.6.4 **Ultraviolet–Visible Absorption Spectroscopy**

Ultraviolet-visible (UV-Vis) absorption spectroscopy has been extensively used for flavonoid structure analysis. This technique can be used to aid in the identification of the flavonoid type and is often used to determine the oxygenation pattern. UV-Vis analysis has been performed on all flavonoids synthesized. This method offers a quick, yet accurate determination of the ring structure due to the differences in absorption profiles of different flavonoids. The UV-Vis absorption spectra of different flavonoid types can be seen in Figure 4.28 [132]. The UV-Vis spectrum of flavonoids is typically determined for a methanol or ethanol solution of the flavonoid. The spectrum typically consists of two absorption maxima in the ranges of 240-285 nm (band II) and 300-550 (band I). Table 4.2 displays the absorption ranges for band II and band I for the classes of flavonoids. Band I is considered to be associated with absorption due to the B-ring while band II with absorption due to the A-ring [128].
Figure 4.28: Ultraviolet-visible spectra of different flavonoid types with equivalent hydroxylation patterns [132].
<table>
<thead>
<tr>
<th>Band II (nm)</th>
<th>Band I (nm)</th>
<th>Flavonoid type</th>
</tr>
</thead>
<tbody>
<tr>
<td>250-280</td>
<td>310-350</td>
<td>Flavone</td>
</tr>
<tr>
<td>250-280</td>
<td>330-360</td>
<td>Flavonols (3-OH substituted)</td>
</tr>
<tr>
<td>250-280</td>
<td>350-385</td>
<td>Flavonols (3-OH free)</td>
</tr>
<tr>
<td>245-275</td>
<td>310-330</td>
<td>Isoflavones</td>
</tr>
<tr>
<td>275-295</td>
<td>300-330</td>
<td>Flavanones and dihydroflavonols</td>
</tr>
<tr>
<td>230-270</td>
<td>340-390</td>
<td>Chalcones</td>
</tr>
<tr>
<td>(low intensity)</td>
<td>380-430</td>
<td>Aurones</td>
</tr>
<tr>
<td>270-280</td>
<td>465-560</td>
<td>Anthocyanidins and anthocyanins</td>
</tr>
</tbody>
</table>

Table 4.2: Ultraviolet-visible absorption ranges for flavonoids [132].

One can easily differentiate a flavonoid from an aurone based upon the band I absorption from the UV spectra. Aurones characteristically have a band I absorption at a longer wavelength position (380-430 nm) as compared to flavones (330-360 nm). The UV-Vis absorption spectrum for 7-methyl-2-phenyl-4'H-1-benzopyran-4-one and 7-methyl-2-phenyl-(2Z)-3-(2'H)-benzofuranone can be seen in Figure 4.29. The band I absorption is shifted in the benzofuranone (371 nm) relative to the benzopyranone (298 nm) which is consistent with the literature. Figure 4.30 presents the UV-Vis absorption spectrum for 6-methoxy-2-phenyl-4'H-1-benzopyran-4-one and 6-methoxy-2-phenyl-(2Z)-3-(2'H)-benzofuranone. Reference spectra for many flavonoids can be found in the literature and are an invaluable tool for the interpretation of UV spectrum [128,132]. Table 4.3 presents the Band I and II absorptions for all synthetic benzopyranones.
Comparison of UV Absorbance for JLW-2-036; Benzopyranone vs Benzofuranone

<table>
<thead>
<tr>
<th>UV Spectral Data (λmax, nm)</th>
<th>Benzopyranone</th>
<th>Benzofuranone</th>
</tr>
</thead>
<tbody>
<tr>
<td>MeOH</td>
<td>234, 255, 267, 298</td>
<td>274, 325, 349, 371</td>
</tr>
</tbody>
</table>

Figure 4.29: UV-Vis absorption spectrum for 7-methyl-2-phenyl-4H-1-benzopyran-4-one and 7-methyl-2-phenyl-(2Z)-3-(2H)-benzofuranone.

Comparison of UV Absorbance for JLW-II-064 vs JLW-II-065; Benzopyranone vs Benzofuranone

<table>
<thead>
<tr>
<th>UV Spectral Data (λmax, nm)</th>
<th>Benzopyranone JLW-II-065</th>
<th>Benzofuranone JLW-II-064</th>
</tr>
</thead>
</table>

Figure 4.30: UV-Vis absorption spectrum for 6-methoxy-2-phenyl-4H-1-benzopyran-4-one and 6-methoxy-2-phenyl-(2Z)-3-(2H)-benzofuranone.
<table>
<thead>
<tr>
<th>Library #</th>
<th>JLW #</th>
<th>Band II (nm)</th>
<th>Band I (nm)</th>
<th>Systematic name</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>I-187</td>
<td>251</td>
<td>294</td>
<td>2-Phenyl-4H-1-benzopyran-4-one</td>
</tr>
<tr>
<td>11</td>
<td>I-247</td>
<td>253</td>
<td>302.06</td>
<td>2-(4'-Methylphenyl)-4H-1-benzopyran-4-one</td>
</tr>
<tr>
<td>12</td>
<td>I-248</td>
<td>253</td>
<td>295</td>
<td>2-(1-Cyclohexen-1-yl)-4H-1-benzopyran-4-one</td>
</tr>
<tr>
<td>13</td>
<td>I-249</td>
<td>224</td>
<td>295</td>
<td>2-(3-Chloropropyl)-4H-1-benzopyran-4-one</td>
</tr>
<tr>
<td>14</td>
<td>I-250</td>
<td>248</td>
<td>298</td>
<td>2-(Diethoxymethyl)-4H-1-benzopyran-4-one</td>
</tr>
<tr>
<td>15</td>
<td>I-190</td>
<td>248</td>
<td>309</td>
<td>3-Bromo-2-phenyl-4H-1-benzopyran-4-one</td>
</tr>
<tr>
<td>20</td>
<td>I-227</td>
<td>265</td>
<td>296</td>
<td>8-Methoxy-2-phenyl-4H-1-benzopyran-4-one</td>
</tr>
<tr>
<td>21</td>
<td>I-229</td>
<td>266</td>
<td>304</td>
<td>8-Methoxy-2-(4'-methylphenyl)-4H-1-benzopyran-4-one</td>
</tr>
<tr>
<td>22</td>
<td>I-225</td>
<td>265</td>
<td>296</td>
<td>2-(1-Cyclohexen-1-yl)-8-methoxy-4H-1-benzopyran-4-one</td>
</tr>
<tr>
<td>23</td>
<td>I-253</td>
<td>251</td>
<td>308</td>
<td>2-(3-Chloropropyl)-8-methoxy-4H-1-benzopyran-4-one</td>
</tr>
<tr>
<td>24</td>
<td>I-230</td>
<td>252</td>
<td>311</td>
<td>2-(Diethoxymethyl)-8-methoxy-4H-1-benzopyran-4-one</td>
</tr>
<tr>
<td>25</td>
<td>II-047</td>
<td>262</td>
<td>307</td>
<td>3-Bromo-8-methoxy-2-(4'-methylphenyl)-4H-1-benzopyran-4-one</td>
</tr>
<tr>
<td>30</td>
<td>I-176</td>
<td>266</td>
<td>306</td>
<td>8-Hydroxy-2-phenyl-4H-1-benzopyran-4-one</td>
</tr>
<tr>
<td>31</td>
<td>II-050</td>
<td>268</td>
<td>306</td>
<td>8-Hydroxy-2-(4'-methylphenyl)-4H-1-benzopyran-4-one</td>
</tr>
<tr>
<td>40</td>
<td>I-172</td>
<td>258</td>
<td>295</td>
<td>8-Methyl-2-phenyl-4H-1-benzopyran-4-one</td>
</tr>
<tr>
<td>41</td>
<td>I-232</td>
<td>258</td>
<td>302</td>
<td>8-Methyl-2-(4'-methylphenyl)-4H-1-benzopyran-4-one</td>
</tr>
<tr>
<td>42</td>
<td>I-233</td>
<td>258</td>
<td>295</td>
<td>2-(1-Cyclohexen-1-yl)-8-methyl-4H-1-benzopyran-4-one</td>
</tr>
<tr>
<td>43</td>
<td>I-121</td>
<td>248</td>
<td>300</td>
<td>2-(3-Chloropropyl)-8-methyl-4H-1-benzopyran-4-one</td>
</tr>
<tr>
<td>44</td>
<td>I-234</td>
<td>245</td>
<td>303</td>
<td>2-(Diethoxymethyl)-8-methyl-4H-1-benzopyran-4-one</td>
</tr>
<tr>
<td>50</td>
<td>I-188</td>
<td>251</td>
<td>307</td>
<td>7-Methoxy-2-phenyl-4H-1-benzopyran-4-one</td>
</tr>
<tr>
<td>51</td>
<td>I-134</td>
<td>253</td>
<td>309</td>
<td>7-Methoxy-2-(4'-methylphenyl)-4H-1-benzopyran-4-one</td>
</tr>
<tr>
<td>52</td>
<td>I-203</td>
<td>234, 252</td>
<td>303</td>
<td>2-(1-Cyclohexen-1-yl)-7-methoxy-4H-1-benzopyran-4-one</td>
</tr>
<tr>
<td>53</td>
<td>I-264</td>
<td>248</td>
<td>293</td>
<td>2-(3-Chloropropyl)-7-methoxy-4H-1-benzopyran-4-one</td>
</tr>
<tr>
<td>54</td>
<td>I-199</td>
<td>248</td>
<td>294</td>
<td>2-(Diethoxymethyl)-7-methoxy-4H-1-benzopyran-4-one</td>
</tr>
<tr>
<td>55</td>
<td>I-191</td>
<td>250</td>
<td>305</td>
<td>3-Bromo-7-methoxy-2-phenyl-4H-1-benzopyran-4-one</td>
</tr>
<tr>
<td>56</td>
<td>II-033</td>
<td>248</td>
<td>292</td>
<td>7-Methoxy-2-propyl-4H-1-benzopyran-4-one</td>
</tr>
<tr>
<td>57</td>
<td>II-037</td>
<td>252</td>
<td>295</td>
<td>3-Bromo-7-methoxy-2-propyl-4H-1-benzopyran-4-one</td>
</tr>
<tr>
<td>60</td>
<td>II-017</td>
<td>251</td>
<td>308</td>
<td>7-Hydroxy-2-phenyl-4H-1-benzopyran-4-one</td>
</tr>
<tr>
<td>61</td>
<td>II-019</td>
<td>253</td>
<td>311</td>
<td>7-Hydroxy-2-(4'-methylphenyl)-4H-1-benzopyran-4-one</td>
</tr>
<tr>
<td>70</td>
<td>II-016</td>
<td>255</td>
<td>298</td>
<td>7-Methyl-2-phenyl-4H-1-benzopyran-4-one</td>
</tr>
<tr>
<td>80</td>
<td>I-273</td>
<td>249</td>
<td>298</td>
<td>7-Chloro-2-phenyl-4H-1-benzopyran-4-one</td>
</tr>
<tr>
<td>81</td>
<td>I-274</td>
<td>250</td>
<td>307</td>
<td>7-Chloro-2-(4'-methylphenyl)-4H-1-benzopyran-4-one</td>
</tr>
<tr>
<td>82</td>
<td>I-278</td>
<td>246</td>
<td>301</td>
<td>7-Chloro-2-(1-cyclohexen-1-yl)-4H-1-benzopyran-4-one</td>
</tr>
<tr>
<td>83</td>
<td>I-280</td>
<td>248</td>
<td>303</td>
<td>7-Chloro-2-(3-chloropropyl)-4H-1-benzopyran-4-one</td>
</tr>
<tr>
<td>90</td>
<td>I-235</td>
<td>254</td>
<td>298</td>
<td>6-Chloro-2-phenyl-4H-1-benzopyran-4-one</td>
</tr>
<tr>
<td>91</td>
<td>I-238</td>
<td>256</td>
<td>307</td>
<td>6-Chloro-2-(4'-methylphenyl)-4H-1-benzopyran-4-one</td>
</tr>
<tr>
<td>92</td>
<td>I-239</td>
<td>252</td>
<td>299</td>
<td>6-Chloro-2-(1-cyclohexen-1-yl)-4H-1-benzopyran-4-one</td>
</tr>
<tr>
<td>93</td>
<td>I-254</td>
<td>287</td>
<td>305</td>
<td>6-Chloro-2-(3-chloropropyl)-4H-1-benzopyran-4-one</td>
</tr>
<tr>
<td>110</td>
<td>I-254</td>
<td>271</td>
<td>303</td>
<td>2,6-Diphenyl-4H-1-benzopyran-4-one</td>
</tr>
<tr>
<td>111</td>
<td>I-257</td>
<td>273</td>
<td>309</td>
<td>2-(4'-Methylphenyl)-6-phenyl-4H-1-benzopyran-4-one</td>
</tr>
<tr>
<td>112</td>
<td>I-258</td>
<td>270</td>
<td>301</td>
<td>2-(1-Cyclohexen-1-yl)-6-phenyl-4H-1-benzopyran-4-one</td>
</tr>
<tr>
<td>113</td>
<td>I-277</td>
<td>250</td>
<td>314</td>
<td>2-(3-Chloropropyl)-6-phenyl-4H-1-benzopyran-4-one</td>
</tr>
<tr>
<td>120</td>
<td>II-065</td>
<td>270</td>
<td>304</td>
<td>6-Methoxy-2-phenyl-4H-1-benzopyran-4-one</td>
</tr>
</tbody>
</table>

Table 4.3: UV absorptions for synthetic benzopyranones.
4.7 High-throughput Synthesis of Benzopyranones

4.7.1 Resin Capture

Our laboratory is interested in the high-throughput solid phase synthesis of a benzopyranone library of compounds. The synthetic methods developed thus far involve the solution phase synthesis of a benzopyranone library and should facilitate the transfer of this chemistry to solid phase. A resin capture strategy has been applied to this chemistry. Resin capture involves the solution phase reaction between an intermediate and a solid support resulting in the product being bound to the resin. In essence, resin capture is often viewed as a purification strategy wherein the desired product from the reaction mixture is trapped onto the solid support, leaving the by-products in solution which can simply be filtered away.

This resin capture strategy was successfully applied to our synthetic methodology resulting in the rapid and high yielding synthesis of substituted benzopyranones. The use of secondary amines for the cyclization step provided an opportunity to use a secondary amine bound to a resin. The resin capture of alkynones with a piperazinyl Merrifield resin in a solution of THF and MeOH resulted in the formation of support bound enaminoketones. Enaminoketones can undergo an on-resin cyclization to release the substituted benzopyranone and regeneration of the resin in yields of 70-82% (Figure 4.31) [105,135].
Figure 4.31: Resin capture of alkynones using a piperazinyl Merrifield resin resulting in the synthesis of 2-substituted-4H-1-benzopyran-4-ones [105,135].

4.7.2 HIGH-THROUGHPUT SYNTHESIS

In an attempt to increase efficiency and determine the feasibility of a high-throughput synthesis, intermediate purification of the alkynones from the Sonogashira couplings was terminated. In lieu of flash chromatography purification of the alkynones, the reaction mixture was filtered through a pad of celite with EtOAc to remove any solids such as the triethylamine salt and palladium catalyst. The reaction mixture was concentrated in vacuo followed by the cyclization protocol resulting in the formation of benzopyranones. This protocol was first applied to the synthesis of 8-methoxy-2-phenyl-4H-1-benzopyran-4-one resulting in the yield of the benzopyranone in 89% from the silated salicylic acid. This yield was dramatically higher as compared to the same reaction conditions except purifying the intermediate alkynone (62%). These results suggest that impurities do not interfere with the cyclization step. Although the stability of the alkynone has been questionable, it has been found to be relatively stable.
stored in the freezer overnight. Direct use of the alkynone could eliminate possible degradation. Because of the slightly acidic nature of silica gel, it has been postulated that the TBS-protecting group could be removed during purification; no experimental proof for this exists. This methodology was then applied to various starting materials resulting in the synthesis of twelve benzopyranones in overall yields ranging from 22-91% (Figure 4.32).

<table>
<thead>
<tr>
<th>Benzopyranone</th>
<th>% Yield of Benzopyranone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( R_2 ) Terminal alkyne</td>
</tr>
<tr>
<td>O( \text{OTBS} )</td>
<td>( \text{CH}_3 )</td>
</tr>
<tr>
<td>O( \text{OTBS} )</td>
<td>( \text{CH}_3 )</td>
</tr>
<tr>
<td>O( \text{OTBS} )</td>
<td>( \text{CH}_3 )</td>
</tr>
</tbody>
</table>

**Figure 4.32:** High-throughput synthesis of substituted-4\( H \)-1-benzopyran-4-ones.
4.8 **Overall Yields of Coupling and Cyclization**

The highest overall yields of the synthesis of substituted-4H-1-benzopyran-4-ones from the protected salicylic acids can be found in Figure 4.33.

![Reaction Scheme](image)

<table>
<thead>
<tr>
<th>alkyne</th>
<th>( \text{Ph} )</th>
<th>( \text{PhCH}_3 )</th>
<th>( \text{C}_6\text{H}_5)</th>
<th>( \text{Cl} )</th>
<th>( \text{OEt} )</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Yield (%)</strong></td>
<td>88%</td>
<td>83%</td>
<td>67%</td>
<td>62%</td>
<td>75%</td>
</tr>
<tr>
<td>( \text{H}_2\text{CO} )</td>
<td>83%</td>
<td>62%</td>
<td>47%</td>
<td>34%</td>
<td>84%</td>
</tr>
<tr>
<td>( \text{Cl} )</td>
<td>85%</td>
<td>77%</td>
<td>68%</td>
<td>39%</td>
<td></td>
</tr>
<tr>
<td>( \text{Cl} )</td>
<td>74%</td>
<td>42%</td>
<td>70%</td>
<td>42%</td>
<td></td>
</tr>
<tr>
<td>( \text{Cl} )</td>
<td>64%</td>
<td>39%</td>
<td>23%</td>
<td>30%</td>
<td></td>
</tr>
<tr>
<td>( \text{OCH}_3 )</td>
<td>80%</td>
<td>91%</td>
<td>83%</td>
<td>40%</td>
<td></td>
</tr>
<tr>
<td>( \text{OCH}_3 )</td>
<td>89%</td>
<td>88%</td>
<td>49%</td>
<td>22%</td>
<td>53%</td>
</tr>
</tbody>
</table>

**Figure 4.33:** Overall cyclization yields of substituted-4H-1-benzopyran-4-ones from the coupling and cyclization of bis-silylated-salicylic acids and terminal alkynes.
4.9 Numbering System of Benzopyranones

The numbering scheme used for the library of benzopyranones is based upon the combination of the numbering of the individual starting materials (i.e.; salicylic acids and terminal alkynes). The first number (Ix) of the benzopyranone is derived from the salicylic acid component while the second number from the alkyne used (x0) (Figure 4.34). For example, if salicylic acid (Ix) were combined with phenylacetylene (x0), the product 2-phenyl-4H-1-benzopyran-4-one would be number 10. Figure 4.25 shows the numbering scheme for the benzopyranones synthesized.

![Diagram of Numbering Scheme](image)

Figure 4.34: Numbering scheme of salicylic acids and terminal alkyne series.
Figure 4.35: Numbering system for the benzopyranone library.
CHAPTER 5

ADDING DIVERSITY TO THE 4H-1-BENZOPYRAN-4-ONE SKELETON

5.1 FUNCTIONALIZING THE 4H-1-BENZOPYRAN-4-ONE SKELETON

In order to understand what functional groups are present on benzopyranones isolated from natural products, one has to look at the biosynthetic pathway of flavonoids (Figure 5.1). The basic skeleton of flavonoids is derived from 4-coumaroyl-CoA and malonyl-CoA and consists of two aromatic rings connected by a three carbon spacer. The hydroxylation patterns of the three rings systems (A, B, and C) is derived from the biosynthetic pathway. The most common hydroxylation pattern of the A-ring is 5,7-hydroxylation; however, occasionally, a 5,7,8- or 5,6,7-hydroxylation pattern is found. The B-ring typically has either a 4'-, a 3',4'-, or a 3',4',5'-hydroxylation pattern. The C-ring is normally a six-membered ring system that contains a carbonyl, hydroxyl, a double bond between carbons 2 and 3, or it can be completely unsubstituted.

Two types of hydroxylases that add hydroxyl groups to the B- and C-rings have been identified: microsomal monooxygenases that are NADPH-dependent cytochrome P-450 enzymes and soluble dioxygenases [136]. Flavonoids can be methylated by O-methyltransferases using S-adenosyl-L-methionine (SAM) as the cofactor. Acylation
Figure 5.1: Overall pathway to the major flavonoids groups [136].
and prenylation of the flavonoids can also occur, however, prenylation is more common in isoflavonoids. Sulfated flavonoids have been isolated and are mainly based on the apigenin or luteolin derivatives. Glycosylation, forming O- and C-glycosides, is an important step that occurs in a late or terminal step of flavonoid biosynthesis resulting in a more water-soluble flavonoid. This is believed to be important for retaining the flavonoids in the vacuole. O-Glycosyltransferases catalyze the reaction using UDP-sugars. Flavones and flavonols, either as aglycones or as the glycosides, are the most variable and abundant of all the flavonoids classes [136]. Although a wide variety of flavonoids with different hydroxylation, methylation, and glycosylation patterns exist, there is also the need to add elements of diversity to the benzopyranone skeleton that are not readily found in nature.

5.2 Reactions to the 4H-1-Benzopyran-4-one Skeleton

Our laboratory has developed a novel solution phase method for constructing 2-substituted-4H-1-benzopyran-4-ones from functionalized salicylic acids and terminal alkynes. The application of this chemistry to solid phase to produce a library of compounds would result in the synthesis of even more compounds that could be screened. Because there are only two sets of building blocks, the salicylic acids and terminal alkynes, the possible combinations of these two building blocks is limited. If the benzopyranones could then be reacted with a third set of building blocks, the number of molecules that could be synthesized could be greatly increased (Figure 5.2).
Figure 5.2: To fully functionalize the 4\(H\)-1-benzopyran-4-one ring system, substitutents are needed at the 3-position.

Direct hydroxylation of 2-phenyl-4\(H\)-1-benzopyran-4-one at the 3-position to form the corresponding flavonols has been described by using hypervalent iodine oxidation (Figure 5.3) [137]. This procedure also has been successful for the oxidation of chromones and \(\alpha\)-naphthoflavone. Alternative methods for the formation of 3-hydroxy-flavones include phenyl iodosyl diacetate and PhI(OAc)\(_2\); presumably by rearrangement of the intermediary epoxides. Epoxidation of the double bond of the benzopyranone ring system could result in useful intermediates in flavone chemistry. Classical oxidants like alkaline H\(_2\)O\(_2\) and \(m\)-CPBA fail to epoxidize flavonoids; while other oxidants like KMnO\(_4\), NiO\(_2\), SeO\(_2\), and Tl(OAc)\(_3\) are inert to the flavone moiety [138]. Dimethyldioxirane in acetone, however, has proved to be a mild and selective oxidant of flavonoids (Figure 5.4) [138,139], isoflavones [140-142], aurones [143], and chalcones [144-146]. Labile flavone epoxides have been isolated (>95%) and upon warming to room temperature afford the 3-hydroxy-flavone or upon treatment with MeOH led to the formation of 3-hydroxy-2-methoxyflavanones [138].
Figure 5.3: C-3 hydroxylation of flavone, α-naphthoflavone, and chromone via iodobenzene diacetate-potassium hydroxide methanol oxidation [137].

Figure 5.4: Dimethyldioxirane epoxidation of flavones [138,139].

Flavonoids halogenated α to the carbonyl (C-3) could be useful intermediates in the synthesis of 3-alkyl, alkenyl, and aryl flavonoids. 3-Iodo derivatives of flavones and thioflavones, and thiochromones can be synthesized by iodine-cerium(IV) ammonium nitrate system under mild conditions (Figure 5.5) [147] or by iodination of
3-lithioflavone [148]. Protocols for the palladium-catalyzed cross coupling of \( \alpha \)-haloenones with organometals containing Zn, Sn, B, and Cu to give \( \alpha \)-organylenones in high yields have been developed [149].

Flavones are readily attacked by lithium diisopropylamide in tetrahydrofuran at -78°C to form 3-lithioflavone, which can react with various electrophiles to form previously unavailable products (Figure 5.6) [148]. In this way, various groups (i.e., CO\(_2\)H, CO\(_2\)Et, SiCH\(_3\), I, SH, SCH\(_3\), and OH) can be introduced at the 3-position with relative ease and in excellent yields. Many of these groups can be further derivatized.

**Figure 5.5:** Iodination of flavones, thioflavones, and thiochromones at the 3-position using iodine-cerium (IV) ammonium nitrate system [147].
<table>
<thead>
<tr>
<th>Electrophile</th>
<th>% Yield</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO₂</td>
<td>92%</td>
<td>COOH</td>
</tr>
<tr>
<td>(EtO)₂CO</td>
<td>51%</td>
<td>CO₂Et</td>
</tr>
<tr>
<td>EtOCCl</td>
<td>48%</td>
<td>CO₂Et</td>
</tr>
<tr>
<td>CH₅SiCl</td>
<td>93%</td>
<td>SiCH₃</td>
</tr>
<tr>
<td>I₂</td>
<td>97%</td>
<td>I</td>
</tr>
<tr>
<td>Sulphur</td>
<td>92%</td>
<td>SH</td>
</tr>
<tr>
<td>CH₅SSCH₃</td>
<td>92%</td>
<td>SCH₃</td>
</tr>
<tr>
<td>Furan-2-CHO</td>
<td>60%</td>
<td>2-C(OH)-Furan</td>
</tr>
<tr>
<td>B(OCH₃)₂H₂O₂</td>
<td>92%</td>
<td>OH</td>
</tr>
</tbody>
</table>

**Figure 5.6**: Lithiation of flavones with lithium diisopropylamide in tetrahydrofuran [148].

### 5.3 Bromination of the Benzopyranone Skeleton

The structural basis for SERM selectivity is believed to stem from the third aryl group that bears a 4-aminoethoxy substitution. This basic amine side chain of tamoxifen and raloxifene has been shown by x-ray co-crystallographic studies to project into the estrogen receptor (ER) displacing helix 12, blocking the interaction between ER and its cellular transcription machinery, giving rise to the antagonistic effects on target genes. The isoflavonoid genistein has been shown to bind to the ER in the orientation shown in Figure 5.7. It has been proposed in our laboratory that adding a basic amine side chain to the 4H-1-benzopyran-4-one skeleton of genistein could give rise to a new class of ER antagonists.

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Figure 5.7: Adding a basic amine side onto the 4H-1-benzopyran-4-one skeleton of genistein.

To synthesize genistein analogs bearing a third aryl group containing a 4-aminoethoxy side chain, introduction of aromatic substitutents at the 3-position of the benzopyranone nucleus is necessary. Figure 5.8 shows the proposed addition of an aromatic group at the C-3 position by bromination of the benzopyranone followed by a Suzuki coupling.

Figure 5.8: Proposed synthesis of 3-aryl-4H-1-benzopyran-4-ones via a Suzuki coupling of the 3-bromo derivative.

The initial approach to make 3-bromobenzopyranones was to treat the enaminoketone intermediate with a chloroform solution of bromine, and in one step perform the cyclization and bromination (Figure 5.9) [125,150]. Initial attempts of this reaction
failed. Thus, an alternative direct synthesis of 3-bromobenzopyranones from the corresponding benzopyranones by pyridinium tribromide/pyridine system was examined [151]. This reaction has worked in all cases with yields ranging from 75-89%. Pyridinium tribromide is an easy to handle stable solid compared to molecular bromine.

$$\text{Br}_2, \text{CHCl}_3$$
$$X = H$$
$$R_1 = 4\text{-OCH}_3$$

Enaminoketone

Benzopyrone

<table>
<thead>
<tr>
<th>$R_1$</th>
<th>$R_2$</th>
<th>Conditions</th>
<th>% Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>H</td>
<td>phenyl</td>
<td>pyridinium tribromide/pyridine</td>
<td>75%</td>
</tr>
<tr>
<td>7-OCH$_3$</td>
<td>phenyl</td>
<td>pyridinium tribromide/pyridine</td>
<td>85%</td>
</tr>
<tr>
<td>7-OCH$_3$</td>
<td>propyl</td>
<td>pyridinium tribromide/pyridine</td>
<td>88%</td>
</tr>
<tr>
<td>8-OCH$_3$</td>
<td>4'-methylphenyl</td>
<td>pyridinium tribromide/pyridine</td>
<td>89%</td>
</tr>
</tbody>
</table>

Figure 5.9: Bromination of benzopyranones.

Current efforts in this synthetic strategy involve the Suzuki palladium-catalyzed cross-coupling reaction of 3-bromo-2-substituted-benzopyranones with arylboronic acids (Figure 5.10) [152]. Suzuki et al. reported the novel synthesis of isoflavones by the coupling of 3-bromochromone and phenylboronic acid in the presence of 3 mol% of Pd(PPh$_3$)$_4$ and Na$_2$CO$_3$ (2M) under refluxing benzene in yields ranging from 71-94% [152]. This methodology was applied to 3-bromo-2-phenyl-4H-1-benzopyran-4-one.
using phenylboronic acid and p-methoxyphenylboronic acid resulting in isolation of starting material. To minimize steric interactions between substituents at the C-2 and C-3, 3-bromo-7-methoxy-2-propyl-4H-1-benzopyran-4-one was used in the Suzuki coupling again resulting in no reaction. The palladium-catalyzed cross-coupling reactions of organoboron compounds has been extensively studied [153,154]. Optimization of the Suzuki coupling needs to be performed pertaining to the base, catalyst, and solvent used (cite). The completion of the Suzuki coupling will result in formation of 2,3-disubstituted-benzopyranones.

\[
\begin{align*}
\text{X} & \quad \text{Br} \quad \text{Pd(PPh}_3)_4 \quad \text{(ZnCO}_3\text{)} / \text{H}_2\text{O} \\
\text{Benzene} & \quad 71 - 94% \\
\text{R} & \quad \text{Ar-B(OH)}_2 \\
\end{align*}
\]

<table>
<thead>
<tr>
<th>R₁</th>
<th>R₂</th>
<th>Ar-B(OH)₂</th>
<th>% Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>H</td>
<td>phenyl</td>
<td>H</td>
<td>noixo</td>
</tr>
<tr>
<td>H</td>
<td>phenyl</td>
<td>4-OCH₃</td>
<td>noixo</td>
</tr>
<tr>
<td>7-OCH₃</td>
<td>propyl</td>
<td>H</td>
<td>noixo</td>
</tr>
</tbody>
</table>

Figure 5.10: Suzuki cross-couplings of arylboronic acids with benzopyranones [152].

5.4 Demethylations of 4H-1-Benzopyran-4-ones

The pharmacophore for 17β-estradiol binding to the estrogen receptor involves an A-ring phenol separated by a hydrophobic spacer of a specified distance to a second
hydroxyl group on the D-ring. Because hydrophobic pockets exist in the α face of the B-ring and β face of the C-ring, larger groups on estradiol can be accommodated at these positions. This information can be applied to functional groups on the benzopyranone ring system. In order to evaluate the synthetic benzopyranones, methoxy groups need to be demethylated to fully determine the structure activity requirements needed to bind to the estrogen receptor. To date, two series of benzopyranones with methoxy substitutions at the 7- and 8-position have been synthesized (Figure 5.11). Using a solution of boron tribromide in dichloromethane, 8-methoxy-2-phenyl-benzopyranone was successfully demethylated in 60% yield as shown in Figure 14 [155]. Because these conditions failed to demethylate 7-methoxy-2-phenyl-benzopyranone, other conditions were examined. Ethanethiol in combination with either the Lewis acid AlCl₃ in CH₂Cl₂ or sodium hydride in DMF have been employed to demethylate both the series of benzopyranones in yields ranging from 2-98%.
### Table: Demethylations of Benzopyranones

<table>
<thead>
<tr>
<th>$R_1$</th>
<th>$R_1$ product</th>
<th>$R_2$</th>
<th>Conditions</th>
<th>% Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>7-OCH$_3$</td>
<td>7-OCH$_3$</td>
<td>phenyl</td>
<td>BBr$_3$, CH$_2$Cl$_2$</td>
<td>No reaction</td>
</tr>
<tr>
<td>8-OCH$_3$</td>
<td>8-OH</td>
<td>phenyl</td>
<td>BBr$_3$, CH$_2$Cl$_2$</td>
<td>60%</td>
</tr>
<tr>
<td>7-OCH$_3$</td>
<td>7-OH</td>
<td>phenyl</td>
<td>EtSH, AlCl$_3$, CH$_2$Cl$_2$</td>
<td>79%</td>
</tr>
<tr>
<td>7-OCH$_3$</td>
<td>7-OH</td>
<td>phenyl</td>
<td>EtSH, NaH, DMF</td>
<td>&gt;95%</td>
</tr>
<tr>
<td>7-OCH$_3$</td>
<td>7-OH</td>
<td>4'-methylphenyl</td>
<td>EtSH, NaH, DMF</td>
<td>92-98%</td>
</tr>
<tr>
<td>8-OCH$_3$</td>
<td>8-OH</td>
<td>4'-methylphenyl</td>
<td>EtSH, NaH, DMF</td>
<td>62%</td>
</tr>
<tr>
<td>7-OCH$_3$</td>
<td>3'-chloropropyl</td>
<td>EtSH, NaH, DMF</td>
<td>unidentified product</td>
<td></td>
</tr>
<tr>
<td>7-OCH$_3$</td>
<td>1-Cyclohexenyl</td>
<td>EtSH, NaH, DMF</td>
<td>unidentified product</td>
<td></td>
</tr>
<tr>
<td>8-OCH$_3$</td>
<td>1-Cyclohexenyl</td>
<td>EtSH, NaH, DMF</td>
<td>unidentified product</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 5.11:** Demethylations of benzopyranones.
CHAPTER 6

BIOLOGICAL TESTING OF SUBSTITUTED-4H-1-BENZOPYRAN-4-ONES

6.1 Molecular Targets of Benzopyranones

Using solution phase chemistry, a library of synthetic 4H-1-benzopyranones has been synthesized in an effort to develop novel agents to treat breast cancer. These compounds have the potential to affect multiple molecular targets in breast cancer including estrogen receptor signaling, estrogen biosynthesis and metabolism, and growth factor signaling cascades (Figure 6.1). Therefore, the screening of benzopyranones against multiple targets needs to be performed. The ability of a benzopyranone to disrupt one or more of these molecular targets could result in potent new therapies for the treatment of hormone dependent breast cancer.

6.2 Initial Screening of Benzopyranone Library

The synthesized benzopyranone analogs were initially evaluated in an cellular proliferation assay in collaboration with S. Joomprabutra to determine the biological effects on human breast cancer cell lines [156]. Cellular proliferation assays are used to assess the cytotoxicity of chemical substances in a simple and rapid fashion. The conditions used in this in vitro assay can be controlled with respect to cell type and
exposure to hormones and/or growth factors. Two different human breast cancer cell lines, estrogen dependent MCF-7 and estrogen independent MDA-MB-231, were used in a primary screening.

Figure 6.1: Molecular targets in breast cancer include inhibition of binding of 17β-estradiol to the estrogen receptor, inhibition of protein tyrosine kinases, inhibition of cyclooxygenases, inhibition of estrogen biosynthesis via aromatase, and metabolism of estradiol to estrogenically weaker or inactive metabolites.
6.2.1 MTS Breast Cancer Cell Cytotoxicity Assay

Various methods for determining cell viability are used including the MTS, BrdU, and $^3$H-thymidine assay. The MTS assay correlates cellular metabolism of tetrazolium salts into colored, water-soluble formazan products to determine cell viability. The amount of formazan produced by dehydrogenase enzymes in the cells is directly proportional to the number of viable cells in culture. The conversion of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) into the water-soluble formazan product is accompanied by a color change from yellow to purple which can be measured at 490 nm (Figure 6.2). This assay has been adapted to 24-well plates and allows for the rapid screening of compounds.

![Figure 6.2: Conversion of MTS into its formazan product is presumed to be accomplished by succinate dehydrogenase enzymes in metabolically active cells.](image)

Various controls were used including 17β-estradiol (E2, 10 nM), 4-hydroxytamoxifen (4-OHT, 1μM), ICI 182,780 (ICI, 1 μM), and genistein (Gen, 1 and 10 μM) (Figure 6.3). By incorporating the biological and molecular modeling data, a pharmacophore
model of the benzopyranone analogs targeting growth inhibition of breast cancer cells can be determined and used for directing future synthetic endeavors.

Figure 6.3: Controls used in the MTS Breast Cancer Cell Proliferation Assay.

6.2.2 RESULTS OF BREAST CANCER CELL CYTOTOXICITY ASSAY

The initial breast cancer cell cytotoxicity assay was used to evaluate the effects of the agents on the growth and cytotoxicity of MCF-7 cells and MDA-MB-231 cells according to the procedure by Joomprabutra [156]. Differential activities were observed with clusters of structurally similar compounds at concentrations of 1.0 μM. The effect of benzopyranones on MCF-7 and MDA-MB-231 breast cancer cell growth can be seen in Figures 6.4 and 6.5. The synthetic 4H-1-benzopyran-4-ones tested had substituents at the 6-, 7-, and 8-positions (H, OCH₃, OH, Cl, Ph) and 2-position (phenyl, 4'-methylphenyl, 3-chloropropyl, cyclohexen-1-yl) and can be seen in Figure 4.35. The effect of benzopyranones on the proliferation of MCF-7 cells varies, with the majority of the compounds showing no effect or a stimulatory effect. In general, the strongest stimulatory effects for each class of compounds was with a 2-phenyl substituent. Only three compounds, 83, 93, and 112, show a decrease in proliferation of MCF-7 cells.
Compound 93 and 112 also show this effect in MDA-MB-231 cells. The benzopyranones demonstrate either no effect or an inhibitory effect on MDA-MB-231 cells. Two compounds, 60 and 80, each possessing a 2-phenyl and 7-substituent (OH and Cl, respectively), show proliferative effects on MDA-MB-231 cells. Inhibitory effects on MDA-MB-231 cells were seen with the following compounds: 15, 22, 23, 41, 43, 55, 82, 83, 90, 91, 93, 110, and 112. Compounds with a 2-(3-chloropropyl) side chain or 3-bromo substituent are inhibitory. In general, a 7-chloro, 6-chloro, or 6-phenyl substituent has greater inhibitory effect than a 7-methoxy, 7-hydroxyl, or 8-hydroxyl group. Preliminary screening in the proliferation assay demonstrates that the compounds do stimulatory and inhibitory effects on human breast cancer cells. Further testing is needed to determine the molecular target of the benzopyranones.
Figure 6.4: Summary of cell proliferation studies on MCF-7 and MDA-MB-231 breast cancer cells using a library of benzopyranones [156].
Figure 6.5: Effects of benzopyranones on A. MCF-7 and B. MDA-MB-231 breast cancer cell proliferation [156].
6.3 RADIOLIGAND ESTROGEN RECEPTOR DISPLACEMENT ASSAY

Results from the breast cancer cell cytotoxicity assay suggest that the biological activities of the benzopyranones may be mediated through various biological targets. A decrease or increase only in MCF-7 cell proliferation (no effect on MDA-MB-231 proliferation) could indicate a dependence on estrogen receptor signaling pathways. In order to determine if the agents bind to the estrogen receptor, a radioligand estrogen receptor displacement assay was performed by S. Joomprabutra [156]. This competitive radioligand-binding assay measures the ability of compounds to bind and compete with ^3H-17β-estradiol for the estrogen receptor. The controls used for this assay were 17β-estradiol (E2, 5 nM), 4-hydroxytamoxifen (4-OHT, 1 μM), ICI 182,780 (ICI, 1 μM), and genistein (Gen, 1 μM). The benzopyranones were tested at 1 μM. The relative binding affinity of benzopyranones for ERα can be seen in Figure 6.6.

6.3.2 RESULTS OF ^3H-E2 DISPLACEMENT ASSAY

The method of the cytosolic ^3H-E2 displacement assay used is that according to Joomprabutra [156] using rat uteri from 3 week old ovariectomized Sprague Dawley. The results from the cytosolic estrogen receptor displacement assay and the molecular docking studies performed by Joomprabutra [156] show that the compounds in native form have relatively low estrogen receptor binding activity (Figure 6.6). Benzopyranones at 1 μM have the ability to bind to ERα in the range of 2% to 20% relative to controls. Genistein displaced 33.5% of ^3H-17β-estradiol. Compounds 30 and 51 show the highest binding abilities of all the compounds tested. The results from
the proliferation and displacement assays for compounds \( \text{30} \) and \( \text{51} \) (growth stimulation in MCF-7 cells, no activity in MDA-MB-231 cells) suggest that they may be weak partial agonists for the estrogen receptor.

**Figure 6.6:** Relative binding affinity to ER\( \alpha \) by synthetic benzopyranones [156].
6.4 AROMATASE AS A POTENTIAL MOLECULAR TARGET

Because the biosynthesis of 17β-estradiol by the enzyme aromatase occurs locally within breast tissue, inhibition of aromatase is an alternative target for treating estrogen-dependent breast cancer (Figure 6.7). Screening of the benzopyranone library (1 µM) in a human placental microsome aromatase assay was performed by J. Baker, Jr. to determine if the synthetic benzopyranones could inhibit aromatase [157].

Figure 6.7: Endocrine control of breast cancer.
6.4.1 Human Placental Microsome Aromatase Assay

The aromatase assay according to the procedure used by Baker [157] uses cytosolic aromatase enzyme from human placental tissues to catalyze the conversion of \([1\beta-^3\text{H}]\)-androst-4-ene-3,17-dione to estrone and \(^3\text{H}_2\text{O}\) in the presence of NADPH and \(O_2\) (Figure 6.8). The tritiated \(H_2O\) released is counted via liquid scintillation and is an index of estrogen formation. A NADPH regeneration system is needed to reduce NADP\(^+\) to NADPH because aromatase is an NADPH dependent enzyme. 7\(\alpha\)-Aminophenylthio-androsta-1,4-diene,3,17-dione (7\(\alpha\)-APTADD) is an enzyme-activated irreversible inhibitor of aromatase and was used as a positive control (Figure 6.9). 7\(\alpha\)-APTADD was designed and initially synthesized in our laboratory [158]. It has an apparent \(K_i\) of 10 nM. Chrysin (5,7-dihydroxyflavone) is marketed in supplement stores as an aromatase inhibitor to increase or maintain higher levels of androgens [83] and was used as a positive control.
Figure 6.8: Aromatase assay.

Figure 6.9: 7α-(Aminophenylthio)-androsta-1,4-diene,3,17-dione is an enzyme-activated irreversible inhibitor of aromatase used as a positive control. Chrysin and apigenin were also used as positive controls in the aromatase assay.
6.4.2 RESULTS OF HUMAN PLACENTAL MICROsome AROMATASE ASSAY

The results from the human placental microsome aromatase assay for the 4H-1-benzopyran-4-one library (1µM) are found in Figure 6.10. IC\textsubscript{50} values were determined for fifteen of the benzopyranones (Table 6.1) [157]. The best aromatase inhibitor of the synthetic benzopyranones was 113, 2-(3-chloropropyl)-6-phenyl-4H-1-benzopyran-4-one (IC\textsubscript{50} = 1.34 µM) followed closely by 52 2-(1-cyclohexen-1-yl)-7-methoxy-4H-1-benzopyran-4-one (IC\textsubscript{50} = 1.72 µM). Changing the 6-phenyl substituent of 113 to a 6-chloro 93 increases the IC\textsubscript{50} to 25.58 µM. Substituents at the 7-position have lower IC\textsubscript{50} values than substituents at the 8-position, which correlates to known SAR of flavonoids for aromatase [64].
Figure 6.10: Effects of benzopyranones on % aromatase activity in a cytosolic aromatase assay. P < 0.05 shown with *. The concentration of 7α-APTADD was 50 nM. Benzopyranones were tested at 1 μM, n = 3 or 4 [157].
<table>
<thead>
<tr>
<th>#</th>
<th>R&lt;sub&gt;1&lt;/sub&gt;</th>
<th>R&lt;sub&gt;2&lt;/sub&gt;</th>
<th>R&lt;sub&gt;3&lt;/sub&gt;</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
<th>95% Conf. Range</th>
<th>1 µM Screen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7α-APTADD</td>
<td>0.05</td>
<td>0.046 - 0.056</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>chrysin</td>
<td>4.74</td>
<td>4.36 - 5.16</td>
<td>79.30</td>
<td>3.68</td>
<td></td>
<td></td>
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<tr>
<td>apigenin</td>
<td>4.30</td>
<td>3.78 - 4.90</td>
<td>82.98</td>
<td>2.64</td>
<td></td>
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<tr>
<td>7-OH-flavone</td>
<td>1.70</td>
<td>1.54 - 1.87</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>10H phenyl</td>
<td>27.39</td>
<td>23.76 - 31.57</td>
<td>100.11</td>
<td>1.97</td>
<td></td>
<td></td>
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<tr>
<td>24 8-OCH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>63.40</td>
<td>51.02 - 78.78</td>
<td>96.69</td>
<td>1.64</td>
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<td></td>
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<tr>
<td>25 8-OCH&lt;sub&gt;3&lt;/sub&gt; 4'methylphenyl Br</td>
<td>6.97</td>
<td>5.35 - 9.08</td>
<td>86.20</td>
<td>1.96</td>
<td></td>
<td></td>
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<tr>
<td>30 8-OH phenyl</td>
<td>29.93</td>
<td>27.14 - 33.00</td>
<td>98.94</td>
<td>1.65</td>
<td></td>
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<tr>
<td>40 8-CH&lt;sub&gt;3&lt;/sub&gt; phenyl</td>
<td>15.87</td>
<td>13.59 - 18.53</td>
<td>87.96</td>
<td>1.10</td>
<td></td>
<td></td>
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<tr>
<td>43 8-CH&lt;sub&gt;3&lt;/sub&gt; 3-chloropropyl</td>
<td>33.28</td>
<td>27.53 - 40.24</td>
<td>89.35</td>
<td>6.51</td>
<td></td>
<td></td>
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<tr>
<td>50 7-OCH&lt;sub&gt;3&lt;/sub&gt; phenyl</td>
<td>8.50</td>
<td>7.86 - 9.20</td>
<td>84.36</td>
<td>2.52</td>
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<tr>
<td>51 7-OCH&lt;sub&gt;3&lt;/sub&gt; 4'methylphenyl</td>
<td>6.84</td>
<td>6.18 - 7.58</td>
<td>90.79</td>
<td>4.63</td>
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<td></td>
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<tr>
<td>52 7-OCH&lt;sub&gt;3&lt;/sub&gt; 1-cyclohexen-1-yl</td>
<td>1.72</td>
<td>1.56 - 1.82</td>
<td>73.50</td>
<td>0.92</td>
<td></td>
<td></td>
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<tr>
<td>60 7-OH phenyl</td>
<td>2.56</td>
<td>2.36 - 2.79</td>
<td>70.47</td>
<td>3.31</td>
<td></td>
<td></td>
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<tr>
<td>61 7-OH 4'methylphenyl</td>
<td>2.83</td>
<td>2.58 - 3.10</td>
<td>85.95</td>
<td>1.26</td>
<td></td>
<td></td>
</tr>
<tr>
<td>70 7-CH&lt;sub&gt;3&lt;/sub&gt; phenyl</td>
<td>2.27</td>
<td>2.03 - 2.53</td>
<td>87.73</td>
<td>1.82</td>
<td></td>
<td></td>
</tr>
<tr>
<td>82 7-Cl 1-cyclohexen-1-yl</td>
<td>2.69</td>
<td>2.52 - 2.88</td>
<td>66.13</td>
<td>1.73</td>
<td></td>
<td></td>
</tr>
<tr>
<td>93 6-Cl 3-chloropropyl</td>
<td>25.58</td>
<td>22.6 - 28.94</td>
<td>100.34</td>
<td>1.99</td>
<td></td>
<td></td>
</tr>
<tr>
<td>113 6-Ph 3-chloropropyl</td>
<td>1.34</td>
<td>1.24 - 1.45</td>
<td>70.47</td>
<td>0.30</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 6.1: IC<sub>50</sub> values for Aromatase Assay [157].
CHAPTER 7

CONCLUSIONS

2,3-Substituted-4H-1-benzopyran-4-ones have been synthesized in moderate to excellent yields from readily available starting materials (salicylic acids and terminal alkynes) via a one-pot acid chlorination, Sonogashira coupling followed by a 6-endo-
trig cyclization. In developing this synthetic strategy, we have accomplished mild, high yielding reactions that are tolerant of various functional groups to give exclusive formation of the 4H-1-benzopyran-4-one ring system while remaining amenable to solid phase.

Silylated salicylic acids are converted to the corresponding acid chlorides under neutral conditions using oxalyl chloride. Quenching of the acid chloride with an ethanol in pyridine/ether mixture resulted in the isolation of the ethyl ester in 77-91%, indicative of the clean formation of the acid chloride. The acid chlorination can be directly followed by the Sonogashira coupling resulting in the one-pot synthesis of alkynone intermediates. The electronic and steric requirements of the alkyne and salicylic acid and their usefulness in the Sonogashira coupling have been determined. Aryl and alkyl alkynes couple in yields ranging from 40-90%. Reaction conditions were optimized
based on both of the reactants while minimizing the competing Glaser homocoupling of alkynes. Four variations for the Sonogashira coupling were employed.

As seen in the literature, a free phenolic hydroxyl can effect either a 6-endo-trig or 5-exo-dig cyclization resulting in the nonselective formation of benzopyranones and benzofuranones, respectively. The benzopyranones have been regioselectively synthesized by converting the alkynones into enaminoketone intermediates and protection of the ortho phenol. Upon removal of the protecting group, the system is prone to Michael addition followed by elimination of the secondary amine to yield the desired benzopyranone as a 6-endo-trig cyclization in yields ranging from 53-96%. A high-throughput synthesis of benzopyranones has also been developed, eliminating the need for intermediate purification of alkynones by flash chromatography. Structure elucidation via heteronuclear shift correlation experiments and UV absorption have confirmed the presence of the benzopyranone ring system. Bromination of the benzopyranone ring system at the three-position via pyridinium tribromide/pyridine system allows for the potential to add aryl groups via a Suzuki coupling in order to make isoflavonoid analogs. Conditions for demethylation of the aryl methylethers have been accomplished via BBr₃ or ethanethiol. Future synthetic efforts need to examine the utilization of other protecting groups for hydroxyl groups at R₁ because of difficulty removing aryl methylethers in the presence of 2-(3-chloropropyl) and 2-(1-cyclohexen-1-yl) substituents.
This chemistry has potential application to solid-phase to make a larger library of novel synthetic benzopyranones. This ring system can also undergo diversification to make a large array of compounds. In the future, the completion of the Suzuki coupling will allow the generation of the isoflavonoid ring system. Although outside the scope of this dissertation, I have proposed to exploit the isoflavonoid template of genistein to develop a series of novel antiestrogenic isoflavonoid analogs as possible therapeutic agents to treat human breast cancer. Pharmacophores present in nonsteroidal or steroidal antiestrogens are basic amine and long unsubstituted aliphatic chains. It is my hypothesis that substitutions at the 2-position of the 4H-1-benzopyran-4-one ring system could mimic the long aliphatic 7α-side chain of the ICI compounds or the basic amine of tamoxifen and raloxifene to produce novel modulators of ERα and ERβ (Figure 7.1, 7.2). The proposed isoflavonoids could enable binding to ER in two distinct orientations, thus, substituents at the 2-position could occupy either of the two large hydrophobic cavities that exist in ER (α face of B-ring or β-face of C-ring). Modifications of 4H-1-benzopyran-4-one ring system at the 2-position, mimicking the side chains of known antiestrogens, should yield novel antiestrogens that could aid in fully understanding the tissue selectivity differences of ERα and ERβ.
1. Basic amine side chains (raloxifene mimics)

\[ R = (\text{CH}_2)_n \text{-OH} \]

where \( n = 0, 1 \)

where \( Y = H, \text{N(CH}_3\text{)}_2, \text{N(Et)}_2, \text{NH} \)

2. Alkyl amine, sulfide, and carboxylic acid side chains

\[ R_1 = \text{CH}_2\text{SCH}_3, \text{CH}_2\text{SCH}_2\text{CH}_3, \text{CH}_2\text{N(CH}_3\text{)}_2, \text{CH}_2\text{N(Et)}_2, \text{CH}_2\text{N} \]

3. Long unsubstituted aliphatic side chains (ICI mimics)

\[ R_1 = (\text{CH}_2)_{10} \]

\[ \text{CH}_3 \]

Figure 7.1: Potential isoflavonoid analogs that could be synthesized based upon the 4H-1-benzopyran-4-one ring system.
Figure 7.2: Synthetic strategy of isoflavonoid analogs as ICI mimics, raloxifene/tamoxifen mimics, and alkyl amine and sulfide derivatives.
CHAPTER 8

EXPERIMENTAL METHODS

8.1 GENERAL METHODS

Chemicals were purchased from Aldrich Chemical Co. (Milwaukee, WI) or Lancaster Chemical Inc. (Windham, NH) and were used as received unless otherwise indicated. Anhydrous solvents were dried by standard procedures. To remove traces of primary and secondary amines, triethylamine was refluxed with phthalic anhydride, then distilled, refluxed with CaH₂, distilled again, and stored over sieves. All reactions were carried out under an inert atmosphere of argon unless otherwise specified. Glassware was flame dried under a flow of argon. Thin layer chromatography was performed on Whatman precoated silica gel F₂₅₄ aluminum foils and were purchased from Fisher Scientific (Fair Lawn, NJ). Visualization was accomplished with a UV lamp and/or staining with 5% ethanolic phosphomolybdic acid or KMNO₄. Intermediates were purified by flash column chromatography using a glass column wet packed with silica gel (32-63 µm particle size, 230-400 mesh) using the indicated solvent systems. ¹H NMR and ¹³C NMR (proton decoupled) spectra were recorded using a Bruker AF 250, Bruker DPX 250, or Bruker DRX 400 model spectrometer in CDCl₃ solutions unless otherwise indicated using the residual protiosolvent signal as internal reference. Two-
dimensional experiments were carried out using a Bruker DRX 400. IR spectra were recorded on a Nicolet Protégé 460 model spectrometer in the phase indicated. UV spectra were recorded on a Perkin Elmer Lambda 10. All melting points were determined in open glass capillaries using a Thomas Hoover apparatus and are uncorrected. Formula conformations (HR) and molecular weight measurements (LR) were obtained at The Ohio State Chemical Instrumentation Center using a Micromass OTOF II ESI, a Bruker Esquire ESI, or a Finnigan MAT900 EI. HPLC-grade MeOH and acetonitrile were obtained from Fisher Scientific. HPLC mobile phase was filtered through a 0.45 μm Nylon-66 membrane filter before use. HPLC was conducted on a Beckman system using a Spectroflow 757 UV detector (254 nm) and a reverse-phase column. Two HPLC solvent systems were used to determine purity: System #1- 70/30 MeOH/H₂O using a Beckman Ultrasphere ODS column (5 micron, 4.5 μm ID x 15 cm) and System #2- 65/35 acetonitrile/H₂O using a Phenomenex Luna phenyl-hexyl column (5 micron, 4.6 μm ID x 25 cm) with a flow rate of 1 mL/min.
8.2 SYNTHETIC METHODS

8.2.1 SYNTHESIS OF PALLADIUM CATALYST

*Dichlorobis(triphenylphosphine)palladium (JLW-I-200):* Following literature procedure of Herrman & Salzer, sodium tetrachloropalladate (II) (0.5 g, 1.7 mmol) and triphenylphosphine (0.95 g, 3.6 mmol) are suspended in EtOH (50 mL) under argon and stirred at room temperature for 24 h [120]. Upon addition of triphenylphosphine to sodium tetrachloropalladate solution, color change from brownish red to yellow. The resulting reaction mixture was filtered by gravity to give a yellow precipitate, which was further washed with H₂O, EtOH, and Et₂O. Solid was recrystallized from CHCl₃/petroleum ether to yield 1.1 g (90%) of the title compound as a yellow powder.

8.2.2 GENERAL METHOD FOR PREPARATION OF 2-[[[(1,1-DIMETHYLETHYL)DIMETHYLSILYL]OXY]-BENZOIC ACID, ETHYL ESTER DERIVATIVES.

Under argon atmosphere, oxalyl chloride (0.10 mL, 1.2 mmol) was added dropwise to a cold (0°C) solution of 2-[[[(1,1-dimethylethyl)dimethylsilyl]oxy]-benzoic acid, (1,1-dimethylethyl)dimethylsilyl ester derivative (0.38 g, 1.0 mmol) in CH₂Cl₂ (4 mL) containing 3 drops of DMF. The resulting solution was stirred at 0°C for 2 h. The reaction was warmed to room temperature and stirred for 17 h followed by concentration *in vacuo*. A 1:1:1 ratio of EtOH:ether:pyridine (3 mL) was added dropwise to the residue and stirred at room temperature under argon for 2 hours. The reaction mixture was concentrated *in vacuo*. Ether was added to the residue and the
precipitate was filtered through a glass sintered filter. The filtrate was concentrated \textit{in vacuo} and purified via flash chromatography (silica gel, 4.5:1 hexanes:EtOAc) to yield desired product.

\[
\begin{array}{c}
\text{OCH}_3 \\
\text{OCH}_3 \\
\text{TBS}
\end{array}
\]

2-[[1,1-Dimethylethyl]dimethylsilyl]oxy]-3-methoxybenzoic acid, ethyl ester (JLW-II-080):

Using the previous procedure and starting from 0.40 g (1.0 mmol) of 2-[[1,1-dimethylethyl]dimethylsilyl]oxy]-3-methoxybenzoic acid, (1,1-dimethylethyl)dimethylsilyl ester, 0.27 g (86%) of the title compound was obtained as a colorless oil: IR (neat, cm\(^{-1}\)) 2953, 2857, 1731, 1288, 1062; \(^1\)H NMR (CDCl\(_3\)) \(\delta\) 7.19 (dd, \(J = 7.1, 2.3\) Hz, 1H), 6.89-6.86 (m, 2H), 4.30 (q, \(J = 7.1\) Hz, 2H), 3.74 (s, 3H), 1.33 (t, \(J = 7.1\) Hz, 3H), 0.96 (s, 9H), 0.13 (s, 6H); \(^13\)C NMR (CDCl\(_3\)) \(\delta\) 166.9, 151.0, 144.4, 124.5, 121.9, 120.6, 114.3, 60.7, 55.1, 25.6, 18.7, 14.2, -4.3; HRMS m/z (M + Na\(^+\)) calculated for C\(_{16}\)H\(_{26}\)O\(_4\)Si was 333.1493, found 333.1489.
2-[[[(1,1-Dimethylethyl)dimethylsilyl]oxy]-4-methoxybenzoic acid, ethyl ester (JLW-II-084):

Using the previous procedure and starting from 0.41 g (1.0 mmol) of 2-[[[(1,1-dimethylethyl)dimethylsilyl]oxy]-4-methoxybenzoic acid, (1,1-dimethylethyl)dimethylsilyl ester, 0.29 g (90%) of the title compound was obtained as a colorless oil: IR (neat, cm⁻¹) 2956, 2858, 1725, 838; ¹H NMR (CDCl₃) δ 7.75 (d, J = 8.8 Hz, 1H), 6.49 (dd, J = 8.8, 2.3 Hz, 1H), 6.35 (d, J = 2.3 Hz, 1H), 4.28 (q, J = 7.1 Hz, 2H), 3.75 (s, 3H), 1.32 (t, J = 7.1 Hz, 3H), 0.98 (s, 9H), 0.20 (s, 6H); ¹³C NMR (CDCl₃) δ 165.9, 163.3, 157.1, 133.2, 115.5, 106.7, 106.6, 60.2, 55.2, 25.7, 18.3, 14.4, -4.4; HRMS m/z (M + Na⁺) calculated for C₁₆H₂₆O₄Si was 333.1493, found 333.1474.

2-[[[(1,1-Dimethylethyl)dimethylsilyl]oxy]-5-methoxybenzoic acid, ethyl ester (JLW-II-081):

Using the previous procedure and starting from 0.42 g (1.1 mmol) of 2-[[[(1,1-dimethylethyl)dimethylsilyl]oxy]-5-methoxybenzoic acid, (1,1-dimethylethyl)dimethylsilyl ester, 0.30 g (91%) of the title compound was obtained as a colorless oil: IR (neat, cm⁻¹) 2956, 2858, 1731, 1284, 1073; ¹H NMR (CDCl₃) δ 7.19 (d, J = 3.2 Hz, 1H), 6.87 (dd, J = 8.9, 3.2 Hz, 1H), 6.75 (d, J = 8.9 Hz, 1H), 4.29 (q, J = 7.1 Hz, 2H), 3.71 (s, 3H), 157
1.31 (t, J = 7.1 Hz, 3H), 0.95 (s, 9H), 0.13 (s, 6H); 13C NMR (CDCl3) δ 166.3, 153.3, 148.6, 123.3, 122.0, 119.2, 114.8, 60.6, 55.5, 25.6, 18.1, 14.2, -4.6; HRMS m/z (M + Na+) calculated for C16H26O4Si was 333.1493, found 333.1475.

2-[[[1,1-Dimethylethyl]dimethylsilyl]oxy]-6-methoxybenzoic acid, ethyl ester (JLW-II-068):

Using the previous procedure and starting from 0.26 g (0.6 mmol) of 2-[[[1,1-dimethylethyl]dimethylsilyl]oxy]-6-methoxybenzoic acid, (1,1-dimethylethyl)dimethylsilyl ester, 0.16 g (77%) of the title compound was obtained as a colorless oil: IR (neat, cm⁻¹) 2957, 2858, 1736, 1311, 1291, 1258, 1069, 839; 1H NMR (CDCl3) δ 7.14 (t, J = 8.3 Hz, 1H), 6.49 (d, J = 8.4 Hz, 1H), 6.44 (d, J = 8.3 Hz, 1H), 4.32 (q, J = 7.2 Hz, 2H), 3.77 (s, 3H), 1.33 (t, J = 7.2 Hz, 3H), 0.94 (s, 9H), 0.19 (s, 6H); 13C NMR (CDCl3) δ 166.6, 157.5, 153.2, 130.3, 116.5, 111.8, 103.9, 61.1, 55.9, 25.5, 18.0, 14.1, -4.4; HRMS m/z (M + Na+) calculated for C16H26O4Si was 333.1493, found 333.15.
2-Hydroxy-4-methoxybenzoic acid (JLW-I-104): Dihydroxybenzoic acid (15.4 g, 0.1 mol) was added to a 20% NaOH solution (50 mL) under argon. The resulting orange-yellow solution was stirred for 30 min until almost all solid was dissolved. Dimethyl sulfate (10.41 mL, 0.11 mol) was added and stirred for 2 h. The reaction was neutralized to pH = 7 with conc. HCl and extracted with Et₂O (3 x 100 mL). The aqueous layer was acidified to pH 1 and the resulting white precipitate was collected by vacuum filtration, washed with H₂O, and dried. Recrystallization from EtOH/H₂O to yield 8.3 g (50%) of the title compound as a white solid; mp 156-158°C; IR (KBr, cm⁻¹) 3441, 1648; ¹H NMR (DMSO-d₆) δ 7.69 (d, J = 9.6 Hz, 1H), 6.50-6.45 (m, 2H), 3.78 (s, 3H); ¹³C NMR (DMSO-d₆) δ 171.7, 165.0, 163.3, 131.5, 106.9, 105.5, 100.6, 55.5; HRMS m/z (M⁺) calculated for C₈H₆O₄ was 168.0420, found 168.0430.
5-(tert-Butyloxycarbonyl)-amino-2-hydroxy-benzoic acid (JLW-I-255):

According to procedure by Chen et al.[110], triethylamine (5.8 mL, 40 mmol) and di-
tert-butylcarbonate (8.73 g, 40 mmol) were added to a mixture of 5-aminosalicylic acid (3.06 g, 20 mmol) in undistilled dioxane (53 mL) and water (27 mL). The reaction was stirred at room temperature under argon for 3 hours. The reaction mixture was concentrated \textit{in vacuo} to reduce the volume by half. The reaction mixture was acidified with 3 M HCl, a precipitate was collected by filtration, washed with water, and dried to afford 4.9 g (96%) of the title compound as a pale brown solid: mp 274-276°C (decomposition); IR (KBr, cm$^{-1}$) 1670.5, 1689.4, 3004.1, 3273.1, 3344.4; $^1$H NMR (DMSO-$d_6$) $\delta$ 9.25 (s, 1H), 7.98 (d, $J = 2.1$ Hz, 1H), 7.48 (dd, $J = 8.9, 2.7$ Hz, 1H), 6.86 (d, $J = 8.9$ Hz, 1H), 1.45 (s, 9H); $^{13}$C NMR (DMSO-$d_6$) $\delta$ 171.8, 156.4, 152.9, 131.2, 126.6, 119.2, 117.1, 112.3, 78.9, 28.1; HRMS $m/z$ (M + Na$^+$) calculated for C$_{12}$H$_{15}$NO$_5$ was 276.0848, found 276.0868.
2-Hydroxy-5-phenyl-benzoic acid (JLW-I-254):

5-Bromosalicylic acid (1.08 g, 5 mmol), sodium carbonate (1.59 g, 15 mmol), and phenylboronic acid (0.67 g, 5.5 mmol) were dissolved in water (25 mL) under argon[109]. Palladium (II) acetate (11.2 mg, 0.05 mmol, 1 mol%) was added and reaction mixture was stirred at room temperature for 1 h to give a gray color with a precipitate present. The slurry was then dissolved in water (300 mL) and treated with HCl until it was acidic to litmus. The precipitate was filtered off and dissolved in ether (20 mL). The ether solution was filtered through a sintered glass funnel to remove Pd-black. The filtrate was concentrated in vacuo and the resulting residue was dissolved in water and treated with dilute NaOH until it was basic to litmus and filtered. The filtrate was treated with HCl, precipitate was filtered off, washed with water, and dried to afford 1.07 g (91%) of the title compound as a white solid: mp 211-212°C; IR (KBr, cm⁻¹) 1666.9, 3037.1, 3250.6, 3500; ¹H NMR (DMSO-d₆) δ 8.02 (d, J = 2.4 Hz, 1H), 7.82 (dd, J = 8.6, 2.4 Hz, 1H), 7.62 (s, 1H), 7.59 (s, 1H), 7.44 (t, J = 7.8, 7.2 Hz, 2H), 7.32 (t, J = 7.2 Hz, 1H), 7.05 (d, J = 8.6 Hz, 1H); ¹³C NMR (DMSO-d₆) δ 171.6, 160.5, 139.0, 133.8, 131.2, 128.9, 127.9, 127.0, 126.1, 117.8, 113.4; HRMS m/z (M + Na⁺) calculated for C₁₃H₁₀O₃ was 237.0528, found 237.0549.
8.2.4 General Method for Preparation of 2-[[((1,1-dimethylethyl)dimethylsilyl]oxy]-benzoic acid, ethyl ester, (1,1-dimethylethyl)dimethylsilyl ester Derivatives.

Et$_3$N (2.8 mL, 20 mmol) was added to a solution of 2-hydroxybenzoic acid derivative (0.84 g, 5 mmol) in freshly distilled CH$_2$Cl$_2$ (13 mL). A solution of tert-butyl(dimethyl)chlorosilane (1.7 g, 11 mmol) in CH$_2$Cl$_2$ (3.5 mL) was cannulated dropwise into the reaction mixture resulting in a cloudy solution. After the reaction was stirred at room temperature for 27 h, toluene (50 mL) was added and the suspension was concentrated to approximately 20 mL in vacuo followed by vacuum filtration to remove the precipitated Et$_3$N-HCl salt. The precipitate was thoroughly washed with hexanes and toluene and the combined organics were concentrated. The residue was purified on a short column (silica gel, 4.5:1 hexane:EtOAc) to yield desired product.
2-[[((1,1-Dimethyl)ethyl)dimethylsilyloxy]-4-methoxybenzoic acid, (1,1-dimethyl-ethyl)dimethylsilyl ester (JLW-I-166):

Using the previous procedure and starting from 2-hydroxy-3-methoxybenzoic acid (0.84 g, 5 mmol), 1.88 g (95%) of the title compound as a pale peach oil: IR (neat, cm⁻¹) 2954, 2932, 2888, 2859, 1711, 1325, 1291, 1255, 1060; ¹H NMR (CDCl₃) δ 7.27 (dd, J = 2.1, 2.0 Hz, 1H), 6.93 (dd, J = 2.1, 2.0 Hz, 1H), 6.86 (t, J = 7.6, 8.1 Hz, 1H), 3.77 (s, 3H), 0.98 (s, 9H), 0.98 (s, 9H), 0.34 (s, 6H), 0.15 (s, 6H); ¹³C NMR (CDCl₃) 165.9, 151.8, 146.0, 125.4, 123.0, 120.7, 115.1, 55.7, 26.2, 26.1, 19.2, 18.2, -3.8, -4.3; HRMS m/z (M⁺) calculated for C₂₀H₂₆O₄Si₂ was 396.2142, found 396.2199.

2-[[((1,1-Dimethyl)ethyl)dimethylsilyloxy]-4-methylbenzoic acid, (1,1-dimethyl-ethyl)dimethylsilyl ester (JLW-I-167):

Using the previous procedure and starting 2-hydroxy-3-methylbenzoic acid (1.53 g, 10 mmol, 3.64 g (95%) of the title compound was obtained as a colorless oil: IR (neat, cm⁻¹) 2955, 2932, 2888, 2859, 1711, 1279, 840, 806; ¹H NMR (CDCl₃) δ 7.92 (dd, J = 7.8, 1.5 Hz, 1H), 7.61 (dd, J = 7.5, 1.1 Hz, 1H), 7.22 (t, J = 7.8, 1H), 2.59 (s, 3H), 1.38 (s,
9H), 1.35 (s, 9H), 0.71 (s, 6H), 0.47 (s, 6H); $^{13}$C NMR (CDCl$_3$) 165.9, 153.9, 134.8, 131.0, 129.0, 124.5, 120.7, 26.0, 25.7, 18.5, 17.8, 17.4, -3.6, -4.7; HRMS $m/z$ (M$^+$) calculated for C$_{20}$H$_{36}$O$_3$Si was 380.2193, found 380.2245.

2-[[[(1,1-Dimethylethyl)dimethylsilyl]oxy]-4-methoxybenzoic acid, (1,1-dimethyl-ethyl)dimethylsilyl ester (JLW-I-118):

Using the previous procedure and starting from 2-hydroxy-4-methoxybenzoic acid (1.7 g, 10 mmol), 3.8 g (95%) of the title compound was obtained as a colorless oil: IR (CCl$_4$, cm$^{-1}$) 2859, 1700, 1607, 1259, 843, 761; $^1$H NMR (CDCl$_3$) $\delta$ 7.75 (d, $J = 8.8$ Hz, 1H), 6.49 (dd, $J = 8.8$, 2.3 Hz, 1H), 6.38 (d, $J = 2.7$ Hz, 1H), 3.78 (s, 3H), 1.00 (s, 9H), 0.98 (s, 8.7H), 0.32 (s, 6.6H), 0.20 (s, 6H); $^{13}$C NMR (CDCl$_3$) $\delta$ 164.5, 163.5, 158.2, 133.4, 116.5, 107.2, 106.7, 55.3, 25.9, 25.8, 18.5, 17.8, -4.4, -4.6; HRMS $m/z$ (M$^+$) calculated for C$_{20}$H$_{36}$O$_4$Si was 396.2142, found 396.2139.
2-[(1,1-Dimethylethyl)dimethylsilyloxy]-4-methoxybenzoic acid, (1,1-dimethyl-ethyldimethylsilyl)dimethylsilyl ester (JLW-II-034):

Using the previous procedure and starting from 4-methylsalicylic acid (5.0 g, 33 mmol), 12.4 g (99%) of the title compound was obtained as a pale yellow oil: IR (neat, cm\(^{-1}\)) 2956, 2930, 2886, 2859, 1712, 1611, 1250, 1081, 840; \(^1\)H NMR (CDCl\(_3\)) \(\delta\) 7.65 (d, \(J = 7.9\) Hz, 1H), 6.75 (d, \(J = 8.0\) Hz, 1H), 6.67 (s, 1H), 2.30 (s, 3 H), 1.00 (s, 9H), 0.98 (s, 9H), 0.33 (s, 6H), 0.19 (s, 6H); \(^13\)C NMR (CDCl\(_3\)) \(\delta\) 165.5, 156.5, 144.2, 132.1, 122.8, 122.1, 121.5, 26.3, 26.2, 21.9, 18.9, 18.2, -3.9, -4.3; LRMS \(m/z\) (M + H\(^+\)) calculated for C\(_{20}\)H\(_{36}\)O\(_3\)Si\(_2\) was 381.2271, found 381.1.

4-Chloro-2-[(1,1-dimethylethyl)dimethylsilyloxy]-benzoic acid, (1,1-dimethylethyl)dimethylsilyl ester (JLW-I-258):

Using the previous procedure and starting from 4-chlorosalicylic acid (1.7 g, 10 mmol), 3.6 g (91%) of the title compound was obtained as a pale yellow-brown oil: IR (neat, cm\(^{-1}\)) 841.3, 955.5, 1094.3, 1141.8, 1254.3, 1363.2, 1403.2, 1591.2, 1715.4, 2859.5-2955.4; \(^1\)H NMR (CDCl\(_3\)) \(\delta\) 7.67 (d, \(J = 8.4\) Hz, 1H), 6.93 (dd, \(J = 8.4, 2.0\) Hz, 1H), 6.87 (d, \(J = 2.0\) Hz, 1H), 1.00 (s, 9H), 0.98 (s, 9H), 0.34 (s, 6H), 0.21 (s, 6H); \(^13\)C NMR
(CDCl₃) δ 164.3, 156.8, 138.3, 132.5, 122.6, 121.8, 121.1, 25.7, 25.7, 18.4, 17.8, -4.4, -4.7; HRMS m/z (M + H⁺) calculated for C₁₉H₃₃ClO₃Sia was 401.1735, found 401.1718.

5-Chloro-2-[(1,2-dimethylethyl)dimethylsilyl]oxy]-benzoic acid, (1,1-dimethylethyl)dimethylsilyl ester (JLW-I-222):

Using the previous procedure and starting from 5-chlorosalicylic acid (0.86 g, 5 mmol), 1.79 g (99%) of the title compound was obtained as a colorless oil: IR (neat, cm⁻¹) 2955, 2931, 2886, 2859, 1716: ¹H NMR (CDCl₃) δ 7.65 (d, J = 2.8 Hz, 1H), 7.28 (dd, J = 8.8, 2.8 Hz, 1H), 6.79 (d, J = 8.8 Hz, 1H), 0.98 (s, 9H), 0.98 (s, 9H), 0.33 (s, 6H), 0.18 (s, 6H); ¹³C NMR (CDCl₃) δ 164.0, 154.5, 132.6, 131.1, 125.5, 125.3, 122.9, 25.7, 18.4, 17.8, -4.4, -4.7; HRMS m/z (M + Na⁺) calculated for C₁₉H₃₃ClO₃ was 423.1554, found 423.1528.

5-(tert-Butoxycarbonyl)-amino-2-[(1,1-dimethylethyl)dimethylsilyl]oxy]-benzoic acid, (1,1-dimethylethyl)dimethylsilyl ester (JLW-I-257):

Using the previous procedure and starting from 5-(tert-butoxycarbonyl)amino-2-hydroxy benzoic acid (2.5 g, 10 mmol), 4.5 g (94%) of the title compound was obtained as a pale brown solid: mp 111-113°C; IR (KBr, cm⁻¹) 842.6, 1252.8, 1503.3, 1529.2,
1697.9, 1713.2, 2860.0, 2933.8, 2957.5, 3354.9; \( ^1H \) NMR (CDCl\(_3\)) \( \delta \) 7.51 (d, \( J = 2.5 \) Hz, 2H), 6.79 (d, \( J = 9.4 \) Hz, 1H), 6.40 (s, 1H), 1.48 (s, 9H), 0.97 (s, 9H), 0.97 (s, 9H), 0.32 (s, 6H), 0.15 (s, 6H); \( ^13C \) NMR (CDCl\(_3\)) \( \delta \) 164.4, 152.5, 151.2, 130.9, 123.7, 123.5, 121.7, 121.5, 80.0, 27.9, 25.4, 25.3, 17.9, 17.4, -4.9, -5.1; HRMS \( m/z \) (M + Na\(^+\)) calculated for C\(_{24}\)H\(_{38}\)NO\(_3\)Si\(_2\) was 504.2578, found 504.2589.

![Chemical structure](https://via.placeholder.com/150)

2-[[(1,1-Dimethylethyl)dimethylsilyl]oxy]-5-phenylbenzoic acid, (1,1-dimethyl-ethyldimethylsilyl]ester (JLW-I-256):

Using the previous procedure and starting from 2-hydroxy-5-phenylbenzoic acid (0.96 g, 4.5 mmol), 1.7 g (88%) of the title compound was obtained as a white solid: mp 88-90°C; IR (KBr, cm\(^{-1}\)) 845.6, 928.1, 1085.3, 1231.3, 1272.5, 1482.0, 1709.0, 2856.8, 2928.6, 2953.8; \( ^1H \) NMR (CDCl\(_3\)) \( \delta \) 8.01 (d, \( J = 2.5 \) Hz, 1H), 7.60 (dd, \( J = 8.6, 2.5 \) Hz, 2H), 7.56 (s, 1H), 7.46 (t, \( J = 7.7 \) Hz, 2H), 7.37-7.35 (m, 1H), 6.98 (d, \( J = 8.5 \) Hz, 1H), 1.05 (s, 9H), 1.04 (s, 9H), 0.40 (s, 6H), 0.27 (s, 6H); \( ^13C \) NMR (CDCl\(_3\)) \( \delta \) 165.3, 155.3, 140.1, 133.7, 131.3, 130.0, 128.8, 127.0, 126.6, 124.4, 121.9, 25.8, 25.8, 18.4, 17.8, -4.3, -4.6; HRMS \( m/z \) (M + Na\(^+\)) calculated for C\(_{25}\)H\(_{38}\)O\(_3\)Si\(_2\) was 465.2257, found 465.2266.
2-[[[(1,1-Dimethylethyl)dimethylsilyl]oxy]-5-methoxybenzoic acid, (1,1-dimethyl-ethyl)dimethylsilyl ester (JLW-II-061):

Using the previous procedure and starting from 5-methoxsalicylic acid (3.4 g, 20 mmol), 7.7 g (97%) of the title compound was obtained as a yellow oil: IR (neat, cm⁻¹) 2931, 2897, 2859, 1713, 1574, 12779, 1072, 841; ¹H NMR (CDCl₃) δ 7.25 (s, 1H), 6.91 (dd, J = 8.9, 3.2 Hz, 1H), 6.79 (d, J = 8.9 Hz, 1H), 3.76 (s, 3H), 0.98 (s, 18H), 0.33 (s, 6H), 0.15 (s, 6H); ¹³C NMR (CDCl₃) δ 165.0, 153.1, 149.8, 124.2, 122.6, 119.1, 115.6, 55.6, 25.9, 25.8, 18.4, 17.8, -4.4, -4.7; LRMS m/z (M + H⁺) calculated for C₂₀H₃₅O₄Si₂ was 397.2220, found 297.17.

2-[[[(1,1-Dimethylethyl)dimethylsilyl]oxy]-6-methoxybenzoic acid, (1,1-dimethyl-ethyl)dimethylsilyl ester (JLW-II-062):

Using the previous procedure and starting from 6-methoxsalicylic acid, 8.0 g (100%) of the title compound was obtained as a colorless oil: IR (neat, cm⁻¹) 2931, 2887, 2859, 1716, 1595, 840; ¹H NMR (CDCl₃) δ 7.11 (t, J = 8.3 Hz, 1H), 6.48 (d, J = 8.4 Hz, 1H), 6.44 (d, J = 8.3 Hz, 1H), 3.76 (s, 3H), 0.95 (s, 9H), 0.94 (s, 9H), 0.33 (s, 6H), 0.20 (s,
6H); $^{13}$C NMR (CDCl$_3$) δ 166.3, 157.3, 152.8, 129.6, 118.5, 112.0, 103.8, 55.7, 25.7, 25.5, 18.2, 17.8, -4.3, -4.6; LRMS m/z (M + H$^+$) calculated for C$_{20}$H$_{36}$O$_4$Si$_2$ was 396.2142, found 397.20.

8.2.5 General Method for Preparation of Acid Chlorides of 2-[[1,1-Dimethylethyl]dimethylsilyl]oxy]-benzoic acid, ethyl ester, (1,1-dimethylethyl)dimethylsilyl ester derivatives.

Under an argon atmosphere, oxalyl chloride (0.1 mL, 1.2 mmol) was added dropwise to a cold (0°C) solution of 2-[[1,1-Dimethylethyl]dimethylsilyl]oxy]-4-benzoic acid, (1,1-dimethylethyl)dimethylsilyl ester derivative (0.40 g, 1.0 mmol) in CH$_2$Cl$_2$ (4 mL) containing 3 drops of DMF. The resulting solution was stirred at 0°C for 2 h. The reaction was warmed to room temperature and stirred for 17 h to afford a pale yellow solution, followed by concentration in vacuo. The residue from the acid chlorination immediately underwent a Sonogashira coupling according to procedures described.

8.2.6 General Method for Preparation of Alkynone 1-[2-[[1,1-Dimethylethyl]dimethylsilyl]oxy]-phenyl]-2-propyn-1-one derivatives via Sonogashira Coupling.

There were four different procedures used for the one-pot acid chlorination Sonogashira couplings used. Detailed experimental conditions including effects of the scale of the reaction (mmol), the reaction time (h), and the amount of alkyne (equiv) can be seen in Tables 3.2 and 3.3.
**Coupling A:** Et$_3$N (4 mL) was slowly added to the residue from the acid chlorination and argon was bubbled into solution for 5 min. The alkyne, Pd(PPh$_3$)$_2$Cl$_2$ (1 mg/mmol alkyne), and CuI (1 mg/mmol alkyne) were added and argon gas was again bubbled through solution for 5 min. The reaction was stirred at room temperature for specified amount of time. The reaction mixture was concentrated *in vacuo* after adding MeOH (5 mL). The residue was taken up into Et$_2$O, washed with H$_2$O, 10% NH$_4$OH, brine, dried (Na$_2$SO$_4$), and concentrated *in vacuo*. The residue was purified via flash chromatography (silica gel, 4.5:1 hexanes:EtOAc) to yield the desired product.

**Coupling A*:** The same as Procedure A, except deoxygenation was not performed.

**Coupling B:** Et$_3$N (4 mL) was slowly added to the residue from the acid chlorination and argon was bubbled into solution for 5 min. The alkyne and CuI (1 mg/mmol alkyne) were added and argon gas was again bubbled through solution for 5 min, followed by the addition of Pd(PPh$_3$)$_2$Cl$_2$ (1 mg/mmol alkyne) and deoxygenation. The reaction was stirred at room temperature for specified amount of time. The reaction mixture was concentrated *in vacuo* after adding MeOH (5 mL). The residue was taken up into Et$_2$O, washed with H$_2$O, 10% NH$_4$OH, brine, dried (Na$_2$SO$_4$), and concentrated *in vacuo*. The residue was purified via flash chromatography (silica gel, 4.5:1 hexanes:EtOAc) to yield the desired product.
**Coupling C:** A solution of alkyne and CuI (1 mg/mmol alkyne) in Et$_3$N (4 mL) under argon was stirred at room temperature for 15 min. This solution was cannulated into the concentrated residue from the acid chlorination, treated with Pd(PPh$_3$)$_2$Cl$_2$ (1 mg/mmol alkyne) and any residual CuI, and argon was bubbled through the solution for 15 min. Reaction progress was followed closely by TLC (4.5:1 hexanes:EtOAc). The reaction was stirred at room temperature for a specified amount of time and concentrated \textit{in vacuo} after addition of MeOH (5 mL). The residue was either adhered to silica gel and purified via flash chromatography (silica gel, 4.5:1 hexanes:EtOAc) to yield the desired product or dissolved in EtOAc, filtered through a pad of celite, concentrated, and used in the subsequent cyclization step without further purification.

**Coupling D:** Et$_3$N (4 mL) was slowly added to the residue from the acid chlorination and argon was bubbled into solution for 5 min. CuI (1 mg/mmol alkyne) Pd(PPh$_3$)$_2$Cl$_2$ (1 mg/mmol alkyne) were added and argon gas was again bubbled through solution for 5 min. Alkyne (1.2 equiv) was added to reaction mixture followed by another 1.2 equiv of alkyne 4 h later. The reaction was stirred at room temperature for a specified time. The reaction mixture was concentrated \textit{in vacuo} after adding MeOH (5 mL). The residue was taken up into Et$_2$O, washed with H$_2$O, 10% NH$_4$OH, brine, dried (Na$_2$SO$_4$), and concentrated \textit{in vacuo}. The residue was purified via flash chromatography (silica gel, 4.5:1 hexanes:EtOAc) to yield the desired product.

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1-[2-[[1,1-Dimethylethyl]dimethylsilyloxy]-3-methoxyphenyl]-3-phenyl-2-propyn-1-one (JLW-I-169):

2-[[1,1-Dimethylethyl]dimethylsilyloxy]-4-methoxy-benzoic acid, (1,1-dimethylethyl)dimethylsilyl ester (0.40 g, 1.0 mmol) was used for the previously described one-pot acid chlorination Sonogashira coupling with phenylacetylene (0.49 mL, 4 mmol) according to Coupling A to yield 0.26 g (70%) of the title compound as a yellow-orange oil: IR (CCl₄, cm⁻¹) 3065, 2931, 2895, 2857, 2203, 1647, 1297, 1259, 1021; ^1H NMR (CDCl₃) δ 7.60-7.56 (m, 2H), 7.51 (dd, J = 7.0, 2.6 Hz, 1H), 7.40-7.33 (m, 3H), 7.10-6.96 (m, 2H), 3.78 (s, 3H), 0.97 (s, 9H), 0.17 (s, 6H); ^13C NMR (CDCl₃) δ 176.9, 151.4, 133.1, 133.0, 130.4, 128.8, 128.5, 123.3, 120.7, 120.7, 115.8, 98.8, 55.3, 25.8, 18.9, -4.0.

1-[2-[[1,1-Dimethylethyl]dimethylsilyloxy]-3-methylphenyl]-3-phenyl-2-propyn-1-one (JLW-I-170):

2-[[1,1-Dimethylethyl]dimethylsilyloxy]-4-methyl-benzoic acid, (1,1-dimethylethyl)dimethylsilyl ester (0.38 g, 1.0 mmol) was used for the previously described one-pot acid chlorination Sonogashira coupling with phenylacetylene (0.49 mL, 4 mmol) according to Procedure A to yield 0.30 g (88%) of the title compound as a yellow-
orange oil: IR (CCl₄, cm⁻¹) 2954, 2930, 2858, 2204, 1645, 1586, 1261; ¹H NMR (CDCl₃) δ 7.64 (dd, J = 7.8, 1.8 Hz, 1H), 7.45-7.41 (m, 2H), 7.40-7.14 (m, 4H), 6.81 (t, J = 7.8 Hz, 1H), 2.09 (s, 3H), 0.86 (s, 9H), -0.03 (s, 6H); ¹³C NMR (CDCl₃) 177.5, 152.2, 135.0, 132.0, 131.4, 130.2, 129.5, 129.5, 128.8, 127.5, 127.4, 120.1, 119.4, 90.4, 87.9, 24.9, 17.5, 16.2, -4.6; HRMS m/z (M⁺) calculated for C₂₂H₂₆O₃Si was 350.1695, found 350.1672.

1-[2-[[[(1,1-Dimethylethyl)dimethylsilyl]oxy]-4-methoxyphenyl]-3-phenyl-2-propyn-1-one (JLW-I-111):

2-[[[1,1-Dimethylethyl)dimethylsilyl]oxy]-4-methoxy-benzoic acid, (1,1-dimethyl-ethy1)dimethylsilyl ester (0.41 g, 1.0 mmol) was used for the previously described one-pot acid chlorination Sonogashira coupling with phenylacetylene (0.64 mL, 5 mmol) according to Coupling A to yield 0.35 g (92%) of the title compound as a brown oil: IR (CCl₄, cm⁻¹) 3063, 2853, 2197, 1638, 1603, 1272, 841; ¹H NMR (CDCl₃) δ 8.05 (d, J = 8.8 Hz, 1H), 7.56-7.53 (m, 2H), 7.32-7.29 (m, 3H), 6.57 (dd, J = 8.8, 2.3 Hz, 1H), 1.27 (d, J = 2.4 Hz, 1H), 3.75 (s, 3H), 1.00 (s, 9H), 0.23 (s, 6H); ¹³C NMR (CDCl₃) δ 175.7, 164.7, 158.2, 135.2, 132.8, 130.1, 128.5, 122.9, 120.9, 107.0, 107.0, 90.5, 88.7, 55.5, 25.9, 18.5, -4.2; HRMS m/z (M⁺) calculated for C₂₂H₂₆O₃Si was 366.1644, found 366.1674.
1-[2-[[1,1-Dimethylethyl]dimethylsilyl]oxy]-4-methoxyphenyl]-3-(4-methylphenyl)-2-propyn-1-one (JLW-I-114):

2-[[1,1-Dimethylethyl]dimethylsilyl]oxy]-4-methoxybenzoic acid, (1,1-dimethyl-ethyl)dimethylsilyl ester (0.60 g, 1.5 mmol) was used for the previously described one-pot acid chlorination Sonogashira coupling with 4-ethynyltoluene (0.95 mL, 7.5 mmol) according to Coupling A to yield 0.40 g (70%) of the title compound as a orange-brown oil: IR (CCl₄, cm⁻¹) 3014, 2859, 2195, 1631, 1604; ¹H NMR (CDCl₃) δ 7.92 (d, J = 8.8 Hz, 1H), 7.33 (d, J = 8.1 Hz, 2H), 6.99 (d, J = 8.0 Hz, 2H), 6.44 (dd, J = 8.8, 2.2 Hz, 1H), 6.24 (d, J = 2.2 Hz, 1H), 3.64 (s, 3H), 2.17 (s, 3H), 0.89 (s, 9H), 0.10 (s, 6H); ¹³C NMR (CDCl₃) δ 175.4, 164.4, 157.8, 140.5, 134.9, 132.7, 132.5, 129.3, 129.1, 122.6, 117.4, 106.8, 106.7, 90.8, 88.2, 55.1, 25.6, 21.3, 18.2, -4.5; HRMS m/z (M⁺) calculated for C₂₃H₂₈O₃Si was 380.1828, found 380.1806.

3-(1-Cyclohexen-1-yl)-1-[2-[[1,1-dimethylethyl]dimethylsilyl]oxy]-4-methoxyphenyl]-2-propyn-1-one (JLW-I-116):

2-[[1,1-Dimethylethyl]dimethylsilyl]oxy]-4-methoxybenzoic acid, (1,1-dimethyl-ethyl)dimethylsilyl ester (0.42 g, 1.0 mmol) was used for the previously described one-
pot acid chlorination Sonogashira coupling with 1-ethynyl-1-cyclohexene (0.76 mL, 6.5 mmol) according to Coupling A to yield 0.39 g (65%) of the title compound as a yellow-orange oil: $^1$H NMR (CDCl$_3$) $\delta$ 7.90 (d, $J = 8.8$ Hz, 1H), 6.48 (dd, $J = 8.8$, 2.2 Hz, 1H), 6.35 (m, 1H), 6.29 (d, $J = 2.3$ Hz, 1H), 3.72 (s, 3H), 2.12-2.05 (m, 4H), 1.55-1.51 (m, 4H), 0.94 (s, 9H), 0.15 (s, 6H); $^{13}$C NMR (CDCl$_3$) $\delta$ 175.6, 164.2, 157.6, 140.6, 135.0, 122.6, 119.2, 106.8, 106.6, 92.6, 86.6, 55.1, 28.2, 25.8, 25.6, 21.8, 21.0, 18.2, -4.5; HRMS m/z (M$^+$) calculated for C$_{22}$H$_{30}$O$_3$Si was 370.1956, found 370.1965.

![Chemical Structure](image)

6-Chloro-1-[2-[[1,1-dimethyllethyl]dimethylsilyl]oxy]-4-methoxyphenyl]-2-hexyn-1-one (JLW-I-110):

2-[[1,1-Dimethyllethyl]dimethylsilyl]oxy]-4-methoxy-benzoic acid, (1,1-dimethyl-ethyl)dimethylsilyl ester (0.42g, 1.1 mmol) was used for the previously described one-pot acid chlorination Sonogashira coupling with 5-chloro-1-pentyne (1.1 mL, 10 mmol) according to Coupling A to yield 0.15 g (42%) of the title compound as a yellow-orange oil: IR (CCl$_4$, cm$^{-1}$) 3012, 2960, 2932, 2859, 2220, 1638, 1601, 1258, 840; $^1$H NMR (CDCl$_3$) $\delta$ 7.93 (d, $J = 8.8$ Hz, 1H), 6.53 (dd, $J = 8.8$, 2.4 Hz, 1H), 6.33 (d, $J = 2.2$ Hz, 1H), 3.78 (s, 3H), 3.64 (t, $J = 6.4$, 6.1 Hz, 2H), 2.60 (t, $J = 6.8$ Hz, 2H), 2.07-2.00 (m, 2H), 0.98 (s, 9H), 0.20 (s, 6H); $^{13}$C NMR (CDCl$_3$) $\delta$ 175.5, 164.5, 158.0, 135.2, 122.5, 106.9, 106.9, 91.2, 81.9, 55.4, 43.4, 30.6, 25.8, 18.5, 16.6, -4.3; HRMS m/z (M$^+$) calculated for C$_{19}$H$_{27}$ClO$_3$Si was 366.1411, found 366.1418.

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4,4-Bisethoxy-1-[2-[[1,1-dimethylethyl]dimethylsilyl]oxy]-4-methoxyphenyl]-2-butyn-1-one (JLW-I-115):

2-[[1,1-Dimethylethyl]dimethylsilyl]oxy]-4-methoxy-benzoic acid, (1,1-dimethyl-ethyl)dimethylsilyl ester (0.40 g, 1.0 mmol) was used for the previously described one-pot acid chlorination Sonogashira coupling with propionaldehyde diethyl acetal (0.72 mL, 5 mmol) according to Coupling A to yield 0.33 g (70%) of the title compound as a orange-yellow oil: IR (CCl₄, cm⁻¹) 3019, 2400, 1700, 1604, 1216; ¹H NMR (CDCl₃) δ 7.86 (d, J = 8.9 Hz, 1H), 6.45 (dd, J = 8.8, 2.4 Hz, 1H), 6.24 (d, J = 2.2 Hz, 1H), 5.32 (s, 1H), 3.68 (s, 1H), 3.66-3.49 (m, 4H), 1.12 (t, J = 7.2, 7.0 Hz, 6H), 0.90 (s, 9H), 0.12 (s, 6H); ¹³C NMR (CDCl₃) δ 174.1, 164.7, 158.0, 135.2, 121.8, 106.7, 106.6, 91.1, 84.9, 82.9, 61.1, 55.1, 25.6, 18.2, 14.7, -4.5; HRMS m/z (M⁺) calculated for C₂₁H₃₂O₅Si was 392.2042, found 392.2028.
1-[2-[(1,1-Dimethylethyl)dimethylsilyl]oxy]-4-methoxyphenyl]-2-hexyn-1-one (JLW-II-032):

2-[(1,1-Dimethylethyl)dimethylsilyl]oxy]-4-methoxy-benzoic acid, (1,1-dimethyl-ethyl)dimethylsilyl ester (0.99 g, 2.5 mmol) was used for the previously described one-pot acid chlorination Sonogashira coupling with 1-pentyne (0.75 mL, 7.6 mmol) according to Coupling A to yield 0.71 g (89%) of the title compound as an orange-brown oil: IR (neat, cm⁻¹) 2962, 2858, 2216, 1644, 1603, 1559, 841; ¹H NMR (CDCl₃) δ 7.93 (d, J = 8.8 Hz, 1H), 6.50 (dd, J = 8.8, 2.1 Hz, 1H), 6.30 (d, J = 2.3 Hz, 1H), 3.75 (s, 3H), 2.33 (t, J = 7.0 Hz, 2H), 1.59 (m, 2H), 0.97 (m, 3H), 0.96 (s, 9H), 0.18 (s, 6H); ¹³C NMR (CDCl₃) δ 176.3, 164.8, 158.3, 135.7, 123.1, 107.4, 107.2, 94.1, 81.8, 55.8, 26.2, 21.8, 21.5, 18.9, 14.0, -3.9; LRMS m/z (M + H⁺) calculated for C₉H₂₈O₃Si was 332.1800, found 333.2.

1-[2-[(1,1-Dimethylethyl)dimethylsilyl]oxy]-4-methoxyphenyl]-3-phenyl-2-propyn-1-one (JLW-II-035):

2-[(1,1-Dimethylethyl)dimethylsilyl]oxy]-4-methylbenzoic acid, (1,1-dimethylethyl)-dimethylsilyl ester (1.14 g, 3.0 mmol) was used for the previously described one-pot
acid chlorination Sonogashira coupling with phenylacetylene (1.10 mL, 9.0 mmol) according to **Coupling A** to yield 0.85 g (81%) of the title compound as a orange-brown oil and 44 mg (6%) of 7-methyl-2-phenyl-3(2H)-benzofuranone: IR (neat, cm⁻¹) 2954, 2929, 2858, 2196, 1644, 1608, 841; ¹H NMR (CDCl₃) δ 7.94 (d, J = 8.0 Hz, 1H), 7.60 (m, 2H), 7.37 (m, 3H), 6.86 (d, J = 6.6 Hz, 1H), 6.70 (broad s, 1H), 2.34 (s, 3H), 1.00 (s, 9H), 0.27 (s, 6H); ¹³C NMR (CDCl₃) δ 177.4, 156.4, 146.1, 133.4, 133.3, 130.7, 128.9, 127.3, 122.6, 122.5, 121.1, 91.4, 89.2, 26.3, 22.2, 18.9, -3.7; LRMS m/z (M + H⁺) calculated for C₂₂H₂₆ClO₂Si was 351.1773, found 351.2.

![Molecule structure](image)

3-(1-Cyclohexen-1-yl)-1-[4-chloro-2-[[1,1-dimethylethyl]dimethylsilyl]oxy]-phenyl]-2-propyn-1-one (JLW-I-275):

4-Chloro-2-[[1,1-dimethylethyl]dimethylsilyl]oxy]benzoic acid, (1,1-dimethylethyl)-dimethylsilyl ester (0.40 g, 1.0 mmol) was used for the previously described one-pot acid chlorination Sonogashira coupling with 1-ethynyl-1-cyclohexene (0.35 mL, 3.0 mmol) according to **Coupling B** to yield 0.36 g (96%) of the title compound as a brown oil: ¹H (CDCl₃) δ 7.82 (d, J = 8.4 Hz, 1H), 6.96 (dd, J = 8.5, 1.8 Hz, 1H), 6.83 (d, J = 1.7 Hz, 1H), 6.44 (m, 1H), 2.19-2.05 (m, 4H), 1.60-1.56 (m, 4H), 0.97 (s, 9H), 0.20 (s, 6H).
1-[2-[[1,1-Dimethylethyl]dimethylsilyl]oxy]-5-phenyl-phenyl]-3-phenyl-2-propyn-1-one (JLW-I-261):

2-[[1,1-Dimethylethyl]dimethylsilyl]oxy]-5-phenylbenzoic acid, (1,1-dimethylethyl)-dimethylsilyl ester (0.44 g, 1.0 mmol) was used for the previously described one-pot acid chlorination Sonogashira coupling with phenylacetylene (0.37 mL, 3.0 mmol) according to Coupling A to yield 0.36 g (96%) of the title compound as an oil: $^1$H NMR (CDCl$_3$) $\delta$ 8.25 (d, $J$ = 2.5 Hz, 1H), 7.68 (dd, $J$ = 8.5, 2.5, 1H), 7.64-7.32 (m, 10H), 7.01 (d, $J$ = 8.5 Hz, 1H), 1.05 (s, 9H), 0.30 (s, 6H).

1-[2-[[1,1-Dimethylethyl]dimethylsilyl]oxy]-5-phenyl-phenyl]-3-(4-methylphenyl)-2-propyn-1-one (JLW-I-263):

2-[[1,1-Dimethylethyl]dimethylsilyl]oxy]-5-phenylbenzoic acid, (1,1-dimethylethyl)-dimethylsilyl ester (0.44 g, 1.0 mmol) was used for the previously described one-pot acid chlorination Sonogashira coupling with 4-ethynyltoluene (0.38 mL, 3.0 mmol)
according to Coupling A to yield the title compound as an oil: $^1$H NMR (CDCl$_3$) δ 8.25 (d, $J = 2.4$ Hz, 1H), 7.69-7.18 (m, 10H), 7.01 (d, $J = 8.5$ Hz, 1H), 2.38 (s, 3H), 1.06 (s, 9H), 0.30 (s, 6H).

\[
\text{3-(1-Cyclohexen-1-yl)-1-[(2-[[1,1-dimethylethyl]dimethylsilyl]oxy]-5-phenyl-phenyl]-2-propyn-1-one (JLW-I-262):}
\]

2-[[1,1-Dimethylethyl]dimethylsilyl]oxy]-5-phenylbenzoic acid, (1,1-dimethylethyl)dimethylsilyl ester (0.44 g, 1.0 mmol) was used for the previously described one-pot acid chlorination Sonogashira coupling with 1-ethynyl-1-cyclohexene (0.38 mL, 3.2 mmol) according to Coupling A to yield the title compound as an oil: $^1$H NMR (CDCl$_3$) δ 8.15 (d, $J = 2.5$ Hz, 1H), 7.61 (dd, $J$ 8.5, 2.5 Hz, 1H), 7.58-7.29 (m, 5H), 6.95 (d, $J = 8.5$ Hz, 1H), 6.47 (m, 1H), 2.21-2.15 (m, 4H), 1.64-1.62 (m, 4H), 1.03 (s, 9H), 0.26 (s, 6H).
6-Chloro-1-[2-[[1,1-dimethylethyl]dimethylsilyl]oxy]-5-phenyl-phenyl]-2-hexen-1-one (JLW-I-270):

2-[[1,1-Dimethylethyl]dimethylsilyl]oxy]-5-phenylbenzoic acid, (1,1-dimethylethyl)-dimethylsilyl ester (0.44 g, 1.0 mmol) was used for the previously described one-pot acid chlorination Sonogashira coupling with 5-chloro-1-pentyne (0.31 mL, 3.0 mmol) according to Coupling A to yield 0.30 g (74%) of the title compound as an orange-brown oil: $\text{^1H NMR (CDCl}_3\text{)}$ $\delta$ 8.14 (d, $J = 2.5$ Hz, 1H), 7.63 (dd, $J = 8.5, 2.5$ Hz, 1H), 7.58-7.32 (m, 5H), 6.95 (d, $J = 8.5$ Hz, 1H), 3.68 (t, $J = 6.2$ Hz, 2H), 2.07 (m, 3H), 1.04 (s, 9H), 0.27 (s, 6H).

1-[2-[[1,1-Dimethylethyl]dimethylsilyl]oxy]-5-methoxyphenyl]-3-phenyl-2-propyn-1-one (JLW-2-064):

2-[[1,1-Dimethylethyl]dimethylsilyl]oxy]-5-methoxybenzoic acid, (1,1-dimethylethyl)-dimethylsilyl ester (1.01 g, 2.6 mmol) was used for the previously described one-pot acid chlorination Sonogashira coupling with phenylacetylene (0.95 mL, 7.8 mmol) according to Coupling A to yield 0.70 g (75%) of the title compound as an orange-brown oil: IR (neat, cm$^{-1}$) 2930, 2857, 2199, 1650; $\text{^1H NMR (CDCl}_3\text{)}$ $\delta$ 7.61 (s, 1H), 7.58 (d, $J = 1.6$ Hz, 1H), 7.46 (d, $J = 3.2$ Hz, 1H), 7.43-7.33 (m, 3H), 6.99 (dd, $J = 8.9$, 2H).
3.2 Hz, 1H), 6.83 (d, J = 8.9 Hz, 1H), 3.81 (s, 3H), 0.97 (s, 9H), 0.18 (s, 6H); \(^{13}\text{C NMR (CDCl}_3\) 8 177.4, 153.5, 149.7, 133.0, 130.4, 129.6, 128.6, 122.6, 121.0, 120.6, 115.5, 91.8, 89.0, 55.8, 25.9, 18.4, -4.2; LRMS \text{m/z (M + Na\(^+\)) calculated for C}_{22}\text{H}_{26}\text{O}_3\text{Si was 389.1644, found 389.16.}
8.2.7 **General Method for Preparation of 4H-1-Benzopyran-4-one Derivatives via Cyclization of Alkynone 1-[2-[[1,1-Dimethylethyl]dimethylsilyl]oxy]-phenyl]-2-propyn-1-one Derivatives.**

**Cyclization A:** Neat anhydrous diethylamine (10 equiv) was added to an alkynone 1-[2-[[1,1-dimethylethyl]dimethylsilyl]oxy]-phenyl]-2-propyn-1-one derivatives under argon and stirred at room temperature for 2 h. The reaction mixture was concentrated *in vacuo* to remove excess diethylamine. The residue was purified via flash chromatography (silica gel, 2:1 hexanes:EtOAc) to yield the desired product.

**Cyclization B:** Anhydrous diethylamine (10 equiv) was added to an alkynone 1-[2-[[1,1-dimethylethyl]dimethylsilyl]oxy]-phenyl]-2-propyn-1-one derivatives in EtOH (1 mL) under argon and stirred at room temperature for 2 h. The reaction mixture was concentrated *in vacuo* to remove excess diethylamine. The residue was purified via flash chromatography (silica gel, 2:1 hexanes:EtOAc) to yield the desired product.

**Cyclization C:** Anhydrous diethylamine (10 equiv) was added to an alkynone 1-[2-[[1,1-dimethylethyl]dimethylsilyl]oxy]-phenyl]-2-propyn-1-one derivatives in EtOH (1 mL) under argon and stirred at room temperature for 2 h. The reaction mixture was concentrated *in vacuo* to remove excess diethylamine. EtOH (1 mL) was added to the residue and heated to reflux overnight, followed by concentration *in vacuo*. The residue was purified via flash chromatography (silica gel, 2:1 hexanes:EtOAc) to yield the desired product.
**Cyclization D:** Neat anhydrous pyrrolidine (10 equiv) was added to an alkynone 1-[2-[[([1,1-dimethylethyl]dimethyl)silyl]oxy]-phenyl]-2-propyn-1-one derivatives under argon and stirred at room temperature for 2 h. The reaction mixture was concentrated *in vacuo* to remove excess pyrrolidine. EtOH (1 mL) was added to the residue and heated to reflux overnight, followed by concentration *in vacuo*. The residue was purified via flash chromatography (silica gel, 2:1 hexanes:EtOAc) to yield the desired product.

**Cyclization E:** Neat anhydrous pyrrolidine (10 equiv) was added to an alkynone 1-[2-[[([1,1-dimethylethyl]dimethyl)silyl]oxy]-phenyl]-2-propyn-1-one derivatives under argon and stirred at room temperature for 2 h. The reaction mixture was concentrated *in vacuo* to remove excess pyrrolidine. The residue was purified via flash chromatography (silica gel, 2:1 hexanes:EtOAc) to yield the desired product.

**Cyclization G:** Anhydrous pyrrolidine (10 equiv) was added to an alkynone 1-[2-[[([1,1-dimethylethyl]dimethyl)silyl]oxy]-phenyl]-2-propyn-1-one derivatives in EtOH (1 mL) under argon and stirred at room temperature for 2 h. The reaction mixture was concentrated *in vacuo* to remove excess pyrrolidine. EtOH (1 mL) was added to the residue and heated to reflux overnight, followed by concentration *in vacuo*. The residue was purified via flash chromatography (silica gel, 2:1 hexanes:EtOAc) to yield the desired product.
10: 2-Phenyl-4H-1-benzopyran-4-one (JLW-I-246):

2-[(1,1-Dimethylethyl)dimethylsilyloxy]-benzoic acid, (1,1-dimethylethyl)dimethylsilyl ester (0.39 g, 1.1 mmol) was used for the previously described one-pot acid chlorination Sonogashira coupling with phenylacetylene (0.37 mL, 2.2 mmol) according to Coupling C. The alkynone obtained from the Sonogashira coupling was used in the subsequent cyclization step according to Cyclization C to yield 0.18 g (73%) of the title compound as a off-white solid: mp 93-94°C; IR (KBr, cm⁻¹) 3072, 1654, 1606, 1376; ¹H NMR (CDCl₃) δ 8.21 (dd, J = 7.9, 0.7 Hz, 1H), 7.92-7.88 (m, 2H), 7.68 (t, J = 7.0 Hz, 1H), 7.56-7.49 (m, 4H), 7.39 (t, J = 7.9, 7.1 Hz, 1H), 6.80 (s, 1H); ¹³C NMR (CDCl₃) δ 178.2, 163.2, 156.1, 133.6, 131.7, 131.5, 128.9, 126.2, 125.6, 125.1, 124.0, 118.0, 107.5; UV (EtOH) λmax 251, 294; HRMS m/z (M⁺) calculated for C₁₅H₁₀O₂ was 245.0578, found 245.0589.

11: 2-(4'-Methylphenyl)-4H-1-benzopyran-4-one (JLW-I-247):

2-[(1,1-Dimethylethyl)dimethylsilyloxy]-benzoic acid, (1,1-dimethylethyl)dimethylsilyl ester (0.42 g, 1.1 mmol) was used for the previously described one-pot acid
chlorination Sonogashira coupling with 1-ethynyl-toluene (0.28 mL, 2.2 mmol) according to Coupling C. The alkynone from the Sonogashira coupling was used in the subsequent cyclization step according to Cyclization C to yield 0.13 g (48%) of the title compound as an off white solid: mp 106-107.5°C; IR (KBr, cm⁻¹) 3059, 2919, 1638, 1228, 1043; ¹H NMR (CDCl₃) δ 8.20 (dd, J = 7.9, 1.6 Hz, 1H), 7.79 (d, J = 8.3 Hz, 2H), 7.66 (t, J = 8.5, 7.0 Hz, 1H), 7.52 (dd, J = 8.4, 0.5 Hz, 1H), 7.38 (t, J = 7.9 Hz, 1H), 7.28 (d, J = 8.4 Hz, 2H), 6.76 (s, 1H), 2.40 (s, 3H); ¹³C NMR (CDCl₃) δ 178.3, 163.6, 156.2, 142.2, 133.6, 129.7, 128.9, 126.2, 125.6, 125.1, 123.9, 112.0, 106.9, 21.4; UV (MeOH) λ max 253, 302; HRMS m/z (M⁺) calculated for C₁₆H₁₂O₂ was 236.0834, found 236.0831.

12: 2-(1-Cyclohexen-1-yl)-4H-1-benzopyran-4-one (JLW-I-248):

2-[[[(1,1-Dimethylethyl)dimethylsilyl]oxy]-benzoic acid, (1,1-dimethylethyl)dimethylsilyl ester (0.35 g, 1.0 mmol) was used for the previously described one-pot acid chlorination Sonogashira coupling with 1-ethynyl-cyclohexene (0.23 mL, 1.9 mmol) according to Coupling C. The alkynone obtained from the Sonogashira coupling was used in the subsequent cyclization step according to Cyclization C to yield 0.15 g (71%) of the title compound as an orange oil: IR (KBr, cm⁻¹) 2932, 1645, 1633; ¹H NMR (CDCl₃) δ 8.12 (dd, J = 7.9, 1.7 Hz, 1H), 7.63-7.56 (m, 1H), 7.40 (d, J = 7.8 Hz, 1H),
7.31 (t, J = 8.0 Hz, 1H), 6.96-6.95 (m, 1H), 6.26 (s, 1H), 2.27-2.25 (m, 4H), 1.80-1.62 (m, 4H); 
$^1$C NMR (CDCl$_3$) δ 179.4, 164.1, 156.4, 134.4, 133.9, 130.0, 125.9, 125.1, 124.2, 118.2, 106.5, 26.4, 24.6, 22.6, 21.9; UV (EtOH) λmax 253, 257, 295; HRMS m/z (M + H$^+$) calculated for C$_{13}$H$_{14}$O$_2$ was 227.1072, found 227.1076.

![Structure of 2-(3-Chloropropyl)-4H-1-benzopyran-4-one](image)

13: 2-(3-Chloropropyl)-4H-1-benzopyran-4-one (JLW-I-249):

2-[(1,1-Dimethyl ethyl)dimethylsilyl]oxy]-benzoic acid, (1,1-dimethyl ethyl)dimethylsilyl ester (0.38 g, 1.3 mmol) was used for the previously described one-pot acid chlorination Sonogashira coupling with 5-chloro-1-pentyne (0.22 mL, 2 mmol) according to Coupling C. The alkynone obtained from the Sonogashira coupling was used in the subsequent cyclization step according to Cyclization C to yield 0.12 g (51%) of the title compound as a orange/brown oil: IR (neat, cm$^{-1}$) 3056, 2924, 1644, 1606; $^1$H NMR (CDCl$_3$) δ 8.12 (dd, J = 7.9, 1.7 Hz, 1H), 7.63-7.56 (m, 1H), 7.39-7.30 (m, 2H), 6.15 (s, 1H), 3.58 (t, J = 6.3 Hz, 2H), 2.78 (t, J = 7.8 Hz, 2H), 2.18 (q, J = 7.8, 6.2 Hz, 2H); $^{13}$C NMR (CDCl$_3$) δ 178.0, 167.6, 156.4, 133.5, 125.6, 125.0, 123.6, 117.7, 110.3, 43.5, 31.4, 29.4; UV (EtOH) λmax 224, 295; HRMS m/z (M$^+$) calculated for C$_{12}$H$_{11}$ClO$_2$ was 222.0445, found 222.0442 (M$^+$, 100%), 224.0417 (M$^+$, 34%).
14: 2-(Diethoxymethyl)-4H-1-benzopyran-4-one (JLW-I-250):

2-[[[(1,1-dimethylethyl)dimethylsilyl]oxy]-benzoic acid, (1,1-dimethylethyl)dimethylsilyl ester (0.39 g, 1.1 mmol) was used for the previously described one-pot acid chlorination Sonogashira coupling with propiolaldehyde diethyl acetal (0.47 mL, 3 mmol) according to Coupling C. The alkynone obtained from the Sonogashira coupling was used in the subsequent cyclization step according to Cyclization C to yield 54 mg (21%) of the title compound as a dark orange oil: IR (KBr, cm$^{-1}$) 3083, 2983, 2946, 2878, 1738, 1664, 1614, 1471, 1323, 1118, 1069, 790, 759 [105]; $^1$H NMR (CDCl$_3$) $\delta$ 8.17 (dd, $J$ = 8.0, 1.6 Hz, 1H), 7.66 (t of d, $J$ = 7.8, 1.7 Hz, 1H), 7.49 (d, $J$ = 8.4 Hz, 1H), 7.38 (t, $J$ = 8.0 Hz, 1H), 6.59 (s, 1H), 5.31 (s, 1H), 3.74-3.61 (m, 4H), 1.26 (t, $J$ = 7.0 Hz, 6H); $^{13}$C NMR (CDCl$_3$) $\delta$ 178.7, 163.7, 156.7, 134.2, 126.1, 125.6, 124.5, 118.7, 110.4, 98.1, 62.6, 15.5; UV (EtOH) $\lambda_{max}$ 298; HRMS $m/z$ (M + Na$^+$) calculated for C$_{14}$H$_{16}$O$_4$ was 271.0946, found 271.0943.
15: 3-Bromo-2-phenyl-4H-1-benzopyran-4-one (JLW-I-189):

Anhydrous pyridine (1 mL, 12.5 mmol) was added dropwise to a solution of 2-phenyl-4H-1-benzopyran-4-one (0.11 g, 0.5 mmol) in CH$_2$Cl$_2$ (5 mL). Upon addition of pyridinium tribromide (0.81 g, 2.5 mmol), the reaction mixture turned a dark brown color. The reaction mixture was stirred at room temperature for 22 h at which time saw little starting material by TLC (2:1 hexanes:EtOAc). Reaction diluted with CH$_2$Cl$_2$ and washed with several portions of 10% Na$_2$S$_2$O$_3$. The organic layer was dried (MgSO$_4$) and concentrated in vacuo. Because there was not good separation of the aqueous and organic layers, the aqueous layer was extracted with EtOAc. The organic layer was then washed with CuSO$_4$, brine, dried (MgSO$_4$), and concentrated in vacuo to afford an orange solid. The solid was purified via flash chromatography (silica gel, 2:1 hexanes:EtOAc) to yield 0.11 g (75%) of the title compound as a white solid: mp 122-123°C; IR (KBr, cm$^{-1}$) 3104, 3064, 1656, 1615, 1067; $^1$H NMR (CDCl$_3$) δ 8.29 (dd, J = 8.0, 1.3 Hz, 1H), 7.85-7.83 (m, 2H), 7.74-7.67 (m, 1H), 7.56-7.42 (m, 5H); $^{13}$C NMR (CDCl$_3$) 173.1, 162.0, 155.7, 134.2, 132.9, 131.1, 129.3, 128.3, 126.6, 125.7, 121.8, 117.9, 109.3; UV (EtOH) λ$_{max}$ 248, 309; HRMS m/z (M$^+$) calculated for C$_{13}$H$_9$BrO$_2$ was 299.9783, found 299.9784 (M$^+$, 100%), 301.9793 (M$^{+2}$, 93%).
20: 8-Methoxy-2-phenyl-4H-1-benzopyran-4-one (JLW-I-171):

Using the previously described **Cyclization C** procedure and starting from 0.14 g (0.4 mmol) of 1-[2-[[1,1-dimethylethyl]dimethylsilyl]oxy]-3-methoxyphenyl]-3-phenyl-2-propyn-1-one, 86 mg (88%) of the title compound was obtained as a white solid: mp 198-198.5°C; IR (KBr, cm⁻¹) 3066, 3009, 2975, 2848, 1641, 1603, 1281, 1061; ¹H NMR (CDCl₃) δ 7.87-7.85 (m, 2H), 7.67 (d, J = 8.0 Hz, 1H), 7.42-7.40 (m, 3H), 7.21 (t, J = 7.9, 7.3 Hz, 1H), 7.07 (d, J = 7.9 Hz, 1H), 6.73 (s, 1H), 3.91 (s, 3H); ¹³C NMR (CDCl₃) 177.4, 161.9, 148.0, 145.5, 130.7, 130.5, 128.0, 125.3, 123.9, 123.8, 115.3, 113.3, 106.2, 55.3; UV (EtOH) λmax 265; HRMS m/z (M⁺) calculated for C₁₆H₁₂O₃ was 252.0783, found 252.0791.

2-[[1,1-Dimethylethyl]dimethylsilyl]oxy]-3-methoxybenzoic acid, (1,1-dimethylethyl)dimethylsilyl ester (0.41 g, 1 mmol) was used for the previously described one-pot acid chlorination Sonogashira coupling with phenylacetylene (0.49 mL, 4 mmol) according to **Coupling C**. The alkynone obtained from the Sonogashira coupling was used in the subsequent cyclization step according to **Cyclization C** to yield 0.23 g (89%) of the title compound as a solid, which was further recrystallized in EtOH to afford pale yellow crystals: mp 198-198.5°C.
21: 8-Methoxy-2-(4’-methylphenyl)-4H-1-benzopyran-4-one (JLW-I-229):

2-[[[(1,1-Dimethylethyl)dimethylsilyloxy]-3-methoxybenzoic acid, (1,1-dimethylethyl)-dimethylsilyl ester (0.41 g, 1 mmol) was used for the previously described one-pot acid chlorination Sonogashira coupling with 4-ethynyltoluene (0.66 mL, 5.2 mmol) according to Coupling C. The alkynone obtained from the Sonogashira coupling was used in the subsequent cyclization step according to Cyclization C to yield 0.24 g (88%) of the title compound as a solid which was further recrystallized (1:1 hexanes:EtOAc) to afford pale yellow crystals: mp 229.5-230°C; IR (KBr, cm⁻¹) 3053, 2698, 2844, 1637, 1377, 1282, 1062; ^1H NMR (CDCl₃) δ 7.85 (dd, J = 6.8, 1.8 Hz, 2H), 7.76 (dd, J = 8.0, 1.8 Hz, 1H), 7.33-7.27 (m, 3H), 7.17 (dd, J = 8.0, 1.4 Hz, 1 H), 6.79 (s, 1H), 4.00 (s, 3H), 2.42 (s, 3H); ^13C NMR (CDCl₃) δ 178.4, 163.2, 149.1, 146.7, 142.1, 129.7, 129.1, 126.3, 125.1, 124.7, 116.5, 114.4, 106.8, 56.4, 21.5; UV (EtOH) λmax 266, 304; HRMS m/z (M + H⁺) calculated for C₁₇H₁₄O₃ was 267.1021, found 267.1026.
22: 2-(1-Cyclohexen-1-yl)-8-methoxy-4H-1-benzopyran-4-one (JLW-I-225):

2-[[1,1-Dimethylethyl]dimethylsilyloxy]-3-methoxybenzoic acid, (1,1-dimethylethyl)dimethylsilyl ester (0.42 g, 1.1 mmol) was used for the previously described one-pot acid chlorination Sonogashira coupling with 1-ethynyl-cyclohexene (0.50 mL, 4.2 mmol) according to Coupling C. to yield 0.26 g (68%) of 3-(1-cyclohexen-1-yl)-1-[2-[[1,1-dimethylethyl]dimethylsilyloxy]-3-methoxy-phenyl]-2-propyn-1-one. The alky none obtained from the Sonogashira coupling was used in the subsequent cyclization step according to Cyclization C to yield 0.12 g (44% overall yield, 65% yield for cyclization) of the title compound as off-white rod-like crystals: mp 137.5-139°C; IR (KBr, cm⁻¹) 3052, 2940, 1643, 1594, 1222, 1059; ¹H NMR (CDCl₃) δ 7.70 (dd, J = 8.0, 1.5 Hz, 1H), 7.24 (t, J = 8.0 Hz, 1H), 7.12 (dd, J = 8.0, 1.4 Hz, 1H), 7.04 (m, 1H), 6.28 (s, 1H), 3.96 (s, 3H), 2.31-2.27 (m, 4H), 1.78-1.66 (m, 4H); ¹³C NMR (CDCl₃) δ 179.4, 163.8, 149.3, 146.8, 134.6, 130.1, 125.2, 124.7, 116.8, 114.7, 106.5, 56.8, 26.4, 24.5, 22.6, 22.0; UV (EtOH) λmax 265; HRMS m/z (M⁺ + H) calculated for C₁₆H₁₆O₃ was 257.1177, found 257.1171.
23: 2-(3-Chloropropyl)-8-methoxy-4H-1-benzopyran-4-one (JLW-I-253):

2-[[[(1,1-Dimethylethyl)dimethylsilyl]oxy]-3-methoxybenzoic acid, (1,1-dimethylethyl)-
dimethylsilyl ester (0.31 g, 0.8 mmol) was used for the previously described one-pot acid chlorination Sonogashira coupling with 5-chloro-1-pentyne (0.24 mL, 2.3 mmol) according to Coupling C. The alkynone obtained from the Sonogashira coupling was used in the subsequent cyclization step according to Cyclization C to yield 43 mg (22%) of the title compound as a off-white solid: mp 82°C; IR (KBr, cm⁻¹) 2970, 2928, 2846, 1664, 1583, 1273, 1057; ¹H NMR (CDCl₃) δ 7.74 (dd, J = 8.0, 1.4 Hz, 1H), 7.30 (t, J =
8.0 Hz, 1H), 7.16 (dd, J = 8.0, 1.3 Hz, 1H), 6.27 (s, 1H), 3.97 (s, 3H), 3.65 (t, J = 6.3 Hz, 2H), 2.87 (t, J = 7.7 Hz, 2H), 2.24 (q, J = 7.7, 6.4 Hz, 2H); ¹³C NMR (CDCl₃)
δ 178.1, 167.5, 148.6, 146.8, 124.7, 116.5, 114.2, 110.3, 56.2, 43.6, 31.4, 29.5; UV
EtOH) λmax 230, 308; HRMS m/z (M + H⁺) calculated for C₁₃H₁₃ClO₃ was 253.0631,
found 253.0634 (M + H⁺, 100%), 255.0650 (M⁺² + H⁺, 29.2%).
24: 2-(Diethoxymethyl)-8-methoxy-4H-1-benzopyran-4-one (JLW-I-230):

2-[[1,1-Dimethylethyl]dimethylsilyl]oxy]-3-methoxybenzoic acid, (1,1-dimethylethyl)-dimethylsilyl ester (0.41 g, 1.2 mmol) was used for the previously described one-pot acid chlorination Sonogashira coupling with propiolardehydediethylacetel (0.73 mL, 5.1 mmol) according to Coupling C. The alkynone obtained from the Sonogashira coupling was used in the subsequent cyclization step according to Cyclization C to yield 0.15 g (53%) of the title compound as a solid which was further recrystallized (1:1 hexanes:EtOAc): mp 64.5-65.5°C; IR (KBr, cm⁻¹) 2977, 2935, 2899, 1650, 1489, 1330, 1275, 1123, 1106, 1059; ¹H NMR (CDCl₃) δ 7.65 (dd, J = 8.0, 1.5 Hz, 1H), 7.23 (t, J = 8.0 Hz, 1H), 7.08 (dd, J = 8.0, 1.4 Hz, 1H), 6.54 (s, 1H), 5.30 (s, 1H), 3.89 (s, 3H), 3.65 (m, 4.5H), 1.19 (t, J = 7.0 Hz, 6.7 H); ¹³C NMR (CDCl₃) δ 178.3, 163.4, 149.0, 146.7, 125.2, 124.8, 116.5, 114.4, 109.8, 97.7, 62.4, 56.3, 25.7, 15.0; UV (MeOH) λmax 229, 252, 311; HRMS m/z (M + Na⁺) calculated for C₁₅H₁₇O₅ was 301.1052, found 301.1050.
Using the same procedure described for 3-bromo-2-phenyl-4H-1-benzopyran-4-one 15, the title compound was obtained from 80 mg (0.3 mmol) of 8-methoxy-2-(4'-methylphenyl)-4H-1-benzopyran-4-one to yield 92 mg (89\%) as a white solid: mp 170.5-171.5°C; IR (KBr, cm$^{-1}$) 1656, 1643, 1275, 1075, 754; $^1$H NMR (CDCl$_3$) $\delta$ 7.81 (dd, $J$ = 8.0, 1.4 Hz, 1H), 7.82 (s, 1H), 7.79 (s, 1H), 7.38-7.31 (m, 3H), 7.18 (dd, $J$ = 8.0, 1.2 Hz, 1H), 3.95 (s, 3H), 2.44 (s, 3H); $^{13}$C NMR (CDCl$_3$) $\delta$ 173.2, 161.8, 148.7, 146.1, 141.7, 130.0, 129.5, 125.4, 122.8, 117.1, 114.5, 109.0, 56.3, 21.6; UV (EtOH) $\lambda_{max}$ 262, 307; HRMS $m/z$ (M + Na$^+$) calculated for C$_{17}$H$_{13}$BrO$_3$ was 366.9946, found 366.9950 (M + Na$^+$, 100\%), 368.9943 (M$^+$ + Na$^+$, 95\%).
30: 8-Hydroxy-2-phenyl-4H-1-benzopyran-4-one (JLW-I-176):

To a solution of 8-methoxy-2-phenyl-4H-1-benzopyran-4-one (0.12 mmol) in CH₂Cl₂ (1.5 mL) at -78°C under argon was added 1.0 M BBr₃ solution in CH₂Cl₂ (0.29 mL, 2.5 equiv) dropwise. The reaction was allowed to stir at -78°C for 2 h, then allowed to warm to room temperature and stirred overnight. The mixture was cooled to 0°C and a small amount of MeOH was added and concentrated *in vacuo*. The residue was taken up into EtOAc, washed with H₂O, dried (Na₂SO₄), and concentrated *in vacuo*. The residue was purified via flash chromatography (silica gel, 1:1 hexanes:EtOAc) followed by recrystallization in EtOH to yield 17 mg (60%) of the title compound as white crystals: mp 243-244°C; IR (KBr, cm⁻¹) 2962, 2637, 1631, 1297, 1048; ¹H NMR (CDCl₃) δ 10.49 (s, 1H), 8.11-8.07 (m, 2H), 7.59-7.57 (m, 3H), 7.45 (m, 1H), 7.29-7.27 (m, 2H), 6.98 (s, 1H); ¹³C NMR (DMSO-ｄ₆) δ 177.3, 162.1, 147.0, 145.4, 131.7, 131.3, 129.1, 126.4, 125.3, 124.7, 119.4, 114.2, 106.6; UV (MeOH) λmax 266; HRMS m/z (M⁺ + Na⁺) calculated for C₁₅H₁₀O₃ was 261.0522, found 261.0501.
31: 8-Hydroxy-2-(4’-methylphenyl)-4H-1-benzopyran-4-one (JLW-II-050):

To a solution of NaH (3.1 equiv of a 60% w/w dispersion) in 2.3 mL DMF at 0°C was added dropwise ethanethiol (3.0 equiv) to generate a 0.5 M NaSeEt solution in DMF. After 30 min, the mixture was warmed to rt cannulated dropwise into a solution of 8-methoxy-2-(4’-methylphenyl)-4H-1-benzopyran-4-one (0.10 g, 0.4 mmol) in DMF (2 mL). The reaction mixture was heated to reflux for 4 h; then cooled in a ice-bath and acidified with dilute (< 1M)HCl to pH 3. The reaction mixture was extracted with EtOAc, washed with H2O, brine, and dried (Na2SO4), and concentrated in vacuo. The aqueous layer was reextracted with CHCl3 and monitored by TLC. The residue was purified via flash chromatography (silica gel, CHCl3/5% MeOH) followed by recrystallization in MeOH to yield 60 mg (62%) of the title compound as a white solid: mp 263-264°C; IR (KBr, cm⁻¹) 3047, 1626, 1249, 1044, 805; ¹H NMR (CDCl3) δ 10.66-10.16 (broad s, 1H), 8.00 (s, 1H), 7.98 (s, 1H), 7.42 (dd, J = 5.8, 3.5 Hz, 1H), 7.36 (s, 1H), 7.34 (s, 1H), 7.24-7.23 (m, 2H), 6.93 (s, 1H), 2.36 (s, 3H); ¹³C NMR (CDCl3) δ 177.2, 162.2, 146.8, 145.3, 141.9, 129.7, 128.5, 127.0, 126.3, 125.2, 124.7, 119.3, 114.2, 106.0, 21.1; UV (EtOH) λmax 268, 306; HRMS m/z (M + Na⁺) calculated for C16H12O3 was 275.0679, found 275.0670.
40: 8-Methyl-2-phenyl-4H-1-benzopyran-4-one (JLW-I-172):

Using the previously described Cyclization C procedure and starting from 0.21 g (0.61 mmol) of 1-[2-[(1,1-dimethylethyl)dimethylsilyl]oxy]-3-methylphenyl]-3-phenyl-2-propyn-1-one, 125.7 mg (87%) of the title compound as a white solid: mp 157-159°C; IR (KBr, cm⁻¹) 3069, 1638, 1600, 1375; ¹H NMR (CDCl₃) δ 7.87 (d, J = 7.8 Hz, 1H), 7.73-7.69 (m, 2H), 7.35-7.30 (m, 4H), 7.09 (t, J = 7.6 Hz, 1H), 6.61 (s, 1H), 2.39 (s, 3H); ¹³C NMR (CDCl₃) 178.3, 162.4, 154.3, 134.4, 131.7, 131.3, 128.8, 127.2, 125.8, 124.4, 123.6, 123.0, 106.9, 15.5; UV (EtOH) λ max 258, 295; HRMS m/z (M⁺) calculated for C₁₆H₁₂O₂ was 236.0834, found 236.0833.

41: 8-Methyl-2-(4'-methylphenyl)-4H-1-benzopyran-4-one (JLW-I-232):

2-[(1,1-Dimethylethyl)dimethylsilyl]oxy]-3-methylbenzoic acid, (1,1-dimethylethyl)-dimethylsilyl ester (0.39 g, 1.0 mmol) was used for the previously described one-pot acid chlorination Sonogashira coupling with 4-ethynyltoluene (0.66 mL, 5.2 mmol) according to Coupling C. The alkynone obtained from the Sonogashira coupling was used without purification in the subsequent cyclization step according to Cyclization C to yield 0.23 g (91%) of the title compound as a solid which was further recrystallized
(1:1 hexanes:EtOAc) to afford very pale orange plate-like crystals: mp 147.5-148.5°C; IR (KBr, cm⁻¹) 3071, 2975, 2925, 1636, 1481, 1371, 1219, 1073; ¹H NMR (CDCl₃) δ 8.06 (d, J = 7.9 Hz, 1H), 7.76 (d, J = 8.2 Hz, 2H), 7.50 (d, J = 7.3 Hz, 1H), 7.30-7.27 (m, 3H), 6.75 (s, 1H), 2.57 (s, 3H), 2.41 (s, 3H); ¹³C NMR (CDCl₃) δ 178.3, 162.6, 154.2, 141.9, 134.2, 129.5, 128.8, 127.2, 125.7, 124.3, 123.5, 122.9, 106.2, 21.2, 15.5; UV (EtOH) 258, 302; HRMS m/z (M⁺) calculated for C₁₇H₁₄O₂ was 250.0990, found 250.0995.

42: 2-(1-Cyclohexen-1-yl)-8-methyl-4H-1-benzopyran-4-one (JLW-I-233):
2-[[1,1-Dimethylethyl]dimethylsilyl]oxy]-3-methylbenzoic acid, (1,1-dimethylethyl)-dimethylsilyl ester (0.39 g, 1.0 mmol) was used for the previously described one-pot acid chlorination Sonogashira coupling with 1-ethynylcyclohexene (0.48 mL, 4.1 mmol) according to Coupling C. The alkynone obtained from the Sonogashira coupling was used without purification in the subsequent cyclization step according to Cyclization C to yield 0.20 g (83%) of the title compound as a yellow solid which was further recrystallized in EtOH: mp 82-84°C; IR (KBr, cm⁻¹) 2931, 2868, 1652, 1595, 1585, 1218, 1021; ¹H NMR (CDCl₃) δ 7.98 (dd, J = 7.4, 0.4 Hz, 1H), 7.44 (dd, J = 6.4, 0.8 Hz, 1H), 7.21 (t, J = 7.6 Hz, 1H), 6.95 (s, 1H), 6.27 (s, 1H), 2.48 (s, 3H), 2.30 (s, 4H), 1.78-1.65 (m, 4H); ¹³C NMR (CDCl₃) δ 179.2, 163.3, 154.4, 134.4, 133.5, 130.0, 199
127.2, 124.2, 123.7, 123.1, 105.9, 26.0, 24.2, 22.2, 21.6, 15.7; UV (MeOH) $\lambda_{\text{max}}$ 258, 295; HRMS $m/z$ ($M^+$) calculated for $C_{16}H_{16}O_2$ was 240.1146, found 240.1149.

43: 2-(3-Chloropropyl)-8-methyl-4H-1-benzopyran-4-one (JLW-I-251):

![Chemical Structure](image)

2-[[[1,1-Dimethylethyl)dimethylsilyloxy]-3-methylbenzoic acid, (1,1-dimethylethyl)dimethylsilyl ester (0.37 g, 0.9 mmol) was used for the previously described one-pot acid chlorination Sonogashira coupling with 5-chloro-1-pentyne (0.29 mL, 3 mmol) according to Coupling C. The alkynone obtained from the Sonogashira coupling was used without purification in the subsequent cyclization step according to Cyclization C to yield 0.09 g (40%) of the title compound as a tan solid: mp 53-54°C; IR (KBr, cm$^{-1}$) 2941, 2928, 1651, 1213, 1070; $^1$H NMR (CDCl$_3$) $\delta$ 7.97 (dd, $J = 7.9$, 1.1 Hz, 1H), 7.44 (dd, $J = 7.3$, 0.8 Hz, 1H), 7.22 (t, $J = 7.6$ Hz, 1H), 6.16 (s, 1H), 3.61 (t, $J = 6.3$ Hz, 2H), 2.81 (t, $J = 7.1$ Hz, 2H), 2.43 (s, 3H), 2.20 (q, 2H); $^{13}$C NMR (CDCl$_3$) $\delta$ 178.4, 167.2, 154.8, 134.4, 127.1, 124.5, 123.5, 123.2, 110.1, 43.5, 31.4, 29.6, 15.5; UV (EtOH) $\lambda_{\text{max}}$ 225, 300; HRMS $m/z$ ($M^++H^+$) calculated for $C_{13}H_{13}ClO_2$ was 237.0682, found 237.0677 ($M^++H^+$, 100%), 239.0667 ($M^{12}^++H^+$, 33%).

200
44: 2-(Diethoxymethyl)-8-methyl-4H-1-benzopyran-4-one (JLW-I-234):
2-[[1,1-Dimethylethyl]dimethylsilyloxy]-3-methylbenzoic acid, (1,1-dimethylethyl)dimethylsilyl ester (0.23 g, 0.8 mmol) was used for the previously described one-pot acid chlorination Sonogashira coupling with propiolaldehyde diethyl acetal (0.57 mL, 4 mmol) according to Coupling C. The alkynone obtained from the Sonogashira coupling was used without purification in the subsequent cyclization step according to Cyclization C to yield the title compound as a dark orange solid: IR (KBr, cm\(^{-1}\)) 2977, 2932, 2889, 1659, 1210, 1111, 1065; \(^1\)H NMR (CDCl\(_3\)) \(\delta\) 8.01 (dd, \(J = 7.9, 1.0\) Hz, 1H), 7.49 (dd, \(J = 7.3, 0.8\) Hz, 1H), 7.26 (t, \(J = 7.8\) Hz, 1H), 6.59 (s, 1H), 5.35 (s, 1H), 3.75-3.62 (m, 4H), 2.47 (s, 3H), 1.26 (t, \(J = 7.1\) Hz, 6H); \(^{13}\)C NMR (CDCl\(_3\)) \(\delta\) 178.6, 163.2, 154.7, 134.6, 127.6, 124.7, 124.0, 123.3, 109.7, 97.7, 62.2, 15.5, 15.1; UV (EtOH) \(\lambda_{max}\) 225, 245, 303; HRMS \(m/z\) (M\(^+\)) calculated for C\(_{15}\)H\(_{18}\)O\(_4\) was 262.1200, found 262.1204.
50: 7-Methoxy-2-phenyl-4H-1-benzopyran-4-one (JLW-I-128):

Using the previously described Cyclization C procedure and starting from 77 mg (0.21 mmol) of 1-[2-[[1,1-dimethylethyl]dimethylsilyl]oxy]-4-methoxyphenyl]-3-phenyl-2-propyn-1-one, 47 mg (90%) of the title compound was obtained as a tan solid, recrystallization in EtOH afforded a white solid: mp 102-104°C; IR (CCl₄, cm⁻¹) 3058, 2959, 2866, 1652, 1602, 1273; ¹H NMR (CDCl₃) δ 8.05 (d, J = 9 Hz, 1H), 7.84-7.80 (m, 2H), 7.46-7.43 (m, 3H), 6.92-6.88 (m, 2H), 6.68 (s, 1H), 3.86 (s, 3H); ¹³C NMR (CDCl₃) δ 177.7, 164.1, 162.9, 157.9, 131.8, 131.3, 128.9, 127.0, 126.1, 117.8, 114.3, 107.5, 100.4, 55.8; UV (MeOH) λmax 251, 307; HRMS m/z (M⁺) calculated for C₁₆H₁₂O₃ was 252.0783, found 252.0782.

51: 7-Methoxy-2-(4'-methylphenyl)-4H-1-benzopyran-4-one (JLW-I-120):

18-Crown-6 (0.41 g, 1.6 mmol) was added to a solution of 1-[2-[[1,1-dimethyl-ethyl]dimethylsilyl]oxy]-4-methoxyphenyl]-3-(4-methylphenyl)-2-propyn-1-one (0.23 g, 0.62 mmol) in anhydrous DMF (8 mL) under argon and stirred for 5 min. The resulting solution was then cooled to 0°C, potassium fluoride (72 mg, 1.2 mmol) added,
and stirred in ice bath for 5 min. The reaction mixture, monitored by TLC (3:1 hexanes:EtOAc), was stirred at room temperature for 3.5 h to give a dark brown color. Saturated NH₄Cl (5 mL) was added to quench reaction and product extracted into EtOAc. Organics were washed with H₂O, brine, dried (Na₂SO₄), and concentrated in vacuo to afford a yellow-orange oil. The residue was purified via flash chromatography (silica gel, 2:1.5 hexanes:EtOAc) to yield 0.10 g (64%) of the title compound as a pale yellow solid: mp 130-131°C; IR (CCl₄, cm⁻¹) 2957, 2923, 2854, 1621, 1600, 1262, 1087; ¹H NMR (CDCl₃) δ 8.01 (d, J = 8.9 Hz, 1H), 7.66 (d, J = 8.1 Hz, 2H), 7.18 (d, J = 8.1 Hz, 2H), 6.82 (m, 2H), 6.60 (s, 1H), 3.82 (s, 3H), 2.32 (s, 3H); ¹³C NMR (CDCl₃) δ 177.5, 163.9, 162.9, 157.7, 141.8, 129.5, 128.7, 126.7, 125.8, 117.6, 114.1, 106.6, 100.2, 55.6, 21.3; UV (MeOH) λmax 309; HRMS m/z (M⁺) calculated for C₁₇H₁₄O₃ was 266.0939, found 266.0949.

Using the previously described Cyclization C procedure and starting from 48 mg (0.13 mmol) of 1-[2-[[1,1-dimethylethyl]dimethylsilyl]oxy]-4-methoxyphenyl]-3-(4-methylphenyl)-2-propyn-l-one, 30 mg (88%) of the title compound was obtained as a white solid: mp 130-131°C; IR (CCl₄, cm⁻¹) 2957, 2923, 2854, 1621, 1600, 1262, 1087; ¹H NMR (CDCl₃) δ 8.09 (d, J = 9 Hz, 1H), 7.76 (d, J = 8.2 Hz, 2H), 7.29-7.26 (m, 2H), 6.96-6.92 (m, 2H), 6.69 (s, 1H), 3.89 (s, 3H), 2.40 (s, 3H); ¹³C NMR (CDCl₃) δ 177.8, 164.1, 163.1, 157.9, 141.9, 129.7, 129.0, 127.0, 126.0, 114.2, 106.9, 100.4, 55.8, 21.4; HRMS m/z (M⁺) calculated for C₁₇H₁₄O₃ was 266.0939, found 266.0949.
52: 2-(1-Cyclohexen-1-yl)-7-methoxy-4H-1-benzopyran-4-one (JLW-I-133):

Using the previously described Cyclization C procedure and starting from 45 mg (0.12 mmol) of 3-(1-cyclohexen-1-yl)-1-[2-[[[1,1-dimethylethyl]dimethylsilyl]oxy]phenyl]-2-propyn-1-one, 23 mg (73%) of the title compound was obtained as a yellow-orange solid: mp 101-102°C; IR (KBr, cm⁻¹) 3051, 2941, 2861, 1622, 1594, 1238, 1213, 1028, 1019; ¹H NMR (CDCl₃) δ 8.04 (d, J = 9 Hz, 1H), 6.90 (dd, J = 8.7, 2.3 Hz, 2H), 6.21 (s, 1H), 3.87 (s, 3H), 2.29-2.27 (m, 4H), 2.27-2.25 (m, 4H); ¹³C NMR (CDCl₃) δ 178.3, 164.0, 163.3, 157.7, 133.3, 129.7, 126.9, 117.7, 113.8, 106.1, 100.2, 55.7, 26.0, 24.2, 22.2, 21.6; UV (MeOH) λmax 234; 303; HRMS m/z (M⁺) calculated for C₁₆H₁₆O₃ was 256.1095, found 256.1098.

53: 2-(3-Chloropropyl)-7-methoxy-4H-1-benzopyran-4-one (JLW-I-132):

Using the previously described Cyclization C procedure and starting from 46 mg (0.13 mmol) of 6-chloro-1-[2-[[[1,1-dimethylethyl]dimethylsilyl]oxy]-4-methoxyphenyl]-2-hexyn-1-one, 25 mg (80%) of the title compound was obtained as a tan solid: mp 104-104.5°C; IR (KBr, cm⁻¹) 3075, 3018, 2965, 2931, 1651, 1604, 1237, 1089; ¹H NMR (CDCl₃) δ 8.05 (d, J = 8.8 Hz, 1H), 6.92 (dd, J = 8.8, 2.3 Hz, 1H), 6.80 (d, J = 2.3 Hz, 204
1H), 6.11 (s, 1H), 3.87 (s, 3H), 3.61 (t, J = 6.5, 6.1 Hz, 2H), 2.76 (t, J = 7.6, 7.3 Hz, 2H), 2.21-2.15 (m, 1H); \(^{13}\)C NMR (CDCl\(_3\)) \(\delta\) 177.5, 167.1, 164.0, 158.2, 127.1, 117.6, 114.2, 110.2, 100.2, 55.8, 43.6, 31.4, 29.5; UV (MeOH) \(\lambda_{\text{max}}\) 216, 241, 248, 293; HRMS \(m/z\) (M\(^+\)) calculated for C\(_{13}\)H\(_{13}\)ClO\(_3\) was 252.0550, found 252.0568 (M\(^+\), 100%), 254.0543 (M\(^+^2\), 32%).

54: 2-(Diethoxymethyl)-7-methoxy-4\(H\)-1-benzopyran-4-one (JLW-I-136):

Using the previously described Cyclization C procedure and starting from 45 mg (0.12 mmol) of 4,4-bisethoxy-1-[2-[[[1,1-dimethylethyl]dimethylsilyl]oxy]-4-methoxyphenyl]-2-butyn-1-one, 32 mg (89%) of the title compound was obtained as a yellow/orange oil: IR (CCl\(_4\), cm\(^{-1}\)) 3076, 2975, 2932, 2897, 1651, 1612, 1279, 1062; \(^1\)H NMR (CDCl\(_3\)) \(\delta\) 8.05 (d, \(J = 8.8\) Hz, 1H) 6.87-6.86 (m, 2H), 6.49 (s, 1H), 5.26 (s, 1H), 3.85 (s, 3H), 3.67-3.61 (m, 4H), 1.23 (t, \(J = 7.0\) Hz, 6H); \(^{13}\)C NMR (CDCl\(_3\)) 177.6, 164.2, 162.7, 158.0, 127.0, 118.0, 114.5, 110.0, 100.5, 97.8, 62.1, 55.8, 25.6, 15.0, 0.9; UV (EtOH) \(\lambda_{\text{max}}\) 241, 248, 294; HRMS \(m/z\) (M\(^+\)) calculated for C\(_{15}\)H\(_{18}\)O\(_5\) was 278.1149, found 278.1130.
55: 3-Bromo-7-methoxy-2-phenyl-4H-1-benzopyran-4-one (JLW-I-191):

Using the same procedure described for 3-bromo-2-phenyl-4H-1-benzopyran-4-one 15, the title compound was obtained from 0.25 g (1.0 mmol) of 8-methoxy-2-phenyl-4H-1-benzopyran-4-one to yield 0.28 g (85%) as white plates: mp 152-154°C; IR (KBr, cm\(^{-1}\)) 3060, 2924, 2854, 1639, 1247, 1068; \(^1\)H NMR (CDCl\(_3\)) \(\delta\) 8.17 (d, \(J = 8.9\) Hz, 1H), 7.84-7.80 (m, 2H), 7.53-7.50 (m, 3H), 7.00 (dd, \(J = 8.9, 2.3\) Hz, 1H), 6.86 (d, \(J = 2.3\) Hz, 1H), 3.89 (s, 3H); \(^13\)C NMR (CDCl\(_3\)) \(\delta\) 172.4, 164.4, 161.5, 157.4, 133.0, 131.0, 129.3, 128.3, 127.9, 115.7, 115.2, 109.3, 99.9, 55.9; UV (MeOH) 250, 305; HRMS m/z (M\(^+\)) calculated for C\(_{16}H_{11}BrO_3\) was 329.9888, found 329.9898 (M\(^+\), 100%), 331.9863 (M\(^+\), 87%).

56: 7-Methoxy-2-propyl-4H-1-benzopyran-4-one (JLW-II-033):

Using the previously described Cyclization C procedure and starting from 0.71 g (2.2 mmol) of 1-2-[(1,1-dimethylethyl)dimethylsilyl]oxy]-4-methoxyphenyl]-2-hexyn-1-one, 0.42 g (76%) of the title compound was obtained as a yellow solid: mp 70-71°C; IR (KBr, cm\(^{-1}\)) 2967, 2932, 2875, 1645, 1602, 1437, 1390, 1266, 1202, 1020, 992; \(^1\)H NMR(CDCl\(_3\)) \(\delta\) 8.07 (d, \(J = 8.9\) Hz, 1H), 6.93 (d, \(J = 8.9, 2.4\) Hz, 1H), 6.81 (d, \(J = 2.4\) Hz, 1H), 3.97 (d, \(J = 6.9\) Hz, 3H), 3.89 (s, 3H), 1.68 (q, \(J = 7.2\) Hz, 2H), 1.27 (t, \(J = 7.2\) Hz, 3H); \(^13\)C NMR (CDCl\(_3\)) \(\delta\) 172.4, 164.4, 161.5, 157.4, 133.0, 131.0, 129.3, 128.3, 127.9, 115.7, 115.2, 109.3, 99.9, 55.9; UV (MeOH) 250, 305; HRMS m/z (M\(^+\)) calculated for C\(_{16}H_{11}BrO_3\) was 329.9888, found 329.9898 (M\(^+\), 100%), 331.9863 (M\(^+\), 87%).
Hz, 1H), 6.11 (s, 1H), 3.88 (s, 3H), 2.55 (t, $J = 7.5$ Hz, 2H), 1.75 (m, 2H), 1.00 (t, $J = 7.4$ Hz, 3H); $^{13}$C NMR (CDCl$_3$) $\delta$ 178.3, 169.5, 164.4, 158.7, 127.4, 117.9, 114.5, 110.1, 100.6, 56.2, 36.5, 20.6, 13.9, UV (EtOH) $\lambda_{max}$ 217, 241, 248, 285; HRMS $m/z$ (M + H$^+$) calculated for C$_{13}$H$_{14}$O$_3$ was 219.1021, found 219.1024.

57: 3-Bromo-7-methoxy-2-propyl-4H-1-benzopyran-4-one (JLW-II-037):

Using the same procedure described for 3-bromo-2-phenyl-4H-1-benzopyran-4-one 15, the title compound was obtained from 92 mg (0.4 mmol) of 8-methoxy-2-propyl-4H-1-benzopyran-4-one to yield 0.11 g (88%) as a white solid: mp 95-95.5°C; IR (KBr, cm$^{-1}$) 2968, 2870, 1638, 1433, 1337, 1240, 1191, 1020; $^1$H NMR (CDCl$_3$) $\delta$ 8.11 (d, $J = 8.9$ Hz, 1H), 6.96 (dd, $J = 8.9$, 2.4 Hz, 1H), 6.81 (d, $J = 2.4$ Hz, 1H), 3.89 (s, 3H), 2.90 (t, $J = 7.4$ Hz, 2H), 1.82 (sixtet, $J = 7.5$ Hz, 2H), 1.04 (t, $J = 7.4$ Hz, 3H); $^{13}$C NMR (CDCl$_3$) $\delta$ 172.1, 166.6, 164.4, 157.4, 128.0, 116.0, 115.0, 109.8, 100.0, 56.1, 36.8, 20.4, 13.9; UV (EtOH) $\lambda_{max}$ 225, 252, 295; HRMS $m/z$ (M + Na$^+$) calculated for C$_{13}$H$_{13}$BrO$_3$ was 318.9946, found 318.9952 (M + Na$^+$, 100%), 320.9935 (M$^{12}$ + Na$^+$, 93.8%).
60: 7-Hydroxy-2-phenyl-4H-1-benzopyran-4-one (JLW-II-017):

To a solution of NaH (3.1 equiv of a 60% w/w dispersion) in 0.7 mL DMF at 0°C was added ethanethiol (3.0 equiv) dropwise to generate a 0.5 M NaSEt solution in DMF. After 30 min, the mixture was warmed to rt and a solution of 7-methoxy-2-phenyl-4H-1-benzopyran-4-one (29 mg, 0.11 mmol) in DMF (0.5 mL) was added dropwise. The reaction mixture was heated to reflux for 3.5 h; then cooled in a ice-bath and acidified with dilute (< 1M)HCl to pH 3. The reaction mixture was extracted with EtOAc, washed with H2O, brine, and dried (Na2SO4), and concentrated in vacuo. The aqueous layer was reextracted with CHCl3 and monitored by TLC. The residue was purified via flash chromatography (silica gel, 2:1 hexanes:EtOAc) followed by recrystallization in MeOH to yield 62 mg (62%) of the title compound as an off-white solid: mp 237-238°C; IR (KBr, cm⁻¹) 3055, 2924, 2594, 1626, 1612, 1261; ¹H NMR (DMSO-d₆) δ 10.80 (s, 1H), 8.03 (dd, 2H), 7.86 (d, J = 8.7 Hz, 1H), 7.56-7.49 (m, 3H), 6.98 (d, J = 2.1 Hz, 1H), 6.90 (dd, J = 8.7, 2.2 Hz, 1H), 6.87 (s, 1H); ¹³C NMR (DMSO-d₆) δ 176.4, 162.7, 161.9, 157.5, 131.5, 131.3, 129.0, 126.5, 126.1, 116.1, 115.1, 106.6, 102.5; UV (EtOH) λmax 251, 308; HRMS m/z (M + Na⁺) calculated for C₁₅H₁₀O₃ was 261.0528, found 261.0527.
61: 7-Hydroxy-2-(4'-methylphenyl)-4H-1-benzopyran-4-one (JLW-II-019):

To a solution of NaH (3.1 equiv of a 60% w/w dispersion) in 0.6 mL DMF at 0°C was added ethanethiol (3.0 equiv) dropwise to generate a 0.5 M NaSEt solution in DMF. After 30 min, the mixture was warmed to rt and added dropwise to a solution of 7-methoxy-2-(4'-methylphenyl)-4H-1-benzopyran-4-one (24 mg, 0.10 mmol) in DMF (0.5 mL). The reaction mixture was heated to reflux for 3.5 h; then cooled in a ice-bath and acidified with dilute (< 1M) HCl to pH 3. The reaction mixture was extracted with EtOAc, washed with H₂O, brine, and dried (Na₂SO₄), and concentrated in vacuo. The aqueous layer was reextracted with CHCl₃ and monitored by TLC. The residue was purified via flash chromatography (silica gel, CHCl₃/5% MeOH) followed by recrystallization in MeOH to yield 21 mg (92%) of the title compound as yellow solid: mp 276-280°C; IR (KBr, cm⁻¹) 3032, 2962, 1625, 1248, 1090; ¹H NMR (DMSO-d₆) δ 10.77 (s, 1H), 7.91 (s, 1H), 7.88 (s, 1H), 7.84 (d, J = 8.7 Hz, 1H), 7.33 (s, 1H), 7.30 (s, 1H), 6.98 (d, J = 2.1 Hz, 1H), 6.90 (dd, J = 8.7, 2.1 Hz, 1H), 6.80 (s, 1H), 2.33 (s, 3H); ¹³C NMR (DMSO-d₆) δ 176.3, 162.5, 162.0, 157.4, 141.6, 129.6, 128.4, 126.4, 126.0, 116.1, 114.8, 105.9, 102.4, 20.9; UV (EtOH) λmax 311; HRMS m/z (M⁺ + H) calculated for C₁₆H₁₂O₃ was 253.0864, found 253.0863.
70: 7-Methyl-2-phenyl-4H-1-benzopyran-4-one (JLW-II-036):

Using the previously described Cyclization C procedure and starting from 0.85 g (2.4 mmol) of 1-[2-[(1,1-dimethylethyl)dimethylsilyl]oxy]-4-methyl[phenyl]-3-phenyl-2-propyn-1-one, 0.46 g (80%) of the title compound was obtained as orange crystals which was further recrystallized with MeOH: mp 118-119°C; IR (KBr, cm⁻¹) 3031, 2919, 1646, 1627; ¹H NMR (CDCl₃) δ 8.09 (d, J = 8.1 Hz, 1H), 7.90 (m, 2H), 7.50 (m, 3H), 7.35 (s, 1H), 7.22 (d, J = 8.8 Hz, 1H), 6.78 (s, 1H), 2.49 (s, 3H); ¹³C NMR (CDCl₃) δ 178.8, 163.5, 156.8, 145.5, 132.3, 131.9, 129.4, 127.1, 126.7, 125.9, 122.1, 118.3, 108.0, 22.3; UV (EtOH) λmax 255, 298; HRMS m/z (M + Na⁺) calculated for C₁₆H₁₂O₂ was 259.0735, found 259.0735.

80: 7-Chloro-2-phenyl-4H-1-benzopyran-4-one (JLW-I-273):

4-Chloro-2-[[1,1-dimethylethyl]dimethylsilyl]oxy]-benzoic acid, (1,1-dimethylethyl)dimethylsilyl ester (0.39 g, 1.0 mmol) was used for the previously described one-pot acid chlorination Sonogashira coupling with phenylacetylene (0.37 mL, 3.0 mmol) according to Coupling B to yield 0.31 g (84%) of an impure sample of 1-[2-[[1,1-dimethylethyl]dimethylsilyl]oxy]-4-chloro-phenyl]-3-phenyl-2-propyn-1-one as a
brown oil, which was used without further purification. The alkynone obtained from the Sonogashira coupling was used in the subsequent cyclization step according to **Cyclization C** to yield 0.12 g (49%) of the title compound as a yellow/brown solid: mp 154°C; IR (KBr, cm⁻¹) 3061, 1638, 1369, 1251, 1070, 813, 768; ¹H NMR (CDCl₃) δ 8.16 (d, J = 8.6 Hz, 1H), 7.91-7.87 (m, 2H), 7.60 (d, J = 1.8 Hz, 1H), 7.54-7.51 (m, 3H), 7.38 (dd, J = 8.3, 1.4 Hz, 1H), 6.80 (s, 1H); ¹³C NMR (CDCl₃) δ 177.5, 163.6, 156.4, 139.8, 131.8, 131.5, 129.1, 127.2, 126.3, 126.1, 122.6, 118.2, 107.9; UV (MeOH) λmax 249, 298; HRMS m/z (M + Na⁺) calculated for C₁₅H₁₀ClO₂ was 279.0189, found 279.0184 (M+Na⁺, 100%), 281.0177 (M⁺ + Na⁺, 34.6%).

81: 7-Chloro-2-(4'-methylphenyl)-4H-1-benzopyran-4-one (JLW-I-274):

4-Chloro-2-[[[1,1-dimethylethyl]dimethylsilyl]oxy]-benzoic acid, (1,1-dimethylethyl)-dimethylsilyl ester (0.40 g, 1.0 mmol) was used for the previously described one-pot acid chlorination Sonogashira coupling with 4-ethynyltoluene (0.47 mL, 3.8 mmol) according to **Coupling B** to yield 0.29 g (77%) of an impure sample of 1-[2-[[[1,1-dimethylethyl]dimethylsilyl]oxy]-4-chloro-phenyl]-3-(4-methylphenyl)-2-propyn-1-one as a yellow solid, which was used without further purification. The alkynone obtained from the Sonogashira coupling was used in the subsequent cyclization step according to **Cyclization C** to yield 0.11 g (42%) of the title compound as a pale yellow solid: mp 211
191-192.5°C; IR (KBr, cm⁻¹) 3064, 1639, 1601, 1434, 1367, 1260, 1039, 818; ¹H NMR (CDCl₃) δ 8.15 (d, J = 8.5 Hz, 1H), 7.80 (s, 1H), 7.77 (s, 1H), 7.59 (d, J = 1.8 Hz, 1H),
7.37 (dd, J = 8.5, 1.9 Hz, 1H), 7.33 (s, 1H), 7.30 (s, 1H), 6.77 (s, 1H), 2.43 (s, 3H); ¹³C NMR (CDCl₃) δ 177.5, 163.8, 156.4, 142.5, 139.6, 129.8, 128.6, 127.1, 126.2, 126.0,
122.6, 118.1, 107.2, 21.5; UV (EtOH) λmax 250, 307; HRMS m/z (M + H⁺) calculated for C₁₅H₁₁ClO₂ was 271.0526, found 271.0510 (M + H⁺, 100%), 273.0501 (M⁺² + H⁺, 35%).

82: 7-Chloro-2-(1-cyclohexen-1-yl)-4H-1-benzopyran-4-one (JLW-I-278):

Using the previously described Cyclization C procedure and starting from 0.36 g (1.0 mmol) of 1-[4-chloro-2-[[[(1,1-dimethylethyl)dimethylsilyl]oxy]-phenyl]-3-phenyl-2-propyn-1-one, 0.12 g (47%) of the title compound was obtained as an orange solid: mp 118-119°C; IR (KBr, cm⁻¹) 2934, 1646, 1605, 1209, 1068, 1017; ¹H (CDCl₃) δ 8.08 (d, J = 8.5 Hz, 1H), 7.46 (d, J = 1.9 Hz, 1H), 7.31 (dd, J = 8.5, 1.9 Hz, 1H), 6.97 (m, 1H),
6.26 (s, 1H), 2.33-2.25 (m, 4H), 1.79-1.64 (m, 4H); ¹³C NMR (CDCl₃) δ 178.0, 163.9,
156.1, 139.5, 134.4, 129.4, 127.0, 125.5, 122.4, 117.9, 106.3, 26.0, 24.2, 22.1, 21.5; UV (EtOH) λmax 246, 301; HRMS m/z (M + H⁺) calculated for C₁₅H₁₃ClO₂ was 261.0681,
found 216.0676 (M + H⁺, 100%), 263.0681 (M⁺² + H⁺, 35%).
83: 7-Chloro-2-(3-chloropropyl)-4H-1-benzopyran-4-one (JLW-I-280):

4-Chloro-2-[(1,1-dimethylethyl)dimethylsilyloxy]-benzoic acid, (1,1-dimethylethyl)dimethylsilyl ester (0.41 g, 1.0 mmol) was used for the previously described one-pot acid chlorination Sonogashira coupling with phenylacetylene (0.47 mL, 3.8 mmol) according to Coupling B. Crude $^1$H NMR before workup reveals absence of TBS peaks and singlet at 5.8 ppm. The residue was dissolved in EtOAc, washed with H$_2$O, 10% NH$_4$OH, brine, dried (Na$_2$SO$_4$), and concentrated in vacuo. The residue was purified via flash chromatography (silica gel, 4:1 hexanes:EtOAc) to yield 0.11 g (42%) of the title compound as a orange/brown oil. This oil solidified over time and was crystallized from EtOH/H$_2$O to give an off-white solid: mp 66-67°C; IR (KBr, cm$^{-1}$) 3061, 1642, 1604, 1431, 1164; $^1$H NMR (CDCl$_3$) $\delta$ 7.96 (d, $J$ = 8.6 Hz, 1H), 7.32 (d, $J$ = 1.5 Hz, 1H), 7.22 (dd, $J$ = 8.7, 1.4 Hz, 1H), 6.08 (s, 1H), 3.54 (t, $J$ = 6.2 Hz, 2H), 2.71 (t, $J$ = 7.7 Hz, 2H), 2.16-2.05 (m, 2H); $^{13}$C NMR (CDCl$_3$) $\delta$ 176.8, 167.7, 156.2, 139.3, 126.8, 125.6, 117.8, 110.3, 43.3, 31.2, 29.1; UV (EtOH) $\lambda_{max}$ 303; HRMS $m/z$ (M + Na$^+$) calculated for C$_{12}$H$_{10}$Cl$_2$O$_2$ was 278.9956, found 278.9960 (M + Na$^+$, 100%), 281.0034 (M$^{+2}$ + Na$^+$, 65.2%), 283.0220 (M$^{+4}$ + Na$^+$, 10.6%).
90: 6-Chloro-2-phenyl-4H-1-benzopyran-4-one (JLW-I-235):

5-Chloro-2-[(1,1-dimethylethyl)dimethylsilyloxy]-benzoic acid, (1,1-dimethylethyl)-dimethylsilyl ester (0.33 g, 1.0 mmol) was used for the previously described one-pot acid chlorination Sonogashira coupling with phenylacetylene (0.47 mL, 3.8 mmol) according to **Coupling C**. The alkynone obtained from the Sonogashira coupling was used without further purification in the subsequent cyclization step according to **Cyclization C** to yield 59 mg (23%) of the title compound which was further washed with petroleum ether to yield 0.15 g (60%) of the title compound which was further recrystallized from EtOH/hexanes to give brown solid: mp 178-179°C; IR (KBr, cm⁻¹) 3086, 1656, 1253, 1022, 772; ¹H NMR (CDCl₃) δ 8.19 (d, J = 2.5 Hz, 1H), 7.92 (d, J = 1.9 Hz, 1H), 7.90-7.88 (m, 1H), 7.64 (dd, J = 8.9, 2.6 Hz, 1H), 7.54-7.50 (m, 4H), 6.82 (s, 1H); ¹³C NMR (CDCl₃) δ 177.3, 163.8, 154.8, 134.1, 132.0, 131.6, 131.4, 129.3, 126.5, 125.4, 125.1, 119.9, 107.7; UV (MeOH) λ max 254, 298; HRMS m/z (M⁺) calculated for C₁₆H₁₄ClO₂ was 256.0289, found 256.0298 (M⁺, 100%), 258.0273 (M⁺², 35%).
Using the previously described Cyclization D procedure and starting from 0.24 g (0.6 mmol) of 1-[2-[[1,1-dimethylethyl]dimethylsilyl]oxy]-5-chlorophenyl]-3-phenyl-2-propyn-1-one, 0.11 g (63%) of the title compound was obtained as a white solid: mp 185-185.5°C; IR (KBr, cm⁻¹) 3068, 2913, 1641, 1614, 816; \(^1\)H NMR (CDCl₃) δ 8.17 (d, \(J = 2.5\) Hz, 1H), 7.81 (s, 1H), 7.77 (s, 1H), 7.62 (dd, \(J = 8.9, 2.5\) Hz, 1H), 7.51 (d, \(J = 8.9\) Hz, 1H), 7.32 (d, \(J = 8.2\) Hz, 1H), 6.78 (s, 1H), 2.43 (s, 3H); \(^13\)C NMR (CDCl₃) δ 177.2, 163.9, 154.6, 142.6, 133.8, 131.1, 129.8, 128.6, 126.3, 125.2, 125.0, 119.8, 106.9, 21.5; UV (EtOH) \(\lambda_{\text{max}}\) 256, 307; HRMS \(m/z\) (M + H⁺) calculated for C₁₆H₁₁O₂Cl was 271.0523, found 271.0524 (M + H⁺, 100%), 273.0485 (M⁺² + H⁺, 23%).
92: 6-Chloro-2-(1-cyclohexen-1-yl)-4H-1-benzopyran-4-one (JLW-I-289):

5-Chloro-2-[[1,1-dimethylethyl]dimethylsilyl]oxy]-benzoic acid, (1,1-dimethylethyl)dimethylsilyl ester (0.40 g, 1.0 mmol) was used for the previously described one-pot acid chlorination Sonogashira coupling with 1-ethynyl-1-cyclohexene (0.47 mL, 4.0 mmol) according to Coupling C. The alkynone obtained from the Sonogashira coupling was used without further purification in the subsequent cyclization step according to Cyclization G to yield 59 mg (23%) of the title compound which was further washed with petroleum ether to yield a orange-brown solid: mp 94.5-97°C; IR (KBr, cm\(^{-1}\)) 2927, 1644, 1632, 1606, 1110; \(^1\)H NMR (acetone-d\(_6\)) \(\delta\) 7.95 (d, \(J = 2.6\) Hz, 1H), 7.74 (dd, \(J = 8.9, 2.6\) Hz, 1H), 7.65 (d, \(J = 8.9\) Hz, 1H), 7.04-7.01 (m, 1H), 6.22 (s, 1H), 2.35- 2.27 (m, 4H), 1.82-1.63 (m, 4H); \(^{13}\)C NMR (acetone-d\(_6\)) \(\delta\) 176.5, 164.0, 154.9, 134.6, 134.1, 130.4, 129.8, 125.2, 124.5, 120.8, 105.9, 26.1, 24.2, 22.4, 21.7; UV (EtOH) \(\lambda_{\max}\) 222, 252, 298; HRMS m/z (M + Na\(^+\)) calculated for C\(_{15}\)H\(_{13}\)ClO\(_2\) was 283.0502, found 283.0502 (M + Na\(^+\), 100%), 285.0489 (M\(^{+2}\) + Na\(^+\), 33%).
93: 6-Chloro-2-(3-chloropropyl)-4H-1-benzopyran-4-one (JLW-I-284):

5-Chloro-2-[[[(1,1-dimethylethyl)dimethylsilyloxy]oxy]benzoic acid, (1,1-dimethylethyl)-dimethylsilyl ester (0.43 g, 1.1 mmol) was used for the previously described one-pot acid chlorination Sonogashira coupling with 5-chloro-1-pentyne (0.69 mL, 6.5 mmol) according to Coupling C to yield 80 mg (29%) of the title compound which was further washed with petroleum ether to yield off-white solid: mp 66-67°C; IR (KBr, cm⁻¹) 3061, 1642, 1604, 1431, 1164; ¹H NMR (CDCl₃) δ 8.12 (d, J = 2.6 Hz, 1H), 7.58 (dd, J = 8.9, 2.6 Hz, 1H), 7.37 (d, J = 8.9 Hz, 1H), 6.21 (s, 1H), 3.61 (t, J = 6.2 Hz, 2H), 2.81 (t, J = 7.8 Hz, 2H), 2.20 (m, 2H); ¹³C NMR (CDCl₃) δ 177.1, 168.3, 154.9, 134.0, 131.2, 1253, 124.8, 119.7, 110.5, 43.7, 31.7, 29.6; UV (EtOH) λmax 227, 305; HRMS m/z (M + H⁺) calculated for C₁₂H₁₀Cl₂O₂ was 257.0136, found 257.0133 (M + H⁺, 100%), 259.0125 (M⁺² + H⁺, 54%), 261.0164 (M⁺³ + H⁺, 5%).
2,6-Diphenyl-4H-1-benzopyran-4-one (JLW-I-264):

2-[[[(1,1-Dimethyl)ethyl]dimethylsilyl]oxy]-5-phenylbenzoic acid, (1,1-dimethyl)ethyl-dimethylsilyl ester (0.44 g, 1.0 mmol) was used for the previously described one-pot acid chlorination Sonogashira coupling with phenylacetylene (0.37 mL, 3.0 mmol) according to Coupling C to yield 0.31 g (76%) of an impure sample of 1-[[[(1,1-dimethyl)ethyl]dimethylsilyl]oxy]-5-phenyl-phenyl]-3-phenyl-2-propyn-1-one. The alkynone obtained from the Sonogashira coupling was used without further purification in the subsequent cyclization step according to Cyclization C to yield 0.17 g (57%) of the title compound which was further washed with petroleum ether to yield 0.14 g (46%) as a pale pink solid; mp 152-153°C; IR (KBr, cm⁻¹) 3061, 1642, 1358, 1249, 769; ¹H NMR (CDCl₃) δ 8.44 (d, J = 2.3 Hz, 1H), 7.95-7.92 (m, 3H), 7.69 (d, J = 1.3 Hz, 1H), 7.65-7.62 (m, 2H), 7.54-7.37 (m, 6H), 6.85 (s, 1H); ¹³C NMR (CDCl₃) δ 178.9, 163.8, 156.1, 139.7, 138.8, 133.0, 132.2, 132.0, 129.5, 129.4, 128.3, 127.6, 126.7, 124.5, 124.0, 119.0, 108.0; UV (EtOH) λmax 271; HRMS m/z (M + H⁺) calculated for C₂₁H₁₄O₂ was 299.1072, found 299.1068.
111: 2-(4'-Methylphenyl)-6-phenyl-4H-1-benzopyran-4-one (JLW-1-267):

2-[[1-Dimethylsilyl]oxy]-5-phenyl-benzoic acid, (1,1-dimethylethyl)-dimethylsilyl ester (0.44 g, 1.0 mmol) was used for the previously described one-pot acid chlorination Sonogashira coupling with 1-ethynyl-toluene (0.38 mL, 3.0 mmol) according to Coupling B. The alkyne obtained from the Sonogashira coupling was used in the subsequent cyclization step according to Cyclization C to yield 0.24 g (77%) of the title compound as a pale yellow solid: mp 202-202.5°C; IR (KBr, cm⁻¹) 3056, 1636, 1615, 1455, 1355, 1253, 1045, 823, 763; ¹H NMR (CDCl₃) δ 8.41 (d, J = 2.3 Hz, 1H); 7.90 (dd, J = 8.7, 2.4 Hz, 1H), 7.81 (s, 1H), 7.75 (s, 1H), 7.66 (m, 1H), 7.63-7.57 (m, 2H), 7.47-7.35 (m, 3H), 7.31 (s, 1H); 7.27 (s, 1H), 6.79 (s, 1H), 2.41 (s, 3H); ¹³C NMR (CDCl₃) δ 178.4, 163.5, 155.6, 142.2, 139.3, 138.2, 132.4, 129.7, 128.9, 127.8, 127.1, 126.6, 126.2, 124.1, 123.5, 118.5, 106.9, 21.5; UV (EtOH) 273, 309; HRMS m/z (M + H⁺) calculated for C₄₂H₂₆O₂ was 313.1228, found 313.1219.
112: 2-(1-Cyclohexen-1-yl)-6-phenyl-4H-1-benzopyran-4-one (JLW-I-266):

2-[[[(1,1-Dimethylethyl)dimethylsilyl]oxy]-5-phenyl-benzoic acid, (1,1-dimethylethyl)-
dimethylsilyl] ester (0.44 g, 1.0 mmol) was used for the previously described one-pot
acid chlorination Sonogashira coupling with 1-ethynyl-cyclohexene (0.38 mL, 3.2
mmol) according to Coupling B. The alkynone obtained from the Sonogashira
coupling was used in the subsequent cyclization step according to Cyclization C to yield
0.21 g (68%) of the title compound as a orange solid: mp 128-129°C; IR (KBr, cm⁻¹)
2919, 1648, 1615, 1455, 1427, 833, 767; ¹H NMR (CDCl₃)  δ 8.34 (d, J = 2.3 Hz, 1H),
7.81 (dd, J = 8.7, 2.4 Hz, 1H), 7.62-7.59 (m, 2H), 7.44-7.31 (m, 4H), 6.93 (m, 1H), 6.26
(s, 1H), 2.26-2.22 (m, 4H), 1.75-1.60 (m, 4H); ¹³C NMR (CDCl₃)  δ 179.3, 164.6, 155.9,
139.7, 138.4, 135.2, 133.1, 130.1, 129.4, 128.2, 127.6, 124.0, 123.8, 118.8, 106.3, 26.5,
24.6, 22.6, 21.9; UV (EtOH) 270; HRMS m/z (M + Na⁺) calculated for C₂₁H₁₈O₂ was
325.1204, found 325.1200.
113: 2-(3-Chloropropyl)-6-phenyl-4H-1-benzopyran-4-one (JLW-I-277):

2-[[((1,1-Dimethylethyl)dimethylsilyl]oxy]-5-phenyl-benzoic acid, (1,1-dimethylethyl)-
dimethylsilyl ester (0.44 g, 1.0 mmol) was used for the previously described one-pot
acid chlorination Sonogashira coupling with 5-chloro-1-pentyne (0.31 mL, 3 mmol)
according to Coupling B to yield 0.30 g (74%) of an impure sample of 6-chloro-1-[2-
[[((1,1-dimethylethyl)dimethylsilyl]oxy]-5-phenyl-phenyl]-2-hexenyn-1-one (JLW-I-
270) as a orange-brown oil. The alkynone obtained from the Sonogashira coupling was
used without further purification in the subsequent cyclization step according to 
Cyclization C to yield 0.12 g (39%) of the title compound as a tan solid: mp 109-111°C;
IR (KBr, cm⁻¹) 2920, 1641, 1473, 1456, 1351, 767; ¹H (CDCl₃) δ 8.39 (d, J = 2.3 Hz,
1H), 7.89 (dd, J = 8.7, 2.3 Hz, 1H), 7.65 (s, 1H), 7.62 (s, 1H), 7.55-7.33 (m, 4H), 6.23
(s, 1H), 3.63 (t, J = 6.2 Hz, 2H), 2.83 (t, J = 7.2 Hz, 2H), 2.23 (q, 2H); ¹³C NMR
(CDCl₃) δ 178.6, 168.2, 156.3, 139.7, 138.7, 132.9, 129.4, 128.3, 127.6, 124.2, 124.0,
118.8, 110.8, 44.0, 32.0, 29.9; UV (MeOH) λmax 250, 314; HRMS m/z (M + H⁺)
calculated for C₁₈H₁₅ClO₂ was 299.0839, found 299.0850 (M + H⁺, 100%), 301.0862
(M⁺² + H⁺, 34.8%).
120: 6-Methoxy-2-phenyl-4H-1-benzopyran-4-one (JLW-II-065):
Using the previously described Cyclization C procedure and starting from 0.69 g (1.9 mmol) of 1-[2-[[1,1-dimethylethyl]dimethylsilyloxy]-5-methoxyphenyl]-3-phenyl-2-propyn-1-one, 0.34 g (71%) of the title compound was obtained as an off-white solid: mp 158.5-159°C; IR (KBr, cm⁻¹) 1640, 1360, 1254, 1076; ¹H NMR (CDCl₃) δ 7.91 (d, J = 2.3 Hz, 1H), 7.89-7.88 (m, 1H), 7.59 (d, J = 3.1 Hz, 1H), 7.54-7.47 (m, 4H), 7.28 (dd, J = 9.1, 3.1 Hz, 1H), 6.80 (s, 1H), 3.90 (s, 3H); NMR (CDCl₃) δ 184.8, 167.1, 149.5, 147.8, 132.9, 132.9, 131.8, 130.1, 129.3, 128.9, 125.3, 124.7, 119.7, 113.5, 112.9, 23.1; UV (MeOH) λmax 270, 304; HRMS m/z (M + Na⁺) calculated for C₁₆H₁₂O₃ was 275.0684, found 275.0671.

7-Methyl-2-phenyl-3(2H)-benzofuranone (JLW-II-035):
¹H NMR (CDCl₃) δ 7.90-7.87 (m, 2H), 7.66 (d, J = 7.9 Hz, 1H), 7.46-7.33 (m, 3H), 7.10 (s, 1H), 6.99 (d, J = 7.9 Hz, 1H), 6.83 (s, 1H), 2.46 (s, 3H); ¹³C NMR (CDCl₃) δ 184.8, 167.1, 149.5, 147.8, 132.9, 132.9, 131.8, 130.1, 129.3, 128.9, 125.3, 124.7, 119.7, 113.5, 112.9, 23.1; UV (MeOH) λmax 325, 371.
7-Chloro-2-(4-methylphenyl)-3(2H)-benzofuranone (JLW-I-272):

IR (KBr, cm⁻¹) 3065, 1707, 1650, 1606, 1258, 1058; ¹H NMR (acetone-d₆) δ 7.74 (d, J = 8.2 Hz, 2H), 7.69 (d, J = 8.2 Hz, 1H), 7.31 (d, J = 1.6 Hz, 1H), 7.23 (d, J = 8.3 Hz, 2H), 7.15 (dd, J = 8.2, 1.6 Hz, 1H), 6.85 (s, 1H), 2.37 (s, 3H); ¹³C NMR (CDCl₃) δ 183.1, 166.0, 146.5, 142.6, 140.8, 131.6, 129.7, 129.2, 125.3, 124.2, 120.4, 114.1, 113.5, 21.6; UV (EtOH) λ max 264, 328, 379.

2-Diethoxymethyl-7-methoxy-3(2H)-benzofuranone (JLW-I-195):

IR (KBr, cm⁻¹) 2979, 2898, 1723, 1614, 1286, 1158, 1053; ¹H NMR (CDCl₃) δ 7.62 (d, J = 8.7 Hz, 1H), 6.67 (dd, J = 8.7, 2.0 Hz, 1H), 6.23 (d, J = 2.0 Hz, 1H), 5.54 (d, J = 7.6 Hz, 1H), 3.87 (s, 3H), 3.74-3.56 (m, 4H), 1.22 (t, J = 7.3 Hz, 6H); ¹³C NMR (CDCl₃) δ 182.4, 169.0, 167.9, 149.4, 126.0, 114.7, 112.1, 109.5, 96.6, 96.1, 61.4, 56.0, 15.2; UV(EtOH) λ max 274, 318.
JLW-II-064 #11-12: 6-Methoxy-2-phenyl-3(2H)-benzofuranone:

$^1$H NMR (CDCl$_3$) $\delta$ 7.90 (s, 1H), 7.87 (s, 1H), 7.46-7.37 (m, 3H), 7.22 (m, 2H), 7.20 (s, 1H), 6.85 (s, 1H), 3.81 (s, 3H); $^{13}$C NMR (CDCl$_3$) $\delta$ 185.0, 161.3, 156.0, 147.7, 132.3, 131.5, 129.8, 128.9, 126.2, 121.7, 113.8, 113.1, 105.2, 55.8; UV (EtOH) $\lambda_{max}$ 259, 336, 381.
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Table 8.1: Numbering system of notebooks (JLW-I-xxx, JLW-II-xxx) and HPLC % purity data for solvent System #1 (70/30 MeOH/H2O) and System #2 (65/35 CH3CN/H2O).
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INFLAMMATION IN ULTRAVIOLET LIGHT INDUCED CARCINOGENESIS, WOUND HEALING, AND FIBROSIS IN ADULT AND FETAL MURINE SKIN

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By
Traci Ann Wilgus, B.S.

The Ohio State University
2001

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Department of Molecular Virology, Immunology and Medical Genetics
ABSTRACT

Inflammation, which involves the induction of vascular permeability, the release of growth factors, pro-inflammatory cytokines and prostaglandins, and the infiltration and activation of inflammatory cells, has been shown to play a role in cancer development, wound healing and fibrosis in the skin. Ultraviolet light B (UVB) radiation is responsible for inducing inflammation following sun exposure and is also the major cause of skin cancer. Recently, an inflammatory enzyme responsible for the production of prostaglandins, (cyclooxygenase-2) COX-2, has been implicated in the development of epithelial cancers, including squamous cell carcinoma in the skin. Nonsteroidal anti-inflammatory drugs (NSAIDs), such as aspirin and ibuprofen, inhibit the COX-2 enzyme and its constitutive correlate, COX-1, and have been shown to prevent the formation of cancer in many organs. Side effects associated with restricting the homeostatic function of COX-1 led to the development of specific COX-2 inhibitors, such as Celecoxib (Celebrex). Although this drug was designed for oral use, we hypothesized that topical administration of Celecoxib would be a better choice for inhibiting
inflammation and the subsequent tumor formation as a result of chronic inflammatory conditions in the skin. Due to the alteration in drug delivery, we characterized the effects of topical Celecoxib treatment on the acute UVB induced inflammatory response. We found that Celecoxib was able to inhibit several parameters of acute inflammation, including vascular permeability, the infiltration and activation of neutrophils, and the production of prostaglandin E\textsubscript{2} (PGE\textsubscript{2}). Additionally, we found that Celecoxib treatment prevented the induction of oxidative damage measured by the detection of 8-oxo-deoxyguanosine DNA adducts and subsequently reduced the number of apoptotic sunburn cells and p53 positive cells in the epidermis. Because topical Celecoxib treatment was shown to have potent anti-inflammatory activity following UVB irradiation, long term studies were performed to examine the ability of topical treatment with this drug to act as a chemopreventative or chemotherapeutic agent after chronic UVB exposure. We found that Celecoxib was able to block chronic inflammation and the formation of papillomas after continued UVB exposure, however, it was unable to regress established tumors. Our data suggest that inflammation may be more of a determinant in the development of UVB-induced tumors than in the maintenance of established tumors.

Because wound healing, like UVB exposure, is a process that involves a robust inflammatory response, we also used topical treatment with Celecoxib to examine the role of COX-2 and inflammation in wound healing and scar formation. The formation of a collagenous scar in adults is the end
result of skin repair processes, but if excessive, scar tissue can be detrimental. It can cause the loss of function, the impairment of joint mobility and psychosocial consequences that result from prominent scars on visible body surfaces. In adult skin, it was found that Celecoxib did not interfere with the reepithelialization process important for wound closure, however, it did have a noticeable effect on the amount of scar tissue formed. Upon further analysis, we found that wounds that had been treated with Celecoxib contained less of the known fibrogenic protein, TGF-β1. Another set of studies looking at the ability of the inflammatory products PGE₂ and H₂O₂ to induce scar formation in a scarless fetal wound healing model revealed the ability of both mediators to induce scarring in fetal skin. Both studies indicate a role for inflammation in the stimulation of scar tissue formation after wounding, exposing a possible new use for anti-inflammatory drugs, the treatment of fibrosis.
This dissertation is dedicated to my son, Ryan. I did this for you as an example of what you can do if you put your mind to something, even if people tell you there is no way you can. Always remember to never give up your dreams and that..."you were born to fly".
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CHAPTER 1

INTRODUCTION

The Skin

The skin is a dynamic organ that functions as a barrier, protecting the body from the outside world and mediating the body's response to the environment. It functions in immunity, temperature regulation and respiration, and protects against fluid loss and mechanical stress (1,2). The skin is composed of two main layers: the epidermis and the dermis. The outermost layer of the skin, the epidermis, is made up of multilayered, stratified squamous epithelium. Cells in the lower basal layer proliferate and migrate outward, differentiating along the way. Basal cells are cuboid, developing into flattened cells as they differentiate. The cells also acquire granules, lose cytoplasmic organelles, and accumulate an excess of keratin and a cornified envelope in this differentiation process (1). The epidermis and dermis are separated by the basement membrane zone, which is important for the adherence of the epidermis to the dermis (2). The dermis, which offers support and elasticity to the skin, contains mostly collagen (~80%), along
with elastin, lipids, hyaluronic acid, proteoglycans, and structural glycoproteins (3). Blood vessels and fibroblasts are also located in the dermis, and along with the epithelial keratinocytes are important for the reaction of the skin to different environmental stimuli, including the response to ultraviolet light (UV) exposure and cutaneous wounds.

**Ultraviolet light and skin cancer**

Ultraviolet light has a major effect on the cellular constituents of the skin. It is a major environmental carcinogen implicated in the development of both basal and squamous cell carcinomas, which together make up a class of cancer known as non-melanoma skin cancer (NMSC). NMSC is the most common type of neoplasm in humans, with over 1 million new cases identified each year (4). The development of the two types of NMSC seem to differ in that BCC is linked to periodic acute sun exposures and sunburn early in life while SCC is associated with chronic intense sun exposure, cumulative over the lifetime of an individual (5-9). Much like the carcinogenesis process in humans, chronic low dose exposures of UVB light can be used to induce SCC in 12-15 weeks in a hairless murine model commonly used for UV carcinogenesis studies (10). Although there are three classes of UV light, UVA (320-400 nm), UVB (290-320 nm) and UVC (200-290 nm), UVB is the most potent inducer of cutaneous damage following both acute and long term sun exposure, making it the most important etiologic agent in skin cancer.
The continued depletion of the ozone layer further increases the potency of UVB as a damaging agent, causing the incidence of skin cancer to grow at an even greater rate (15).

Skin Carcinogenesis

The process of skin carcinogenesis is a multistep process, first defined as a result of chemical carcinogenesis studies carried out in murine skin (16). This step-wise progression to skin cancer involves three distinct steps, initiation, promotion, and progression (17).

Initiation is the first step of the carcinogenesis process, induced by a permanent and heritable change in the genomic DNA. This phenotype can be inherited or induced, either as a result of spontaneous mutation or exposure to environmental carcinogens, such as UV light. This DNA alteration or mutation does not cause the cell to become malignant, but may provide a growth advantage to the initiated cell. The mutation in the initiated cell often occurs in growth regulatory genes, such as tumor suppressor genes or oncogenes, such that uncontrolled proliferation in the presence of a growth stimulus occurs. In the case of ultraviolet light, mutations are often induced in the tumor suppressor gene p53 (15).

In the next phase of carcinogenesis, promotion, epigenetic changes stimulate the growth of initiated cells. While most carcinogens can be classified as either initiators or promoters, ultraviolet light is classified as a complete carcinogen, that is, it is capable of acting as both an initiator and a
promoter (18). It both causes mutations in cellular DNA that forms initiated cells and it induces the production of a host of growth factors and cytokines that contribute to the growth of initiated cells. One potent promoter induced by UVB is prostaglandin E₂ (19,20). Promotion results in focal proliferation of initiated cells, resulting in the formation of benign papillomas in the skin. UV also suppresses local and systemic immune responses, which impairs the ability of immune system to destroy and detect transformed keratinocytes thus allowing the development of tumors (21).

The last phase of the carcinogenesis process, progression, involves the acquisition of even more mutations, resulting in transformation of the previously benign cell into one that is malignant. This malignant cell and its progeny are characterized by an accelerated growth rate, the ability to invade local tissue and the potential to metastasize to distant sites in the body. Although it is rare for SCC to metastasize, if left untreated it can metastasize to local lymph nodes.

**Ultraviolet Light-induced DNA damage: direct damage/p53 and indirect damage/inflammation**

There are several mechanisms by which UVB contributes to the process of skin carcinogenesis. Ultraviolet light exposure causes both direct and indirect DNA damage. If this damage is not properly repaired before replication ensues, mutations are acquired in the DNA. These mutations can
lead to the inactivation of protooncogenes and/or the constitutive activation of oncogenes, contributing to the process of carcinogenesis by allowing mutated cells to proliferate in an uncontrolled fashion (13,22).

Direct DNA damage occurs as a result of photons of ultraviolet light energy being absorbed directly by the DNA, causing signature adducts such as pyrimidine dimers and 6,4-photoproducts (22,23). Failure to correct these DNA adducts results in C to T and CC to TT transitions during DNA replication. These UV-induced mutations are commonly found in the p53 gene. The accumulation of p53-mutant cells is an early event in the process of skin carcinogenesis resulting in dysfunctional cell cycle regulation and the accumulation of even more DNA damage (18,22,23). The importance of p53 in the skin's response to UV damage is highlighted by the fact that p53 is mutated in over 90% of SCCs (22). The p53 tumor suppressor gene encodes a 53 kDa molecular weight protein that functions as a transcription factor, regulating many genes involved in cell cycle regulation and apoptosis, such as bax, bcl-2, and p21^{Waf-1/Cip1} (24,25). The levels of p53 protein increase normally after UVB exposure via decreased degradation of the protein (26). This results in a G_{1} to S phase halt in the cell cycle and the induction of repair proteins so that the DNA damage can be repaired before replication, or if the damage is too extensive to be repaired, the induction of pro-apoptotic proteins (27). The cells in the epidermal layer of the skin that are induced to undergo apoptosis by p53 after UV exposure can be distinguished morphologically and are often referred to as sunburn cells. These sunburn
cells can be distinguished from normal keratinocytes in hemotoxylin and eosin stained tissue sections by their contracted nuclei and highly eosinophilic cytoplasm, and their formation is thought to be a mechanism to rid the skin of cells with the potential for transformation (25,28-30). The induction of apoptosis by p53 is an attempt to avoid the creation of DNA mutations upon replication. However, after repeated exposures, the p53 gene may become mutated leading to a dysfunctional protein product. The mutant protein becomes resistant to degradation, losing its ability to halt cell cycle progression and resulting in the accumulation of p53-positive proliferating cells (28).

Indirect damage occurs as a result of secondary processes induced by ultraviolet light such as the activation and subsequent release of reactive oxygen species (ROS) by inflammatory cells and other cell types within the skin, which can cause oxidative DNA damage. It has become increasingly apparent that solar radiation-induced inflammation and the oxidative DNA damage that results play an important role in skin carcinogenesis (31,32,33). The hallmark of oxidative DNA damage is the formation of 8-oxo-deoxyguanosine (8-oxo-dG) adducts (34). Its ability to cause G to T transitions without repair makes it one of the most mutagenic DNA lesions. Although not traditionally thought of as UV induced, it is becoming more apparent that oxidative damage likely plays a larger role in photocarcinogenesis than previously believed. Besides the normal events involved in the inflammatory response, including increased blood flow and
vascular permeability resulting in edema and erythema, the infiltration and activation of inflammatory cells, and the induction of pro-inflammatory cytokines and growth factors, UVB induces the production of reactive oxygen species both by resident skin cells and infiltrating inflammatory cells (33,35,36). This inflammatory response and the generation of ROS can be heightened by the products of the cyclooxygenase-2 (COX-2) enzyme, particularly prostaglandin E₂ (PGE₂) (37). The increase in prostaglandin production in response to UVB is a result of both an increased release of arachidonic acid (AA) from membrane phospholipids by phospholipase enzymes, as well as increased expression of the COX-2 enzyme which is responsible for the biosynthesis of prostaglandins from AA (38-41). UVB as well as several of the pro-inflammatory cytokines induced by UVB, including TNF-α and IL-1β, have been shown to induce COX-2 expression (42,43). This process of prostaglandin production seems to be critical to the damaging effects of inflammation in many organs, including the damaging effects of UVB-induced inflammation in the skin, processes that upon becoming chronic conditions often lead to the development of cancer (44-46). The process of inflammation and prostaglandin production was first linked to cancer development by epidemiological studies connecting frequent use of nonsteroidal anti-inflammatory drugs (NSAIDs) that block inflammation through the inhibition of the COX enzymes, with decreased risk of various types of epithelial based cancers (47-51). In addition, a number of studies have shown that tumors in a variety of organs, including SCCs in the skin,
produce greater amounts of the COX-2 enzyme and its prostaglandin products than normal tissue of the same type (52-54) with the prostaglandins acting as promoters in the carcinogenesis process (19).

Since the realization that COX-2 is overexpressed in colon cancer and that nonsteroidal anti-inflammatory drugs can reduce the incidence and mortality from colorectal cancers, increasing emphasis has been placed on the role of COX-2 in cancer. The prostaglandins produced by COX-2 are thought to contribute to cancer development in several ways. Prostaglandins have been shown to act as tumor promoters or co-carcinogens (19), enhancing DNA, RNA and protein synthesis and behaving as a mitogen for several different cell types including epithelial cells (55,56). In addition, COX-2, which is induced by UVB irradiation (41,52) can contribute to the formation of oxidative damage in two ways. COX-2 can cause indirect oxidative damage by promoting the inflammatory process, including the infiltration, activation and release of reactive oxygen species by inflammatory cells (56). It can also cause oxidative DNA damage directly by generating reactive oxygen species upon reaction with its substrate, arachidonic acid (57-59).

During the production of prostaglandins from AA, (Fig 1.1) oxygen is incorporated to produce PGG2. This intermediate is then reduced by the peroxidase activity of COX-2 to PGH2. During this reaction, oxygen-derived free radicals are produced that can cause oxidative DNA damage, known to contribute to the carcinogenesis process. Furthermore, it has been shown that the overexpression of COX-2 leads to decreased apoptosis as a result of
increased bcl-2 expression (42,60-63). This can extend the survival of initiated cells, increasing time to acquire more mutations and potentially become transformed into a cancer cell. The COX product PGE\textsubscript{2}, which may be instrumental in the ability of COX-2 to contribute to cancer, is not only mitogenic for epithelial cells, but has been shown to have angiogenic activity (64). The ability to induce blood vessel formation allows PGE\textsubscript{2} to contribute to a tumor's growth requirements, thus supporting invasion and metastasis. The involvement of COX-2 and its products in various aspects of cancer suggests that modification of its activity could be beneficial to the treatment or prevention of epithelial derived cancers.

**Cyclooxygenase enzymes**

Two cyclooxygenase enzymes exist, cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2), both of which catalyze the conversion of AA to prostaglandins. COX-1 is a constitutively expressed protein involved in the maintenance of homeostatic conditions. It plays a cytoprotective role in organs such as the skin and gastrointestinal tract, which contain epithelial cells that proliferate and differentiate on a continual basis (65). COX-1 is also involved in platelet aggregation and kidney and renal function. In contrast, COX-2 is an inducible enzyme involved in the immediate-early gene response to various stimuli, including hormones, mitogens, growth factors, and UVB light (43,52,66-68). These enzymes have become of increasing
interest since the discovery that they are the targets for the action of nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin and ibuprofen (69,70).

The COX enzymes, which share similar structures, function as homodimers attached to a lipid bilayer by hydrophobic helices located in the membrane binding domain of the protein (71). The enzymes are located in the endoplasmic reticulum and nuclear membrane and are membrane associated so that AA released from damaged membranes adjacent to the enzyme can enter a hydrophobic channel leading to the active site of the enzyme and be converted to PGs (72). Each subunit of the enzyme contains three major folding domains. An amino terminal epidermal growth factor domain located at the dimer interface functions in proper enzyme folding, a membrane binding domain attaches the protein to a lipid bilayer and forms a channel around the active site of the enzyme into which the AA substrate or inhibitory agents (NSAIDs) enter, and a large carboxy terminal globular catalytic domain contains the cyclooxygenase and peroxidase activities of the enzyme separated by a heme group (73).

**Mechanism of prostaglandin production by cyclooxygenases**

The conversion of AA to prostaglandins and other pathway end products involves several steps. AA released by phospholipases upon membrane damage or one of various other stimuli that induce phospholipases enters the COX active site via the hydrophobic channel. The
first reaction is the cyclooxygenase reaction (bis-oxygenation) of arachidonic acid by the cyclooxygenase function of the enzyme. The insertion of 2 oxygen molecules occurs at the tyr 385 residue located in the hydrophobic channel, and the formation of a 5 member ring structure forms an unstable endoperoxide intermediate, PGG₂. The second reaction involves the formation of more stable PGH₂ from PGG₂ by the peroxidase function of the COX-enzyme, utilizing the heme group in a 2 electron reduction reaction (74). Terminal reductases/synthetases are then responsible for the production of the individual prostaglandin and thromboxane products from PGH₂.

Prostaglandins exit by carrier-mediated transport from the cell in which they were produced, where they interact either in an autocrine or paracrine manner with G-protein-linked receptors (75,76). The binding of prostaglandins to their receptors activates one of several possible second messenger systems, depending on the receptor. For example, PGE₂ can bind one of four receptors, each of which is coupled to different intracellular effector molecules, which can result in the activation or inhibition of adenylate cyclase, activation of PI-specific phospholipase C, or the induction of intracellular calcium levels. The binding of PGE₂ to its receptors mediates the myriad of its effects on the body, including the induction of fever or edema, mediation of the pain response, or the proliferation, migration or activation of several different cell types (75,76).
NSAIDs and cyclooxygenases

Nonsteroidal anti-inflammatory drugs (NSAIDs) like ibuprofen and aspirin, which have analgesic, antipyretic and anti-inflammatory actions, function by inhibiting the cyclooxygenase (COX) enzymes (69,70,77). They prevent the formation of prostaglandins from AA by binding at Arg 120 located about halfway down the hydrophobic channel that surrounds the active site (Fig 1.2) (78). This prevents the entrance of AA into the channel, and subsequently its conversion to prostaglandins.

Recent studies have demonstrated that by inhibiting COX-mediated inflammation, NSAIDs can inhibit tumor formation and induce regression of established tumors in some models (46-51). Limiting the therapeutic potential of these drugs, however, are the significant side effects associated with long-term use of NSAIDs (79). Problems with chronic NSAID use, which develop as a result of inhibiting the homeostatic functions of the COX-1 (70) enzyme include renal dysfunction (80), aberrant platelet aggregation (81) and GI manifestations, including: epigastric pain/indigestion, upper GI tract dyspepsia, and life-threatening peptic ulceration, hemorrhage and perforation (82-83). In US alone, NSAID use is associated with 41,000 hospitalizations and 3300 deaths a year (83). Because of the serious side effects resulting from COX-1 inhibition, COX-2 specific inhibitors such as Celecoxib (Celebrex®, Fig 1.2) were developed. These drugs enable patients who need long-term anti-inflammatory treatments to alleviate pain and inflammation with reduced risk of complications (84-87).
COX-2 Inhibitors

Celecoxib is a diaryl substituted pyrazole-based drug with a sulfonamide substituent, a so-called tricyclic COX-2 inhibitor (Fig 1.2) (88). These drugs take advantage of minor differences in the amino acid sequences of the active site between the COX-1 and COX-2 enzymes. By virtue of a single amino acid substitution, essentially a methyl group, between the two isozymes at position 523 (Ile in COX-1 and Val in COX-2), COX-2 contains a gap in the wall of the channel giving access to a side pocket into which specific COX-2 inhibitors can bind (Fig 1.2) (72,88,89). The importance of the amino acid at position 523 can be demonstrated by studies showing that substitution of Ile with another amino acid at this position in COX-1 allows this enzyme to also be inhibited by COX-2 specific inhibitors (89,90). In addition, COX-2 has a slightly larger hydrophobic channel and a substitution Arg 513 in COX-2 instead of His 513 in COX-1 causes a stable positive charge in the side pocket. These minor changes in COX-2 allow specific COX-2 inhibitors to enter the side-pocket. The diarylheterocyclic COX-2 inhibitors are characterized by polar moieties which form a rigid side extension, usually sulphonyl, sulphone or sulphonamide groups, that can access the side pocket where they interact with Arg 513, preventing the entrance of AA into COX-2 but not COX-1 (Fig 1.2) (72,88,91).
COX-2 inhibitors and skin cancer

As with colon and other types of epithelioid cancers, COX-2 and its prostaglandin products have been shown to be important for UVB induced skin cancer formation (52-54). Prostaglandin production is induced following UV irradiation as a result of both increased release of AA by phospholipases and by the induction of COX-2 message and protein levels (38-41). COX-2 overexpression has been demonstrated in all types of skin cancers, including premalignant lesions such as actinic keratosis (AK), although they contain less COX-2 than malignant lesions (52-54). This points to COX-2 overexpression being an early event in UV induced skin carcinogenesis. In addition, this also suggests a positive correlation with increasing levels of COX-2 expression and increasing potential for invasion and metastasis, or severity of the lesion (52-54). Many older studies have proven the effectiveness of NSAIDs in the prevention of photocarcinogenesis (49-51), but the spawn of specific COX-2 inhibitors has contributed to an increase in skin studies evaluating the potential for these new drugs. In vitro studies have shown the efficacy of specific COX-2 inhibitors in inhibiting the growth of skin cancer cell lines (52,53). In addition, in vivo studies have shown that oral treatment, despite not having an effect on several measures of acute skin inflammation, has the ability to prevent the formation of skin tumors (92,93). The ability of these new inhibitors in both the chemoprevention of
skin cancer show promise, although ideas are still evolving about the best doses and best routes of administration to get the maximum benefit from their actions.

**Cutaneous wounds and the phases of adult repair**

In addition to mediating the ultraviolet light induced inflammatory response, prostaglandins also mediate the inflammatory phase of wound healing, and by doing so, the outcome of the wound healing process, including the restoration of the epidermal lining and scar tissue formation. Like ultraviolet light exposure, skin wounds are a daily occurrence for most and inflammation is a pivotal component of the skin’s response to both events. Cutaneous wounds affect 35 million Americans a year, costing over 7 billion dollars in medical treatment (94). They cause a breach in the skin barrier, allowing microbes and other foreign particles to invade the tissue, eventually causing a permanent scar in the area of the wound. The scar tissue, besides never achieving the same strength as the original tissue (95), impairs the many functions of the skin in the area where the scar tissue is present. Scarring often leads to restricted joint movement, a clear problem for burn victims, impaired growth in children, common in the midfacial area after surgeries to fix cleft lips and palates, and psychosocial consequences of disfigurement (96,97). In addition to causing problems in the skin, scar tissue is the source of fibrotic diseases that can occur in virtually any organ in the body.
The inflammatory phase

The repair of wounds in adult skin is a complex process comprised of three major overlapping phases: inflammation, proliferation, and remodeling. The first requirement after wound healing is to reestablish hemostasis and to stop the bleeding. This is accomplished through the aggregation of platelets, which initiates the inflammatory phase. The wound causes tissue injury and damage to endothelial cells in the blood vessel lining, resulting in the extravasation of plasma proteins and blood cells into the wound tissue. After this initial permeability of the blood vessels, they constrict to help regain hemostasis. The exposure and subsequent binding of platelets to the subendothelial matrix, particularly collagen, causes their activation and aggregation (98). These activated platelets orchestrate the formation of a fibrin clot, which plugs damaged vessels to reestablish hemostasis and forms a provisional matrix into which other cell types and blood vessels can migrate (99). Besides reestablishing hemostasis, activated platelets also initiate the first phase of repair, inflammation. They achieve this by releasing cytokines and growth factors that attract and activate inflammatory cells, particularly platelet derived growth factor (PDGF) (100). This growth factor, which is chemotactic for neutrophils and macrophages, also activates and causes the migration of fibroblasts. The fibroblasts are responsible for regenerating the extracellular matrix (ECM) in the wound area to reestablish dermal integrity.
Neutrophils are the first phagocytic cell to infiltrate the wound site. Neutrophils populate the wound area in response to bacterial products, platelet activating factor (PAF) released by endothelial cells and platelets, platelet derived growth factor (PDGF) also released by platelets, and tumor necrosis factor (TNF) and interleukin-1 (IL-1) released by endothelial and epidermal cells (98,101,102). The neutrophils function to eliminate debris and foreign particles present in the wound area. The activation of neutrophils causes the production of potent oxygen radicals such as hydrogen peroxide (H$_2$O$_2$) and hypochlorous acid (HOCl) used to kill any microbes that may have entered wound site (103). Activated neutrophils also release collagenase and elastase from granules, which degrade the ECM components collagen and elastin. This enables the neutrophils to migrate and to remove damaged tissue (103).

The appearance of monocytes/macrophages in the wound site follows the presence of neutrophils. These inflammatory cells are recruited by ECM fragments, bacterial components, PDGF, and transforming growth factor-β (TGF-β) released by platelets and neutrophils (101,104,105). The high levels of growth factors in the area (PDGF and TGF-β) cause these cells to become "reparative" macrophages, which function to phagocytose and kill invading microorganisms (98,99). The macrophage is believed to be the primary effector cell of adult wound healing because when they are depleted in mice, the animals exhibit defective wound repair (106).
The proliferation phase and collagen deposition

One of the most important purposes of the wound macrophage is to induce the proliferative phase of wound healing, which results in granulation tissue formation (99). They release growth factors that stimulate neovascularization and endothelial cell proliferation (vascular endothelial growth factor/VEGF, fibroblast growth factor/FGF, PDGF and TGF-β), fibroblast migration and proliferation (PDGF, FGF and TGF-β), and epithelial cell proliferation (VEGF, PDGF, and FGF) and migration (TGF-β) (107-116). Granulation tissue, which makes up the wound area in the transition period between the presence of the provisional matrix and collagen scar, is rich in blood vessels, macrophages and fibroblasts (117). The new blood vessels feed the wounded tissue with oxygen and nutrients, the epithelial cells proliferate and migrate to regenerate the barrier of skin over the wound area (reepithelialization), and the fibroblasts repair the damaged ECM. During this process, activated fibroblasts generate even more inflammatory and fibrogenic factors, recruiting more fibroblasts to the area and activating them (118-121) leading to an overproduction of disorganized collagen that makes up scar tissue. A certain percentage of fibroblasts in the wound area are specialized contractile fibroblasts called myofibroblasts, that express α-smooth muscle actin (122). These myofibroblasts, which are induced by TGF-β and are associated with chronic inflammation, help to pull the wound margins together with their contractile filaments (123,124). These cells normally undergo apoptosis after wound closure, but sometimes they persist
The prolonged presence of these cells is associated with fibrocontractive diseases in the skin (122,125,126), lung (127), liver (128), intestine (124), kidneys (129) and heart (130), in addition to the development of abnormal scars in the skin (hypertrophic scars and keloids) (131). They can also cause excessive scar contracture, a particularly serious problem for scars on the skin of burn victims (132).

The remodeling phase and scar maturation

Once the fibroblasts have deposited enough collagen to reestablish dermal integrity, the remodeling phase can begin. Several events occur in the remodeling phase. Collagen maturation occurs with the formation of crosslinks between the collagen fibers and the organization of collagen fibers into bundles, which fibroblasts accomplish by using specialized clefts (95,132). Matrix metalloproteinases (MMPs) are secreted by macrophages, endothelial cells, epidermal cells and fibroblasts, which degrade the collagen (98). During the remodeling phase, which can last for up to 20 years after the initial injury (133), there is a constant cycle of collagen synthesis and degradation. The ultimate result is a mature scar, which in essence patches the break in the tissue. The scar tissue is marked by disorganized dermal collagen arranged in bundles parallel to the skin and the loss of subepidermal appendages such as hair follicles (134). The scar functions to
increase the tensile strength of the skin in the wounded area, however, the
tensile strength of wound tissue never exceeds 70% of the strength of
uninjured skin (135).

**Scarless fetal healing**

In addition to understanding the process of adult wound healing,
knowledge of the events that occur during fetal wound healing is becoming
increasingly important. The number and types of surgeries and invasive
procedures being performed in utero is growing at an alarming rate. In utero
surgical procedures are already established for the repair of hydronephrosis
(136), diaphragmatic hernia (137), sacrococcygeal teratoma (138), and cystic
adenomatoid malformation of the lung (139). In addition, invasive procedures
used for embryonic testing such as amniocentesis can result in fetal wounds,
making it important to understand the potential dangers of inducing fetal
wounds. It is now known that fetal wounds heal in a fundamentally different
manner than adult wounds, resulting in scarless healing of the skin.
However, the information about how the two processes differ is very limited,
making the comparison of the wound healing process in both adults and the
fetus an essential task. A more extensive understanding of the process of
scarless healing could have therapeutic implications in the adult for many
types of fibrotic diseases, eliminating the loss of organ function associated
with excess scar tissue formation in addition to lessening the morbidity
associated with excessive scarring.
In contrast to the adult repair process, first and second trimester fetuses heal skin wounds in a scarless manner. The first observation of this phenomenon in the late 70's (140) sparked the induction of the field of fetal wound healing research. Since then, it has been established that in contrast to the adult, the early fetus repairs skin at an accelerated rate, with no inflammatory phase and little neovascularization, and results in the restoration of skin of the same quality and structure that existed prior to wounding (134). This regenerative process does not lead to the production of an unorganized collagen "patch" as in the adult, but instead leads to a "basketweave" collagen arrangement present in normal skin, with regrowth of hair follicles and other subepidermal appendages (141). The phenomenon of scarless healing is limited both by time and by tissue type. There is a switch late in gestation from scarless healing to adult-like fibrotic healing (141-143), proving skin regeneration is an age related process. In addition, scarless healing seems to be unique to the skin, as diaphragmatic (144-145), stomach (122) and intestinal tract wounds (146) have all been shown heal with a scar, even during the time when the skin heals scarlessly. There are several unique characteristics of the fetus and the fetal environment that could account for scarless healing, including the unique fetal wound matrix, amniotic fluid environment, growth factor profile, or the lack of inflammation.
Fetal wound matrix

The wound matrix in fetal skin has several unique features, namely an abundance of hyaluronic acid and a different ratio of the two major types of collagen found in healing wounds (types I and III). Hyaluronic acid (HA) is a matrix protein that promotes cell migration and proliferation (147) and is deposited during times of high proliferation and regeneration (148). It is produced in both adult and fetal wounds, but in higher amounts and for a prolonged period of time in fetal wounds (149,150), with a higher baseline level in normal fetal skin compared to the adult (151,152). In both types of wounds, HA is deposited early, but it is quickly degraded by hyaluronidase in adult wounds (153,154). In contrast, much more HA is produced in fetal wounds and hyaluronidase is not produced, so HA levels remain elevated (153). In addition to hyaluronate being present in high levels in fetal tissue, it is also present in the amniotic fluid, which may contribute to its rapid deposition during fetal wound healing (155). A factor with hyaluronic acid stimulating activity (HASA) has recently been isolated and has been detected at high levels in amniotic fluid (156) and fetal wounds (149,157) and serum (158), which is not present in adult wounds (149,157). These studies indicate that the high levels of HA found in fetal wound tissue is likely a combination of the high levels of HA in amniotic fluid, normal fetal skin and serum, the lack of hyaluronidase, and the presence of HASA. Studies demonstrating an increase in fibrosis after depleting HA from the fetal wound environment have cemented the idea that HA is an important mediator of scarless healing.
(159). However, topical application of HA to adult wounds cannot block scar formation, so other factors are likely to be involved in this process (160). HA is thought to promote regeneration by providing a matrix suitable for cell migration and proliferation (149), but HA may also be acting to decrease fibrosis in other ways, including by inhibition of the inflammatory phase of wound healing (161,162) or by inhibition of myofibroblast induction (163). However, the precise role of HA in scarless healing must still be defined.

Another matrix component with a different pattern of deposition in fetal wounds is collagen. Fibrillar collagens (types I and III) are the major constituent of the dermal layer of the skin and the disorganized bundles of collagen that form scars. Due to the fact that fetal wounds heal without a scar, it is probable that differences in the way collagen is produced or remodeled exist in fetal wound healing, but this issue has not been well studied. There are discrepancies about the rate and overall amount of collagen deposition in fetal wounds compared to adult wounds. Some groups have shown that fetal fibroblasts are capable of depositing large amounts of collagen efficiently and quickly after wounding (141,164), while other groups have shown the opposite (165). Differences in the ratios of collagen type I to type III present in fetal wounds compared to adult wounds have been demonstrated. Collagen type I, which has a tendency to form thick fiber bundles, makes up the majority of collagen in normal skin and in scar tissue in adults (95). In contrast, there is more collagen type III produced than type I after fetal wounding and it is also more abundant in normal fetal skin.
compared to adults (165,166). In addition, an increase in collagen I deposition correlates to the transition to fibrotic healing (167), indicating that the differences in the ratio of collagen type I to III may be critical to the process of scarless healing. More studies need to be performed to further examine the role of the different collagen types in this process. If the hypothesis that collagen is deposited more efficiently in fetal wounds is correct, then potentially an increase in remodeling or collagen degradation may contribute to scarless healing, although this has not yet been examined.

Fetal environment

The fact that the fetus is bathed in warm, sterile amniotic fluid has also been thought to contribute to scarless healing. Amniotic fluid is rich in growth factors and matrix components such as HA (155). Amniotic fluid has also been shown to inhibit hyaluronidase and increase collagenase (122), both of which could contribute to scarless healing. However, experiments have shown that adult skin heals with a scar even in a fetal environment with amniotic fluid exposure (168). This suggests scarless healing is an intrinsic property of fetal skin rather than the effect of an extrinsic amniotic fluid environment.

Growth factor profile

The growth factor profile of fetal wounds also differs between scarless and fibrotic wounds. One of the most studied wound healing factors to date is
TGF-β. Three isoforms of the growth factor have been shown to be important to the wound healing process, TGF-β 1, 2 and 3, however the most studied isoform is the one most abundant in all tissues, TGF-β 1 (169). These molecules, which are secreted as latent proteins, have been shown to be activated by reactive oxygen species, among other things (169). TGF-β, a chemotactic factor for macrophages and fibroblasts and a mitogen and stimulator of collagen production for fibroblasts, has been shown to promote scar formation in vivo and mice lacking this factor display minimal scar formation after wounding (109,170). In addition, TGF-β is not present in early fetal wounds (171), and adding it to early fetal wounds has been shown to induce inflammation and subsequent scar formation in wounds that would otherwise heal scarlessly (171-174). TGF-β also causes the induction of myofibroblasts (175), and the persistence of these cells has also been shown to be associated with the switch to fibrotic healing in the fetus (176). It is thought that TGF-β promotes fibrosis through a combination of stimulating matrix synthesis (177,178) and inhibiting matrix degradation (179), but as with HA and the fetal amniotic fluid environment, inhibiting TGF-β in adult wounds cannot completely abrogate scar formation (180).

Inflammation and fetal wound repair

Of all of the factors that may contribute to scarless healing, the lack of an acute inflammatory response after fetal wounding appears to be one of the most important. Although the specific components of the inflammatory
response that contribute to scarring have not been defined, there are several studies suggesting the importance of the inflammatory response in fibroplasia and scarring. The first indication that inflammation may contribute to scarring in the skin is the positive correlation between the presence of inflammation in fetal wounds and the switch from scarless to fibrotic healing (181-185). The expression of several mediators that promote inflammation have been found to be absent in fetal wounds, including IL-6 (186) and IL-8 (187). The induction of an acute inflammatory response in fetal wounds, accomplished by adding various inducers of inflammation (carrageenan or turpentine (188), killed or live bacteria (189), and inflammatory cytokines and growth factors like PDGF (190), and TGF-β (171-174), transforms the process of scarless healing into one of fibrosis. This demonstrates the ability of the fetus to mount an inflammatory response, so the reason for a lack of inflammation in fetal wounds is not due to immature inflammatory cell activity. In addition, elevated levels of cytokines that inhibit the inflammatory response, such as interleukin-10 (IL-10) may play a role in the scarless phenotype. Fetal skin from IL-10 knockout mice heals with inflammation and fibrosis when transplanted onto the backs of adult mice, whereas fetal skin from the background strain heals in a scarless fashion (191). Moreover, the anti-inflammatory and antioxidant pentoxifylline has been shown to decrease the production of collagen giving further evidence for inflammation as a regulator of fibrosis (192). Although several studies suggest a role for
inflammation in fibrosis, this idea must be investigated further to define what specific components of inflammation are responsible for promoting fibrotic healing.

Two specific mediators of inflammation likely to be involved in the promotion of scar formation in adult skin by inflammation are prostaglandin E₂ (PGE₂) and hydrogen peroxide (H₂O₂). PGE₂ is a lipid mediator formed from the conversion of arachidonic acid by the cyclooxygenase enzymes. Inflammatory stimuli induce the release of arachidonic acid from membrane phospholipids by inducing the activity of phospholipases and also induce the production of the cyclooxygenase-2 enzyme, both of which contribute to an increase in PGE₂ during inflammatory events (38-41). In the skin, PGE₂ has been shown to contribute to the immunosuppressive effects of UVB light exposure (193), but this contradicts other studies that suggest a role for PGE₂ as a proinflammatory molecule (194,195). PGE₂ is present in high levels in amniotic fluid and is thought to be immunosuppressive, functioning to decrease the likelihood of fetal rejection by the mother (196). Although PGE₂ was initially thought to contribute to the lack of inflammation seen during fetal wound healing through inhibiting the immune response (196), it was later shown to induce inflammation in fetal wounds (197). However, this later study did not examine the effect of the PGE₂ induced inflammation on the induction of wound fibrosis. It is possible that the activity of this molecule differs depending on the types of receptors present, the organ in which it is being produced, or the age of the host. In the adult, PGE₂ and other lipid
peroxidation products have been shown to increase collagen production and contribute to liver fibrosis (198,199). In addition, prostaglandin production by the cyclooxygenase enzymes generates as byproducts reactive oxygen intermediates (200) which may also contribute to fibrosis (198,199). Therefore, the true role of PGE₂ in both adult and fetal wound healing remains to be clarified.

In addition to pro-inflammatory mediators, reactive oxygen species generated by activated inflammatory cells may contribute to scar formation. Reactive oxygen intermediates such as H₂O₂ are produced by inflammatory cells to kill microbes that may have entered the skin upon wounding. Activated fibroblasts and other cell types important for wound healing can also release reactive oxygen species (201), which are also thought to mediate the actions of the pro-fibrogenic TGF-β (201,202). These substances, although important for eliminating infection can also damage surrounding tissue and host cells, initiating further inflammation and oxidant production (203). Oxidants produced by inflammatory processes have long been thought to contribute to uncontrolled proliferation involved in the development of cancer, although their importance in the uncontrolled proliferation and collagen production in fibroblasts associated with fibroplasia is not well documented. However, there are a few studies supporting the idea of reactive oxygen species contributing to fibrosis in that antioxidants have been shown to be anti-fibrogenic (204-206) and are even being tested as a treatment for liver fibrosis (207). Although the suggestion has been made
that reactive oxygen species may contribute to fibrosis, the present studies are the first to examine their role in scar formation using a cutaneous fetal wound healing model.

The goals of the studies presented in the following chapters were to examine of the role of specific components of the inflammatory response on both ultraviolet light induced skin carcinogenesis and on the process of scar formation during cutaneous wound healing. Specifically, the hypothesis for these experiments were that the COX-2 enzyme is involved in the UVB-induced carcinogenesis process and in scar formation as a result of cutaneous wounds. The results of these studies show that both tumor formation and scar formation in the skin can be altered through the inhibition of the inflammatory response, specifically through the modulation of the cyclooxygenase-2 enzyme.
Figure 1.2. The structure of the cyclooxygenase enzymes and chemical structure of Celecoxib. The important binding sites and structural differences of the COX isoforms is demonstrated in A. The chemical structure of the heterocyclic COX-2 inhibitor Celecoxib is shown in B.
Membrane-bound Phospholipid

\[ \text{DAMAGE} \rightarrow \text{Phospholipases (PLA)} \]

Arachidonic Acid

\[ \text{COX-1} \]

\( \text{COX-2} \)

(cyclooxygenase reaction)

\[ \text{COOH} \]

\[ \text{Peroxidase reaction} \]

\[ \text{COOH} \]

\[ \text{OOH} \]

\[ \text{OH} \]

\[ \text{PGH}^2 \]

Individual synthase enzymes

\[ \text{PGE}_2 \]

\[ \text{PGF}_{2\alpha} \]

\[ \text{PGI}_2 \]

\[ \text{TXA}_2 \]

\[ \text{PGD}_2 \]

Figure 1.1. Pathway of prostanoid synthesis. During inflammation, phospholipase enzymes release AA from membrane phospholipids, where it undergoes a series of modifications by the COX enzymes, ultimately leading to the formation of prostaglandins and thromboxanes.

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MATERIALS AND METHODS

A. Quantitation of myeloperoxidase in cutaneous tissue

Myeloperoxidase (MPO), an enzyme that converts hydrogen peroxide to hypochlorous acid, is released by activated neutrophils during inflammatory events. The levels of MPO in cutaneous tissue were determined and used as a measure of the extent of neutrophil activation. Skin punches were isolated from three mice per group using a 10-mm-diameter cork borer. The punches were homogenized in 1.25 mL of 0.5% hexadecyltrimethylammonium bromide in 50 mM potassium phosphate buffer, pH 6.0. The skin was homogenized at 4°C and subjected to three cycles of sonication, freezing, and thawing. The samples were then centrifuged for 30 min at 14,000 rpm and 4°C. The supernatants were transferred to new tubes and 10 µL of each was transferred to individual wells of a 96-well microtiter plate with 290 µL of substrate (0.167 mg/mL 0-dianisidine dihydrochloride and 0.0005% H₂O₂ in 50 mM potassium phosphate buffer).
phosphate buffer, pH 6.0). MPO activity was measured spectrophotometrically over a 5 min period at 450 nm with a programmable microplate reader (Molecular Devices, Menlo Park, CA). The data are expressed as mean units of MPO activity from three 10-mm skin punches, where the amount of MPO required to degrade 1 μmol of peroxidase/min at 25°C is equal to one unit of MPO activity. The content of MPO activity in each sample was calculated based on a standard curve.

B. Histology, immunohistochemistry and in situ

Immediately following sacrifice, skin sections (0.5 cm²) were placed in 10% neutral buffered formalin for two hours, washed with PBS, processed and embedded in paraffin blocks. Tissue sections (5 μm) were cut and mounted onto ProbeOn Plus microscope slides (Fisher Scientific, Pittsburgh, PA). The tissue sections were then deparaffinized using Clear-Rite 3 (Richard-Allan Scientific, Kalamazoo, MI) and rehydrated in a graded series of alcohols. Histochemical and immunohistochemical staining utilized the Microprobe Manual Staining System (Fisher Scientific).

C. Immunohistochemical detection of COX-1, COX-2 and Ly-6G protein

Following rehydration, tissues were either rinsed in Automation Buffer (Biomeda Corp, Foster City, CA) for the detection of neutrophils using an Ly-6G antibody, or heat-treated for 15 min in distilled, deionized water microwaved to boiling for COX-1 and COX-2 detection. Non-specific binding was blocked with Casein Solution (Vector Laboratories, Burlingame, CA) for
30 min. After every step that follows, slides were rinsed in Automation Buffer. Primary antibodies were placed on the tissue under the following conditions: monoclonal rat anti-mouse Ly-6G (PharMingen, San Diego, CA), 1:400 dilution, 1 hr; polyclonal rabbit anti-mouse COX-1 (Cayman Chemical, Ann Arbor, MI), 1:50 dilution, 2 hr; polyclonal goat anti-rat COX-2 (Santa Cruz Biotechnologies, Santa Cruz, CA), 1:800 dilution, 1 hr. The tissue was then incubated with a 1:200 dilution of the appropriate biotinylated secondary antibody (Vector) for 30 min, followed by an avidin/biotinylated horseradish peroxidase complex (ABC Elite, Vector) for 30 min. The chromagen, 3, 3’ diaminobenzidine solution (DAB, Vector), was placed on the tissue for 4 min (COX-1 and COX-2) or 10 min (Ly-6G). DAB development was stopped by rinsing with distilled water, then the tissue was counterstained with Harris hematoxylin (Shandon, Pittsburgh, PA) for 1 min. Slides were then dehydrated, mounted, viewed and photographed. The ImagePro (Media Cybernetics, Atlanta, GA) analysis program was used to quantitate the number of positive cells in ten 60X fields in each tissue section, one tissue section per mouse.

D. Hematoxylin and Eosin Staining and Measurement of Apoptotic Sunburn Cells

After rehydration, tissue sections were stained for 1 minute in Hematoxylin 2 (Richard-Allan Scientific, Kalamazoo, MI). Following a 2 minute wash in distilled water, the slides were dipped into Clarifier 2
(Richard-Allan Scientific), water-rinsed for 30 seconds, dipped 10 times in ammonia water, then rinsed again for 30 seconds in water. After 10 dips in 70% alcohol, the tissue was stained for 30 seconds in Eosin-Y (Richard-Allan Scientific), dehydrated in a series of alcohols, and placed in Clear-Rite 3 (Richard-Allan Scientific) for 10 minutes. Apoptotic sunburn cells were identified in the epidermis by their intensely eosinophilic cytoplasm and small, dense nuclei and quantitated using Image Pro (Media Cybernetics) image analysis software. The data are expressed as the mean number of sunburn cells counted in ten 60X fields per tissue section, one tissue section per mouse.

E. Immunohistochemical detection of p53 and PCNA protein

Following rehydration, an antigen retrieval process was performed by microwaving twice for 5 minutes in antigen unmasking fluid (Vector, Burlingame, CA) with a 3 minute cooling period in between. After cooling at room temperature for 15 minutes, the sections were washed in automation buffer (Biomeda Corp., Foster City, CA) and blocked with casein (Vector). The tissue was incubated with primary anti-mouse p53 (1:300; Vector) or PCNA (1:100; Signet Pathology Systems, Dedham, MA) antibody diluted in casein for 90 minutes at room temperature. For the control, sections were incubated with rabbit IgG (p53) or mouse IgG2a (PCNA) under the same conditions. The tissue was washed with automation buffer then incubated for 30 minutes at room temperature with Rabbit link solution (BioGenex, San
Ramon, CA), then label solution (BioGenex) for p53 or for 30 minutes with ABC solution (Vector) for PCNA. After washing with automation buffer, incubation with DAB solution (Vector) for 5 minutes and a final wash in distilled water, the tissue was counterstained with hematoxylin, dehydrated and mounted. The ImagePro (Media Cybernetics, Atlanta) analysis program was used to quantitate the number of positive cells in ten 60X fields per section.

F. In situ detection of 8-oxo-dG adducts

The following protocol utilized the TSA amplification system (NEN Life Sciences, Cambridge, MA) for the detection of antibody binding as described by Hougaard et al for in situ hybridization (208). Following rehydration, sections were incubated with 3% H$_2$O$_2$ for 15 minutes to quench endogenous peroxidase activity followed by washes in PBS and 0.1M Tris-HCl, pH 8.0. For long-term UVB samples which had a thickened epidermal layer, an additional step was necessary to achieve permeabilization of the skin. For these samples, the sections were boiled twice for 5 minutes in antigen unmasking fluid (Vector) before incubation with 3% H$_2$O$_2$. The tissue was then incubated with 10 µg/ml proteinase K (Sigma, St. Louis, MO) in 0.1 M Tris-HCl containing 0.05M EDTA, pH 8.0 (1 hour, 37°C), washed in PBS, then incubated with the following: 4N NaOH (7 min), 50 mM Tris-base (5 min), 10% FBS in 10 mM Tris-HCl, pH 7.5 (1 hour, 37°C), and anti-8-oxo-dG antibody (Trevigen, Gaithersburg, MD) diluted 1:150 in 10% FBS/Tris-HCl.
pH 7.5 (overnight in humid chamber, 4°C). The following day, the sections were washed in PBS, incubated with biotinylated rabbit anti-mouse IgG (DAKO Corp., Carpinteria, CA) diluted 1:500 in PBS-BSA (10% BSA in PBS; 30 minutes), washed with PBS, and incubated with TNB buffer (0.1M Tris-HCl, pH 7.5, 0.15M NaCl, 0.5% Dupont blocking reagent) for 30 minutes followed by HRP-labeled streptavidin (DAKO Corp.) diluted 1:500 in TNT buffer (0.1M Tris-HCl, pH 7.5, 0.15M NaCl, 0.05% Tween 20; 30 minutes). For fluorescent detection of HRP, the fluorescent tyramide amplification and detection system was used (NEN, Boston, MA). The sections were washed in TNT buffer, incubated with fluorescent tyramide diluted 1:50 in amplification diluent (10 minutes), washed in TNT buffer, and mounted with Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL).

G. Visualization of collagen: Masson's trichrome

Reagents from the Accustain Masson's trichrome stain kit (Sigma, St. Louis, MO) were utilized for the following procedure. Rehydrated wound sections were incubated for 15 minutes in preheated Bouin's solution (56°C). The slides were then cooled in distilled water for 2 minutes, and washed in distilled water prior to staining for 5 minutes in Weigert's iron hematoxylin solution. After washing again in distilled water, the sections were stained with Bebrich scarlet-acid fuchsin for 5 minutes and washed in distilled water, followed by 5 minute stainings with phosphotungstic/phosphomolybdic acid solution then aniline blue solution. After a 2 minute incubation in 1% acetic
acid, the slides were dehydrated and mounted. The tissue was visualized using a Nikon DXM1200 digital camera and scar width was measured with a stage micrometer.

H. RNA extraction and reverse-transcription-polymerase chain reaction

Total RNA from dorsal skin that had been snap frozen in liquid nitrogen was isolated using TRIzol reagent (Gibco BRL, Grand Island, NY). The tissue was homogenized in 3.5 mL of TRIzol, 0.7 mL of chloroform was added, then the samples were centrifuged to separate the organic and aqueous phases. The aqueous phase was transferred to a new tube and the RNA was precipitated with isopropanol. The RNA was centrifuged, washed with ethanol, and pelleted. The pellets were air dried, resuspended in Molecular Biology Grade water (5'→3', Boulder, CO) and heated to promote resuspension. Spectrophotometric analysis was used to determine the RNA concentration of the samples, then 2 µg of RNA was electrophoresed on a 1.2% agarose-formaldehyde gel and stained with ethidium bromide to determine the integrity of the 18S and 28S ribosomal RNA. All of the following RT-PCR reactions were carried out using a Hybaid PCR Express Thermocycler (Marsh, Rochester, NY). RNA samples (2 µg/10 µL) were denatured for 5 min at 65°C, and cDNA was generated using a mixture of 1X PCR Buffer II (Gibco BRL), 5 mM MgCl₂, 1 mM dNTP’s, 2.5 µM Oligo dT’s, 1 unit RNasin (Gibco BRL) and 100 units Maloney Murine Leukemia Virus-Reverse Transcriptase (MMLV-RT, Gibco BRL). Denatured RNA (10 µL) was
added to 30 µL of the above mixture, and incubated at 37°C for 60 min to elongate, then 95°C for 5 min to inactivate the enzymes. HPRT (2.5 µL), COX-1 and COX-2 (5 µL) cDNA was amplified twice per sample using 1X PCR Buffer II, 2.5 mM MgCl₂, 0.2 mM each of dATP, dCTP, dGTP, and dTTP (Promega, Madison, WI), 200 pmol 5′ primer, 200 pmol 3′ primer and 0.25 units Platinum Taq DNA polymerase (Gibco BRL). Amplification was carried out using the following protocol: 1) initial denaturation: 95°C for 30 sec; 2) 28 (COX-1), 30 (HPRT), or 35 (COX-2) cycles of a 95°C 30 sec denaturation, 60°C (COX-2 and HPRT) or 62°C (COX-1) 30 sec annealing step, and a 72°C 30 sec elongation; 3) final elongation: 72°C for 7 min. The sequences for the primers used are as follows: HPRT (5′-GTA ATG ATC AGT CAA CGG GGG AC-3′, 5′ primer; 5′-CCA GCA AGC TTG CAA CCT TAA CCA-3′, 3′ primer), COX-1 (5′-AGG AGA TGG CTG CTG AGT TGG-3′, 5′ primer; 5′-AAT CTG ACT TTC TGA GTT GCC-3′, 3′ primer), and COX-2 (5′-GGG CCA TGG AGT GGA CTT AAA TCA-3′, 5′ primer; 5′-TTC CTT CTC TCC TGT AAG TTC TTC-3′ primer). HPRT (177 bp), COX-1 (604 bp) and COX-2 (790bp) were visualized on ethidium bromide stained 2.0% agarose gels (Sigma, St. Louis, MO). Images of the gels were captured using a Kodak DC120 digital camera and analyzed using Kodak1D image analysis software (Eastman Kodak, Rochester, NY). The net intensities of the PCR products of COX-1 or COX-2 were compared to those of HPRT to give a ratio of gene
expression compared to the housekeeping gene. Each measurement is the average of two separate RT-PCR reactions of each sample isolated from each treatment group.

I. Protein isolation and enzyme immunoassays

Total protein was isolated from dorsal skin that had been snap frozen in liquid nitrogen by homogenization in buffer containing the following final concentrations: 2 mM PMSF, 5 mM each of DTT, EDTA and EGTA, 10 mM sodium molybdate, 60 mM Tris-HCl, 300 mM sucrose, 20 µg/mL aprotinin, and 200 µg/mL leupeptin (209). After adjusting the pH to 8.6, the buffer was filter sterilized. Following homogenization, the tissue was sonicated, centrifuged, and the supernatants were transferred to new tubes. After a final centrifugation and transfer, protein concentrations were determined using the BIO-RAD protein assay (BIO-RAD, Hercules, CA). Biotrak enzyme immunoassays (EIA) (Amersham-Pharmacia, Piscataway, NJ) were used to determine the concentrations of PGE2 (a major product of COX-2). Eight micrograms of total protein was used for the EIA, which was carried out as outlined in the manufacturer’s protocols. A SpectraMax 190 microplate reader (Molecular Devices, Sunnyvale, CA) was used to read the EIA plates.

Due to the extremely small amounts of tissue isolated for fetal wounds, the protein was isolated from frozen skin by grinding in liquid nitrogen. The powder was then dissolved in 750 µl of the homogenation buffer and the rest of the procedure remained as described above.
J. SDS-PAGE, Westerns and image analysis

Detection of COX-2 and TGF-β1 protein was carried out using the Criterion gel apparatus and Semi-dry transfer cell from Bio-Rad. Laemmli buffer containing β-mercaptoethanol (Bio-RAD, Hercules, CA) was added to samples of 40 μg of total protein or biotinylated protein standards (Bio-Rad) and boiled for 3 minutes. The samples were then loaded in a 10% Tris-HCl Criterion pre-cast gel and electrophoresed at 200V for 50 minutes (COX-2) or 30 minutes (TGF-β1). The gels were removed and were equilibrated along with pre-cut Immobilon-P (Millipore, Bedford, MA) membranes in transfer buffer (Tris/Glicine, Bio-Rad) for 15 minutes. The proteins were transferred from the gel onto the membrane in the semidry transfer cell at 20V for 30 minutes. After the transfer, the gels were soaked in Coomassie stain for 30 minutes and destained overnight while the membranes were blocked with TBS/Casein/Tween 20 for 15 minutes, washed 3 times with TBS/Tween 20 and incubated with primary antibody overnight with agitation at 25 degrees (COX-2, Cayman, 1:100 dilution; TGF-β1, Santa Cruz, 1:150 dilution). The next day, the membrane was washed in TBS/Tween 20, incubated with 1 hour with the appropriate biotinylated secondary antibody (Vector, 1:200 dilution), for 30 minutes with ABC solution (Vector), then for 10 minutes with DAB solution (Vector) with washes between each step. DAB development was stopped after 10 minutes by washing with distilled water, then the
membrane was dried. The coomassie stained gels and developed membranes were scanned and analyzed using Kodak 1D image analysis software (Eastman Kodak).

K. DNA isolation and 8-oxo-deoxyguanosine (8-oxo-dG) quantification

DNA was isolated from tissue that had been snap frozen in liquid nitrogen using the QIAamp DNA Mini Kit (Qiagen Inc., Valencia, CA) as directed by the manufacturer for tissue samples. After isolation, the DNA was hydrolyzed and used for ELISA analysis of 8-oxo-dG adducts with the highly sensitive 8-OHdG ELISA kit as specified by the manufacturer (Japan Institute for the Control of Aging, Shizuoka Pref., Japan). This kit has been used extensively in studies for the detection of 8-oxo-dG in various tissues and body fluids (210). Briefly, the standards and samples were added to wells of the supplied 8-OHdG coated 96-well plate. An anti-8-OHdG antibody was added to the wells and the plate was incubated overnight at 4°C. The next day, the plate was washed with PBS, and then a 1 hour incubation with the secondary antibody was performed at room temperature. After a second wash, the enzyme substrate solution was added to the plate and incubated for 15 minutes in the dark. Approximately 10 minutes after the addition of the reaction terminating solution, the absorbance was read at 450 nm. A standard curve was constructed from which the sample concentrations (ng/ml) were calculated.
L. Determination of hydroxyproline content

10mm punches that had been frozen in liquid nitrogen were homogenized in 2 ml PBS and stored overnight at 4°C. The following day, 1 ml of the homogenate was hydrolyzed in 0.5 ml 6N HCl at 120°C for 4.5 hours. After allowing the samples to cool for 10-15 minutes, 20 μl of each sample was added to a 96 well plate. 50 μl of Chloramine T solution (282 mg Chloramine T (Sigma), 2 ml n-propanol, 2 ml distilled water, 16 ml citrate-acetate buffer (5% citric acid, 7.24% sodium acetate, 3.4% sodium hydroxide, and 1.2% glacial acetic acid)) was added to each sample and incubated for 20 minutes at room temperature, after which 50 μl Ehrlich’s solution (2.5 g 4-(dimethylamino)benzaldehyde, 9.3 ml n-propanol, and 3.9 ml 70% perchloric acid was added. The samples were then incubated at 65°C for 15 minutes, cooled for 10 minutes and the plate was read at 550 nm on a SpectrMax 190 (Molecular Devices) microplate reader. A standard curve of hydroxyproline (Sigma) was constructed and the concentrations of hydroxyproline in each sample was calculated.

M. Statistical analysis

The StatView program (Abacus Concepts, Berkeley, CA) was used to statistically analyze differences in the data via the paired student’s t-test. Statistical significance refers to a p value < 0.05 using the paired student’s t-test.
CHAPTER 3

INHIBITION OF ACUTE UVB-MEDIATED INFLAMMATION BY CELECOXIB

Introduction and Rationale:

Ultraviolet B (UVB) radiation is responsible for the majority of cutaneous damage following both acute and long-term exposure, and is believed to be the most important etiologic agent in human skin cancer. UVB carcinogenesis initially induces an inflammatory response characterized by edema, dermal infiltration of leukocytes, as well as the production and release of prostaglandins, which may be critical to the observed damaging effects of UVB light on skin. This inflammatory response is now thought to contribute to tumor formation in the skin. Recently, a specific cyclooxygenase-2 (COX-2) inhibitor, Celecoxib, was developed. This drug was designed to inhibit COX-2-mediated inflammation without inhibiting the cytoprotective function of cyclooxygenase-1 (COX-1), thereby eliminating the side effects associated with COX-1 inhibition. Studies have demonstrated that oral administration of Celecoxib decreased the incidence of skin and colon tumors, providing further support for the idea that inflammation...
contributes to carcinogenesis. We hypothesized that topical administration of Celecoxib would be a better choice for inhibiting tumor formation in the skin than oral administration, as topical treatment would target the organ of interest directly and eliminate any problems that may occur with high systemic doses of this drug. Because this drug was designed for oral use, we first had to characterize its effects on the skin with topical use. The goal of the present studies was to characterize the effects of topical Celecoxib treatment on the acute UVB induced inflammatory response so that if effective, further studies examining the ability of topical treatment with this specific COX-2 inhibitor to act as a chemopreventative and chemotherapeutic agent could be carried out.

Experimental design:

To examine the ability of Celecoxib (Celebrex®, Searle-Monsanto, St. Louis, MO) to decrease acute inflammation following exposure to UVB light, female Skh/hr hairless mice (8-10 weeks, 26-28 g, Charles River, Wilmington, MA) were divided into five treatment groups (where n=8 mice per treatment group). The mice were housed in the vivarium at the Ohio State University according to the requirements established by the American Association for Accreditation of Laboratory Animal Care. All procedures were approved prior to beginning the study by the appropriate Institutional Animal Care Utilization Committee. The control mice were treated topically with either acetone vehicle (0.2 ml) or 500 µg of Celecoxib dissolved in acetone.
(0.2 ml). Irradiated mice were exposed dorsally to Phillips FS40UVB lamps (American Ultraviolet Company, Murray Hill, NJ), which provide predominantly UVB light (290-320 nm). Immediately after exposure to 2240 J/m² of UVB light (one minimal erythemic dose), mice were treated topically with 0.2 ml of acetone as a vehicle control or 100 or 500 µg of Celecoxib in 0.2 ml of acetone. The mice were sacrificed 24 hours after a single treatment or at 1 week, 24 hours after a total of three treatments. Four mice per group were sacrificed for each timepoint. Directly after sacrifice, dorsal skin thickness was measured with calipers. In addition, skin sections were either used immediately for MPO measurement, frozen in liquid nitrogen for later RNA, DNA and protein isolation, or fixed in 10% neutral buffered formalin for histochemical analysis.

Results:

A. Measurement of skin thickening

Skin thickness was used as a measure of vascular permeability and edema, two hallmarks of the UVB-induced cutaneous inflammatory response. Dorsal exposure of Skh/hr hairless mice to UVB resulted in a time dependent increase in skin thickening, which was significant compared to Acetone controls (**p<0.05; Fig. 3.1). Topical treatment with Celecoxib immediately following UVB exposure resulted in a dose dependent decrease in UVB mediated edema. The decrease in skin thickening compared to
UVB/Acetone controls was statistically significant following a topical treatment with 500 µg of Celecoxib at both 24 hours and 1 week (*p<0.05).

B. Biochemical determination of the inhibitory effect of Celecoxib on neutrophil-associated myeloperoxidase levels

The ability of a topical application of Celecoxib to inhibit the activation of neutrophils, measured by the determination of myeloperoxidase (MPO) levels was evaluated. MPO is an enzyme released by activated neutrophils that converts hydrogen peroxide to hypochlorous acid, a potent microbicidal agent. As shown in Fig. 3.2, exposure to UVB light (UVB/Acetone) significantly (**p<0.05) increased the amount of MPO detected within the skin of mice at 24 hours and 1 week following exposure as compared to levels detected in skin isolated from unirradiated mice treated with Acetone or Celecoxib. Topical treatment with Celecoxib decreased the MPO levels induced by UVB in a dose-dependent manner at 24 hours and both doses diminished the activity to near control levels at 1 week (*p<0.05).

C. Quantitative assessment of the effects of Celecoxib on UVB induced dermal neutrophil infiltration

To determine whether the decrease in MPO levels with Celecoxib treatment was a result of decreased neutrophil activation or to decreased neutrophil presence in the skin, immunohistochemical analysis in conjunction with an Ly-6G antibody was used to identify dermal neutrophils. Quantitation
of the number of Ly-6G positive cells within 10 high power (60X) fields demonstrated a significant increase in the number of neutrophils within the dermis at 24 hours following UVB exposure compared to the number of neutrophils within the dermis of non-irradiated skin (**p<0.05, Fig. 3.3A and B). Topical treatment with 500μg of Celecoxib (Fig. 3.3A and C) significantly inhibited neutrophil infiltration as detected by a decrease in the number of Ly-6G positive cells.

D. Effect of Celecoxib on COX gene expression following UVB exposure

The ability of Celecoxib to inhibit COX-2 gene expression was determined by RT-PCR analysis. Exposure to UVB irradiation resulted in a time dependent increase in COX-2 gene expression in Skh/hr skin following UVB exposure while COX-1 mRNA levels remained unchanged (Fig. 3.4). Topical treatment with Celecoxib did not affect COX-1 or COX-2 gene expression at any time point examined, which was expected as the drug acts by inhibiting COX-2 enzyme activity rather than decreasing its transcription.

E. Immunohistochemical localization of COX protein

Immunohistochemical analysis of skin isolated from Skh/hr hairless mice demonstrated an increase in COX-2 protein levels within the epidermis at 24 hours and 1 week following UVB exposure. At 24 hours following UVB exposure/topical treatment, COX-2 specific cytoplasmic and perinuclear
staining was evident within the epidermis and hair follicles (Fig. 3.5) while only low levels were detected in unirradiated skin. Topical treatment with Celecoxib (500 μg) had no affect on the level of epidermal COX-2 staining compared to UVB/Acetone skin. No changes in COX-1 staining were detected in response to UVB exposure or topical treatment with Celecoxib.

F. EIA detection of PGE₂ in UVB irradiated and Celecoxib treated skin

Levels of PGE₂ in treated murine skin were determined as an indirect measure of COX-2 activity. Exposure to UVB irradiation resulted in an increase in PGE₂ levels in isolated skin at 24 hours. Statistically significant inhibition of UVB induced PGE₂ levels following treatment with 500 μg Celecoxib was first evident 24 hours following a single treatment (Fig. 3.6, *p<0.05). Although there was no difference in the levels of PGE₂ between unirradiated and irradiated skin at 1 week, topically applied Celecoxib was able to decrease PGE₂ levels below those seen following Acetone treatment alone at 1 week. Acetone, the vehicle used in the present study, is itself an irritant capable of stimulating the production of low levels of PGE₂ within the skin. Topically applied Celecoxib was able to decrease PGE₂ levels below the slight increase in PGE₂ seen with Acetone treatment alone at 1 week.

G. Detection and quantitation of epidermal sunburn cells

Exposure of the skin to UVB results in the development of sunburn cells, shown to be apoptotic cells within the epidermis (28-30). At 24 hours
image analysis assisted quantitation of the number of sunburn cells within 10 high power (60X) fields revealed a high presence of sunburn cells in mice exposed to UVB (UVB/Acetone, Fig. 3.7). Topical treatment with 500 µg of Celecoxib (*p<0.05) significantly decreased the number of sunburn cells within the epidermis compared to vehicle treated mice.

**H. Detection and quantitation of p53 positive epidermal cells**

Immunohistochemical techniques and image analysis were used to examine the level of p53 expression in epidermal cells as a general indication of the amount of DNA damage. The antibody used does not distinguish between wild-type and mutated p53, although at early timepoints such as these it is not likely that the p53 protein is mutated. UVB exposure resulted in the presence of p53 positive epidermal cells compared to unirradiated skin that had none (Fig. 3.8). Topical treatment with Celecoxib immediately after irradiation diminished UVB-induced p53 protein levels in a dose-dependent manner, suggestive of a decrease in DNA damage.

**I. Detection and measurement of 8-oxo-dG DNA adducts**

To ensure that the decrease in p53 with Celecoxib treatment was a result of the cells containing less DNA damage, we investigated by two different methods the formation of the most common oxidative DNA adduct, 8-oxo-dG. This adduct can be formed as a result of reactive oxygen species such as hydrogen peroxide released by inflammatory cells and by epidermal...
keratinocytes upon activation by an inflammatory stimulus. Topical treatment with Celecoxib resulted in a dose dependent reduction of 8-oxo-dG adducts detected by both ELISA and immunohistochemical analysis in comparison to the irradiated control animals (Fig. 3.9 and 3.10). Immunohistochemical analysis demonstrated that adducts were localized to the basal epidermal cells and the bulge region of the hair follicles of irradiated skin.

Discussion

It is now recognized that inflammation is a major component of carcinogenesis. The current study demonstrates the efficacy of topically applied Celecoxib on the UVB-induced cutaneous inflammatory response, suggesting this may be a useful treatment for inhibiting the development of skin tumors following chronic UVB exposure. Topical treatment with Celecoxib significantly reduced edema, myeloperoxidase levels, and PGE$_2$ levels in the skin induced by UVB. In contrast to the data presented here, a recent study by Fischer et al (93) showed that oral treatment with Celecoxib had no effect on acute UVB-induced inflammation. In addition, several older studies using oral NSAID administration have shown that unlike topical treatment, the oral route of administration is not an effective means of inhibiting cutaneous inflammation following UVB exposure (49-51). These studies and our data support the hypothesis that topically applied Celecoxib, with its ability to inhibit the UVB mediated acute inflammatory response, may be better at controlling the formation of tumors after repeated exposure to
UVB. In addition, the drug was applied after UVB exposure, suggesting that this could be an effective secondary means of eliminating skin inflammation and the resulting skin damage following irradiation if sunscreen was not used or was not effective. By inhibiting PGE$_2$ levels in the skin, known to be a component of the pain response to inflammation, Celecoxib could not only be used to reduce the edema, vascular permeability, cytokine production, and leukocyte infiltration seen following sun exposure, but it could also be useful in managing the pain of a sunburn.

The acute UVB-induced inflammatory response in the skin is characterized by the rapid induction of COX-2 gene expression with the subsequent production of prostaglandins, including PGE$_2$, resulting in dermal edema and the infiltration of activated neutrophils into the dermis (56). Edema or swelling, which results from an increase in vascular permeability is one of the first physical signs of an acute UVB mediated cutaneous inflammatory response. This increase in vascular permeability allows inflammatory cells, such as neutrophils, from the peripheral circulation to migrate into the local tissue site. The magnitude of the edematous response can be measured by an increase in skin thickening. In the present study the increase in the edematous response was abated by topical treatment immediately following UVB irradiation with Celecoxib. Neutrophils, key components of an acute inflammatory response, are the first cell type to infiltrate into the dermal region following exposure to UVB light. Because neutrophil derived reactive oxidants are cytotoxic, immunosuppressive, and carcinogenic, these agents
are potential mediators of UVB-mediated tissue damage and tumorigenesis. Myeloperoxidase (MPO), an enzyme which converts hydrogen peroxide released by neutrophils to hypochlorous acid, has been used as a marker for neutrophil activation (36). We used an assay to quantify MPO levels as a measure of neutrophil activation within the dermis and found that the increase in MPO activity observed following UVB exposure was significantly decreased by topical treatment with Celecoxib. However, immunohistochemical analysis demonstrated that Celecoxib inhibited the number of neutrophils that had infiltrated the dermis, suggesting this drug not only inhibits the activation of neutrophils, but also inhibits the infiltration of neutrophils into the skin, as opposed to other drugs which have shown to decrease neutrophil infiltration without affecting their activation (211). This data suggests that topical treatment with Celecoxib may diminish the damaging effects of neutrophil products on the skin during an acute inflammatory response.

Exposure to UVB light results in an immediate induction of COX-2 gene expression followed by increases in protein levels within the skin. Stimulation with UVB light also increases the release of arachidonic acid from membrane phospholipids by phospholipases (38-41). Together, these events contribute to the production of high levels of prostaglandins, particularly PGE₂, which plays an important role in the skin. While low levels of PGE₂ in the skin are needed for the normal homeostasis of this organ, elevated levels of PGE₂ have been shown to have a number of detrimental
effects. PGE$_2$ has been shown to play a key role in enhancing the UVB induced inflammatory process, including the induction of erythema and pain (76) and mediating the suppression of contact hypersensitivity seen in UVB exposed skin (193). In addition, studies have shown that PGE$_2$, which stimulates DNA, RNA, protein and collagen synthesis, can act as a tumor promoter in murine skin and may also play a role in tumor progression (55). In fact high levels of PGE$_2$ have been detected in human actinic keratosis and squamous cell carcinomas (52-54). In addition to producing elevated levels of prostaglandins which impair immune surveillance and the killing of malignant cells (193), COX catalyzed reactions may contribute to tumor formation by converting a variety of chemicals to mutagens (75). The peroxidase function of COX contributes to the activation of pro-carcinogens and results in the generation of reactive oxygen intermediates (ROI) which themselves can damage DNA. While topical treatment with Celecoxib after UVB exposure had no inhibitory effect on the UVB mediated increase in COX-2 mRNA and protein within the skin, it did significantly decrease detectable cutaneous PGE$_2$ levels compared to UVB/Vehicle treated skin.

One of the most characteristic features of UVB induced skin damage is the development of sunburn cells within the epidermis (28-30). These cells have been shown to be keratinocytes undergoing apoptosis. Recent studies by Tsujii et al have demonstrated that overexpression of COX-2 in epithelial cells inhibits apoptosis (60) thus allowing cells that may be neoplastically transformed to escape elimination. Previous studies have reported inhibition
of sunburn cell formation following topical application of the non-selective NSAID indomethacin prior to UVB exposure (50-51). The present study demonstrated that topical application of Celecoxib immediately following UVB exposure was effective in diminishing the number of sunburn cells present within the epidermis at 24 hours. In addition, Celecoxib reduced the number of p53 positive cells in the epidermis after UVB exposure. The ability of Celecoxib to inhibit increases in sunburn cell formation and p53 levels correlates with their abilities to decrease dermal neutrophil infiltration following UVB irradiation. It is possible that by inhibiting the infiltration of activated neutrophils into the skin following UVB irradiation, Celecoxib is inhibiting the levels of oxidants released by these cells, inhibiting oxidative damage and in turn the need to undergo apoptosis, hence the decrease in sunburn cells and p53 positive cells. Although a decrease in apoptotic sunburn cells and p53 could be harmful if damaged cells continue to proliferate, we hypothesized that the decreases were due to the fact that there was less oxidative damage in the animals treated with Celecoxib due to the inhibition of the activation and infiltration of inflammatory cells after irradiation as a result of a decrease in the activity of the UVB-induced COX-2 activity. To determine whether this decrease in apoptotic cells was because less damage had accrued in these cells and to rule out the possibility that we were inhibiting the destruction of severely damaged cells, we examined p53 protein levels and oxidative DNA adduct formation in treated skin. A recent report demonstrated the formation of oxidants and subsequent 8-oxo-dG
adduct formation by the COX-2 enzyme itself after incubation with arachidonic acid (57). The authors suggest that the damaging agents are generated from one of three reactions catalyzed by the COX-2 enzyme: the peroxidatic reaction required for xenobiotic metabolism, the cyclooxygenase reaction that converts arachidonic acid to the intermediate prostaglandin G₂ (PGG₂) or the peroxidase reduction of PGG₂ to prostaglandin H₂ (PGH₂). All of these reactions occur before the formation of the final end products (i.e., PGE₂) from PGH₂. Celecoxib only inhibits the reaction forming the final products, not the preliminary reactions. Therefore, it is likely that Celecoxib reduces the amount of oxidative adduct formation by preventing inflammatory phagocytes from infiltrating into the dermis and releasing potentially damaging reactive oxygen species, a phenomenon illustrated in our previous work (195). If this is the case, oral administration of the drug may not be effective in inhibiting 8-oxo-dG formation since a previous study by Fischer et al. showed that this route of administration did not inhibit the UVB-induced cutaneous inflammatory response (93). Although the anti-inflammatory drug nimesulide, which also has antioxidant activity at higher doses has been shown to inhibit 8-oxo-dG formation in a model of colon tumorigenesis (212), this is the first report to our knowledge that Celecoxib can inhibit oxidative adduct formation. Because oxidative adducts have been shown to contribute to the process of carcinogenesis, this newly identified capacity for Celecoxib to inhibit oxidative adduct formation suggests a novel explanation for the chemopreventative activity of this drug.
Taken together, the data presented in this chapter suggest that topical treatment with a specific COX-2 inhibitor may be an effective measure for the treatment of sunburn in humans by its ability to inhibit inflammation, sun-induced damage, and possibly pain after exposure to an erythemic dose of sunlight. Ultimately, treatment with a drug such as this after UVB exposure may also be effective in preventing the development of skin cancer resulting from excessive sun exposure.
Figure 3.1. Dorsal skin thickness as a measure of vascular permeability and edema. UVB irradiation caused a significant increase in the amount of skin thickening compared to control animals (**p<0.05 compared to Acetone). Topical treatment with Celecoxib (500 μg) immediately following UVB exposure significantly decreased the skin thickness as compared to UVB/Acetone treated skin at both 24 hours and 1 week (*p<0.05 compared to UVB/Acetone).
Figure 3.2. Myeloperoxidase as a measure of neutrophil activation. UVB irradiation resulted in a significant increase in MPO activity compared to animals treated with the vehicle control (**p<0.05 compared to Acetone). Topical treatment with 100 µg or 500 µg of Celecoxib immediately following exposure to UVB light resulted in a significant decrease in cutaneous MPO levels compared to UVB/Acetone treated skin at 24 hours and 1 week (* p<0.05 compared to UVB/Acetone).
Figure 3.3. Immunohistochemical quantitation of dermal neutrophil number. The number of Ly-6G-positive cells (neutrophils) was determined immunohistochemically and quantitated using an image analysis system. Representative photomicrographs of stained tissue sections show the induction of dermal neutrophil infiltration after UVB exposure at 24 hours, as seen by the significant increase in the number of Ly-6G-positive cells compared to unirradiated controls (C; **p<0.05). Topical treatment with Celecoxib (500 µg, B) reduced the level of neutrophil infiltration in a dose-dependent manner, which was statistically significant (* p<0.05) compared to UVB/Acetone controls (A).
Figure 3.4. RT-PCR analysis of COX gene expression. Quantitation of COX-1 and COX-2 message levels at 24 hr (A) and 1 week (B) shows that exposure to UVB induced COX-2 gene expression in a time dependent manner while having no effect on the expression of COX-1. Topical treatment with Celecoxib had no effect on COX-1 or COX-2 gene expression at either time point examined.
Figure 3.5. Immunohistochemical localization of COX protein. Immunohistochemical analysis of tissue isolated 24 hours after a single exposure to UVB had no effect on COX-1 protein production in the skin while it increased protein levels of COX-2. Topical treatment with Celecoxib had no effect on protein levels of either enzyme.
Figure 3.6. EIA analysis of PGE$_2$ concentration in dorsal skin. Exposure to UVB light induced the production of PGE$_2$ levels in the skin. Topical treatment with Celecoxib caused a significant inhibition in the formation of PGE$_2$ at 24 hours (500 µg dose) and 1 week (both doses). (* p<0.05 compared to UVB/acetone)
Figure 3.7. Quantitation of epidermal sunburn cells. The number of apoptotic sunburn cells at 24 hours was determined in H&E stained tissue sections using an image analysis program. A photomicrograph of the epidermis of skin isolated from the UVB/Acetone group is shown to illustrate a sunburn cell (A). The data, shown graphically (B), demonstrates a dose dependent decrease in the number of sunburn cells formed in skin that had been treated topically with Celecoxib after irradiation with UVB compared to control irradiated skin (*p<0.05 compared to UVB/Acetone).
Figure 3.8. Immunohistochemical quantitation of p53 protein levels. Immunohistochemical analysis was used to examine p53 protein levels in the skin. Ultraviolet light exposure causes a dramatic increase in p53 protein levels in the epidermal layer of the skin 24 hours after exposure (UVB/Acetone; B,E) in comparison to non-irradiated controls (Acetone; A,E). Topical treatment with Celecoxib immediately after irradiation resulted in a dose-dependent decrease in the number of p53 positive epidermal cells (UVB/100 µg Celecoxib; C,E and UVB/500 µg Celecoxib; D,E) compared to the irradiated vehicle control skin (UVB/Acetone; B,E). Image analysis software was used to quantitate the number of p53 positive cells, shown in E. Celecoxib treatment (500 µg) resulted in a statistically significant inhibition of UVB-induced p53 expressing cells (E). **p<0.05 compared to Acetone; *p<0.05 compared to UVB/Acetone.
Figure 3.9. Enzyme-linked immunoanalysis of oxidative DNA adducts. An ELISA was used to quantify the number of 8-oxo-dG adducts from DNA isolated from dorsal skin at 24 hours and 1 week. Exposure to UVB light (UVB/Acetone) caused an increase in the number of 8-oxo-dG adducts detected compared to non-irradiated mice (Acetone). Topical treatment with Celecoxib significantly inhibited the level of 8-oxo-dG adduct formation following UVB exposure (*p<0.05 compared to UVB/Acetone).
Figure 3.10. Fluorescent immunohistochemical localization of 8-oxo-dG adducts in the skin. UVB irradiation induced the formation of 8-oxo-dG adducts in basal keratinocytes and epidermal cells in the bulge region of hair follicles (UVB/Acetone, B) which can be seen from the increase in positive cells compared to unirradiated controls (Acetone, A). Topical Celecoxib treatment resulted in a dose-dependent reduction in the number of 8-oxo-dG positive cells detected at 1 week (100 μg; C and 500 μg; D), with a substantial decrease using 500 μg (D) compared to the irradiated vehicle control skin (UVB/Acetone, B).
CHAPTER 4

EFFICACY OF CELECOXIB AS AN ANTI-INFLAMMATORY, CHEMOPREVENTION OR CHEMOTHERAPEUTIC AGENT AFTER CHRONIC UVB EXPOSURE

Introduction and Rationale:

Ultraviolet (UV) radiation is the most prominent cause of nonmelanoma skin cancer worldwide. Exposure of the skin to UV light causes an increase in prostaglandin production resulting from both increased release of arachidonic acid from membrane phospholipids, as well as increased biosynthesis of prostaglandins from arachidonic acid by the COX-2 enzyme (38-41). Two isoforms of COX exist, cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2). COX-1 is constitutively expressed and is involved in homeostasis and cytoprotection in organs such as the skin and gastrointestinal tract (65). COX-2, on the other hand, is an inducible enzyme involved in the immediate-early gene response to various stimuli, including UV light (43,52,66-68). The induction of COX-2, the subsequent production of prostanoids, and the inflammatory response prompted by these events have recently been linked with the process of tumor formation (52-54). In
addition, tumors in a variety of organs produce greater amounts of prostaglandins than do normal tissues of the same type (52-54). Besides causing direct DNA damage, such as thymidine dimer formation, UVB can cause indirect DNA damage. This occurs through both the activation of keratinocytes and inflammatory cells (respiratory burst) and the conversion of arachidonic acid to prostaglandins both of which lead to the production of reactive oxygen intermediates (ROIs) (33,35-37). These ROIs can cause oxidative DNA adduct formation, such as 8-oxo-deoxyguanosine (8-oxo dG), which if not repaired before replication could lead to permanent DNA mutations and ultimately carcinogenesis (31-34). The cellular response to DNA damage includes the induction of p53 protein expression and subsequent cell cycle arrest in an attempt to avoid replicating damaged DNA.

The data presented in Chapter 3 shows that topical application of Celecoxib, a specific COX-2 inhibitor, reduces acute inflammation (24 hours and 1 week) in the skin following UV irradiation. This is in contrast to oral studies, which demonstrated Celecoxib did not inhibit UVB-induced skin inflammation. The hypothesis for the studies presented here is that topical treatment with Celecoxib, by inhibiting UVB-induced inflammation, would be a more effective means of preventing chronic inflammation and the formation of tumors after chronic UVB exposure (20 weeks). In addition, we also tested the ability of topical Celecoxib treatment to act as a chemotherapeutic agent, or to regress established tumors.
Experimental design

A. Chemoprevention study

Long term studies were performed to evaluate the ability of topically applied Celecoxib (Celebrex®, Searle-Monsanto, St. Louis, MO) to prevent UVB-induced chronic inflammation and papilloma development. Mice were housed in the vivarium at the Ohio State University according to the requirements established by the American Association for Accreditation of Laboratory Animal Care. All procedures were approved prior to beginning the study by the appropriate Institutional Animal Care Utilization Committee. Female Skh/hr hairless mice (8-10 weeks, 26-28 g, Charles River, Wilmington, MA) with 9 animals per treatment group were exposed dorsally to 2240J/m² of UVB light (one minimal erythemic dose). Phillips FS40UVB lamps (American Ultraviolet Company, Murray Hill, NJ) which provide predominantly UVB light (290-320 nm) were used as the UV light source. To ensure that the light emitted was only in the UVB range, Kodacel filters (Eastman Kodak, Rochester, NY) were used to eliminate UVC light. Immediately after exposure, irradiated mice were treated topically with 0.2 ml of acetone as a vehicle control or 500 µg of Celecoxib in 0.2 ml of acetone. The control unirradiated mice were also treated topically with either acetone or 500 µg of Celecoxib. The mice were subjected to this treatment regimen three times a week for 20 weeks. The number of papillomas per mouse and

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their sizes were recorded weekly. Mice were sacrificed after 20 weeks, 24 hours after the last treatment. Directly after sacrifice, skin thickness was measured with calipers and dorsal skin and papillomas were isolated. Fresh skin sections were examined for myeloperoxidase levels and skin sections and papillomas were also snap frozen in liquid nitrogen for later protein isolation or fixed for histochemical analysis.

**B. Chemotherapy study**

Long term studies were performed to evaluate the ability of topically applied Celecoxib (Celebrex®, Searle-Monsanto, St. Louis, MO) to regress established papillomas that had developed as a result of chronic UVB exposure. Female Skh/hr hairless mice (8-10 weeks, 26-28 g, Charles River, Wilmington, MA) were used with 10 animals per treatment group. Mice were exposed dorsally to 2240J/m² of UVB light (one minimal erythemic dose) provided by Phillips FS40UVB lamps (American Ultraviolet Company, Murray Hill, NJ) with Kodacel filters (Kodak), which eliminated UVC light. The control mice were not irradiated during this time. After 16 weeks, with 87% of the irradiated mice bearing papillomas, irradiations were stopped and the mice were treated topically with 0.2 ml of acetone as a vehicle control or 500 µg of Celecoxib in 0.2 ml of acetone three times a week. Control mice also began receiving treatments at 16 weeks with either 0.2 ml of acetone as a vehicle control or 500 µg of Celecoxib in 0.2 ml of acetone three times a week. The mice were subjected to this treatment regimen for 6 weeks. The number of
papillomas and their sizes were recorded weekly. Mice were sacrificed at 22 weeks, 24 hours after the last treatment. Directly after sacrifice, skin thickness was measured with calipers and dorsal skin and papillomas were isolated. Fresh skin sections were examined for myeloperoxidase levels and skin sections and papillomas were also snap frozen in liquid nitrogen for protein or fixed for histochemical analysis.

Results

A. Papilloma development

Exposure to UVB three times a week resulted in the development of papillomas beginning 12 weeks after exposure. The number of papillomas per mouse were counted weekly over the timecourse of UVB exposure and topical treatment with either Acetone or Celecoxib (Fig. 4.1 and 4.2). Unirradiated mice treated with Acetone or Celecoxib never developed tumors. Treatment with Celecoxib after each irradiation for 20 weeks (chemoprevention) resulted in a two week delay in the onset of papillomas compared to UVB/Acetone mice (Fig. 4.1). In addition, mice treated with Celecoxib had significantly less papillomas than Acetone treated mice at every timepoint examined (*p<0.05). Topical treatment with Celecoxib immediately after irradiation beginning at week 16 (UVB/Celecoxib, chemotherapy) stopped the development of new papillomas compared to the
vehicle treated mice (UVB/Acetone), which continued to develop new papillomas (Fig. 4.2). However, Celecoxib was not able to decrease the number of tumors that were already present.

B. Papilloma size

The growth rate of papillomas was determined by taking weekly measurements of diameter for each individual papilloma. Papillomas that developed in Celecoxib treated animals after 20 weeks of UVB exposure and treatment (chemoprevention) were significantly smaller than those that developed in the Acetone treated control mice (Fig. 4.3; *p<0.05 compared to UVB/Celecoxib). In the chemotherapy study, although it did reduce the number of new papillomas formed compared to Acetone treated mice, topical treatment with Celecoxib starting at week 16 was unable to regress the papillomas that were present (Fig. 4.4)

C. Measurement of chronic inflammation

Chronic inflammation induced by UVB light was initially assessed by measuring dorsal skin thickness. After 20 weeks of UVB irradiation (chemoprevention), a significant increase was seen in skin thickening compared to Acetone controls (UVB/Acetone, **p<0.05; Fig 4.5A). Topical treatment with Celecoxib following each UVB exposure significantly inhibited UVB-mediated skin thickness compared to vehicle treated mice
(UVB/Acetone, *p<0.05). In addition to skin thickness, MPO levels were also used to assess the level of cutaneous inflammation, which measures the activation of dermal leukocytes. As shown in Fig. 4.5B, UVB irradiation (UVB/Acetone) significantly (**p<0.05) increased the amount of MPO detected within the skin of mice following 20 weeks of exposure compared to levels detected in skin isolated from unirradiated control mice (Acetone). Topical treatment with Celecoxib resulted in a statistically significant decrease (*p<0.05) in MPO levels compared to irradiated vehicle controls (UVB/Acetone).

In contrast to the mice in the chemoprevention study that had been irradiated for 20 weeks at the time of sacrifice, the mice in the chemotherapy study, which had not been irradiated for 6 weeks prior to sacrifice, did not have significant signs of skin inflammation as measured by either skin thickness or MPO (Fig. 4.6A and B). Celecoxib treatment had no effect under these conditions.

D. Western blot detection of COX-2 protein

SDS-PAGE and Western blot analysis were used to examine the levels of COX-2 protein in the skin. Exposure to UVB for 20 weeks (chemoprevention) resulted in an induction of COX-2 protein levels in comparison to unirradiated skin (Fig. 4.7A). Similarly, the mice that had been irradiated for 16 weeks (chemotherapy) also showed increased COX-2
protein levels, even 6 weeks after the irradiations had ended (Fig. 4.7B). Treatment with Celecoxib had no effect on COX-2 protein levels compared to the UVB/Acetone controls in either study.

E. EIA detection of cutaneous PGE$_2$ levels

PGE$_2$ levels in the skin were assessed to determine COX-2 activity. UVB exposure induced the production of PGE$_2$ compared to unirradiated controls (Acetone) after both 20 weeks of exposure (chemoprevention; Fig. 4.8A) or 16 weeks of exposure with 6 weeks of no irradiation before sacrifice (chemotherapy; Fig. 4.8B). However, the mice that had not been irradiated immediately before sacrifice (Fig. 4.8B) had less PGE$_2$ than mice that had been irradiated 24 hours before sacrifice (Fig. 4.8A). While PGE$_2$ levels remained elevated in skin isolated from mice in the chemotherapy arm of the study, these levels were lower than that seen in skin isolated from mice in the chemoprevention arm which had been irradiated 24 hours prior to sacrifice. This demonstrates the ability of UVB to induce COX-2 to produce PGE$_2$ even following chronic UVB exposure. Topical treatment with Celecoxib significantly decreased UVB-induced PGE$_2$ levels in the chemoprevention study compared to UVB/Acetone treated skin (Fig. 4.8A; *p<0.05) but did not decrease PGE$_2$ levels in the chemotherapy study (Fig. 4.8B).

In addition, PGE$_2$ levels in papillomas were also examined. In papillomas isolated from mice that had been exposed to UVB for 20 weeks (chemoprevention), both treatment groups (UVB/Acetone papillomas and
UVB/Celecoxib papillomas) had higher levels of PGE$_2$ than skin isolated from mice that had been treated with either Acetone or Celecoxib after UVB exposure. Papillomas isolated from mice that had been treated with Celecoxib contained significantly less PGE$_2$ than the vehicle treated counterparts (UVB/Acetone papillomas, **p<0.05). In contrast, the papillomas isolated from mice that had been exposed to UVB for 16 weeks followed by 6 weeks of drug treatment (chemotherapy) did not have elevated PGE$_2$ levels compared to the skin, and Celecoxib treatment did not significantly alter papilloma PGE$_2$ levels.

F. Immunohistochemical detection of p53 protein

The levels of p53 protein were examined by immunohistochemistry and quantitated using an image analysis system. The antibody used detects both wild-type and mutant p53, however, it is likely that the majority of p53 detected after chronic UVB exposure as in this study is mutated (28). Exposure to UVB for 20 weeks (chemoprevention) resulted in the induction of p53 protein expression compared to nonirradiated mice (Fig. 4.9A and B). p53 levels were also elevated in papillomas isolated from mice that had been exposed to UVB. However, mice that had been treated topically with Celecoxib after every irradiation (Fig. 4.9D) had significantly lower levels of p53 than UVB/Acetone control skin (Fig. 4.9C). In addition, the papillomas isolated from these mice (Fig. 4.9F) had significantly less p53 staining than
UVB/Acetone papillomas (Fig. 4.9E). Staining with the isotypic control antibody is shown (Fig. 4.9G) to demonstrate the specificity of the p53 antibody.

In contrast, p53 levels were only slightly elevated in mice in the chemotherapy arm of the study that had been exposed to UVB for 16 weeks and treated for 6 weeks (Fig. 4.10 C and D) compared to nonirradiated controls (Fig. 4.10 A and B). Papillomas isolated from the irradiated mice had slightly more p53 than the skin, and Celecoxib had no effect on p53 levels in the skin or in papillomas.

G. Immunohistochemical localization of PCNA positive cells

As an attempt to explain both the decrease in papilloma development and p53 expression with Celecoxib treatment in the chemoprevention mice, the number of cells demonstrating positive staining for the proliferation marker PCNA (proliferating cell nuclear antigen) were analyzed by immunohistochemistry and image analysis (Fig. 4.11). As with the p53 protein expression, UVB exposure for 20 weeks (chemoprevention) resulted in a substantial increase in the number of PCNA positive epidermal cells (UVB/Acetone) compared to nonirradiated mice (Acetone, Celecoxib). Topical treatment with Celecoxib after each irradiation significantly reduced the number of proliferating cells in the skin (UVB/Celecoxib). In addition, the number of proliferating cells in papillomas isolated from Celecoxib treated mice were significantly lower than those isolated from mice treated with
Acetone. This is in contrast to the inability of Celecoxib to reduce the number of proliferating cells in papillomas isolated from chemotherapy mice (Fig. 4.12), which may serve as one explanation for Celecoxib's efficacy in inhibiting the size of papillomas in the chemoprevention arm but not in the chemotherapy arm.

DISCUSSION

As demonstrated in Chapter 3, topical Celecoxib treatment is able to alleviate the acute UVB-induced inflammatory response, decreasing the negative effects of inflammation in the skin, including the induction of oxidative DNA damage. This has led to the examination of the effectiveness of COX-2 inhibitors such as Celecoxib to prevent or regress tumors in a variety of organs. Recent studies have demonstrated the effectiveness of orally administered Celecoxib as both a chemotherapeutic and chemopreventative agent in a Skh/hr hairless mouse UVB skin carcinogenesis model (92,93). However, the systemic oral administration of the inhibitor failed to block the characteristic cutaneous inflammatory response seen in the skin following exposure to UVB light. In addition, the oral route of administration limited the concentration of drug that could be achieved at the site of inflammation. The goal of the experiments presented in this chapter was to take those studies a step further, to determine the
efficacy of topical treatment with Celecoxib as an anti-inflammatory, chemopreventative and chemotherapeutic agent in UVB induced skin carcinogenesis.

The chemoprevention arm of the chronic irradiation studies demonstrated the ability of topically applied Celecoxib to inhibit chronic inflammation, to decrease the formation of pre-malignant papillomas, and to slow the growth of the papillomas that did develop. An increased level of inflammation, demonstrated by the induction of skin thickness and MPO, found in the skin isolated from irradiated mice, was reduced by Celecoxib treatment. In addition, the increased level of inflammation observed in the skin and papillomas isolated from irradiated mice correlated with increases in PGE$_2$ levels which were significantly reduced by treatment with Celecoxib, both in the skin and papillomas. A similar pattern was observed when the numbers of p53 and PCNA positive cells were analyzed. An increase in the number of p53 positive and proliferating cells was observed in the irradiated skin and papillomas, whereas the skin and papillomas from irradiated mice that had been treated with Celecoxib showed a much less extensive increase in p53 and PCNA protein levels. It has been suggested that the majority of p53 detected after long-term UV exposure is a mutant form of the protein (28). It would be interesting to speculate that the ability of Celecoxib to inhibit inflammation and the resulting DNA damage (Chapter 3) has lead to a decrease in mutant p53 levels in these studies, hence the decreased PCNA protein levels and the reduction in the formation and growth rate of
papillomas. Benign papillomas cannot be distinguished from squamous cell carcinomas on a gross level, but if the idea that Celecoxib could inhibit the induction of mutations in p53 through the inhibition of inflammation and subsequent DNA damage, the majority of the growths isolated from this group should be papillomas. The lesions isolated from irradiated mice treated with Acetone, on the other hand, may contain carcinomas. Although these ideas have not been explored to date, they will be the focus of future work. However, further supporting this idea are studies that have shown that the levels of COX-2 activity seem to increase with invasive potential and seriousness of skin tumors, with normal skin having very low levels of COX-2 and PGE$_2$, premalignant human actinic keratosis lesions having increased levels of COX-2 and PGE$_2$, and squamous cell carcinomas having the highest levels of detectable COX-2 and PGE$_2$(52-54).

In contrast to the chemoprevention study in which Celecoxib was effective in inhibiting papilloma formation and growth, the chemotherapy arm of the present studies showed that while able to inhibit the development of new papillomas, Celecoxib was not able to regress established papillomas. These mice, which had been irradiated for the first 16 weeks of the study but not for a 6 week period prior to sacrifice, instead were treated with either Acetone or Celecoxib during this last phase. It makes sense, therefore, that the lack of inflammatory stimuli resulted in low levels of inflammation found in the skin of these mice as detected by skin thickness or MPO. Interestingly, however, is the fact that PGE$_2$ levels were significantly elevated in these mice
even 6 weeks following the last exposure to ultraviolet light. This is most likely due to the fact that COX-2 protein levels remained elevated even after this reprieve from UVB exposure. It has been shown that COX-2 levels become constitutive after repeated irradiation (52-54). The constitutive production of PGE$_2$ even after the inflammatory stimulus (UVB) was removed may help to explain how skin cancer develops in people some 10, 20 or 30 years after having been exposed to high levels of UVB. The relatively small dose of Celecoxib that was used in the topical application may explain the lack of regression of these tumors. Alternatively, previous studies by Pentland et al using oral dosing of the drug showed similar results, suggesting the dependence on inflammation for tumor development, but not growth of established tumors (92). The inability of Celecoxib to regress established tumors suggests that the mechanism of its chemopreventative activity may be more related to its ability to prevent DNA damage than previously believed. In fact, our studies are the first to show that Celecoxib can inhibit oxidative damage and the induction of p53 suggests that "direct damage" or p53 mutations induced by UVB may have more of an oxidative component than previously thought. Therefore, without the ability to directly kill these damaged cells or to reverse the damaged condition, Celecoxib would not be expected to regress these tumors. The fact that the DNA damage and mutations in proliferation control proteins such as p53 have
already accumulated in established papillomas and cannot be prevented by Celecoxib treatment explains the differences in the data between the two arms of the study.

It is now well accepted that inflammation is key to cancer development in many organs, including the skin (31-33,44-46). The fact that anti-inflammatory drugs, both non-specific COX inhibitors and specific inhibitors of COX-2, can decrease the development of cancer in the skin further supports this idea (47-51). However, the effectiveness of anti-inflammatory drugs on specific organs is dependent on the route of administration. The data in Chapter 3 and several previous studies suggest the most effective way of inhibiting UVB-induced skin inflammation may be by directly targeting the skin with topical treatment (49-51). This topical route of administration may be a more effective means of inhibiting skin tumor development, both by directly targeting the organ involved and by providing the ability to use larger doses of drug in the skin. By using a topical application, one can eliminate side effects associated with a large systemic dose. In addition, as COX-1 is important to homeostasis in epithelial organs such as the skin, anti-inflammatory drugs that target the COX-2 enzyme specifically may be a better alternative for skin treatment rather than inhibitors that target both enzymes.

Although the development of specific COX-2 inhibitors represents progress compared to non-specific NSAIDs, neither type of drug inhibits both of the COX catalyzed reactions that lead to the production of reactive oxygen
species and prostaglandins. Because reactive oxygen species are known to contribute to the process of carcinogenesis, the chemopreventative activity of COX inhibitors may be enhanced if they were modified to also inhibit the peroxidase reaction in addition to the cyclooxygenase reaction, which generated reactive oxygen species that may be encouraging the carcinogenesis process. This idea is supported by previous work demonstrating the ability of an antisense COX-2 oligo which blocks COX-2 production, to better inhibit the growth of a squamous cell carcinoma cell line in vitro than a specific COX-2 functional inhibitor, NS-398 (53). Therefore, a drug that inhibits not only the conversion of the final prostaglandin products from PGH$_2$ but also the precursor reactions which form the PGG$_2$ and PGH$_2$ intermediates may have even more potent chemopreventative activity.

Several mechanisms have been proposed to explain the ability of COX-2 overexpression to promote the process of skin carcinogenesis, including the enhancement of PGE$_2$ levels which can promote the growth of initiated cells, the survival of sunburn cells normally discarded by apoptosis, increased ability for invasion, and/or the formation of DNA adducts or decreased repair of damaged DNA (92). The studies presented here support or negate many of these proposed mechanisms through the use of specific COX-2 inhibitors. Perhaps the most important hypothesis brought about by our studies is the idea that Celecoxib is able to inhibit the damage that accrues in cells exposed to continued inflammation, leading to a reduction in
the need to undergo apoptosis by these cells, less of a need for induced p53 protein levels, and a decrease in the proliferative capacity of these cells.
Figure 4.1. Timecourse of papilloma development (chemoprevention). Exposure to UVB three times a week resulted in the development of papillomas beginning 12 weeks after exposure (UVB/Acetone). Topical treatment with Celecoxib immediately after irradiation caused a two week delay in the appearance of papillomas. In addition, Celecoxib treatment significantly lowered the number of papillomas per mouse compared to vehicle treated mice at every timepoint examined (*p<0.05 compared to UVB/Celecoxib).
Figure 4.2. Timecourse of papilloma development (chemotherapy). Exposure to UVB three times a week resulted in the development of papillomas beginning 12 weeks after exposure. Topical treatment with Celecoxib immediately after irradiation beginning at week 16 (UVB/Celecoxib) inhibited the development of new papillomas when compared to the vehicle treated mice (UVB/Acetone), which continued to develop new papillomas. The graphs represent papilloma percentages as a percent of the number of papillomas at the beginning of the topical Acetone or Celecoxib treatments (week 16).
Figure 4.3. Timecourse of papilloma growth (chemoprevention). The growth rate of papillomas was determined by taking weekly measurements of diameter for each papilloma. Papillomas that developed in Celecoxib treated animals were significantly smaller than those that developed in the Acetone treated control mice (*p<0.05 compared to UVB/Celecoxib).
Figure 4.4. Timecourse of papilloma growth (chemotherapy). The growth rate of papillomas was determined by taking weekly measurements of diameter for each papilloma. At week 0 on the graph, UVB irradiations were stopped and topical treatments with either Acetone or Celecoxib were begun. Although it did reduce the number of papillomas formed (Fig. 4.3), topical treatment with Celecoxib for 6 weeks did not have an effect on the size of the papillomas that had been induced by 16 weeks of UVB exposure. The graph represents size percentages as a percent of the size of papillomas at the beginning of the topical Acetone or Celecoxib treatments (week 0).
Figure 4.5. Skin thickness and myeloperoxidase as measurements of chronic inflammation (chemoprevention). 20 weeks of UVB irradiation resulted in significant increases in skin thickness (A) and MPO activity (B) compared to the skin of animals treated only topically with the vehicle control (**p<0.05 compared to Acetone). Topical treatment with Celecoxib immediately following each exposure to UVB light resulted in a significant decrease in both parameters of inflammation (*p<0.05 compared to UVB/Acetone).
Figure 4.6. Effect of topical Celecoxib treatment on chronic inflammation (chemotherapy). While the skin isolated from mice in the chemoprevention arm of the study (Fig. 4.5) displayed significant inflammation by both skin thickness and MPO, these mice had been irradiated up to 24 hours prior to sacrifice. In contrast, the skin of mice from the chemotherapy arm, which had not been irradiated for the 6 weeks before sacrifice, did not show signs of inflammation by either parameter, skin thickness (A) and MPO (B).
Figure 4.7. Analysis of COX-2 protein levels. SDS-PAGE and Western blotting was used to detect COX-2 protein in skin isolated from Skh/hr mouse skin. Photomicrographs of representative COX-2 Western blots are shown above. Exposure to UVB for 20 weeks (A, chemoprevention) or for 16 weeks with 6 weeks of no exposure (B, chemotherapy) resulted in an increase in COX-2 protein levels compared to unirradiated controls. Topical treatment with Celecoxib had no effect on COX-2 protein levels in either arm of the study.
Figure 4.8. EIA analysis of cutaneous PGE\textsubscript{2} levels. Exposure to UVB light for 20 weeks caused a significant increase in PGE\textsubscript{2} levels in the skin (A, chemoprevention, \( \ddagger \ p<0.05 \) compared to Acetone). Topical treatment with Celecoxib significantly inhibited the induction of PGE\textsubscript{2} compared to vehicle treated skin (UVB/Acetone, \( \ast \ p<0.05 \)). In addition, the papillomas isolated from animals that had been treated with Celecoxib after irradiation had significantly less PGE\textsubscript{2} than papillomas isolated from mice treated with acetone after irradiation (A, **\( p<0.05 \) compared to UVB/Acetone papillomas). Similarly, in the chemotherapy study (B), PGE\textsubscript{2} levels were significantly higher in the skin of irradiated mice that had been treated with the vehicle control (UVB/Acetone, \( \ddagger \ p<0.05 \)) compared to their unirradiated counterparts (Acetone), although the total amounts were about half of those seen in the chemoprevention samples. However, there were marked differences between the two studies in that the PGE\textsubscript{2} levels in the papillomas isolated from the chemotherapy mice were not significantly different from those in the skin, and topical treatment with Celecoxib had no effect on the levels found in either the skin or the papillomas.
Figure 4.9. Immunohistochemical analysis of p53 protein (chemoprevention). P53 positive cells were detected by immunohistochemical techniques and quantitated using image analysis. Unirradiated skin (A, Acetone and B, Celecoxib, contained significantly fewer p53 positive cells in comparison to irradiated skin (C, UVB/Acetone *p<0.05) and papillomas (E, UVB/Acetone papillomas *p<0.05). Topical treatment with Celecoxib was able to significantly reduce the number of p53 positive cells in both the skin and papillomas compared to the vehicle treated counterparts (**p<0.05, D, UVB/Celecoxib; ***p<0.05, F, UVB/Celecoxib papillomas). G represents the isotype control and H is a graph of the number of positive cells.
Figure 4.10. Immunohistochemical detection of p53 protein (chemotherapy). The graph above represents the mean number of positive p53 cells for each treatment group. There were no statistical differences in the levels of p53 protein detected in this arm of the study.
Figure 4.11. Immunohistochemical analysis of PCNA protein (chemoprevention). PCNA positive cells were detected by immunohistochemical techniques and quantitated using image analysis. The number of PCNA positive cells was used as a measure of epidermal cell proliferation. Unirradiated skin (A, Acetone and B, Celecoxib, contained significantly fewer proliferating cells in comparison to irradiated skin (C, UVB/Acetone *p<0.05) and papillomas contained more than irradiated skin (E, UVB/Acetone papillomas, **p<0.05). Topical treatment with Celecoxib was able to significantly reduce the number of proliferating cells in both the skin and papillomas compared to vehicle treated mice (***p<0.05, D, UVB/Celcoxib and ****p<0.05, F, UVB/Celecoxib papillomas). G represents the isotype control and H is a graph of the number of positive cells.
Figure 4.12. Immunohistochemical detection of PCNA protein (chemotherapy). The graph above represents the mean number of PCNA positive cells for each treatment group. Although there was an induction of PCNA in irradiated skin and papillomas, Celecoxib was not able to reduce these levels.
CHAPTER 5

INFLAMMATION IN WOUND HEALING AND SCAR FORMATION IN FETAL AND ADULT SKIN

Introduction and rationale

Wound healing is a process everyone deals with on a continual basis, translating into a cost of over 7 billion dollars a year for Americans. This process is complex and highly regulated, ultimately leading to the formation of a collagenous scar in adults. Scar tissue functions to restore the strength of the skin, however, it can be detrimental to its function, leading to loss of joint mobility and psychosocial consequences for those with evident scars on exposed areas of the body. This is a particularly serious problem for burn victims. Treatment options for the problem of excessive scarring are limited, but the knowledge of the wound healing process in early embryos offers hope. Wound healing in the early fetus (first to second trimester) is scarless. It occurs more rapidly, with no inflammation, and less neovascularization, regenerating skin of the same quality as before wounding. This is different from adult repair, which has an evident inflammatory response after wounding and ends with scar formation and the loss of subepidermal
appendages. However, the mechanism of how adult fibrotic healing and scarless fetal healing differ remains elusive. Most of the studies to date examining fetal wound healing have focused on the extracellular matrix component hyaluronic acid and members of the TGF-β (transforming growth factor-beta) growth factor family. Although the high levels of hyaluronic acid and the lack of TGF-β are important components of scarless healing, the fact that modulating the levels of these proteins in adult healing has not been able to eliminate scarring in adult wounds, giving evidence that there are other components contributing to this phenomenon, leaving much left to be uncovered about this process. There is a transition from scarless healing to adult-like fibrotic healing late in gestation, which corresponds to the presence of an inflammatory response to wounding. Although it is well accepted that the lack of inflammation is important to the process of scarless healing in the fetal wound healing literature, the adult wound healing field views inflammation as a necessary process for successful wound healing. While a complete lack of inflammation may be detrimental in adult wound healing due to the risk of infection, a balance between the severity of inflammation and the formation of scar tissue seems the best choice for wound treatment in a normal, immune competent adult. The goal of the studies presented in this chapter was to better define the components of the inflammatory response that contribute to scar tissue formation by utilizing fetal scarless healing as a model to examine components of the inflammatory response for their ability to induce scar tissue and to translate this information into a treatment to
minimize scar formation in adult wounds. Furthermore, due to the known involvement of COX-2, its products, and the microbicidal reactive oxygen intermediate H$_2$O$_2$ in inflammation and cellular proliferation, the role of this enzyme in wound repair and scar formation in both fetal and adult healing was examined.

**Experimental design**

**A. Fetal wound healing studies**

Scarless fetal wound healing was used as a model to measure the ability of the inflammatory mediators PGE$_2$ and H$_2$O$_2$ to cause scar formation in fetuses that would normally heal without a scar. Female and male mice were mated, and surgery was performed on the pregnant female 15 days after the detection of a vaginal plug, designated day 0. This timepoint represents an age at which scarless healing occurs (173). Wounding was also performed on fetuses at E17, after the transition to fibrotic healing as a comparison to E15. Following isofluorane anesthetization of pregnant females, a midline laparotomy was performed. During the surgery, the abdomen and uterus were bathed in warm, sterile PBS to avoid dehydration. After exposure of the uterine horns, incisions were made in the uterine wall and amniotic sac to access the fetus (Fig. 5.1A) and a full thickness dorsal wound was made in the dorsal skin of the fetus with microsurgical scissors. India ink combined with either PBS, PGE$_2$ (2 μg) or H$_2$O$_2$ (1 %) was injected subcutaneously at
the wound site. A total volume of 1 μl was injected (Fig. 5.1B). The India Ink, commonly used in fetal healing studies, allows visualization of the injection into the wound site and can also be used to mark the wound site after healing had occurred (Fig. 5.1D). (173). In addition, it has been shown not to induce inflammation or interfere with scarless healing (173). For control wounds, sterile PBS was injected into the wound site in order to confirm scarless healing in the absence of the inflammatory mediators. After the injections into the wound, the uterus was sutured (Fig. 5.1C) and replaced, then the muscle and skin layers of the mother were sutured. The formation of a scar upon injection of an inflammatory mediator demonstrates that the mediator is a positive regulator of fibrosis, because under normal conditions, they would heal with a scarless phenotype. Fetal skin was harvested at 24 hours to examine inflammation and at 3, 5 or 7 days after wounding to analyze scar tissue formation. The skin was either fixed in formalin for subsequent histological analysis or snap frozen for protein isolation.

B. Adult wound healing studies

Three days prior to beginning the wound healing studies, the dorsal skin of female FVB/N mice (6-8 weeks of age, Taconic Farms, Germantown, NY) were shaved and divided into treatment groups (4 mice per group). The mice were housed in the vivarium at the Ohio State University according to the requirements established by the American Association for Accreditation of Laboratory Animal Care. All procedures were approved prior to beginning the
study by the appropriate Institutional Animal Care Utilization Committee. Animals were fed Purina Pico Chow (Barnes Supply, Durham, NC) and basal Teklad 22/5 rodent diet (Harlan Industries, Indianapolis, IN) ad libitum and kept in rooms maintained on a 12 hr light/dark cycle. Under isofluorane anesthetic, mice were given 3 cm full thickness dorsal wounds which were closed with 9 mm stainless steel wound clips (Fisher Scientific, Pittsburgh, PA).

I. Timecourse of FVB/N wound healing

5 mice per group were either left unwounded or wounded and sacrificed at various timepoints during the inflammatory phase of wound healing, including 6, 12, 24 and 48 hours post wounding. After sacrifice, skin sections were isolated from unwounded mice or from the wound area of mice that had been wounded and fixed in formalin for subsequent detection of COX-2 protein levels by immunohistochemical analysis.

II. Effect of Celecoxib on wound healing and scar formation

5 mice per group were either left unwounded or wounded, then treated immediately with 200 μl of the vehicle control (K-Y Jelly; Ortho Pharmaceutical Corp., Raritan, NJ) or with 1 mg of Celecoxib (Celebrex®, Searle, St. Louis, MO) dissolved in 200 μl of the vehicle. The mice were treated daily for up to 14 days with the vehicle or drug, and euthanized by CO₂ inhalation at 48 hours to assess inflammation, at 7 days to analyze reepithelialization, and at 14 days to assess the
pattern of scar formation. After sacrifice, skin from the wound site was immediately assessed for myeloperoxidase levels, snap frozen in liquid nitrogen for subsequent protein isolation and hydroxyproline determination, or fixed in formalin for immunohistochemical analysis.

Results

A. Differential inflammatory response to wounding and scar tissue formation between E15, E17 and adult wounds

H&E staining was used to analyze the wound area 24 hours after wounding in E15 (Fig. 5.2A) or E17 (Fig. 5.2C) fetal wounds or adult wounds (Fig 5.2E). A noticeable absence of inflammation was noted in E15 wounds, while E17 and adult wounds contained a significant inflammatory cell infiltrate. The lack of inflammation in E15 fetal wounds correlated with scarless healing, demonstrated by Masson’s trichrome staining of skin isolated 5 days after wounding (Fig. 5.2B). Very little disorganized collagen deposition occurred in these wounds with the maintenance of normal tissue architecture in comparison to E17 (Fig. 5.2D) and adult wounds (Fig. 5.2E), which contained a striking level of fibroplasia and a concomitant destruction of normal tissue architecture.

B. Effects of PGE2 on scar formation

To examine the effects of PGE2 on scar formation, fetal wounds were injected with either PBS (Fig. 5.3A) or PGE2 (Fig. 5.3B) and examined 3 days
after wounding by Masson's trichrome staining for the formation of a scar. The induction of scar tissue generation by PGE\(_2\) was evident when comparing the level of disorganized collagen deposition in the wound site compared to the control wounds injected with PBS.

C. Effects of hydrogen peroxide on scar formation and TGF-β1 protein production

As with PGE\(_2\), hydrogen peroxide was examined for its capacity to promote scar formation in E15 fetal wounds. The control wounds, which had been injected with PBS (Fig. 5.4B) retained normal tissue architecture 7 days after wounding and were very similar to unwounded skin (Fig. 5.4A). In contrast, wounds that had been injected with hydrogen peroxide, an oxidant known to be released by activated inflammatory phagocytes, caused a break in the tissue architecture of the skin with the deposition of excess collagen (Fig. 5.4D) in comparison to normal skin (Fig. 5.4C). The ability of this inflammatory product to promote scar formation may be partially explained by its ability to increase the levels of a protein known to contribute to the formation of scar tissue, TGF-β1 (Fig. 5.4E).

D. Timecourse of COX-2 expression in adult wound healing

Immunohistochemistry was used to examine COX-2 expression during the inflammatory phase of wound healing (Fig. 5.5). Unwounded skin (A) had few COX-2 expressing epidermal cells, which was significantly increased
upon wounding (B-E). In addition, COX-2 expression was localized to epidermal cells adjacent to the wound site (marked with arrows). The number of COX-2 positive cells were quantitated and graphed in F, which demonstrates the induction of COX-2 6 hours after wounding, with a gradual decrease over a 48 hour period.

E. Effect of Celecoxib on neutrophil infiltration and activity in skin wounds

We examined the effect of topical Celecoxib treatment, an anti-inflammatory drug that specifically inhibits the function of COX-2, on the inflammatory, reepithelialization and scarring phases of wound healing. The inflammatory phase, which is marked by a rapid infiltration of neutrophils into the skin after wounding, was examined using immunohistochemistry with an Ly-6G antibody to detect neutrophils. There is an evident difference between unwounded skin (A), in which there is an absence of neutrophils, and wounded skin (B, D), where a massive infiltration of neutrophils can be seen. Topical treatment with Celecoxib immediately after wounding was able to significantly decrease the amount of neutrophil infiltration (Fig. 5.6 C and D; *p<0.05), however, the presence of some neutrophils should be noted, as a complete lack of neutrophils in wound tissue may pose a risk for infection. In addition to decreasing the number of neutrophils in wound tissue, Celecoxib treatment was able to decrease the levels of MPO, an enzyme released by neutrophils upon activation (Fig. 5.6E; *p<0.05). This enzyme, which converts hydrogen peroxide to hypochlorous acid, a potent oxidant that can
cause oxidative DNA damage, is one component of the phagocytic respiratory burst that is capable of causing host damage under inflammatory conditions.

F. Effect of celecoxib on 8-oxo-dG oxidative DNA adduct formation

The level of oxidative damage in wound tissue was examined by measuring the number of 8-oxo-dG adducts in the DNA by ELISA (Fig. 5.7B). The pattern of 8-oxo-dG adducts detected in the skin followed the same general pattern as the MPO (Fig. 5.6E), with an increase in adducts after wounding and a decrease in Celecoxib treated wounds. The reduction in 8-oxo-dG adducts with Celecoxib treatment, however, was not statistically significant.

G. Western analysis of COX-2 expression and measurement of PGE₂ levels

SDS-PAGE and Western blotting of total protein was used to examine the effect of COX-2 protein levels by Celecoxib. As demonstrated in Fig. 5.8A, there was no difference in the levels of COX-2 detected by Western blot. The protein bands were quantitated using image analysis software, confirming the fact that there was no difference in COX-2 levels between wounds that had been treated with the vehicle or with Celecoxib. Although Celecoxib had no effect on COX-2 protein levels, it did reduce the ability of COX-2 to form PGE₂ (Fig. 5.8B). The reduction in PGE₂ in wound tissue that had been treated with Celecoxib was not as dramatic as that seen in 24 hour
wound tissue (data not shown), where there was a more dramatic induction of PGE$_2$ after wounding. This is to be expected, as the levels of COX-2 protein 48 hours after wounding are on their way to returning to normal (Fig. 5.5).

H. Reepithelialization

To ensure that Celecoxib was not having a detrimental effect on wound closure, H&E stained tissue sections were examined to analyze reepithelialization of the wounds. The wounds (marked by an arrow), which show no significant differences between the two treatments, can be distinguished from unwounded skin (Fig. 5.9A) by the thickened epithelial layer in the wound site and the loss of hair follicles in the area (Fig. 5.9 B and C).

I. Masson’s trichrome staining and determination of hydroxyproline levels

Masson’s trichrome staining was used to identify collagen (blue staining) in the skin sections, which marks the scar tissue. The presence of a mass of disorganized blue collagen in the dermal layer of the skin marks the wounds (Fig. 5.10 B and C; marked by arrows), which is absent in unwounded skin (Fig. 5.10A). The width of the scar tissue was measured to detect any changes in the amount of scar tissue formed after healing and is
represented graphically in D. Compared to wounds treated with the vehicle (B), wounds treated with the anti-inflammatory drug Celecoxib (C) developed significantly less scar tissue (*p<0.05).

Collagen production was also measured by the amount of hydroxyproline detected in the tissue (Fig. 5.10E). Compared to unwounded skin, wounded skin treated with the vehicle contained significantly higher levels of hydroxyproline (**p<0.05). Celecoxib treatment was able to significantly reduce the amount of hydroxyproline compared to vehicle treated wounds (*p<0.05).

**J. Measurement of TGF-β**

To determine the mechanism by which Celecoxib was decreasing the amount of scar tissue/collagen formation, the levels of the pro-scarring inflammatory molecule, TGF-β1 were examined (Fig. 5.11). Image analysis of Western blots revealed the ability of Celecoxib treatment to significantly decrease the levels of TGF-β1 compared to vehicle treated wounds, which had higher levels of TGF-β1 than unwounded skin.

**Discussion**

The ideas about the role of inflammation and its importance in the process of wound healing is in a current state of flux. Experiments carried out in fetal skin have clearly demonstrated the correlation between inflammation and the formation of scar tissue during wound healing (134,140,141).
this, inflammation is often thought to be necessary for proper wound healing in adult skin. The studies presented here, however, point to high levels of inflammation as not a requirement for wound healing, but rather a promoter of scar formation. Although scar tissue in the adult wound is formed to increase the tensile strength of a wound, it regains only 70% of the strength of unwounded skin. The studies in this chapter addressed the hypothesis that if adult wounds were allowed to heal in an environment with a decreased level of inflammation, perhaps they would heal with less scar tissue deposition and display a more fetal like pattern of true regeneration instead of repair.

In a general sense, the induction of inflammation has been shown to encourage the formation of scar tissue in fetal wounds that would have normally healed in a scarless manner. However, aside from TGF-β, the specific components of the inflammatory response that cause the formation of scar tissue have not been identified. The data presented in this chapter tested the ability of two products of the inflammatory response, PGE₂ and hydrogen peroxide, to induce the formation of scar tissue in a fetal murine model of scarless healing. Both of these substances have been implicated as causative agents in the development of cancer in the skin, a disease of the uncontrolled proliferation of epithelial cells (55-59). The high rate of proliferation seen in tumor formation and wound re-epithelialization suggests that these two processes have much in common. Although different cell
types are implicated, both processes involve an overproliferation of cells, leading to the development of a tumor in one case and the development of a scar in the other.

The ability of hydrogen peroxide to promote scar formation in a scarless model can be explained, at least in part, by its ability to induce the production of a known fibrogenic agent, TGF-β1 as shown in Fig 5.4. In contrast, Celecoxib, which decreased the formation of scar tissue (Fig. 5.10) and caused a reduction in TGF-β1 levels (Fig. 5.11), likely acts by decreasing the concentration of hydrogen peroxide in the wound by inhibiting the infiltration of inflammatory cells (Fig. 5.6). The data presented here suggests that as in the development of cancers in the skin, inflammation also seems to be important in the proliferation/activation of fibroblasts in the promotion of scar tissue during the process of wound healing. Just as COX-2 can be localized in epithelial skin cells that have been irradiated by UVB, it can be localized to epithelial cells adjacent to a wound site in the skin (Fig. 5.5). Although at first glance it would seem that COX-2 induction which induces proliferation of epithelial cells would be important for reepithelialization of the wound site, the data presented in this chapter indicates a different role for COX-2, since inhibiting its activity did not affect wound reepithelialization (Fig. 5.9). It is likely that the products of the COX-2 enzyme produced by stimulated epithelial cells, instead of acting in an autocrine manner and being
needed for epithelial proliferation, act in a paracrine manner, causing the proliferation, migration and production of collagen by neighboring fibroblasts of the dermis.

Attempts to mimic scarless healing in adults using the current information available about scarless fetal healing has proven unsuccessful. If we are to ever achieve the goal of making adult wounds heal without excessive scarring, it is imperative that the sequence of event and the players in the two processes be elucidated. The studies presented in this chapter were designed to begin the process of identifying the components of inflammation involved in the transition of scarless to fibrotic healing in fetal wounds and to apply this knowledge to modulate scar formation in adult wounds. The results obtained from these studies will give us a better understanding of the factors that promote the formation of scar tissue and will put us a step forward in achieving the ultimate goal of inducing regeneration rather than fibrosis in post-natal wounds.
Figure 5.1. Microsurgical Technique. Microsurgical techniques were used to wound fetal skin at either embryonic day 15 (E15) or E17. India ink combined with either PBS, PGE_2, or H_2O_2 was then injected into the wound site (B) to mark the wound area after opening the uterus and amniotic sac (A) to expose the fetus. The wound will then be made in the area of dye injection, the uterus sutured (C) and returned to the abdominal cavity. After suturing the mother, the pregnancy continues normally until sacrifice. This technique allows for the visualization of the wound site (D) even after scarless healing if performed at E15 (wound is marked with an arrow).
Figure 5.2. Inflammation and scar tissue formation in fetal and adult wounds. Hematoxylin and eosin stained tissue sections (60X) are shown to demonstrate the level of inflammation seen 24 hours after wounding in E15 (A), E17 (C), or adult (E) skin wounds. The lack of inflammation seen in early (E15) fetal wounds correlates with later scarless healing. The absence of scar tissue 5 days after wounding in E15 fetuses can be seen in 60X Masson's trichrome stained tissue sections (B) in comparison to the overproduction of scar tissue and fibroplasia in E17 and adult wounds (D and F). Arrows are used to indicate the wound site (2 arrows) or wound margin of one side of a wound (1 arrow).
Figure 5.3. Effect of PGE$_2$ on scar formation in fetal wounds. E15 fetal wounds were injected with either PBS (A) or PGE$_2$ (B). Masson's trichrome staining was used to visualize scar formation in tissue sections from skin isolated 3 days after wounding. The ability of the inflammatory mediator PGE$_2$ to intensify scar tissue formation be clearly seen (B) compared to control wounds which healed without a prominent scar (A). The wound areas are marked with arrows.
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CHAPTER 6

CONCLUSIONS

The skin is one of the most complex organs of the body and perhaps the most abused, whether it is from assault by environmental factors such as ultraviolet light or from direct trauma such as wounding. The response of the skin to both of these processes, which in differing magnitudes are everyday events for most people, are mediated by inflammation. At first glance the processes of ultraviolet light induced skin carcinogenesis, and wound healing look like very different processes, however, a deeper look reveals the striking similarities between the two and how they are linked by inflammation and cellular proliferation. The response of the skin to insult, whether it be UVB exposure or to wounding, involves a complex series of regulated events that if uncontrolled lead to problems. The response to UVB involves epidermal hyperplasia, the controlled proliferation of epidermal cells and the infiltration of inflammatory cells. The hyperplastic skin response is an attempt to create a thickened skin barrier so that the harmful UVB rays will not penetrate as
deeply into the skin and harm other layers of the skin or other organs. The inflammatory cells mediate cell proliferation by releasing growth factors and cytokines that cause the proliferation of the epidermal and dermal cells while clearing away the damaged tissue. However, if chronic exposure occurs, resulting in the prolonged presence of inflammatory cells, the epidermal cells may acquire DNA mutations that can lead to uncontrolled proliferation. In addition, the release of oxidants from inflammatory cells, meant to kill bacteria and other foreign invaders, can also cause damage particularly to the epidermal cells. These events may eventually result in the development of benign papillomas and ultimately skin carcinomas.

The same principles apply to wound healing, which involves the proliferation of both epithelial cells and fibroblasts. The overstimulation of fibroblasts, causing excessive proliferation and collagen production, leads to the excessive production of scar tissue after wounding, much like the uncontrolled proliferation of epithelial cells results in tumor formation. This can lead to the growth of unsightly hypertrophic scars or in some cases the growth of benign lesions such as keloids. While these studies focused on the skin, excessive scarring can lead to problems in practically every organ in the body.

The processes of tumorigenesis and wound healing are also similar in other ways. Chronic wounding has been shown to lead to the development of squamous cell carcinomas in the skin, likely due to repeated inflammation damaging the cells in the area of the wound and then promoting the growth
of the damaged or mutated cells. In addition, one of the most serious consequences of developing squamous cell carcinoma is not, as one would think, the potential for invasion and metastasis, but rather the process of removing the lesion itself, which generates a wound and often an unsightly scar. This is of particular concern since the majority of squamous cell carcinomas arise in conspicuous places that are exposed to the sun that are highly visible, such as the face.

The studies presented here focus on the anti-inflammatory drug Celecoxib, which specifically targets the COX-2 enzyme and inhibits the formation of prostaglandins. We characterized the ability of this drug used in a new way, topically, to inhibit UVB induced inflammation and the damage to epidermal cells that results. In addition, we showed that this mode of administration was effective in inhibiting chronic inflammation and the development of pre-malignant papillomas after repeated UVB exposure. Although Celecoxib was able to block the formation of papillomas, it was not effective in regressing established tumors, emphasizing the role of inflammation in tumor development as opposed to the continued growth of tumors that have already been formed. Topical application of Celecoxib was also tested in a novel way, for its effect on the wound healing process. Our data demonstrate that this anti-inflammatory drug may be useful clinically to reduce the formation of excessive scar formation. These are the first studies to demonstrate the effectiveness of inhibiting COX-2 mediated inflammation on scar formation in adult skin. In retrospect given the previously reported
data examining the lack of inflammation in scarless healing and our data demonstrating the ability of PGE$_2$ and H$_2$O$_2$ to promote scar formation, the fact that Celecoxib, an anti-inflammatory agent was able to reduce scarring in adult wounds is not a surprising one.

As we learn more about how the skin responds to inflammation, develops tumors and heals wounds, and dissect the mechanisms that control them, we will be able to take ideas from one focused area of research and apply them to others. For instance, drugs that were initially designed to inhibit inflammation in osteoarthritis and to treat the pain associated with this condition have now been shown to be useful in the inhibition of cancer development in a number of organs. Similarly, we may be able to apply these same principles to the process of wound healing and scar formation. The studies presented here are the first step in demonstrating the usefulness of not only using anti-inflammatory drugs in a targeted way (topically), but to also use these drugs in a new capacity all together, to modulate scar formation. The results of these studies suggest that drugs used to regulate inflammation and more recently, to inhibit the development of epithelial cancers, may be also be useful in inhibiting the growths associated with excessive scar formation, such as keloids or hypertrophic scars, in those with a predisposition to their development.


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POSITIONAL CLONING OF A NOVEL HERITABLE, RARE, FOLATE-SENSITIVE FRAGILE SITE (FRAXG) IN Xp22.1 ASSOCIATED WITH SHORT STATURE IN A FINNISH KINDRED AND CLONING OF AN ASSOCIATED CANDIDATE GENE (FXGAG)

DISSEPTION

Presented in Partial Fulfillment of the Requirement for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

Shanxiang Zhang, M.S.

* * * * *

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2001

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ABSTRACT

Fragile sites are chromosomal nonstaining gaps or breaks that become apparent when cells are cultured under special inducing conditions. Based on their frequency, fragile sites are classified as common or rare fragile sites. Common fragile sites are part of normal chromosomal structures, which may be involved in chromosomal recombination, deletions and instability. Rare fragile sites occur in less than 1% of individuals. Rare, heritable folate-sensitive fragile sites (RHFFS), which are induced by low folic acid and/or thymidine in culture medium, have been clearly linked to several clinical phenotypes, including fragile X syndrome, the most common inherited form of mental retardation in children, and Jacobsen's syndrome. Molecular characterization of a total of five RHFFS to date has indicated a common underlying mutation and pathophysiological mechanism: the expansion of an unstable (CCG)n/(CGG)n trinucleotide repeat in the 5' promoter region together with the hypermethylation of an adjacent CpG island result in transcriptional silencing of a downstream gene and the clinical phenotype in patients.

The genetic basis for short stature (SS) in general and in Turner syndrome (TS) in particular has been investigated for at least five decades. One or multiple genes in the short arm, particularly Xp22, of the X chromosome have been implicated. However, the underlying gene(s) has been elusive, as most of the studies were based on the observed cytogenetic deletions that encompass several genes.
Here, we report the association of a novel RHFFS in Xp22.1 with the SS phenotype in a Finnish proband. Through positional cloning, the novel RHFFS, termed GRAXG, was characterized. Like other RHFFS, FRAXG is also caused by the expansion of a normally polymorphic (CGG)n trinucleotide repeat. When mutated, the (CGG)n repeat and the adjacent CpG island are hypermethylated. This finding provides further evidence for the proposed general etiological mechanism of RHFFS.

In addition, a novel gene FXGAG associated with the (CGG)n repeat has been identified. Based on its associated phenotype of SS in the proband and its genomic location in Xp22.1, we propose that FXGAG constitutes a novel candidate gene for SS and TS.
Dedicated to my Family
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CHAPTER 1

LITERATURE REVIEW

1.1 CHROMOSOMAL FRAGILE SITES

Chromosomal fragile sites are sites that show an increased frequency of gaps and breaks when the cells from which the chromosomes are prepared are exposed to specific conditions of tissue culture or chemical agents (Fig. 1.1) (Sutherland, 1979b).

The first chromosomal fragile site was reported by Dekaban (Dekaban, 1965) on the long arm of a C-group chromosome in 1965. In 1968, Lejeune first reported a heritable fragile site in 2q1 in a woman and her daughter (Sutherland, 1979b). Since then many fragile sites were reported. However, most fragile sites were regarded as preparational artifacts or common human chromosomal variants of little or no clinical significance (Sutherland, 1979b). In 1969, Lubs reported the observation of fra(X), or marker X, in the long arm of the X chromosome in two males with mental retardation and their unaffected mother, when their blood lymphocytes were cultured in medium 199 (Lubs, 1969). However this otherwise groundbreaking discovery, which would lay the foundation for the characterization of fragile X syndrome and other

trinucleotide
Figure 1.1 Examples of some common fragile sites. Human chromosome metaphases were GTG-banded. Fragile sites were shown as nonstaining chromosome gaps or breaks (indicated by arrows) (Wells et al., 1997).
repeat diseases, could not be confirmed in many other large families with mental retardation segregating as an X-linked trait, and was dismissed as a serendipitous finding (Jacky, 1996). It was not until 1977 that Sutherland showed that the expression of the fragile site on the X chromosome and other apparently rare and heritable fragile sites on other chromosomes was a function of the type of medium used in the cell culture for chromosome preparation (Sutherland, 1977a). He demonstrated that the fra(X) and other heritable fragile sites were generally expressed in only a portion of cells analyzed but at significantly higher frequency in tissue culture medium 199 as opposed to, for instance, RPMI 1640 or Ham’s F10 (Sutherland, 1977a; 1979a). In 1979, Sutherland summarized the inducing conditions for fra(X) and other heritable fragile sites, the group currently known as rare heritable folate-sensitive fragile sites (RHFFS) (Sutherland, 1979a). It was the relative deficiency of folic acid and a pyrimidine, thymidine, in medium 199, which were responsible for its effectiveness in eliciting fragile site expression, when compared to other tissue culture media. Media containing normal levels of folic acid and/or thymidine suppressed the expression of fra(X), which explains the absence of fra(X) in some families with familial mental retardation when cells were cultured in regular media. Similarly this type of fragile site could be suppressed by thymidine analogue, such as 5-bromodeoxyuridine. Folate antagonists, such as methotrexate, and the thymidylate synthetase antagonist 5-fluorodeoxyuridine could effectively induce folate/thymidine depletion and elicit expression of the fragile site (Sutherland, 1979a). Based mainly on his work, Sutherland put forward the current working definition of heritable fragile sites on human chromosomes: (1) they are nonrandom achromatic lesions bridging one or both
chromatids of a chromosome arm; (2) they are usually present in only a portion of cells analyzed, but when expressed, are present at exactly the same chromosome position in all cells from an individual and other individuals within the same kindred; (3) fragile sites are indeed fragile as evidenced by deletion of the chromosome fragment distal to the fragile site, or by the formation of triradial type structures; and (4) the fragile site in a chromosome is heritable and segregates in a mendelian codominant fashion within a kindred (Sutherland, 1979b).

The clarification of fragile site definition and inducing conditions led to the discovery of over 100 fragile sites to date (Sutherland et al., 1999). Fragile sites are named following two forms of nomenclature: one is cytogenetic nomenclature using “fra” followed in parentheses by the chromosome band in which the fragile site is present. For example, the rare fragile site in band 27.3 on the long (q) arm of X chromosome is thus fra(X)(q27.3). Another nomenclature is a gene symbol for each fragile site, which is given by “FRA,” the chromosome number, and an assigned alphabet based on the order of their consideration by the international committee on human gene mapping (Berger et al., 1985; Sutherland et al., 1987; Sutherland et al., 1988).

According to their frequency and the culture conditions that induce their expression, fragile sites are generally divided into two classes (Sutherland GR, 1996; Sutherland et al., 1999) common or rare fragile sites. Common fragile sites appear to be part of the normal chromosome structure and are believed to be present in all individuals (Sutherland et al., 1999). As summarized in Table 1.1,
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1. folate sensitive</td>
<td>low folate and thymidine, FudR, MTX, high thymidine</td>
<td>23</td>
<td>5</td>
<td>CCG</td>
</tr>
<tr>
<td>2. distamycin A inducible</td>
<td>1. distamycin A, other minor groove-binding oligopeptide antibiotics, and BrdU</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2. as above, but not BrdU (recorded only in the Japanese)</td>
<td>2</td>
<td>1</td>
<td>33 bp AT-rich</td>
</tr>
<tr>
<td>3. BrdU inducible</td>
<td>BrdU or BrdC</td>
<td>2</td>
<td>1</td>
<td>~42 bp variable AT-rich</td>
</tr>
<tr>
<td>Common</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. aphidicolin inducible</td>
<td>aphidicolin for at least 24 hr</td>
<td>76</td>
<td>3</td>
<td>none obvious</td>
</tr>
<tr>
<td>5. 5-azacytidine inducible</td>
<td>5-azacytidine for no more than 8 hr</td>
<td>4</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>6. BrdU inducible</td>
<td>BrdU for no more than 8 hr</td>
<td>6</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>7. Adenovirus 12 inducible</td>
<td>adenovirus 12 in U1 small nuclear RNA genes in p53-expressing cells</td>
<td>4</td>
<td>0</td>
<td></td>
</tr>
</tbody>
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Table 1.1 Classification and Some Properties of Fragile Sites (Sutherland et al., 1999)
four subgroups of common fragile sites are currently identified (Sutherland et al., 1999). The first one is aphidicolin-inducible. Aphidicolin is an inhibitor of DNA polymerase alpha and delta (Glover et al., 1984). Aphidicolin induces the majority of common fragile sites, including the most prevalent common fragile site FRA3B in 3p14.2 (Sutherland et al., 1999). The second one is 5-azacytidine-inducible. Five-azacytidine, an analogue of cytidine, has been reported to induce the elongation of centric heterochromatin of chromosomes 1, 7, 9, and 16; segmentation, and at higher concentration, pulverization of chromosomes (Fucik et al., 1970; Viegas-Pequignot et al., 1976; 1981). The third one is bromodeoxyuridine (BrdU)-inducible. BrdU is incorporated into DNA during DNA replication in place of thymidine. It can induce both common and rare fragile sites (Sutherland GR, 1996; Sutherland et al., 1999). The last subgroup in common fragile sites is adenovirus 12-inducible (Sutherland et al., 1999). This is a special group of fragile sites. They occur in the small nuclear RNA gene clusters but only in cells expressing p53. To date, four such fragile sites have been reported. It is postulated that an adenovirus 12 protein causes p53 to undergo a gain of function, leading either to perturbed transcription by RNA polymerase II or to inefficient chromatin condensation (Bailey et al., 1995; Li et al., 1998a; Li et al., 1998b).

In contrast to common fragile sites, rare fragile sites vary in frequency from only a handful of case reports up to 1 in 40 chromosomes (Sutherland, 1982; Sutherland GR, 1996). Based on the inducing conditions or agents used, three subgroups are identified (Table 1.1) (Sutherland, 1996). The first one is folate-sensitive. This group of fragile sites is induced by low folate and thymidine in the culture medium, and/or in
the presence of folate antagonists, such as methotrexate (MTX), and thymidylate synthetase antagonist 5-fluorodeoxyuridine (5-FUdR). Excess of thymidine may also induce this group of fragile sites. Folic acid is a cofactor for the conversion of uridine monophosphate to thymidylate. Thymidine provides thymidine triphosphate for DNA synthesis. At high concentrations, thymidine inhibits ribonucleotide reductase, which converts cytidine diphosphate to deoxycytidine diphosphate. Thus the induction of this group of fragile sites requires a relative deficiency of deoxycytidine 5'-triphosphate (dCTP) or deoxythymidine 5'-triphosphate (dTTP) at the time of DNA synthesis (Sutherland, 1977a; 1979a). The second one is distamycin A-inducible. Distamycin A is an oligopeptide antibiotic that binds to the minor groove of AT-rich DNA in a sequence-specific manner (Schmid et al., 1984; Sutherland et al., 1984). In this subgroup, two distinctive subclasses are present. One is induced by other minor groove-binding oligopeptide antibiotics in addition to distamycin A, such as berenil, and BrdU; in the other group, fragile sites are induced by distamycin A and other minor groove-binding oligopeptide antibiotics only, but not BrdU (Sutherland GR, 1996). The last subgroup is BrdU-inducible. This subgroup is induced only by BrdU and its related compound bromodeoxycytidine (BrdC) (reviewed by Sutherland et al. 1999).

Their molecular characterization indicates that common and rare fragile sites are different at the molecular level (Sutherland et al., 1995; Sutherland et al., 1998). To date, four common fragile sites, FRA3B in 3p14.2, FRA7G in 7q31.2, FRA7H in 7q32.3, and FRA16D in 16q23.2 have been characterized (Wilke et al., 1994; Rassool et al., 1996; Wilke et al., 1996; Huang et al., 1998b; Mishmar et al., 1998; Krummel 1999).
et al., 2000). As common fragile sites are part of normal chromosome structures, positional cloning approaches cannot be used to identify the underlying sequences which are responsible for the fragile sites. Instead fluorescence in situ hybridization (FISH) was used with the contiged physical clones, like yeast artificial chromosome (YAC) and phage lambda clones, to define the region containing the gaps and/or breaks for the corresponding fragile sites (Wilke et al., 1994; Rassool et al., 1996; Wilke et al., 1996; Huang et al., 1998b; Mishmar et al., 1998; Krummel et al., 2000). It was also based on the observations that they are the preferential integration sites of some DNA viruses and they are mapped to the same regions with many chromosomal rearrangements and cancer breakpoints (Seki et al., 1992; Wilke et al., 1996; Mishmar et al., 1998). Generally, common fragile sites seem to be large fragile regions, spanning from ~150 to over 1000 kb in size. Sequence analyses and comparisons of the regions for these four cloned common fragile sites have indicated that those regions tend to be AT-rich in sequence and show the high-flexibility, low-stability, and may form unusual DNA structures, like non-B- DNA structure. However, no special sequences, such as expanded microsatellite repeats identified in the rare fragile sites, have been identified, which may clearly explain the fragility of these regions (Seki et al., 1992; Wilke et al., 1996; Mishmar et al., 1998).

The biological functions of the common fragile sites have not been convincingly demonstrated beyond being a basic component of chromosome (Wells et al., 1998). Overall the common fragile site regions are delayed in replication, less well condensed, and prone to break (Wilke et al., 1994; Rassool et al., 1996; Wilke et al., 1996; Huang et al., 1998b; Mishmar et al., 1998; Krummel et al., 2000). Based on its
fragility, common fragile site regions have been suggested to be preferential sites of DNA recombination, deletion, and some viral integrations (Wells et al., 1998).

Deletion of the region containing FRA3B, located in gene FHIT (for fragile histidine triad), has been observed in many solid tumors, including those of digestive tract, cervix, lung, breast, and head and neck squamous cell carcinoma (Brachman, 1994; Sozzi et al., 1996; Greenspan et al., 1997; Hayashi et al., 1997). FHIT has thus been extensively studied as a candidate tumor suppressor gene. However, no clear conclusions of FHIT as a tumor suppressor gene can be made from the functional study of Fhit protein (Mao, 1998). The locus of FRA16D, the second most frequent common fragile sites behind FRA3B, has also been observed to be deleted in many tumors, including prostate, breast, ovarian, liver cancers and multiple myeloma etc. (Smith et al., 1998; Bednarek et al., 2000; Krummel et al., 2000; Mangelsdorf et al., 2000; Paige et al., 2000; Ried et al., 2000; Yakicier et al., 2001). The gene FOR (fragile site FRA16D oxidoreductase), which spans FRA16D, has thus been proposed to contribute in those tumors with DNA instability at FRA16D (Ried et al., 2000).

Expression of common fragile sites have also been linked to intrachromosomal mammalian gene amplification and proposed to be involved in the amplification of some oncogenes during tumor progression (Coquelle et al., 1997). Combining these observations, it was suggested that the intrinsic instability at these fragile site regions might be involved mechanistically in the frequent deletions and amplifications in tumors (Smith et al., 1998). The observations that FRA3B and FRA16D are the preferential integration sites of viruses HPV16 and simian virus 40 respectively may reflect the intrinsic property of common fragile sites of forming unusual DNA
structure and being delayed in DNA replication and less well condensed (Wilke et al.,
1996; Paige et al., 2000).

Rare fragile sites were cloned by positional cloning approaches. In 1991, rare,
folate-sensitive fragile site FRAXA was the first fragile site to be cloned. Expansions
of polymorphic (CGG)n trinucleotide repeats were identified to co-segregate with the
expressions of FRAXA in the families with Fragile X syndrome (Heitz et al., 1991;
Kremer et al., 1991a; Verkerk et al., 1991; Yu et al., 1991). To date, a total of seven
rare fragile sites have been characterized at the molecular level (Sutherland et al.,
1999). Five of them: FRAXA, FRAXE, FRAXF, FRA11B, and FRA16A, which are
all rare, folate-sensitive fragile sites, are caused by expansions of polymorphic
(CCG)n/(CGG)n repeats (Kremer et al., 1991a; Oberle et al., 1991; Verkerk et al.,
1991; Yu et al., 1991; Knight et al., 1993; Nancarrow et al., 1994; Parrish et al., 1994;
Jones et al., 1995). The other two, FRA16B and FRA10B are distamycin A inducible
and bromodeoxyuridine requiring rare fragile sites respectively, and are caused by the
expansion of polymorphic AT-rich minisatellite repeats (Yu et al., 1997; Hewett et al.,
1998).

To date, a total of six fragile sites have been reported on the X chromosome
(Jacky, 1996). As shown in Fig.1.2, they are FRAXA through FRAXF following the
order they were considered by the committee at the Eighth and Ninth International
Workshops on Human Gene Mapping (HGM8/9) (Berger et al., 1985; Sutherland et
al., 1987; Sutherland et al., 1988). Three of them: FRAXB, FRAXC, and FRAXD are
common fragile sites. The other three are rare, folate-sensitive fragile sites. FRAXB is
located in the short arm, Xp22.31 (Fig1.2). The other five are located in the long arm
Figure 1.2 Gene mapping nomenclature for X chromosome fragile sites, both common: FRAXB, FRAXC, FRAXD, and rare: FRAXA, FRAXE, FRAXF, and their distribution along the chromosome are shown.
of X chromosome, FRAXA in Xq27.3; FRAXC in Xq22.1; FRAXD in Xq27.2; FRAXE in 28; and FRAXF in Xq28 (Jacky, 1996). FRAXA, FRAXE, and FRA11B have been linked to clinical phenotypes. FRAXA is linked to fragile X syndrome, the most common form of inherited mental retardation in children (Lubs, 1969; Sutherland, 1977a; b; Tommerup, 1989). Fragile X syndrome is caused by the functional deficiency of FMR1 gene product. More than 95% of this deficiency is caused by an expansion of unstable (CGG)n trinucleotide repeat in the 5' UTR region of FMR1, as shown in Fig.1.3. The mutant expansion of (CGG)n repeats is associated with the hypermethylation of the expanded repeat and adjacent CpG island, which results in the downregulation of transcription of FMR1 (Fu et al., 1991; Kremer et al., 1991a; Kremer et al., 1991b; Oberle et al., 1991; Verkerk et al., 1991; Yu et al., 1991). The presence of the expanded (CGG)n in mutant FMR1 transcripts can also interfere with the translation of the FMRP protein (Feng et al., 1995a; Feng et al., 1995b). Small deletions and point mutations which cause the functionally deficient FMR1 in fragile X syndrome patients confirmed that Fragile X syndrome is due to a single gene FMR1 defect (Wohrle et al., 1992; De Boulle et al., 1993). FRAXE is linked to a nonspecific mild mental retardation. Similar to fragile X syndrome, the expansion of an unstable (CCG)n repeat in the 5'UTR of FMR2 gene causes the downregulation of transcription and translation of FMR2, which results in FRAXE-related mental retardation (Knight et al., 1993; Gecz et al., 1996; Gu et al., 1996). FRA11B is caused by the expansion of (CCG)n repeat in the 5'UTR of a proto-oncogene, CBL2, which is involved in Jacobsen syndrome patients (Jones et al., 1994; Jones et al., 1995; Michaelis et al., 1998; Tunnacliffe et al., 1999; Jones et al., 2000).
Figure 1. Expansion of the (CCG)n repeat and hypermethylation of the adjacent CpG island in Fragile X syndrome.
The mechanism of expanded (CCG)n/(CGG)n repeats resulting in downregulation of an associated gene is not totally clear (Jin et al., 2000). Based on mostly studies in FRAXA, the following model has been proposed (Chiurazzi et al., 1998; Chiurazzi et al., 1999; Jin et al., 2000). The expansion of (CCG)n/(CGG)n repeats induces the hypermethylation of itself and the associated CpG island by 5' DNA methyltransferase. Then a methyl-cytosine-binding protein (MeCP2) binds to hypermethylated CGG repeats and forms a complex with histone deacetylase (HDAC) via the transcription repressor Sin3. HDAC deacetylates histones H3 and H4 around the CGG repeat. Histone deacetylation then leads to chromatin condensation and transcriptional silencing. In addition to this major mechanism, the (CCG)n/(CGG)n repeat expansion in symptomatic premutation carriers may interfere with the translatability of mutant transcripts into protein (Feng et al., 1995a; Feng et al., 1995b). The overall effect is the reduced level or complete deficiency of the FMRP protein.

To date, only RHFFSs have been clearly linked to clinical phenotypes (Sutherland et al., 2000). The identification and characterization of more such fragile sites at molecular level is of great interest. Based on the common molecular mechanisms involved in the five cloned RHFFSs, it is proposed that the expansion of unstable (CCG)n/(CGG)n repeats may underlie the majority if not all other RHFFSs.
1.2 DYNAMIC MUTATIONS AND CLINICAL DISEASES

Dynamic mutations are abnormal expansions of normally polymorphic microsatellite or minisatellite repeats, which are the underlying causes of diseases (Robert D. Wells, 1998). To date, a total of 17 disorders have been linked to the dynamic mutations (Lalioti et al., 1997; Virtaneva et al., 1997; Cummings et al., 2000; Matsuura et al., 2000; Liquori et al., 2001). Fourteen of them (summarized in Table 1.2) are caused by the expansion of trinucleotide repeats (Cummings et al., 2000), and the other three, myotonic dystrophy type 2 (DM2), spinocerebellar ataxia type 10 (SCA10), and progressive myoclonus epilepsy of the Unverricht-Lundborg (EPM1) are caused by the expansion of (CCTG)n, (ATTCT)n, and (CCCCGCCCCCGCG)n repeats respectively (Lalioti et al., 1997; Virtaneva et al., 1997; Matsuura et al., 2000; Liquori et al., 2001). The 14 trinucleotide repeat diseases are summarized in Table 1.2. Based on the locations of the trinucleotide repeats to their associated genes, they are divided into two groups (Wells, 1998). As illustrated in Fig. 1.4, the first group is with the repeat in the coding region of a gene, including spinobulbar muscular atrophy (Kennedy disease), Huntington’s disease, dentatorubral-pallidoluysian atrophy, spinocerebellar ataxia type 1, 2, 3, 6, and 7. Though there are different genes involved in individual diseases in this group, they all have expansions of (CAG)n repeats in the coding region, which encodes a polyglutamine tract, and similar pathogenesis: progressive neuronal dysfunction, which typically begins in mid-life and results in severe neurodegeneration. The “gain of function” from the polyglutamine tract may be the common mechanisms underlying this group of disease.
as well. The second group is with the trinucleotide repeat in the non-coding region of a
gene: including fragile X syndrome (FRAXA), fragile XE syndrome (FRAXE),
Friedreich ataxia (FRDA), myotonic dystrophy (DM), spinocerebellar ataxia type 8
(SCA8), and 12 (SCA12). Different trinucleotide repeats as well as their different
locations in the genes are identified. FRAXA and FRAXE are caused by expansions of
(CG)n/(GCC)n repeat in the 5'UTR of \textit{FMR1} and \textit{FMR2} genes respectively. FRDA
is caused by an expansion of (GAA)n in the intron of \textit{X25} gene. DM is caused by an
expansion of (CTG)n in the 3'UTR of \textit{DMPK} gene. SCA8 is caused by an expanded
CTG repeat in its terminal exon of the transcript. SCA12 is caused by a (CAG)n repeat
in the 5'UTR region of the \textit{PPP2R2B} gene. Except in DM, which both loss-of-
function and gain-of-function mechanisms have been proposed, a loss-of-function of
its corresponding gene has been suggested to be the causes of diseases (Cummings \textit{et al.}, 2000). While the clinical phenotypes of the 14 trinucleotide repeat diseases vary,
they share the same characteristics of anticipation: that is the reduced age of onset
and/or increased severity of a disorder phenotype in successive generations
(Cummings \textit{et al.}, 2000). This phenomenon, termed Sherman paradox in fragile X
syndrome (Sherman, 1981), is the hallmark of dynamic mutation (Wells, 1998). In
normal populations, the trinucleotide repeats are polymorphic with some interrupted
sequences (Fu \textit{et al.}, 1991; Snow \textit{et al.}, 1993; Eichler \textit{et al.}, 1994; Kunst \textit{et al.}, 1994;
Snow \textit{et al.}, 1994; Zhong \textit{et al.}, 1995). However, once the copy number of the pure
triplet repeats exceeds a certain threshold, and/or the interrupted sequences are lost,
the repeat becomes highly unstable when passed through the germline to its offspring,
<table>
<thead>
<tr>
<th>Disorder</th>
<th>Inheritance</th>
<th>Gene locus</th>
<th>Chromosomal localization</th>
<th>Protein product</th>
<th>Expansion size</th>
<th>Repeat location</th>
<th>Mutation type</th>
<th>Parental gender bias</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fragile X syndrome</td>
<td>X-linked dominant</td>
<td>FMR1 (FRAX A)</td>
<td>Xq27.3</td>
<td>FMRP</td>
<td>(CGG)$<em>{62}$ (CGG)$</em>{100-200}$ (premutation) (CGG)$_{230-1000}$ (full)</td>
<td>5'-UTR</td>
<td>LOF</td>
<td>Maternal</td>
</tr>
<tr>
<td>Fragile XE mental retardation</td>
<td>X-linked dominant</td>
<td>FMR2 (FRAX E)</td>
<td>Xq28</td>
<td>FMR2 protein</td>
<td>(GCC)$<em>{62}$ (GCC)$</em>{100-200}$ (premutation) (GCC)$_{230-750}$ (full)</td>
<td>5'-UTR</td>
<td>LOF</td>
<td>ND</td>
</tr>
<tr>
<td>Friedreich's ataxia</td>
<td>Autosomal recessive</td>
<td>X25</td>
<td>9q13-21.1</td>
<td>Frataxin</td>
<td>(GAA)$<em>{24}$ (GAA)$</em>{50}$ (premutation) (GAA)$_{112-170}$ (full)</td>
<td>Intron 1</td>
<td>LOF (partial)</td>
<td>Paternal</td>
</tr>
<tr>
<td>Myotonic dystrophy</td>
<td>Autosomal dominant</td>
<td>DMPK</td>
<td>19q13</td>
<td>Myotonic dystrophy protein kinase</td>
<td>(CTG)$<em>{5-7}$ (CTG)$</em>{50,3000}$</td>
<td>3'-UTR</td>
<td>Coding</td>
<td>ND</td>
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<td>Kennedy syndrome (SBMA)</td>
<td>X-linked recessive</td>
<td>AR</td>
<td>Xq13-21</td>
<td>Androgen receptor</td>
<td>(CAG)$<em>{11,13}$ (CAG)$</em>{38,66}$</td>
<td>Coding</td>
<td>LOF (partial)</td>
<td>Paternal</td>
</tr>
<tr>
<td>Huntington's disease</td>
<td>Autosomal dominant</td>
<td>IT15</td>
<td>4p16.3</td>
<td>Huntingtin</td>
<td>(CAG)$<em>{29}$ (CAG)$</em>{30,121}$ (CAG)$<em>{35}$ (CAG)$</em>{31,88}$</td>
<td>Coding</td>
<td>Coding</td>
<td>Paternal</td>
</tr>
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<td>Dentatorubral-pallidolouysian atrophy/Haw River syndrome</td>
<td>Autosomal dominant</td>
<td>DRPLA (B37)</td>
<td>12p13.31</td>
<td>Atrophin-1 (drplap)</td>
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<td>Coding</td>
<td>Coding</td>
<td>Paternal</td>
</tr>
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<td>Spinocerebellar ataxia type 1</td>
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<td>SCA1</td>
<td>6p23</td>
<td>Ataxin-1</td>
<td>(CAG)$<em>{0,39}$ (CAG)$</em>{41,81}$</td>
<td>Coding</td>
<td>GOF</td>
<td>Paternal</td>
</tr>
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<td>SCA2</td>
<td>12q24.1</td>
<td>Ataxin-2</td>
<td>(CAG)$<em>{14,31}$ (CAG)$</em>{55,64}$</td>
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<td>GOF</td>
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<td>Spinocerebellar ataxia type 3/Machado-Joseph disease</td>
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<td>SCA3 (MJD1)</td>
<td>14q32.1</td>
<td>Ataxin-3</td>
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<td>GOF</td>
<td>Paternal</td>
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<td>Autosomal dominant</td>
<td>SCA6</td>
<td>19p13</td>
<td>Voltage-dependent (CAG)$<em>{4}$ (CAG)$</em>{20,23}$ (EA2) (CAG)$_{21}$ (St'A6)</td>
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<td>SCA7</td>
<td>3p12-13</td>
<td>Ataxin-7</td>
<td>(CAG)$<em>{7,11}$ (CAG)$</em>{18,130}$</td>
<td>Coding</td>
<td>GOF</td>
<td>Paternal</td>
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<tr>
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<td>SCA8</td>
<td>13q21</td>
<td>none</td>
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<td>3'-UTR</td>
<td>unknown</td>
<td>ND</td>
</tr>
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<td>PPP2R2B</td>
<td>5q31-33</td>
<td>PP2A-PR55β</td>
<td>(CAG)$<em>{7,28}$ (CAG)$</em>{60,78}$</td>
<td>5'-UTR</td>
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<td>ND</td>
</tr>
</tbody>
</table>

Table 1.2 Trinucleotide Repeats in Human Genetic Disease (Wells et al., 1998)
which generally leads to a dramatic increase of the repeat copy number in patients (Wells, 1998). In FMRI locus, normal individuals have six to 52 copies of (CGG)n repeats that are interrupted by cryptic AGG in FMRI locus (Fu et al., 1991; Snow et al., 1993; Eichler et al., 1994; Kunst et al., 1994; Snow et al., 1994; Zhong et al., 1995). When the copy number of (CGG)n repeat exceeds 60, or with the loss of interrupted AGG, the repeat becomes unstable when passed through germline to offspring (Eichler et al., 1994; Hirst et al., 1994; Kunst et al., 1994; Snow et al., 1994; Zhong et al., 1995). In females, this instability tends to express as the expanded (CGG)n repeat in offspring (Fu et al., 1991; Heitz et al., 1992; Yu et al., 1992; Fisch et al., 1995; Nolin et al., 1996), while in males the (CGG)n repeat tends to contract, especially when the male has a (CGG)n repeat in the full mutation range (Nolin et al., 1996). The difference of this inheritance between female and male is probably due to the selection against the sperms with (CGG)n in full-mutation in males (Willems et al., 1992; Rousseau et al., 1994b). However, if the copy number is less than 200, the individuals harboring the repeat within this range (52-200) are usually phenotypically normal (pre-mutation carrier) (Feng et al., 1995a). In Fragile X patients, they usually have more than 230 copies of (CGG)n repeats (full mutation) (Oberle et al., 1991; Rousseau et al., 1991; Hansen et al., 1992; Hornstra et al., 1993).

The mechanisms underlying dynamic mutations are currently unclear (Richards et al., 1997). Two relevant aspects have been under intensive studies in the past ten years: one is what factors determine the stability of a trinucleotide repeat; another is how the observed instabilities of the trinucleotide repeats in disease patients occur (Wells, 1998). Generally it is believed that the instability of a trinucleotide repeat is
from the DNA itself instead of protein defects, as no instabilities of other microsatellite repeats in these patients are present (Wells, 1998). The limits of DNA instability to the specific trinucleotide repeat in this group of trinucleotide repeat diseases separate them from another group of DNA instability diseases showing overall DNA instabilities at the microsatellite repeat loci due to the defects of DNA repair enzymes, like defect of MSH2 in some colon cancer patients (Glover, 1998).

In Fragile X syndrome, population and in vitro studies have shown that the stability of (CGG)\textsuperscript{n} repeat is both length- and purity-dependent (Eichler \textit{et al.}, 1994; Hirst \textit{et al.}, 1994; Kunst \textit{et al.}, 1994; Snow \textit{et al.}, 1994; Zhong \textit{et al.}, 1995). It has been reported that a perfect (CGG)\textsuperscript{n} repeat with as little as 34 conferred the instability and the disease transmission (Eichler \textit{et al.}, 1994). Besides the repeat itself, it is also suggested that the stability of the repeat is related to its flanking sequences or haplotypes (Richards \textit{et al.}, 1992; Oudet \textit{et al.}, 1993). Linkage studies with a polymorphic dinucleotide marker 7 kb upstream of the (CGG)\textsuperscript{n} repeat in \textit{FMR1} locus and two single nucleotide polymorphic loci downstream of the (CGG)\textsuperscript{n} repeat showed that certain haplotypes were over-represented in the Fragile X syndrome patients independent of the length and purity of the (CGG)\textsuperscript{n} repeat (Richards \textit{et al.}, 1992; Oudet \textit{et al.}, 1993; Kunst \textit{et al.}, 1994). Similarly, the stability of other trinucleotide repeats, like (CAG)\textsuperscript{n} and (CTG)\textsuperscript{n}, have been shown to be dependent on their repeat length and purity, and may be affected by its flanking sequences (Brock \textit{et al.}, 1999; White \textit{et al.}, 1999; Rolfsmeier \textit{et al.}, 2000).

To date, several mechanisms have been proposed to explain how the trinucleotide repeats expand (Wells, 1998). Generally it involves the formation of
alternative DNA structures within triplet repeats, thus resulting in aberrant DNA replication, recombination and repair (Sinden, 1999). For (CGG)n and (CAG)n repeats, it is believed that they may form hairpin structure in vivo due to the intramolecular base-pairings among the repeats. When the repeat is long enough, i.e. when the copy number of the repeat exceeds the threshold for the instability or the interrupted stabilizing sequence is lost, the formed secondary structures become highly stabilized (Mitas, 1997; Darlow et al., 1998; Pearson et al., 1998a; Pearson et al., 1998b; Pearson et al., 1998c). The DNA replication in the locus containing these stable secondary structures may be staggered or blocked, which may lead to the repeat expansion or contraction (Sinden, 1999). One proposed mechanism is through DNA slippage during DNA replication. Depending on whether it is the DNA template strand or primer strand the formed secondary structure is located on, the repeat may contract or expand respectively during DNA replication. Other mechanisms, like gene conversion, DNA slippage synthesis etc. have also been proposed (Sinden, 1999).

1.3 GENETICS OF SHORT STATURE

Growth is a fundamental aspect of the development of an organism. Height, a major index of human growth, represents a multifactorial trait influenced by both environmental and genetic factors. Environmental factors, such as nutrition, living conditions, and infections have been associated with final adult height. Remarkable increases in average adult height observed over the last century have been ascribed to improved nutrition, living conditions, and the control of most common infections.
(Vogel et al., 1997). Genetic factors, such as growth hormone (GH), insulin-like growth factor I (IGF-I), and genes involved in skeletal development have been identified to be important in the achievement of final adult height (Phillips et al., 1981; Leung et al., 1987; Martial et al., 1992; Rousseau et al., 1994a; Shiang et al., 1994; Briggs et al., 1995; Goddard et al., 1995; Hecht et al., 1995; Kotzot et al., 1995; Muenke et al., 1995).

Short stature is defined as a condition in which the height of an individual is 2 standard deviation (SD) below the corresponding mean height for a given age, sex, and population group (Ranke, 1996). It affects about 3% of the population (Ranke, 1996). While some short stature cases are related to known environmental or/and genetic factors, the majority of them do not have any known etiology. So-called idiopathic short stature (ISS) is based on the exclusion of other likely causes of short stature, as well as on meeting the following minimal criteria: normal size (>=–2 SDs) for gestational age at birth; normal body proportions; no evidence of chronic organic disease; no psychiatric disease or severe emotional disturbance; normal food intake; and no evidence of endocrine deficiency. As part of ISS, familial short stature (FSS) is an assumed diagnosis for children whose measured height at any given age is below the normal range (mean –2 SDs) of the corresponding population, but lies within the “normal” range for parental height (Ogata et al., 1993; Preece, 1996; Price, 1996; Ranke, 1996; Kelnar et al., 1999).

Based on the fact that a strong resemblance exists between the height of family members, and the extent of this similarity increases, as the family relationship becomes closer, it is suggested that there is a genetic basis to adult height (Preece, 1996).
1996). Considering that adult stature is continuously and normally distributed, a polygenic model of inheritance has been proposed (Preece, 1996). It assumes that the effects of different genes involved are additive with an appropriate scale of measurement (Preece, 1996). Identifying the underlying genes has been the focus of much research in the past decades.

Short stature has been consistently linked with a well-known syndrome, Turner syndrome (TS) (Ullrich, 1930; Turner, 1938). Turner syndrome was first described by Otto Ullrich and Henry Turner (Ullrich, 1930; Turner, 1938). In 1959, Ford et al. first reported a TS patient with a 45, X karyotype (Ford, 1959). Since then, the lack of one X-chromosome in females (monosomy X) has been linked to TS. It is estimated that 1-2% of all human conceptions contain the monosomy X (Hassold et al., 1980; Hassold et al., 1992). About 99% of 45, X fetuses do not survive to term: most die by 28 weeks of gestation (Zinn et al., 1993). However, about 1/5,000 live born females have a single X-chromosome (Hook et al., 1983). Their main features include short stature, prepubertal ovarian failure, and anatomical abnormalities, including webbed neck, increased carrying angle of the elbow (cubitus valgus), congenital swelling of the hands and feet (lymphedema), aortic narrowing (coarctation), high arched palate etc. Other features include glucose intolerance, hypothyroidism and cognitive deficit in the ability to analyze visual-spatial relationships. Individual TS patients may have different combinations of the above features, but invariably include short stature and ovarian failure (Zinn et al., 1993). The mechanisms underlying these symptoms are currently unknown (Zinn et al., 1993). Based on the monosomy of X in TS, several hypotheses have been proposed to explain the TS phenotype (Zinn et al., 1998a). The
first one is chromosome aneuploidy, including nonspecific effects, such as
chromosome imbalance (Ogata et al., 1995a), or specific effects such as impaired
meiosis caused by unpaired chromosomes (Zinn et al., 1998a). The second one is that
the loss of one X chromosome might unmask X-linked recessive mutations, or,
alternatively, may cause phenotypic expression of imprinted X-linked loci (Zinn et al.,
1998a). Currently the most widely accepted hypothesis is that haploinsufficiency of
specific genes in X chromosome may cause the TS phenotype (Zinn et al., 1998a).

Haploinsufficiency of a gene implies that diploid dosage of the gene is required
for normal function (Ashburner, 1989). Accordingly for TS, it is believed that one
functional copy of X-chromosome gene(s) is not sufficient in 45, X, even though one
X chromosome is randomly inactivated in individual cells in female (X-chromosome
inactivation). This implies that the gene(s) for TS must escape X-chromosome
inactivation. Also implied in the haploinsufficiency hypothesis is that there is a
functional homologue of the TS gene(s) in the Y chromosome, which explains why
there is no TS phenotype in a male, even though only one X chromosome is present in
normal males (Zinn et al., 1993; Zinn et al., 1998a).

In accordance with the above-proposed criteria for TS and its underlying gene(s),
genes in the pseudoautosomal region of the X chromosome have been the focus of
study (Ogata et al., 1992a; Joseph et al., 1996; Schwinger et al., 1996; Spranger et al.,
1997). The pseudoautosomal region in the X and Y chromosomes is a region of about
2.6 Mb in the tip of short arms, which recombine during meiosis (Strachan et al.,
1996). Genes there have been shown to escape X chromosome inactivation, and have
functional homologues in Y chromosome (Rappold, 1993). Many cytogenetic studies
of TS patients indicate that most TS patients have only partial X chromosomal deletions. By genotype/phenotype correlation studies, it has been established that different phenotypes, such as short stature, ovarian failure in TS, are caused by the loss of different loci (Ferguson-Smith, 1965; Kalousek et al., 1979; Fryns et al., 1981; Goldman et al., 1982; Simpson, 1988; Jacobs et al., 1990; Temtamy et al., 1992; Ogata et al., 1995a). Short stature is most likely linked to the deletion of the pseudoautosomal region in Xp22.31 (Ballabio et al., 1989; Ogata et al., 1992a; Ogata et al., 1992c; Ogata et al., 1995a). These studies led to the cloning of SHOX/PHOG from this region in 1997 as the short stature candidate gene. SHOX stands for short stature homeobox-containing gene; PHOG stands for pseudoautosomal homeobox-containing gene. The same gene was cloned independently by Rao et al. and Ellison et al., respectively (Ellison et al., 1997; Rao et al., 1997). Both groups showed that SHOX/PHOG escapes X inactivation and that there is a functional homologue in the Y chromosome (Ellison et al., 1997; Rao et al., 1997). A functionally significant mutation in SHOX was also identified in one out of 91 screened patients with ISS. This mutation cosegregates with short stature in that family (Rao et al., 1997).

Combining its wide expression with the highest expression in bone marrow fibroblast, SHOX/PHOG has been considered as a very strong candidate gene for short stature (Ellison et al., 1997; Rao et al., 1997; Clement-Jones et al., 2000).

However, additional data indicate that there are other gene(s) besides SHOX/PHOG in Xp likely involved in ISS and TS (Zinn, 1997; Zinn et al., 1998a; Zinn et al., 1998b). Firstly, only about 1% of patients of ISS contain mutations in SHOX (Rao et al., 1997). Secondly, deletion of Xp22.3, which includes SHOX, does
not always correlate with short stature. Thirdly, the bigger the region deleted, the more severe the short stature phenotype is (Ellison et al., 1997; Zinn, 1997). By using a statistical treatment of the correlation between short stature with different partial deletions in Xp observed in patients, Zinn et al. identified that a deletion of a region in Xp11-p22.1 between markers SYP and PDHAL is significantly linked to short stature (illustrated in Fig. 1.5) (Zinn et al., 1998b). The region is estimated to be 30 Mb. Using similar approach, Wandstrat et al. (Wandstrat et al., 2000) reported that there might be a common region of approximately 2 Mb in Xp22.12 that contains the breakpoints for short stature. However, other than SHOX, the culprit gene(s) remains elusive, partly because the genetic defects very often express as contiguous deletion, which contains several genes (Ballabio et al., 1989; Ballabio et al., 1991; Ballabio et al., 1992; Wandstrat et al., 2000). Identification of patients with normal cytogenetics may eventually help to pinpoint the underlying gene(s).

In this thesis, we report the association of a novel RHFFS in Xp22.1 with the SS phenotype in a Finnish proband. Through positional cloning, the novel RHFFS, termed GRAXG, was characterized. Like other RHFFS, FRAXG is also caused by the expansion of a normally polymorphic (CGG)n trinucleotide repeat. When mutated, the (CGG)n repeat and the adjacent CpG island are hypermethylated. This finding provides further evidence for the proposed general etiological mechanism of RHFFS. In addition, a novel gene FXGAG associated with the (CGG)n repeat has been identified. Based on its associated phenotype of SS in the proband and its genomic location in Xp22.1, we propose that FXGAG constitutes a novel candidate gene for SS and TS.
Figure 1.4 Location of trinucleotide expansions in humans. A summary of the locations of the known trinucleotide expansions is indicated within this factitious gene consisting of a coding sequence (gray) distributed between three exons with two intervening introns. The sequence of the involved repetitive triplet is indicated above the named diseases. CGG expansions occur in the 5' UTR in Fragile X syndrome, fragile XE mental retardation, and fragile site 11B, which has been associated with disease only indirectly through its predisposition to Jacobsen syndrome, a chromosomal deletion disorder. FRAXF and FRA16A (not indicated) are also fragile sites associated with CGG expansions, but they have not been associated with a clinical phenotype and no nearby genes have been associated with them yet. Other diseases with repeat expansions occurring in noncoding regions include Friedreich's ataxia (an intronic GAA repeat), and myotonic dystrophy, spinocerebellar atrophy 8 (CTG expansion in the 3'UTR). The expansion of CAG repeats in Huntington's disease, dentatorubral-pallidoluysian atrophy (DRPLA), spinobulbar muscular atrophy (SBMA), and the spinocerebellar atrophies (SCAs) results in proteins with elongated tracts of glutamine (Q) because of the expansion's location within a coding segment of the exon. SCA12 is caused by expansion of CAG repeat in the 5'UTR of PPP2R2B gene (Wells et al., 1998).
Figure 1.5 Short stature (SS) locus mapping
CHAPTER 2

MATERIALS AND METHODS

2.1 YAC clones

A total of six YAC clones in Xp22.1 were selected (Alitalo et al., 1995; Ferrero et al., 1995). Clones y911G5, y827E10, y946F5, and y811D11 were purchased from Research Genetics (Huntsville, AL). Clones y295D1 and y517G4 were obtained from CEPH (France). YAC clones were plated on AHC agar plates (1.7 g of YNB, Difco, Detroit MI, supplemented with 5 g of ammonium sulfate, Sigma, St. Louis, MO, and 20 g of agar-B, Bio101, Inc., Vista CA, per liter of dH2O). Isolated single clones were picked and expanded in YPD broth (Difco, Detroit MI).

2.2 Isolation of YAC DNA

A “rapid YAC DNA preparation” method was used to isolate YAC DNA. Briefly, 5 ml of overnight YPD culture at 30°C from a single YAC colony on AHC plate was pelleted. After washing in dH2O, the pellet was resuspended in 200 µl of GDIS (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris pH 8.0, and 1 mM EDTA), 200 µl of phenol-chloroform alcohol (25:24:1), and 0.35 g acid-washed glass
beads (Sigma, St. Louis, MO). Vortex the mixture rigorously for 2.5 min, then add
200 μl of dH2O and spin for 4 min at maximum speed (14,000rpm). Transfer the
aqueous layer to a new eppendorf tube, add equal volume of 100% of EtOH and let it
sit at room temperature for 2 min to precipitate the DNA. Spin down the pellet and
wash it once with 70% of EtOH. Resuspend the final DNA pellet in 200 μl of dH2O.
Use 1 μl for PCR.

2.3 Synthetic DNA primers

Oligonucleotides for PCR primers were designed using the software DNAStar
(Madison, WI), and synthesized by Fisher-Genosys (Pittsburgh, PA). A total of 30
pairs of primers for the STS markers used to map the YACs are listed in Table 2.1.

The Intron-Alu PCR primers for YAC fingerprinting were those designed by Liu
et at. (Liu et al., 1993).

<table>
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<th>Sequence</th>
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<tr>
<td>Alu1_P</td>
<td>GGA TTA CAG GYR TGA GCC A</td>
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<tr>
<td>Alu2_P</td>
<td>RCC AYT GCA CTC CAG CCT G</td>
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</table>

2.4 STS content mapping of YACs by PCR

To test whether a YAC is positive for a STS marker is tested by PCR
amplification of the marker with the corresponding synthetic PCR primers from the
YAC using the HotStar Taq Master Mix Kit (Qiagen). PCR was carried out as
followes: 1 cycle at 95 °C for 15 min; 35 cycles at 94 °C for 30 sec, Tx(°C) for 45 sec,
72 °C for 45 sec; 1 cycle at 72 °C for 10 min. Tx (°C) was determined for each pair of
PCR primers using the method as described (Innis, 1990). The amplified PCR product
was analyzed by 1.5% agarose gel electrophoresis, and visualized by ethidium
bromide staining.
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<td>AGG GGA AAG ATC TTA TTT AC</td>
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<td>GAG AAG AGA CGG GAC AGC GGG AG</td>
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<td>DXS8268</td>
<td>TGA GAT CTG TTT CAG AGC CAG C</td>
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<td>AGA GAC CAG GCA GCA GAG G</td>
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Table 2.1 STS Primer Sequences

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<td>TCA CCC TGG AAG CAC ATG G</td>
<td>ATG GGA AAA GCG TAG TAT CTG G</td>
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<td>DXS365</td>
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<td>TGA ACA CCC ATT CTA GGT</td>
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<td>DXS8265</td>
<td>CCC ACA GAA ATA GCT GAC CTG</td>
<td>TGC TGG CCT CCC AAC TA</td>
</tr>
</tbody>
</table>
2.5 Fingerprinting of YACs by Alu-PCR

The procedure was as described (Liu et al., 1993). Briefly, 50 μl of PCR reaction was carried out with either primer Alu1_P only, or Alu2_P only or Alu1_P plus Alu2_P using the HotStar Taq Master Mix Kit (Qiagen). The PCR conditions were: 1 cycle at 95°C for 15 min; 35 cycles at 94°C for 60 sec, ramp: 0.2 °C/sec to 37 °C, 37 °C for 30 sec, ramp: 0.2 °C/sec to 72 °C, 72 °C for 5 min; 1 cycle at 72 °C for 10 min. The amplified PCR product was analyzed by 1.0% agarose gel electrophoresis, and visualized by ethidium bromide staining. The image was captured using EagleEye.

2.6 Fluorescence in situ hybridization (FISH)

The procedures were basically as described (Kievits et al., 1990). Briefly, slides with normal male and female metaphase spreads were purchased from Vysis (Downers Grove, IL). Briefly, 1 μg of inter-Alu PCR-amplified PCR product from individual YAC clone or 1 μg of extracted BAC DNA was labeled with fluorescin-12-dUTP (NEN Life Science Products, Boston, MA) by nick translation (Nick Translation Kit, Vysis, Downers Grove, IL). CEPX Alpha, SpentraGreen (Vysis, Downers Grove, IL), specific for X chromosome centromere was included for X chromosome identification. Texas Red-5-dUTP (NEN Life Science Products, Boston, MA)-labeled BAC b733018, mapped to Xp22.3, was included for the identification of the telomeric part of the X chromosome. DNA concentrations were 10 ng/μl for YAC and b733O18, and 1/10 dilution of CEPX Alpha. Human Cot-1 competitor DNA (GIBCO BRL, Rockville, Maryland) was used at 400 ng/μl. Before hybridization, metaphase spread slides were denatured by 70% formamide (Pittsburgh, PA) and...
dehydration by serial treatment in 70%, 85%, and 100% of EtOH. Hybridization mix
(20 μl) containing probes and Cot-1 was denatured and preannealed for 30 min at
37°C, then added onto the pretreated slides. The slides were hybridized in the moisture
chamber for overnight at 37°C. Slides were then washed at 44°C in 2X SSC plus 50%
formamide, and further washed to 0.1X SSC. Slides were finally rinsed in 1X PBD
(0.1 N NaH₂PO₄ + 0.1 N Na₂HPO₄ + 0.1% IGEPAL CA-630 (Sigma, St. Louis, MO)
before they were mounted in antifade solution with DAPI (Vector Laboratories,
Burlingame, CA). Axioplan 2 and Axioshot 2 Universal Microscope (Zeiss, Germany,
HBO 100) was used for image capture, and the computer software ISIS (Metasystem,
MA) for analysis.

2.7 Preparation of STS probes for library screening

The 18 STS markers positively mapped to the YAC contig were used for the
BAC library screening. These STS fragments (Table 2.1) were amplified with the
corresponding primers by PCR using the HotStar Taq Master Mix Kit (Qiagen). The
PCR conditions were the same as for the YAC mapping. Amplified PCR products
were separated in 0.8% LMP agarose (SeaPlaque GTG, FMC BioProducts, Rockland,
ME) gel electrophoresis, and bands of the expected size were cut out and extracted
from the gel using QIAquick Gel Extraction Kit (Qiagen). The purified DNA was [α-
32P]dCTP- labeled by nick translation using Prime-IT II Random Primer Labeling Kit
(Stratagene, Cedar Creek, TX, 300385).
2.8 BAC library screening

Human BAC library RPCI-11 (BAC/PAC Resource, Children’s Hospital, Oakland, CA) was used. Each set of RPCI-11 contains 24 filters. In general, three filters were initially screened with each probe using RapidHyb buffer (Amersham Life Science, England) following the manufacturer’s protocol. If less than two positive clones were identified, additional filters were screened until at least two positive clones were identified.

2.9 BAC DNA extraction and end-sequencing

Positive BACs by hybridization were obtained from the BAC/PAC Resource (Children’s Hospital, Oakland, CA). Each clone was further tested by PCR with the same primers for the generation of the respective probes. BAC DNA from confirmed positive clones was extracted from the cells using Qiagen-tip 100 columns (Qiagen) following the manufacturer’s protocol. End sequences for each BAC were determined by sequencing on an automated system (ABI Prism, Foster City, CA).

2.10 DNA probes for chromosomal walking

DNA sequences from the BAC ends were BLAST searched against the NCBI nucleic acid database (www.ncbi.nlm.nih.gov) to identify nonrepetitive region. PCR primers were selected from unique sequences using DNA Star program, and were synthesized by Fisher-Genosys (Pittsburgh, PA). New probes from the BAC end-sequences were generated, and used for further BAC library screening. The primers used were listed in Table 2.2.

34
<table>
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<th>Primer</th>
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2.11 BAC analysis by field inversion gel electrophoresis (FIGE)

To estimate the sizes of BACs, 0.5 μg of each BAC was digested with *Not* I, and separated on 1.0% pulsed field certified agarose gel (SeaPlaque GTG, FMC BioProducts, Rockland, ME) using FIGE mapper (Bio-Rad, Hercules, California) with program 4 (switch time ramp: 0.1-3.5 sec, linear shape; forward voltage: 180, reverse voltage: 120; run time: 16 hr at room temperature.) following the manufacturer's protocol. Gels were stained by ethidium bromide and images were captured using EagleEye.

2.12 Induction of FRAXG from the proband's peripheral blood lymphocytes

The induction was carried out in Finland following the recommended procedures (Jacky et al., 1991). Briefly, 0.5 ml whole blood from each family member was cultured at 37 °C with 5% CO₂ for 4 days in medium 199 (Gibco BRL) supplemented with 6% of fetal calf serum and 1% PEST (Gibco BRL) in the total volume of 8 ml. Cells were treated with colcemid for 2 hr before harvesting. 0.75M KCl was used as hypotonic solution for 10 min at 37 °C and the cells were fixed in acetic acid/methanol (1:3) fixtative in standard way.

2.13 Induction of *FRAXG* from the proband's lymphoblastoid cell line

All lymphoblastoid cell lines (LBCLs) were established from peripheral blood lymphocytes at Finland according to the established protocols (Jacobs et al., 1982; Abruzzo et al., 1986). The LBCL from the proband was maintained in medium RPMI 1640 (Mediatech, VA) supplemented with 15% fetal bovine serum (GIBCO BRL), 2
mM L-glutamine, 20 mM Hepes buffer, pH 7.2, and penicillin/streptomycin. The FRAXG was induced by suspending 2 \times 10^5 \text{cells/ml} or 5 \times 10^5 \text{cells/ml} in medium 199 (GIBCO BRL) supplemented with 5% fetal bovine serum, 20 mM Hepes buffer, pH 7.2, and penicillin/streptomycin. The cultures were treated with either 10^{-6}, 5 \times 10^{-7} \text{ or } 10^{-7} \text{M FudR (5-fluoro-2'-deoxyuridine, Sigma, MO) for 24 and 48 hrs; or } 10^{-7} \text{M MTX (methotrexate, Bedford Laboratories, OH) for 24 and 48 hrs. Colcemid (GIBCO BRL) was added to the culture at 100 \mu g/ml for the last 45 min of the incubation period and the cells were harvested by standard cytogenetic techniques (Rooney, 1997).}

2.14 Giemsa staining of metaphase slides

Cells in Carnoy’s fixative (methanol and acetic acid, 3 to 1 in volume) were washed several times with fresh Carnoy’s fixative until the pellet was clear. The pellet was resuspended in 1 ml of Carnoy’s fixative. 1 to 3 drops were dropped onto slides treated with 95% EtOH. Slides were air-dried and stained by Giemsa dye (Sigma) (Rooney, 1997).

2.15 Synthetic DNA primers for genotyping of \textit{FRAXG} (CGG)n locus and cloning of \textit{FXGAG} gene

Listed in Table 2.3.
<table>
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<tr>
<th>Primer</th>
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<td>GCCTCATCTCTGCTACTCGTCTT</td>
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Table 2.3 Synthetic DNA Primers Used in FRAXG (CGG)ₙ analysis cloning of FXGAG

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2.16 Southern blot for BAC or genomic DNA

1 µg of BAC DNA or 10 µg of genomic DNA was digested to completion with the appropriate restriction enzymes, resolved on a 0.8 % agarose gel, blotted onto Zeta-Probe membranes (BIO-RAD, CA, 162-0197). The membranes were hybridized with appropriate \([\alpha^{-32P}] dCTP\)-labeled probes, and autoradiographed according to standard procedures (Southern, 1975). For the Screening of \((CCG)n/(CGG)n\) trinucleotide repeats in BACs, the BAC DNA filters were hybridized by \([\alpha^{-32P}] \) ATP end-labeled \((CGG)_7\) probe using RapidHyb (Amersham) solution following the manufacturer's protocol.

2.17 Cloning of \((CCG)\tau\)-positive fragments from BAC DNA

The identified positive BACs for \((CCG)\tau\) probe by Southern blot based screening were digested with the same restriction enzymes, resolved on 0.8 % low-melting-point GTG agarose (FMC BioProducts, Rockland, ME). The corresponding fragments positive for \((CCG)_7\) were cut from the gel. The DNA was extracted with Qiagen Gel-extraction kit (Germany), and cloned into \(pZErO\)-2 vector (Invitrogen, Carlsbad, CA). The presence of inserts with correct size was verified by restriction enzyme analysis, and the presence of \((CCG)n\) repeat was further confirmed by Southern blot analysis with the \([\alpha^{-32P}] \) ATP labeled \((CCG)_7\). The inserts from confirmed clones were sequenced using an automated system (ABI Prism, Foster City, CA).
2.18 PCR amplification of FRAXG (CCG)n locus

The FRAXG (CCG)n locus was amplified with synthetic PCR primers M13F393H10_PF and M13R393H10_PR using PLATINUM Taq PCRx DNA Polymerase system with the enhancer solution (Life Technologies, Rockville, Maryland). The PCR conditions were: 1 cycle at 95°C for 5 min; 35 cycles at 94°C for 1 min, 65°C for 2 min, and 72°C for 5 min; 1 cycle at 72°C for 10 min.

To genotype the FRAXG (CCG)n locus, the fluorescence labeled primer FAM_393H10_PF was used in place of M13F393H10_PF in the above PCR reactions. The amplified PCR products were resolved using an automated system (ABI Prism, Foster City, CA).

2.19 Shotgun sequencing of BAC b393H10

Six μg of extracted b393H10 DNA was sheared by sonication in the presence of 1X Mung Bean buffer in a volume of 48 μl with a Sonicator (Misonix Incorporated). The conditions were: power level 3, three seconds for three times followed by two seconds once. After sonication, the DNA was treated with 80 units of Mung Bean exonuclease for 30 min at 30 °C. After phenol/chloroform extraction and ethanol precipitation, the DNA was treated with five units of Pfu polymerase (GIBCO BRL) for 45 min at 72 °C. The DNA was then separated on 0.8 % low melting point GTG agarose (FMC BioProducts, Rockland, ME). The 1-2 kb and 2-4 kb fractions were cut from the gel. The DNAs were extracted with Qiagen gel extraction kit (Qiagen) and cloned into EcoRV site of pZErO-2 vector (Invitrogen, Carlsbad, CA). The presence of inserts was verified by restriction analysis from a selected set of clones. DNAs from
a total of 1,700 randomly picked clones were extracted with a Qiagen BIO ROBOT 3000 following the manufacturer’s suggestions. The DNAs were sequenced on an automated DNA sequencing system (ABI Prism, Foster City, CA).

2.20 Total RNA extraction from LBCLs

Total RNAs were extracted from the LBCLs from the family following the established protocol (Farrell, 1993). Briefly, six T-75 flasks of cultured cells were pelleted by centrifugation. The cellular pellets were resuspended completely in 10 ml of RNA STAT-60 (Tel-Test, TX), followed by the addition of 2 ml chloroform. After incubation at room temperature for 3 min, the mixture was centrifuged at 12,000 rpm for 15 min at 4 °C. Following the centrifugation, equal volume of isopropanol was added to the upper aqueous phase of the sample in a new 50 ml conical tube. The sample was stored at -20 °C for 30 min, and centrifuged at 12,000 rpm for 60 min at 4 °C. The precipitated RNA was washed with cold 70% ethanol. The final RNA was harvested by centrifugation at 8,000 rpm for 45 min at 4 °C. After the pellet was air-dried, the RNA samples were resuspended in 250 μl DEPC-treated dH2O, and stored at -80 °C.

2.21. Analysis of the expression of candidate genes by RT-PCR, and confirmed by TA cloning and sequencing

cDNA was synthesized from 1 μg of total RNA in a 20 μl of reaction using the cDNA synthesis kit (GIBCO BRL) with either oligo dT or random hexamer as the primer, following the manufacturer’s protocol. 1 μl of the product was used in the
following PCR with the appropriate gene specific primers. The PCR product was analyzed by 1% agarose gel electrophoresis.

To clone the PCR product, either the total PCR product, or PCR fragment cut from low-melting-point agarose gel (Nusieve GTG, FMC Bioproducts, ME), was cloned into the TA vector using TOPO PCR Cloning Kit (Invitrogen) following the manufacturer's protocol. The cloned product was sequenced using an automatic ABI sequencer.
CHAPTER 3

PHYSICAL AND TRANSCRIPT MAP OF A 2 MB REGION IN XP22.1 CONTAINING CANDIDATE GENES FOR X-LINKED MENTAL RETARDATION AND SHORT STATURE

3.1 ABSTRACT

Genetic loci for several diseases, including X-linked nonspecific mental retardation and short stature, have been mapped to Xp22.1. In spite of the recent publications of two draft sequences for the human genome, this region appears to be largely unmapped and unsequenced. Here we report an integrated physical and transcript map of ~2-Mb from DXS8004 to DXS365. Using STS-content mapping and chromosome walking, we assembled a genomic clone contig of 54 BACs and one cosmid with an estimated 4.5-fold coverage of this region. The minimum tiling path consists of 23 BACs and one cosmid. Onto this contig, we mapped 30 new STSs derived from the unique end-sequences of the BACs, three ESTs, five genes and seven CpG islands. This integrated map provides us a unique resource for the positional cloning of candidate disease genes mapping to Xp22.1 and is therefore of value for the completion of the genomic sequence of this region. It also provides the essential
resources in our positional cloning of the novel rare, heritable folate-sensitive fragile site associated with short stature in Xp22.1.
3.2 INTRODUCTION

Genetic loci for several diseases have been mapped to the Xp22.1 region, including those for X-linked mental retardation (XLMR) (Merienne et al., 1999; Ronce et al., 1999; Chiurazzi et al., 2001), idiopathic short stature (Zinn et al., 1998b; Wandstrat et al., 2000), Coffin-Lowry syndrome (CLS) (Trivier et al., 1996; Jacquot et al., 1998), and X-linked juvenile retinoschisis (Sauer et al., 1997). Mental retardation (MR) is in most cases linked to a deficit in the adaptive behavior. About 1% of general population is reported to be functioning two standard deviations below the average based on their overall adaptive performances and their IQ of less than 70, with excess of affected males than females observed (Lehrke, 1972; Turner et al., 1974; Chiurazzi et al., 2000). Several genetic defects have been identified to underlie most of them (Lehrke, 1972; Turner et al., 1974; Neri et al., 1991; Neri et al., 1999; Chiurazzi et al., 2000). The underlying mutations in X-chromosome genes have been proposed to interpret that there are more patients with mental retardation in males than in females. Deleterious mutations in X-chromosome genes cannot be compensated in hemizygous males, thus resulting in clinical phenotypes. In females, the presence of a normal copy gene in the other X chromosome may protect the females from being affected (Lehrke, 1972; Neri et al., 1999; Chiurazzi et al., 2001).

Based on whether XLMR is linked to other conditions, two major groups are identified (Neri et al., 1999; Hamel et al., 2000a; Hamel et al., 2000b): (1) nonspecific XLMR and (2) specific or syndromal XLMR. Nonspecific XLMR, indicated by the acronym MRX, does not have any other associated conditions except mental deficit;
while specific XLMR, indicated as MRXS, associates with one or more other conditions. To date, over 200 XLMR conditions have been reported, with three MRXS and seven MRX mapping to Xp22.1. However, the majority of the underlying genes have not been identified (Chiurazzi et al., 2001).

Short stature (SS) is defined as height two standard deviations below the average (Ranke, 1996). It affects approximately 3% of general populations (Ranke, 1996). Both environmental and genetic factors have been suggested to affect the final adult height. Poor nutrition, improper living conditions, and chronic infections have been linked to the reduced height in adults (Vogel et al., 1997). Growth hormone, insulin-like growth factor 1 (IGF-1), and other gene products involved in the skeletal development have been identified to be the important genetic factors in the achievement of final adult height (Phillips et al., 1981; Leung et al., 1987; Martial et al., 1992; Rousseau et al., 1994a; Briggs et al., 1995; Goddard et al., 1995; Hecht et al., 1995; Kotzot et al., 1995; Muenke et al., 1995).

However, the majority of short stature cases (idiopathic short stature, ISS) currently do not involve any known genetic or environmental factors that may affect the adult stature (Ranke, 1996). Based on the observation that a strong resemblance exists between the height of family members, and the extent of this similarity increases, as the family relationship becomes closer, it has been suggested that there are genetic bases underlying ISS (Preece, 1996).

Short stature has been consistently linked with a well-known syndrome, Turner syndrome (TS) (Ullrich, 1930; Turner, 1938). TS is caused by the loss of one X chromosome in female (45, X) (Ford, 1959; Zinn et al., 1993). The major clinical
features for TS include SS, sexual infantilism and anatomical abnormalities such as a webbed neck and increased angle of the elbow (Ullrich, 1930; Turner, 1938). As one of the two X chromosomes is randomly inactivated in female, and there is only one X chromosome in male (Strachan et al., 1996), it is proposed that the genes underlying TS escape X-chromosome inactivation, and there are functional homologues in Y chromosome (Zinn et al., 1998a). In accordance with this hypothesis, genes in pseudoautosomal region (PAR) in Xp22.31 were proposed to be involved, as genes there have been shown to have both functional copies in the X and Y chromosomes and they escape the X-chromosome inactivation in females (Ogata et al., 1992a; Ogata et al., 1992c; Joseph et al., 1996; Schwinger et al., 1996; Spranger et al., 1997).

Cytogenetic studies in TS patients identified that most TS patients had only partial X chromosomal deletions. By genotype/phenotype correlation study, it is established that SS, ovarian failure, and other symptoms in TS are caused by loss of different loci (Ferguson-Smith, 1965; Kalousek et al., 1979; Fryns et al., 1981; Goldman et al., 1982; Simpson, 1988; Jacobs et al., 1990; Temtamy et al., 1992; Ogata et al., 1995a). These studies led to the cloning of SHOX/PHOG from PAR, and the identification of a functionally significant mutation of SHOX in SS patients (Ellison et al., 1997; Rao et al., 1997). Thus, SHOX/PHOG has been proposed as a strong candidate gene for SS (Ellison et al., 1997; Rao et al., 1997).

However, other studies indicated that there were other gene(s) in additional to SHOX in Xp involved in ISS and TS (Zinn, 1997; Zinn et al., 1998a; Zinn et al., 1998b). By using a statistical treatment of the correlation between short stature and the partial deletions of Xp in TS patients, Zinn et al. identified that a deletion of a region
in Xpl1-p22.1 between markers SYP and PDHA1 was significantly linked to SS (Zinn et al., 1998b). The region was estimated to be around 30 Mb (Zinn et al., 1998b). With similar approaches, Wandstrat et al. reported further that a region of approximately 2 Mb in Xp22.12 contained the breakpoints for SS (Wandstrat et al., 2000).

To identify candidate disease genes mapping to Xp22.12, such as genes for short stature and nonspecific mental retardation, it is essential to have a high-resolution physical and transcript map for the region. Although yeast artificial chromosome (YAC) contigs spanning this region have been assembled (Alitalo et al., 1995; Ferrero et al., 1995; Trump et al., 1996; Van de Vosse et al., 1996), this region contains surprisingly few mapped markers. Considering the innate instability and susceptibility to chimerism in YACs, a high-resolution physical map with more stable clones, such as PI artificial chromosome (PAC) or bacterial artificial chromosome (BAC) is needed for the localization and isolation of candidate disease genes mapped to this region. We selected six overlapping YACs (Alitalo et al., 1995; Ferrero et al., 1995) covering a region of about 2 Mb in Xp22.1 from DXS3424 to DXS365 as a basis for the construction of a high-resolution physical and transcript map by combining sequence-tagged site (STS) mapping, expressed sequence tag (EST) mapping, clone end sequencing, chromosome walking, and public database searches (Lander et al., 2001; Venter et al., 2001).
3.3 RESULTS

3.3.1 STS mapping of the YACs

In order to fine map the STS markers, a group of six contiged YAC clones, y911G5, y827E10, y946F5, y295D1, y517G4, and y811D11 from the retinoschisis region in Xp22.1 (Alitalo et al., 1995; Ferrero et al., 1995) was used. Total YAC DNA was extracted, and used as the template in PCR reaction to map 30 STS markers mapped into that region (WUSTL database, www.wustl.edu). Fig. 3.1 summarized the ordered YAC contig based on STS content mapping. A total of 18 STSs were positive in the six YAC clones. Based on this mapping, it is clear that y827E10 and y811D11 are chimeric. However this is the basis for the assembly of high-resolution physical map based on BACs and PACs.

3.3.2 Inter-Alu PCR (Alu-PCR) content mapping and amplification of YAC insert

To further validate the overlapping of the six YACs, and to amplify the YAC DNAs for FISH mapping, inter-Alu PCR (Alu-PCR) content mapping and amplification of YAC inserts were performed (Liu et al., 1993). Alu repeats are one class of interspersed repeat element (IRE) (Strachan et al., 1996). It occurs on average about once every 4 kb in the human genome. Alu-PCR is a PCR reaction in which an oligonucleotide primer with a sequence derived from the Alu repeat is used to amplify sequences between pairs of neighboring Alu sequences which are in converging opposite orientation (Strachan et al., 1996). It has been used successfully to amplify the content of human YAC clones (Liu et al., 1993; Strachan et al., 1996). Fig. 3.2 is
Figure 3.1 STS content mapping of YACs in Xp22.1. STSs mapped to Xp22.1 were mapped to the six contiged YACs by PCR. A sticker symbol indicates the presence of that marker in a YAC, a cross symbol indicates the absence of that marker in a YAC. The sizes for each YAC were indicated in kb in parentheses.
Figure 3.2 Alu-PCR fingerprinting of YACs. Alu PCR primers Alu1, Alu2 or both were used as primers in PCR with individual YAC as the template. PCR products were separated on 1% agarose gel, and visualized by ethidium bromide staining.

Lane 1: dH2O; Lane 2: y911G5; Lane 3: y827E10; Lane 4: y946F5; Lane 5: y295D1; Lane 6: y517G4; Lane 7: y811D11.
the Alu-PCR product of the six YACs with Alu1, or Alu2 primer alone, or Alu1 and Alu2 primers together. The smear with distinct bands shows typical amplification pattern for such PCR (Liu et al., 1993). The overlapping pattern observed from Lane 2-7 verifies the overlapping of these six YAC clones.

3.3.3 A complete BAC-cosmid contig spanning the region of 2 Mb in Xp22.1

To have a high-resolution physical map with more stable clones than YACs, bacterial artificial chromosome (BAC) was chosen because such clones tend to be more stable, and less chimeric. Moreover, BAC has been selected as the clones in the generation of whole human genome physical map. To start, pools of 3 STS markers from the 18 STSs mapped to the six YACs were used to screen the human BAC RPCI-11 library filters. Positive BAC clones were ordered from the human BAC/PAC resource (Children’s Hospital, Oakland, CA). The clones were further verified for the presence of the respective STS markers by PCR. Confirmed BACs were end-sequenced. Their sequences have been deposited into GenBank (G72396-G72425). New STSs were generated from the non-repetitive sequences of the BAC ends. They were used to isolate more BACs to fill in apparent gaps. During this process, both public database (Lander et al., 2001) and private database (Venter et al., 2001) were searched as well to identify any overlapping BACs. Fig. 3.3. summarizes the finished BAC contig. A total of 54 BACs and one cosmid cover the whole region by about 4.5 folds. The minimal tiling path is covered by 23 BACs and one cosmid (Fig. 3.4).
Fig. 3.3 Complete BAC-cosmid contig of 2 Mb region in Xp22.1
Fig. 3.4 Transcript map of a 2 Mb region in Xp22.1
The region was well represented by the overlapping BACs except one small region covered by a single cosmid LLNLc110F2323Q2 (cB4) (RZPD, Germany). BAC filters were screened extensively with the flanking markers, but no BAC was identified to span the region. This suggests that the region is difficult to clone, or unstable in the cloning vectors, as the single cosmid clone is not stable either during the amplification (data not shown). To estimate the region more accurately, the sizes of the 23 BACs in the minimal tiling path were determined by FIGE analysis of NotI-digested BAC DNA. They are indicated in parentheses in Fig. 3.4. To confirm that all the 23 BACs are not chimeric, each of the BACs was tested by FISH on normal metaphase spreads and was identified to map to one single region in Xp. Shown in Fig. 3.5 is FISH image with b393H10 on both normal female (A) and male (B) metaphase spreads.

3.3.4 Transcript map of the 2-Mb region in Xp22.1

To locate candidate disease genes in this region, ESTs, genes and potential CpG islands as landmarks for 5' promoter region (Bird, 1986) were mapped onto the BAC contig. The 23 BAC and one cosmid clones were tested by PCR with 36 ESTs and the three genes assigned to the region based on GeneMap99 (Deloukas et al., 1998). A total of three ESTs, and three genes were precisely mapped (Fig. 3.4). BLAST sequence analysis mapped two more genes. Table 3.1 summarizes the known information about these three mapped ESTs and five genes.

To construct a long-range restriction enzyme map across the region and to identify potential CpG islands, the 23 BACs were used for rare-cutter restriction
Fig. 3.5 Fluorescence in situ hybridization (FISH) on normal female (A) and male (B) metaphases showing FITC-labeled b393H10 located on Xp. A X-chromosome centromere specific probe CEP-X was included as control.
<table>
<thead>
<tr>
<th>EST/Gene</th>
<th>GenBank Acc. No.</th>
<th>Gene Similarity</th>
<th>Known Function</th>
<th>Disease/Phenotype (OMIM No.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>stSG2954</td>
<td>Z19300</td>
<td>RPS6KA3</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>stSG13075</td>
<td>G33067</td>
<td>low similarity to RPS6KA3</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>stSG62525</td>
<td>N50138</td>
<td>NA*</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>PHKA2</td>
<td>X80497</td>
<td>NA</td>
<td>phosphorylase kinase</td>
<td>X-linked liver glycogenosis (306000)</td>
</tr>
<tr>
<td>AK027409</td>
<td>AK027409</td>
<td>low similarity to human E-MAP-115</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>EIFIAX</td>
<td>G34989</td>
<td>EIFI A</td>
<td>stabilizes the binding of the initiator Met-tRNA to 40S ribosomal subunits</td>
<td>putative Turner syndrome candidate gene (300186)</td>
</tr>
<tr>
<td>RPS6KA3</td>
<td>NM_004586</td>
<td>NA</td>
<td>growth-factor induced kinase involved in MAP kinase pathway</td>
<td>CLS (303600); XI.MR, type 19 (300075)</td>
</tr>
<tr>
<td>KIAA0902</td>
<td>AB020709</td>
<td>high similarity to R. norvegicus T18293 guanylate kinase-interacting protein 1/Maguin-1, membrane-associated</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

Table 3.1 Transcripts assigned to the reported Xp22.1 BAC contig. *NA, not applicable.
Figure 3.6 Field inversion gel electrophoresis (FIGE) of NotI-digested BAC DNA
0.6 μg of each BAC was digested with NotI to completion, and was separated on
1% FIGE gel.
<table>
<thead>
<tr>
<th>BAC</th>
<th>NotI</th>
<th>Sall</th>
<th>Eagl</th>
<th>BssHII</th>
</tr>
</thead>
<tbody>
<tr>
<td>128B23</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>497J19</td>
<td>+</td>
<td>+</td>
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<td>491N21</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
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<tr>
<td>501O24</td>
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<td>+</td>
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</tr>
<tr>
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<td>+</td>
<td>+</td>
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<tr>
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<td>+</td>
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<td>-</td>
</tr>
<tr>
<td>450P7</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 3.2 Summary of BAC DNA digestion by restriction enzyme cutting GC-rich region
analysis with the enzymes NotI, SacII, and EagI (New England, BioLab) as described (Trump et al., 1996). Their sizes were determined by FIGE mapping. Fig. 3.6 shows the NotI digestion of part of the 23 BACs. The total results were summarized in Table 3.2. A total of seven CpG islands were identified (Fig. 3.4). Six of them cluster in two small regions (about 300 kb each), which suggests that these regions are gene-rich.

3.4 DISCUSSION

Many disease loci have been mapped to Xp22.1, including X-linked nonspecific mental retardation and short stature (Zinn et al., 1998b; Merienne et al., 1999; Ronce et al., 1999; Wandstrat et al., 2000; Chiurazzi et al., 2001). By 2000, eight MRX families have been mapped to Xp22.1 (Chiurazzi et al., 2001). However, no distinct candidate genes have been identified from this region. Some of these may prove to be due to mutations in the same gene (Chiurazzi et al., 2001). Similarly short stature has been linked to gene(s) in Xp22.1 (Zinn et al., 1998b; Wandstrat et al., 2000). The assembled high-resolution physical and transcript map in this region serves as the foundation for the cloning of these candidate genes.

From the gene, EST, and CpG island mapping shown in Fig. 2.4, it is interesting to note that they cluster into two small regions, with each about 300 kb in size. More ESTs and genes in these two regions may be identified once sequences for these regions are available. Two known genes RSK2 and EIF1AX are located in these two cluster regions (Chang et al., 1995; Lahn et al., 1997). RSK2 has been linked to Coffin-Lowry syndrome and MRX 19 (Trivier et al., 1996; Merienne et al., 1999).
\textit{EIF1AX} was proposed to be a candidate gene for TS based on its chromosomal location, as well as the fact that there is a functional copy \textit{EIF1AY} in the Y chromosome (Lahn et al., 1997). Moreover \textit{EIF1AX} escapes X-chromosome inactivation (Lahn et al., 1997). Interestingly, \textit{RSK2} partially escapes X-chromosome inactivation as well (Carrel et al., 1999). It seems tempting to speculate that based on their location, the genes clustering with \textit{EIF1AX} and \textit{RSK2} covered by b393H10 and b228D12 may escape X-chromosome inactivation. This possibility makes the genes in the region rational candidates for TS.

Interestingly both \textit{KIAA0902} and \textit{RSK2} (Fig. 1B) contain long trinucleotide repeats in their respective 5'UTR or promoter regions. \textit{KIAA0902} with unknown function (Nagase et al., 1998) contains a \((CAG)_{12}(CCG)_{5}\) repeat in its 5'UTR (GenBank: AB020709). \textit{RSK2}, the gene mutated in Coffin-Lowry syndrome CLD) and nonspecific mental retardation (Trivier et al., 1996; Merienne et al., 1999) contains a novel \((CCG)_{3}(CTG)_{5}(CCGCTG)_{3}(CCG)_{8}(CTG)_{1}(CCG)_{1}\) repeat in its promoter or 5'UTR. Interestingly, an estimated 66% of CLS patients do not have mutations in the \textit{RSK2} coding region, some of these show reduced or no \textit{RSK2} protein (Jacquot et al., 1998; Delaunoy et al., 2001). It is possible that changes in the copy number or the abnormal expansion of the \((CCG)_{n}\) repeat in the putative promoter/5'UTR region of \textit{RSK2} may result in abnormal expression in CLS patients without coding mutations. Based on the sequence of the complex repeat arrays in their promoters/5'UTR, \textit{KIAA0902} and \textit{RPS6KA3} constitute good candidate genes for diseases mapping to this region, especially those associated with anticipation and/or
rare, folate-sensitive chromosomal fragile sites (Sutherland et al., 1998; Cummings et al., 2000).

To the best of our knowledge, our high-resolution physical and transcript map is the only complete BAC contig map covering this region in Xp22.1. The BAC contig available from the public Human Genome Project as well as both public and private genomic sequences are currently highly incomplete for this region. To date, only five BACs from this region have been partially sequenced. The relatively deep coverage of clones and the high density of ordered markers in our contig map provide a unique resource for the mapping and identification of candidate disease genes mapping to Xp22.1. Thus, our map should be of considerable value for the completion of the genomic sequence of this region of the human genome. The complete BAC contig also serves as the foundation for us to fine map and eventually identify the molecular basis for the newly identified rare heritable folate-sensitive fragile site in this region, which is associated with short stature in the proband.
CHAPTER 4

POSITIONAL CLONING OF A NOVEL HERITABLE, RARE, FOLATE-SENSITIVE FRAGILE SITE (FRAXG) IN XP22.1 ASSOCIATED WITH SHORT STATURE IN A FINNISH KINDRED AND CLONING OF AN ASSOCIATED CANDIDATE GENE (FXGAG)

4.1 ABSTRACT

Fragile sites are chromosomal nonstaining gaps or breaks that become apparent when cells are cultured under special inducing conditions. Based on their frequency, fragile sites are classified as common or rare fragile sites. Common fragile sites are part of normal chromosomal structures, which may be involved in chromosomal recombination, deletions and instability. Rare fragile sites occur in less than 1% of individuals. Rare, heritable folate-sensitive fragile sites (RHFFS), which are induced by low folic acid and/or thymidine in culture medium, have been clearly linked to several clinical phenotypes, including fragile X syndrome, the most common inherited form of mental retardation in children, and Jacobsen’s syndrome. Molecular characterization of a total of five RHFFS to date has indicated a common underlying mutation and pathophysiological mechanism: the expansion of an unstable (CCG)n/(CGG)n trinucleotide repeat in the 5’ promoter region together with the
hypermethylation of an adjacent CpG island result in transcriptional silencing of a downstream gene and the clinical phenotype in patients.

The genetic basis for short stature (SS) in general and in Turner syndrome (TS) in particular has been investigated for at least five decades. One or multiple genes in the short arm, particularly Xp22, of the X chromosome have been implicated. However, the underlying gene(s) has been elusive, as most of the studies were based on the observed cytogenetic deletions that encompass several genes.

Here, we report the association of a novel RHFFS in Xp22.1 with the SS phenotype in a Finnish proband. Through positional cloning, the novel RHFFS, termed GRAXG, was characterized. Like other RHFFS, FRAXG is also caused by the expansion of a normally polymorphic (CGG)n trinucleotide repeat. When mutated, the (CGG)n repeat and the adjacent CpG island are hypermethylated. This finding provides further evidence for the proposed general etiological mechanism of RHFFS.

In addition, a novel gene FXGAG associated with the (CGG)n repeat has been identified. Based on its associated phenotype of SS in the proband and its genomic location in Xp22.1, we propose that FXGAG constitutes a novel candidate gene for SS and TS.
4.2 INTRODUCTION

4.2.1 Human fragile sites

Chromosomal Fragile sites on chromosomes are sites that show an increased frequency of gaps and breaks when cells from which the chromosomes are prepared are exposed to specific conditions of tissue culture or chemical agents (Sutherland, 1979b; a). Based on their frequency, fragile sites are divided as common or rare fragile sites (Sutherland GR, 1996; Sutherland et al., 1999). Common fragile sites are present probably on all chromosomes, which is part of normal chromosome structure. However, the proportion of metaphases in which any common fragile site is expressed cytogenetically varies from person to person (Sutherland et al., 1995; Sutherland GR, 1996). Rare fragile sites vary in frequency from only a handful of case reports to 1 in 40 chromosomes (Sutherland et al., 1995; Sutherland GR, 1996).

There are more than 80 common fragile sites reported to date (Sutherland et al., 1999). Based on the conditions of tissue culture required to induce their cytogenetic expression, common fragile sites are further divided as aphidicolin inducible, 5-azacytidine inducible, and bromodeoxyuridine inducible (Sutherland et al., 1995). However, the molecular basis for them has not been understood yet, despite that the complete sequence for common fragile site FRA3B and partial sequences for common fragile sites FRA7G and FRA7H have been determined (Boldog et al., 1997; Inoue et al., 1997; Huang et al., 1998a; Huang et al., 1998b; Mishmar et al., 1998; Mimori et al., 1999). Common fragile sites have been proposed to be involved in chromosomal deletions, rearrangements, and to be the preferential site of viral integration (Rassool
et al., 1991; Wang et al., 1993; Glover, 1998). As deletions in or around common fragile sites FRA3B and FRA16D have been observed in many solid tumors including breast, lung, head and neck, and cervical cancers, common fragile sites have been suggested to be involved in tumorigenesis (Negrini et al., 1996; Sozzi et al., 1996; Virgilio et al., 1996; Druck et al., 1997; Smith et al., 1998; Krummel et al., 2000; Mangelsdorf et al., 2000; Paige et al., 2000; Ried et al., 2000).

Rare fragile sites are further divided into folate sensitive, distamycin A inducible, and bromodeoxyuridine requiring fragile sites. There are more than 25 reported to date (Sutherland et al., 1995). Seven of them have been characterized at the molecular level (Sutherland et al., 1995). Five of them FRAXA, FRAXE, FRAXF, FRA11B, and FRA16A are rare, heritable folate-sensitive fragile sites. They are all caused by expansion of a normally polymorphic (CCG)n/(CGG)n trinucleotide repeat. (Kremer et al., 1991a; Oberle et al., 1991; Verkerk et al., 1991; Yu et al., 1991; Knight et al., 1993; Parrish et al., 1994; Jones et al., 1995; Nancarrow et al., 1995). The other two FRA16B and FRA10B are distamycin A inducible and bromodeoxyuridine requiring common fragile sites respectively. They are caused by expansion of AT-rich minisatellite repeats (Yu et al., 1997; Hewett et al., 1998).

FRAXA, FRAXE, and FRA11B have been linked to clinical phenotypes (Sutherland et al., 2000). FRAXA, located in Xq27.3, is linked to fragile X syndrome; the most common inherited mental retardation in children (Lubs, 1969; Sutherland, 1977a; b; Tommerup, 1989). Fragile X syndrome is caused by a functional deficiency of FMRI gene. More than 95% of this deficiency is caused by an expansion of an unstable (CGG)n trinucleotide repeat in the 5' UTR region of FMRI gene. The
expansion of (CGG)n repeat induces the hypermethylation of itself and an adjacent CpG island, which results in downregulation of transcription of \textit{FMR1} gene (Fu \textit{et al.}, 1991; Kremer \textit{et al.}, 1991a; Oberle \textit{et al.}, 1991; Verkerk \textit{et al.}, 1991; Yu \textit{et al.}, 1991). The presence of expanded (CGG)n in the mutant \textit{FMR1} transcripts can also interfere with the translation of \textit{FMR1} (Feng \textit{et al.}, 1995a; Feng \textit{et al.}, 1995b). Small deletions and point mutations which cause the functionally deficient \textit{FMR1} in fragile X syndrome patients have also been reported (Wohrle \textit{et al.}, 1992; De Boulle \textit{et al.}, 1993). FRAXE is linked to a nonspecific mild mental retardation. Similar to fragile X syndrome, expansion of an unstable (CCG)n repeat in the 5'UTR of \textit{FMR2} gene causes the downregulation of the transcription and translation of \textit{FMR2}, which results in FRAXE-related mental retardation (Knight \textit{et al.}, 1993; Gecz \textit{et al.}, 1996; Gu \textit{et al.}, 1996). FRA11B is caused by an expansion of (CCG)n repeat in the 5'UTR of proto-oncogene \textit{CBL2}, which may be involved in some of Jacobsen syndrome (Jones \textit{et al.}, 1994; Jones \textit{et al.}, 1995; Michaelis \textit{et al.}, 1998; Tunnacliffe \textit{et al.}, 1999; Jones \textit{et al.}, 2000).

\textbf{4.2.2 Rare, folate-sensitive fragile site FRAXA and Fragile X syndrome}

FRAXA is the first fragile site to be extensively analyzed. As FRAXA is linked to fragile X syndrome, the most common form of inherited mental retardation in children, and its high frequency in human population, it is possible to have enough cases and families for the analysis in detail. The molecular cloning and characterization of FRAXA lead to directly the cloning of other rare folate-sensitive fragile sites. From the study of FRAXA, many aspects including induction,
cytogenetic diagnosis, have been standardized for other RHFFS as well as FRAXA (Jacky, 1996; Wells, 1998).

As discussed in the first chapter, expression of FRAXA and cytogenetic detection require that cells cultured under conditions that either restrict the availability of folic acid entering or being processed through the folate pathway (Sutherland, 1977a; 1979a) or more specifically, interfere with the production of thymidylate and its availability for DNA replication (Glover, 1981; Tommerup et al., 1981). Practically this is achieved by using culture medium either low in folic acid, like TC 199, or entirely lacking folic acid, like MEM-FA, in which proliferating cells over time deplete folate reserves and effectively induce restricted conditions. Alternatively, FRAXA is induced by using medium containing normal levels of folic acid but incorporating a folate or thymidylate antagonist for a significant period before chromosome harvest to induce restricted conditions. Neither culture system should contain exogenous thymidine, which would metabolically permit bypassing of depletion or antagonist methods (Jacky, 1996).

As peripheral blood lymphocytes (PBL) are the most accessible source of cells for culture and induction, it has become standard to use PBL clinically in the induction and diagnosis of FRAXA (Jacky, 1996), and this routine has been followed in the induction of other RHFFS as well (Jacky, 1996). Several alternative methods as the following have been suggested, and generally they require using two or more methods for the induction to reduce the false-positive rate. One of which at least should use a method including folate or thymidylate antagonist (Jacky et al., 1991).

Folate/Thymidine depletion: TC 199 or MEM-FA, or EMPI 1640-FA (Irvine), or
RPMI-FA 1640-FA (Irvine), Medium M (GIBCO), with 2-5% FBS. Dihydrofolate Reductase inhibitors: $10^{-3}$ to $10^{-5}$ M methotrexate, 16 mg/L aminopterin, or 13-26 mg/L trimethoprim, or 300-600 mg/L Bactrim (Roche; roughly equivalent to 50-100 mg/L trimethoprim). Thymidylate Synthetase Inhibitors: RPMI 1640, or Ham’s F10 + 0.005-0.01 mg/L, FudR, or FCdR. Ribonucleotide Reductase Inhibitors: 300-1200 mg/L excess thymidine. Simple folate restriction conditions, like method 1 above, are usually imposed at culture initiation, whereas specific folate/thymidine antagonists are added 24 hours or 48 hours before chromosome harvest. Decreasing pH in medium over the culture may inhibit the expression of fragile site, and generally the culture medium is buffered to pH 7.6 with 25 mM HEPES.

After the proper induction, the cells are harvested and processed following standard cytogenetic protocols. To analyze the expression of FRAXA and similarly other fragile sites, the following guidelines were proposed (Jacky et al., 1991): At least 100 cells should be scored for males and 150 for females. The presence of a suspicious fragile site should be confirmed with chromosome banding. When a frequency of less than 4% is obtained in FRAXA and probably in other fragile sites as well, a repeat specimen should be requested. If no higher frequency of expression is obtained, the results should be interpreted with caution and on a case-by-case basis. Proper cytogenetic ISCN nomenclature should be used [(i.e. 46, fra(X)(q27.3,Y), and 46,X, fra(X)(q27.3) for FRAXA in male and female respectively]. Fragile X studies for suspected X-linked mental retardation should include a standard constitutional chromosome analysis.
While cytogenetic diagnosis of FRAXA is still in wide use, the molecular
detection of the expansion of (CGG)n trinucleotide repeat by PCR and genomic DNA
Southern blot has become the gold standard in the diagnosis of Fragile X syndrome
(Wells, 1998). The combination of PCR and Southern blot can essentially eliminate
the false negative in the cytogenetic diagnosis of Fragile X syndrome (Wells, 1998).

Fragile X syndrome is the representative of a group of inherited diseases with the
common genetic basis: dynamic mutations of trinucleotide repeats. Currently much of
our understanding of the unstable trinucleotide repeats involved in this group of
diseases stems from the analysis of Fragile X syndrome. This is due to not only that
Fragile X syndrome is the first one to be characterized in detail, but also that the high
frequency of the disease and mutation in the human population, which allows a large
number of patients and families to be studied (Wells, 1998).

Fragile X syndrome is inherited in an X-linked dominant fashion with reduced
penetrance (Webb et al., 1986). It is clinically characterized by mild to severe mental
retardation, macro orchidism, and mild facial dysmorphism (narrow faces with
prominent jaw and forehead and large ears) (Wells, 1998). Fragile X syndrome was
originally identified through its association with rare, folate-sensitive fragile site
FRAXA in Xq27.3 (Sutherland, 1977a). In 1991, it was identified that expansion of an
unstable (CGG)n trinucleotide repeats in the 5' UTR of FMR1 gene and subsequent
hypermethylation of the repeat and adjacent CpG island caused most of the Fragile X
syndrome diseases (Kremer et al., 1991a; Oberle et al., 1991; Verkerk et al., 1991; Yu
et al., 1991). FMRP, the product of FMR1 gene, is an RNA-binding protein with
selective bindings to some mRNAs in the brain and its own mRNA (Ashley et al.,
1993; Siomi et al., 1993). It shuttles between cytoplasm and nucleus, and it associates with the polyribosomes in the cytoplasm (Eberhart et al., 1996; Khandjian et al., 1996; Tamanini et al., 1996). It is suggested that FMRP may play a role in local protein translation at neuronal dendrites and in dendritic spine maturation (Comery et al., 1997; Feng et al., 1997; Braun et al., 2000; Nimchinsky et al., 2001), which may explain that the loss of FMRP causes the mental retardation in Fragile X syndrome patients.

Based on the numbers of (CGG)n trinucleotide repeats at FRAXA locus, four different categories with different characteristics are classified (Wells, 1998). Normal alleles contain from six to 45 copies of (CGG)n repeats with zero to three AGG interruptions. The major allele within this category is (CGG)9 AGG (CGG)9 AGG (CGG)9-10. Within this range, the (CGG)n repeat is stable in transmission. No somatic instability is present. The allele in the active X chromosome is unmethylated, and another allele in the inactive X chromosome is methylated due to the random X chromosome inactivation. Normal amounts of FMR1 transcript and FMR1 protein are present from the active allele (Fu et al., 1991; Snow et al., 1993; Eichler et al., 1994; Kunst et al., 1994; Snow et al., 1994; Zhong et al., 1995). Grey zone alleles contain 45 to 60 copies of (CGG)n repeats with zero to two AGG interruptions. The major allele for this group is (CGG)9 AGG (CGG)x (CGG)9 AGG (CGG)9 AGG (CGG)x. For this range, the (CGG)n repeats show very limited instability during transmission dependent on the AGG interspersion. The longer the perfect CGG repeat in the 3’-end, the higher instability the (CGG)n repeat in the transmission. The somatic instability is absent and there is no methylation of the (CGG)n repeat in the active X chromosome. Normal
amounts of FMR1 transcript and FMR1 protein are present from the active allele (Eichler et al., 1994; Murray et al., 1997). Premutation alleles contain about 60 to 200 copies of (CGG)n repeats. Alleles within this range are unstable during transmission. Upon maternal transmission, premutations have a strong tendency to increase in size, either to larger premutations or full mutation. The risk of transition to full mutation is strongly dependent on the size of maternal premutation. It appears that the risk is probably less than 5% for alleles with less than 60 repeats; about 50% for alleles with around 80 repeats, and above 95% for alleles with more than 100 repeats. The interspersed AGG also affects this instability. The loss of AGG interruption has been shown to enhance the transmission from a premutation to full mutation for an allele with 59 pure (CGG)n repeats and no AGG during two generations. In contrast, premutations show a much more limited instability upon paternal transmission with no obvious trend toward preferential increase or decrease in size. Gain or loss of more than 20 copies of CGG repeats is very rare. So far there is no single report of a transmission from premutation to full mutation upon paternal transmission. Except this germinal instability for premutation, there is very limited somatic instability. Generally normal amounts of FMR1 transcript and FMR1 protein are present from the active allele, which is unmethylated. Individuals with (CGG)n repeats within the ranges for normal alleles, grey zone alleles and premutation alleles are clinically normal (Fu et al., 1991; Heitz et al., 1992; Snow et al., 1993; Eichler et al., 1994; Fisch et al., 1995). Full mutation alleles contain ~ 230 to more than 1000 copies of (CGG)n repeats. They are highly unstable both at germinal and somatic levels. Similar to premutations, full mutations show different characteristics between maternal and
paternal transmissions. Upon maternal transmissions, full mutations almost invariably result in offspring with full mutations. However, only premutations in daughters of affected fathers with full mutations have been reported to date. The contraction of (CGG)n repeats in male transmission appears to be specific to germ-line, and is believed due to the selection against the sperms with full mutations during spermatogenesis. Alleles with full mutations are methylated even in active X chromosome, and usually no FMR1 transcript and FMR1 protein are present. Full spectra of clinical phenotypes of Fragile X syndrome are present. Mosaics alleles with different combinations usually show characteristics of individual alleles (Oberle et al., 1991; Rousseau et al., 1991; Yu et al., 1991; Hansen et al., 1992; Willems et al., 1992; Hornstra et al., 1993; Rousseau et al., 1994b).

Detection and mutation analyses of expansion of (CGG)n repeats have been performed mainly by Southern blot of genomic DNA from peripheral blood lymphocyte. In normal individuals, there is usually a single band. In individuals with full mutations, there are often multiple discrete bands, sometimes a smear, and less frequently a single apparently homogenously band, which all migrate slower than the normal band. Large premutations may be similarly detected by Southern blot as slower migrating band or a fuzzy band when somatic instability is present. Smaller changes as in normal alleles, grey zone alleles, and small premutations may be detected by PCR and polyacrylamide gel analysis and/or sequencing. Abnormal methylation of the (CGG)n repeat and its adjacent CpG island is detected also by Southern blot when a methylation-sensitive restriction enzyme, like NolI, EagI, BssHII, and SacII, and a
common methylation-insensitive restriction enzyme are used (Bell et al., 1991; Heitz et al., 1991; Oberle et al., 1991; Moutou et al., 1997).

The characteristics of premutation instability account for the complex inheritance of fragile X syndrome that had been previously uncovered by segregation analysis in affected families, and in particular for the fact that the penetrance (or the risk of having mentally retarded children) is much lower for mothers of normal transmitting males than for their daughters. Female carriers with a small premutation tend to have offspring with larger premutation, including normal transmitting males. The latter will pass, in general with little changes, this premutation to their daughters, who in turn will be at much greater risk to have affected offspring with a full mutation (Sherman et al., 1984; Sherman et al., 1985).

4.2.3 Genetics of short stature

Short stature is defined as a condition in which the height of an individual is two standard deviations (SD) below the corresponding mean height for a given age, sex, and population group (Ranke, 1996). It affects about 3% of population (Ranke, 1996). While some short stature cases are related to known environmental or/and genetic factors, majority of them do not have any known underlying pathogenesis or etiology (idiopathic short stature, ISS) (Ranke, 1996).

Short stature has been consistently linked with Turner syndrome (TS) (Ullrich, 1930; Turner, 1938). TS is caused by loss of one X chromosome in female (45, X) (Ford, 1959). Based on the monosomy of X in TS, haploinsufficiency of specific genes in X chromosome is proposed to cause the TS phenotype. .Gene(s) underlying
TS are postulated to be expressed form both X chromosomes in female, and there are corresponding functional copies in the Y chromosome in males (Zinn et al., 1993; Zinn et al., 1998a).

Many cytogenetic studies of TS patients indicate that most TS patients have only partial X chromosomal deletions. Based on genotype/phenotype correlation study, it is established that SS is most likely linked to deletion of the pseudoautosomal region (PAR) in Xp22.3 (Ballabio et al., 1989; Ogata et al., 1992a; Ogata et al., 1992b; Ogata et al., 1995b). SHOX/PHOG, cloned as SS candidate gene from PAR, was identified to contain mutations in about 1% of SS patients. It has also been shown that SHOX/PHOG escapes X chromosome inactivation and there is a functional homologue in Y chromosome (Ellison et al., 1997; Rao et al., 1997). Thus, SHOX/PHOG has been considered as a strong candidate gene for short stature (Ellison et al., 1997; Rao et al., 1997).

However, further evidences indicate that there are other gene(s) in Xp in addition to SHOX/PHOG involved in SS (Zinn et al., 1998b; Wandstrat et al., 2000). By using a statistical treatment of the correlation between short stature and partial deletions in Xp identified in SS patients, Zinn et al. suggested that a deletion of the region in Xp11-p22.1 between markers SYP and PDHA1 is significantly linked to short stature. The region is estimated to be 30 Mb (Zinn et al., 1998b). With similar approach, Wandstrat et al. reported that there might be a common region of approximately 2 Mb in Xp22.12 that contains the common breakpoints for SS (Wandstrat et al., 2000). However, the culprit gene(s) for SS remain elusive, partly because the genetic defects very often express as a block deletion, which may contain several genes. Identification
of SS patients with no apparent chromosome deletion may be needed to isolate the underlying gene(s).

4.3. RESULTS

4.3.1 Case study of the Finnish family

The proband was a Finnish girl of seven years old when she was brought into the physician’s attention due to her short stature. No other complaints were mentioned. No abnormal eating, sleeping habit mentioned. No chronic fever, diarrhea, or chronic pain in the body mentioned. She was delivered naturally without any incidents. Her body weight and height were within normal range at birth. Physical examinations were generally normal except her height. Her height was below five percentile of her peers’. The ratio of her upper body length over her lower limb length was normal. No physical dysmorphia was identified. Her intelligence and speech were normal. Hair and skin were normal. No brittle hair and no abnormal skin temperature were observed. Her external sex organ was normal. Body temperature, heart rate, and blood pressure were normal. Regular laboratory tests including serum sodium, potassium, chloride, and calcium were normal. She has two sisters. Both are normal with normal height. Her parents are normal with normal height. Her initial diagnosis was idiopathic short stature. To exclude the possible chromosomal abnormality, including Turner syndrome, her peripheral blood lymphocyte was subject to cytogenetic study. She had a normal karyotype with 46, XX. However a nonstaining gap or break in the short arm of X chromosome were observed in about 2-4% of her peripheral blood lymphocyte
metaphases stained by Giemsa solution. Further Trypsin-Giemsa staining indicated the presence of a possible novel fragile site in Xp22.1. As no other fragile sites have been reported to date in the short arm of the X chromosome, except common aphidicolin inducible fragile site FRAXB located in Xp22.31 (Sutherland GR, 1996), this novel fragile site might be a rare fragile site. To determine which group this novel fragile site belongs to, different culture conditions for the induction of rare fragile sites, including rare, folate-sensitive fragile sites, were tested. The culture condition for RHFF enhanced the expression of this novel fragile site dramatically. Thus a novel rare, folate-sensitive fragile site in Xp22.1 was identified in the proband with idiopathic short stature. Subsequently the proband's parents and her two sisters were examined similarly for the presence of this novel fragile site. Her mother and one of her sisters had the same RHFF in Xp22.1. Her father and another sister did not have this novel fragile site (summarized in Fig. 4.1). It was hypothesized that this novel fragile site was inheritable, and the proband and one of her sisters inherited it from their mother. Genotyping of the X chromosomes of the family members using X-chromosome specific polymorphic microsatellite markers confirmed this hypothesis (data not shown).

4.3.2 Cytogenetic characterization of the novel, rare, folate-sensitive fragile site.

To cytogenetically characterize the novel fragile site, peripheral blood lymphocytes from the family members were cultured under the standard folate-sensitive fragile site inducing conditions (Jacky et al., 1991). Cells were harvested and
Figure 4.1 Pedigree of the finish family. Filled circles indicate the expression of FRAXG. The arrow indicates the proband with short stature (S.S.)
metaphase spreads were prepared following the standard cytogenetic procedures (Jacky, 1996). Metaphase slides were first stained with Giemsa to identify the fragile site. As shown in Fig. 4.2 (A), a nonstaining gap in the distal region of Xp was identified. In Fig. 4.2 (B), a single sister chromatid break can be seen. To further localize the cytogenetic location of the fragile site, metaphase spreads from the proband were G-banded with trypsin and Giemsa stain. As shown in Fig. 4.3, the novel fragile site is located in Xp22.1. According standardized nomenclature for fragile sites, this RHFFS was named as FRAXG. To determine the expression frequency of FRAXG, a total of 100 metaphase spreads with clear visible single chromosomes were identified from each sample, and images were captured. The presence of fragile site as a nonstaining gap, constriction, or chromosome break in Xp22 was determined by at least two individuals independently. The fragile site index was calculated as the # of observed fragile sites over all metaphase spreads counted.

As summarized in Table 4.1, the expression frequency of FRAXG in the proband is 27%, similar to her mother and one sister.

4.3.3 Induction of FRAXG from the proband’s lymphoblastoid cell line.

Lymphoblastoid cell lines (LBCL) for all family members were established from peripheral lymphocytes. Two inducing conditions for RHFFS in LBCL were used (Jacobs et al., 1982; Abruzzo et al., 1986). One is medium 199 plus FudR at concentration of $10^{-6}$, $5 \times 10^{-7}$ or $10^{-7}$ M for 24 or 48 hours, another is medium 199 plus MTX at concentration of $10^{-7}$ M for 24 or 48 hours. As shown in Fig. 4.4,
Figure 4.2 Giemsa staining of metaphase spreads from the proband's peripheral blood lymphocytes cultured under folate-sensitive fragile site inducing condition showing FRAXG as a nonstaining gap (A) and a chromatid breakage (B)
Figure 4.3 Trypsin-Giemsa staining of metaphase spreads from the proband’s peripheral blood lymphocytes cultured under folate-sensitive fragile site inducing condition locating FRAXG to Xp22.1
<table>
<thead>
<tr>
<th>Member</th>
<th>Gaps/Breaks</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Father</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mother</td>
<td>26</td>
<td>26</td>
</tr>
<tr>
<td>Daughter 1</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>Daughter 2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Daughter 3</td>
<td>27</td>
<td>27</td>
</tr>
</tbody>
</table>

Table 4.1 Frequency of FRAXG in the Finnish Family. Fresh peripheral lymphocytes from the family members were cultured under the folate-sensitive fragile site inducing conditions. Metaphase spread slides were prepared, and stained by Giemsa. A total of 100 metaphases were examined under microscope. The nonstaining gaps or breaks in Xp22 were counted as the expression of FRAXG.
Figure 4.4 Giemsa staining of metaphase spreads from the proband's lymphoblastoid cell line cultured under folate-sensitive fragile site inducing condition showing FRAXG as a chromatid breakage (A) and a nonstaining gap (B) (indicated by arrows)
FRAXG was observed as both chromatid break (Fig. 4.4A) and non-staining gap (Fig. 4.4B). Under the inducing conditions tested, medium 199 plus $10^{-7}$ M FudR gave the highest induction rate of FRAXG (5-7%). Compared to PBL, this is about 25% of that from fresh PBL. The lower induction rate observed for FRAXG from LBCL is consistent with other published reports on FRAXA induction (Abruzzo et al., 1986). However, successful induction of FRAXG in LBCLs not only confirmed the expression of this novel fragile site in the Finnish kindred, but also provided us with sufficient samples for the subsequent fine FISH mapping of FRAXG.

4.3.4 Mapping of FRAXG by FISH to a region about 1 Mb in Xp22.1 with a contig of YACs

Since fragile sites express cytogenetically as chromosome breakages or non-staining gaps, FISH has been used to define the region a fragile site spans with mapped clones, such as YACs or BACs (Verkerk et al., 1992; Wilke et al., 1994). Based on the GTG banding of metaphase chromosomes expressing FRAXG, FRAXG was tentatively mapped to Xp22.1. To further fine map FRAXG, a contig of six YACs from Xp22.1 was used in FISH to determine the location of FRAXG (Alitalo et al., 1995; Ferrero et al., 1995). Five of the YACs were individually labeled by FITC-dUTP, and used in FISH. To determine the relative location of a YAC to FRAXG, at least five metaphase spreads expressing FRAXG and a good FISH signal from the respective YAC were identified and captured. Each signal was designated as centromeric when the signal was centromeric to FRAXG; telomeric when the signal was located telomeric to FRAXG; and on gap when the signal and FRAXG were
located in the same position. The position of a YAC to FRAXG was determined based on the location of the majority of FISH signals relative to FRAXG. As shown in Fig. 4.5, y827E10 is located right onto the broken chromatids (indicated by green arrow). The red signal was from b733O18, which has been mapped to Xp22.31. It was used to identify the telomeric part of Xp – either still attached or broken off. An X-chromosome centromere specific probe, CEPX alpha (Vysis), was also included to identify the X chromosome. Shown in Fig. 4.6A and Fig. 4.6B are two representative FISH images showing y911G5 and y946F5 located telomeric and centromeric to FRAXG, respectively. Fig. 4.7 summarized the FISH results of the locations of the five YACs relative to FRAXG. Based on these mapping data, FRAXG is clearly located in a region in Xp22.1, which is covered telomERICALLY by y911G5 and centromERICALLY by y946F5, a critical region of about 1 Mb (indicated by the solid bar in Fig. 4.7). The FISH mapping with the YAC contig not only defines the region of FRAXG, but also provided a solid basis for the further fine mapping and eventual cloning of FRAXG.

4.3.5 Further fine mapping of FRAXG to a region of less than 200 kb by FISH with BAC contig

As YACs on average have inserts of hundreds of kb, and are highly chimeric and unstable, it is recommended to use other clones that have smaller inserts, but are more stable and less chimeric to further fine map FRAXG. BACs were chosen as they are highly stable and less chimeric. They have on average insert size of 150 kb-250 kb. BAC DNA can also be directly sequenced. During the course of this project,
Figure 4.5 Fluorescence *in situ* hybridization (FISH) mapping of FRAXG with YAC y827E10. The FRAXG was shown as a chromatid breakage indicated by the white arrow (1). YAC 827E10 was located on the gap (green arrow, 2). CEP-X, a marker for X-chromosome centromere, and BAC b733O18, located on Xp22.31 were included as the control for the X-chromosome centromere and telomere respectively.
Figure 4.6 Fluorescence in situ hybridization (FISH) mapping of FRAXG with YAC y911G5 and y946F5. y911G5 was located telomeric to FRAXG (A), and y946F5 centromeric to FRAXG (B) (indicated by arrows). CEP-X, a marker for X-chromosome centromere, and b733O18, located on Xp22.31 were included as the control for the X-chromosome centromere and telomere respectively.
Figure 4.7 Mapping of FRAXG to a critical region of about 1 Mb in Xp22.1 by FISH with a contig of six YAC clones. The numbers in brackets are the estimated sizes in kb for YACs. Cen: centromeric to FRAXG; Tel: telomeric to FRAXG; N.D.: not done.
a complete BAC-cosmid contig was assembled to cover the region covered by the YAC contig (Chapter 3). A total of 23 BACs covers this region with minimal overlapping. They were used in the FISH mapping of FRAXG. As summarized in Fig. 4.8, all BACs centromeric to b228D12, including b228D12, were centromeric to FRAXG, while all BACs telomeric to b692N21, including b692N21, were telomeric to FRAXG. Therefore, FRAXG was mapped to a region of less than 200 kb covered by two overlapping b393H10 and b2406. As shown in Fig.4.9, b393H10 is located right on the unstaining gap of FRAXG, which indicates that b393H10 contains the region of FRAXG.

4.3.6 (CCG)n trinucleotide repeats in the region covered by the BAC contig

To date all five RHFFS have been shown to be caused by the expansion of normally polymorphic (CCG)n trinucleotide repeats (Kremer et al., 1991a; Oberle et al., 1991; Verkerk et al., 1991; Yu et al., 1991; Knight et al., 1993; Nancarrow et al., 1994; Parrish et al., 1994; Jones et al., 1995). Based on this, we hypothesized that FRAXG is similarly caused by the expansion of a (CCG)n trinucleotide repeat. To identify potential (CCG)n repeats in the region, a Southern blot based approach with radiolabeled (CCG)n oligonucleotide was developed. To establish the specificity of the (CCG)n probe with different lengths, a pilot test Southern blot was carried out. As shown in Fig. 4.10, four Southern blots were prepared with two DNA samples on each blot. One is a cloned 5.1 kb EcoRI fragment form FMRI gene that contains a (CCG)_{19} ( ). This clone was used as the positive control for the detection of (CCG)n repeat tracts by Southern blot. Another is a 5.0 kb EcoRI fragment from Xp22.1 with no
Figure 4.8 Mapping of FRAXG to a critical region of less than 200 kb in Xp22.1 by FISH with a contig of 23 BAC clones. The critical region of FRAXG is indicated by the solid bar. All BACs right to 228D12 (including 228D12) are located centromeric to FRAXG, and those left to 692N21 (including 692N21) are located centromeric to FRAXG. BAC 393H10 is located right on the gap.
Figure 4.9 Fluorescence *in situ* hybridization (FISH) mapping of FRAXG with BAC 393H10. The FRAXG was shown as a non-staining gap indicated by the white arrow. BAC 393H10 was located right on the gap (indicated by arrow). CEP-X, a marker for X-chromosome centromere, and BAC 733O18, located on Xp22.31 were included as the control for the X-chromosome centromere and telomere respectively.
Figure 4.10 Characterization of the specificity of the probes (CCG)n with different lengths in Southern blot. (CCG)n with copy number at 5, 7, 9, and 10 were end-labeled by [γ-32P] ATP, and used as the probes. pE5.1, a 5.1 kb fragment containing (CCG)_{19} in FMR1 gene. pZ5.0, a 5.0 kb fragment containing no (CCG)n (n<3) repeat in Xp22.1.
(CCG)n \((n \leq 3)\) repeats (cloned and sequenced in our lab, data not shown), which served as a negative control. Four probes of (CCG)n with five, seven, nine, and ten copies of CCG were end-labeled with \([\gamma-^{32}\text{P}]\text{ATP}\). The same amount of labeled probe was used in the four parallel Southern blot experiments. Clearly \((\text{CCG})_7\) gave the best signal with minimal background under the hybridization conditions tested.

All 23 BACs in the minimal tiling path for the region were digested with \textit{EcoRI} and hybridized with the radiolabeled \((\text{CCG})_7\) probe. As shown in Fig. 4.11, three distinct \((\text{CCG})_n\)-positive fragments from \(b1139J14\), \(b1037J10\), and \(b393H10\) were detected. To clone the \((\text{CCG})_n\)-positive fragments, \(b1139J14\) was further restriction mapped and analyzed by Southern blot to identify the smallest restriction fragment containing the \((\text{CCG})_n\) repeat for easy cloning and later sequencing. A 2.5 kb \textit{XhoI} fragment positive for the \((\text{CCG})_7\) probe was identified (Fig. 4.12). The corresponding fragment was identified from the gel and cut out, extracted from the gel and cloned into the \textit{XhoI} site of plasmid vector \(p\text{Zero2}\). The presence of 2.5 kb insert was verified by \textit{XhoI} digestion of DNA from the picked clones. The presence of \((\text{CCG})_n\) repeat in the clones was further confirmed by Southern blot analysis with the same labeled \((\text{CCG})_7\) probe. Then the clone was sequenced completely. As shown in Table 4.2, a \((\text{CAG})_{12}(\text{CCG})_5\) was identified in the sequence. BLAST sequence analysis identified that this repeat was located in the 5' UTR region of gene \textit{KIAA0902}. \textit{KIAA0902} was identified during a large-scale cDNA sequencing project (Nagase \textit{et al.}, 1998). Its function is currently unknown. Although \(b1139J14\) was mapped centromerically to \textit{FRAXG}, expansion of \((\text{CAG})_{12}(\text{CCG})_5\) from \(b1139J14\) in the proband was further excluded by Southern blot analysis.
Figure 4.11 Detection of (CCG)n-positive fragments in b1139J14, b1037J10, and b393H10 by Southern blot. The probe was [γ-32P] ATP-labeled (CCG)_n.
Figure 4.12 Restriction mapping of (CCG)n-containing fragment in BAC 1139J14 by Southern blot. 1 µg of 1139J14 DNA was digested by different restriction enzymes, separated on 0.8% agarose gel, and hybridized by [γ-32P]ATP labeled (CCG)₇.
<table>
<thead>
<tr>
<th>BAC</th>
<th>Repeats</th>
<th>Gene</th>
<th>Location</th>
<th>Expanded</th>
</tr>
</thead>
<tbody>
<tr>
<td>1139J14</td>
<td>(CAG)$<em>{12}$[(CCG)$</em>{5}$]</td>
<td>KIAA0902</td>
<td>5' UTR</td>
<td>No</td>
</tr>
<tr>
<td>1037J10</td>
<td>(CCG)$<em>{3}$[(CTG)$</em>{5}$][(CCGCTG)$_{3}$]</td>
<td>RSK2</td>
<td>5' UTR/promoter</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>(CCG)$<em>{8}$[(CTG)$</em>{1}$][(CCG)$_{1}$]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>393H10</td>
<td>(CCG)$_{17}$</td>
<td>FRAXG</td>
<td>?</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Table 4.2 Summary of the Identified Trinucleotide Repeats in the BACs
Similarly the (CCG)n-containing fragment in b1037J10 was mapped to a 2.7 kb XhoI fragment (Fig. 4.13). After cloning and sequencing, a (CCG)$_3$(CTG)$_3$(CCGCTG)$_3$(CCG)$_8$(CTG)$_1$(CCG)$_1$ repeat was identified. BLAST sequence analysis identified that this sequence was in the promoter or 5'UTR region of RSK2 gene. RSK2 encodes a growth-factor induced kinase (Chang et al., 1995). Mutations of RSK2 cause Coffin-Lowey syndrome (Trivier et al., 1996), and nonspecific X-linked mental retardation (Merienne et al., 1999). b1037J10 containing this repeat is very close to the mapped FRAXG critical region (Chapter 3). However, genomic DNA Southern blot analysis confirmed that there was no expansion of this repeat in the FRAXG-expressing individuals (data not shown).

b393H10 was shown to contain the region for FRAXG by FISH. The (CCG)n-containing fragment was further mapped to a 1.6 kb EcoRI-NotI fragment (Fig. 4.15A). After it was cloned into EcoRI-NotI site of pZero2 vector, the whole 1.6 kb fragment was sequenced. A (CCG)$_17$ was identified. The whole sequence is listed in Fig. 4.14 with the (CCG)$_17$ repeat in bold. As illustrated in Fig. 4.15B, the (CCG)$_17$ is located 261 bp downstream the NotI site. A 770 bp Hpal-EcoRI fragment which does not contain the (CCG)$_17$ from this 1.6 kb was used in subsequent Southern blot analyses. BLAST sequence analysis identified no known homologous sequences. Further sequence analysis indicates that this repeat is within a CpG island. Fig. 4.16 is an EcoRI-digested genomic DNA Southern blot, hybridized with the radiolabeled 770 bp Hpal-EcoRI fragment. In lanes 2, 3, and 5, a higher molecular weight fragment in addition to the common fragment was detected. Samples in these three lanes were from the proband's mother (Lane 2), sister (Lane 3) and the proband (Lane 5). All
Figure 4.13 Restriction mapping of (CCG)n-containing fragment in BAC 1037J10 by Southern blot.

1 μg of 1037J10 DNA was digested by different restriction enzymes, separated on 0.8% agarose gel, and hybridized by [γ-32P]ATP labeled (CCG)₇.
Figure 4.14.1.6 kb *EcoRI-N*otI sequence from BAC 393H10 showing the CCG repeat (italic and bold) and the locations of primers 393H10_PIF and 393H10_PIR (sequence underlined)
Figure 4.14: (continued)

1101 ACAATTACA AACTGAGGAG AGAAATCAGG AAGGCCTCTTT TAGGCTCCTC
1151 GCCAGGCAGC CTTTTTAGCA TTTTTCTGAA TGTGAACAGG TCCAAACGAC
1201 AGAGCAGAAT TTGAAAAGAA AGGTTTTGCT GCAGATTCAT AACGAGGACT
1251 TTATTTTTCA TTGGTATTTT AATGGAATGT ATATAGMLAA TTTTTTTATT
1301 TTAATGGTTT TTTTAATGG CTACTAAAAT TTTATTTTGA AATCAGAGCT
1351 GTTCAGTCG CCCCCAAGGAA ACCCTTTAAA CGTCTTTATTA GAAACATTTT
1401 TATATATAGG AACATTTAGC CATTATTACA CATTCTTAG ATAGAAATCA
1451 CAATGGAAGAT ACGTTGGTTC ACTATTCCAA GTCGGAAGT ACGATGGTGA
1501 ATATGTTTTC CCCATTCGAT AGTAACCATGT GTGAATGGG TTATACACCT
1551 CCAGTCTTTT TCTATGCGA GCTTAATACA TGGAGTTTTTC TAAAGTGGAAT
1601 TC
Figure 4.15 (A) Southern blot analysis of b393H10 with [γ-32P] ATP-labeled (CCG)$_7$ as the probe. 
(B) A restriction map of the region around the (CCG)$_7$ repeat. Figure drawn on scale, probe HpRI is the probe used in the genomic DNA Southern blots shown in figures 4.16, 4.18 and 4.19. 
H, HindIII; N, NotI; RI, EcoRI; RV, EcoRV.
Figure 4.16 Genomic DNA Southern blot showing expansion in FRAXG-expressing individuals. Genomic DNA was digested by EcoRI, and the samples were hybridized by 0.77 kb HpaI-EcoRI fragment.
three had been shown to express FRAXG. The proband’s father and another sister did not express FRAXG, and no second band was detected. A single approximately 12 kb EcoRI fragment was detected in a normal male control. Genotyping of the X chromosomes in this family with 11 X-chromosome microsatellite markers indicated that the three X chromosomes with the expansion were the same chromosomes inherited (data not shown).

To have a better separation of the expanded fragment from the normal one, genomic DNA Southern blots with different restriction enzymes were carried out. First a restriction map of the region containing the cloned (CCG)$_{17}$ repeat from BAC 393H10 was established by Southern blot analysis of b393H10 digested by different restriction enzymes with the (CCG)$_{17}$ as the probe (Fig. 4.17A). The (CCG)$_{17}$ is located within an estimated 2.0 kb HindIII-EcoRI fragment or a 2.6 kb HindIII fragment. Fig. 4.18 is a genomic DNA Southern blot with HindIII-EcoRI double digestion, hybridized by the 770 bp HpaI-EcoRI probe. In all the five family members and one normal unrelated male, a common fragment about 2.0 kb in size was detected. In the proband and her FRAXG-expressing mother and sister, there were additional smears around 3.5-5.5 kb. This suggested an expansion of the HindIII-EcoRI fragment in the FRAXG-expressing individuals. The smear also suggests the somatic instability of the expansion. This HindIII-EcoRI fragment was completely sequenced. The (CCG)$_{17}$ is the only repeat within this fragment, which suggests that the expansion is most probably from the (CCG)$_{17}$. To investigate whether the expansion may affect the methylation of an adjacent CpG island, a methylation-sensitive restriction enzyme NotI was included in the genomic DNA Southern blot. When the two Cs in
Figure 4.17 Partial restriction map of the region around the (CGG)_n repeat and the CpG island (A) and Southern blot mapping of the expansion (B)
Figure 4.18 Genomic DNA Southern blot showing expansion in the FRAXG-expressing individuals. Genomic DNA was digested by EcoRI and HindIII, and the samples were hybridized by 0.77 kb Hpal-EcoRI fragment.
GCGGCCGC NotI site are methylated, the NotI cleavage at this site is blocked (New England BioLabs). As shown in Fig. 4.19, in HindIII single digest, a common 2.6 kb fragment was present in all individuals. The expanded fragments and smears were detected in the FRAXG-expressing individuals. As shown in lanes 3, 4, and 6 in the left half, the expansion was further expanded as the maternal X chromosome was passed to her daughters, which suggested the germline instability of the expansion. The largest expansion was observed in the proband. There are three major expanded fragments together with the smear in the proband: by +1.5 kb, +2.7 kb, and +3.6 kb. The calculated increase of the copy number of (CCG)n would be around 500, 900, and 1200. In the HindIII and NotI double digestion, the right half in Fig. 4.19, lane one is a non-related normal female. Half of her X chromosome DNA was methylated due to the random X chromosome inactivation, as indicated by about equal amount of 2.6 kb HindIII and 2.0 kb HindIII and NotI fragment. Lane 7 is a normal male control. All his X chromosome is unmethylated, as indicated by no remaining 2.6 kb HindIII fragment after the HindIII and NotI double digestion. In lanes 3, 4, and 6, the NotI sites in all the expanded fragments were methylated as indicated by the same amount of remaining fragments compared with those in the HindIII single digestion. Lane 5 is the normal sister of the proband. The methylation pattern is same as the normal female control (Lane 1), indicating the random X chromosome inactivation.

4.3.7 Population study of FRAXG (CGG)n locus

To exclude the possibility that the expansions observed in the FRAXG-expressing individuals are rare polymorphism, studies of the population distribution of
Figure 4.19 Hypermethylation of the associated CpG island in FRAXG-expressing individuals. Genomic DNA was digested by HindIII or HindIII and NotI. The samples were hybridized first by 0.77 kb HpaI-EcoRI fragment, and then by a 0.60 kb fragment from 11q22 to adjust the loading amount.
(CGG)n triplets and the inheritance of the FRAXG (CGG)n locus in normal families were carried out. Two normal CEPH control families were selected, CEPH 1331 and CEPH 1347 for the inheritance study. The copy numbers of (CGG)n triplets at FRAXG loci were determined. As shown in Fig. 4.20, the FRAXG (CGG)n repeats in CEPH 1347 were stably transmitted. In CEPH 1331, there are both decrease and increase by two CGG triplets during the transmission from one generation to another. This may reflect that the (CGG)n repeat is prone to errors in DNA replication, resulting in these small changes of the copy number of CGG triplets. For the population study, a total of 286 normal, unrelated Finnish individuals were genotyped for the copy numbers of (CGG)n triplets at FRAXG loci by PCR. Figure 4.21 summarizes the results. The Finnish population likely contains nine to 21 copies of CGG triplets at FRAXG loci, based on this sample study. Almost half of the population studied has 13 copies of CGG triplets. More than 85% of the population contains 12-16 copies of the CGG triplets.

4.3.8 Identification and characterization of EST388929

To search for the gene(s) associated with the CpG island and the (CCG)n repeat, flanking regions were sequenced. First, a high density filter with EcoRV-NotI human genomic DNA plasmid pBluescript clones (courtesy of Dr. Christoph Plass) was screened by hybridization with the radiolabeled 770 bp HpaI-EcoRI fragment. A single positive clone p68H2 was identified (data not shown). However, this clone was highly unstable in the host E. Coli during the growth, as only 15% of the clones still contained the insert after overnight growth (data not shown). By reducing the growth
Figure 4.20 Inheritance of FRAXG (CGG)n locus in two CEPH families. A, part of family 1331; B, part of family 1347. The copy number of (CGG)n triplet (indicated in parentheses) at FRAXG associated locus were determined by genotyping the samples with FAM-labeled 393H10_PF primer, and comparing them to a sequenced clone containing (CGG)17 repeat.
Figure 4.21 Distribution of the polymorphic FRAXG (CGG)n triplet in Finnish population (n = 286)
time, and increasing the concentration of antibiotic used in the growth medium, about 1 μg of plasmid DNA with the correct insert was isolated. The clone was sequenced using a transposon-based shotgun sequencing method (New England Biolab, Beverly, MA) following the manufacturer’s protocol. Then DNAStar software was used to assemble the contig. Three contigs were assembled with two gaps among them. The largest contig, which contains the (CGG)n repeat and the CpG island, was 5878 bp in size (data not shown). BLAST sequence analysis against NCBI human EST database identified an EST388929. The EST clone (IMAGE 2660055) was purchased from Research Genetics (Huntsville, AL), and sequenced completely. The whole insert contains 680 bp, with about 450 bp of Alu repeat at its 3' end. The 680 bp EST aligned to two regions of the 5878 bp contig with a 2321 bp intron in-between (Fig. 4.22). To confirm the EST, an RT-PCR was carried out with the primers 2660055_F and 2660055_R (Fig. 4.22). As shown in Fig. 4.22, three fragments were amplified from the total RNA of lymphoblastoid cell line. They were cloned into TA PCR cloning vector (Invitrogen, TOPO PCR Cloning Kit, Carlsbad, CA), and sequenced. The 239 bp fragment was expected from EST388929. The fragments 392 bp and 439 bp were alternative splice products, which give two possible alternative ESTs at 833 bp and 880 bp respectively. Sequence analysis of ESTs 680 bp, and 833 bp, 880 bp, however, identified no open reading frames. To further investigate the expression and the size of endogenous transcript, Northern blot analysis was carried out with a human multiple tissue Northern blot (Clontech, Palo Alto, CA). However, the Northern blot analysis was not satisfactory (data not shown), possibly due to the short probe used (about 200 bp).
Figure 4.22 Novel gene associated with FRAXG-associated CpG island. Primers from EST 2660055 from different exons were used in the RT-PCR to study its expression. Total RNAs from the five members of the Finnish family were used. PCR product was separated on 1% agarose gel.
4.3.9 Shotgun sequencing of b393H10 and identification of partial putative FXGAG gene.

To identify the gene(s) associated with the FRAXG (CGG)n repeat and the CpG island, as well as the flanking sequences, b393H10 was shotgun sequenced. As there are no sequences for this region from the public human genome project and private company Celera, it is also important to have the region sequenced. By sequencing from the both ends a total of 1700 plasmid clones from the sheared DNA of b393H10, a total of 7-fold coverage of b393H10 was obtained. Assembly of these sequences generated mainly 10 contigs, with the largest one over 30 kb in size. A contig of about 6.8 kb DNA sequence contains the (CGG)n repeat and the associated CpG island (data not shown). Sequence BLAST analysis of this 6.8 kb did not identify any additional ESTs. Further sequence analysis with the program FirstEF for the prediction of 5' UTR and first exon (Davuluri et al, 2001) identified a common promoter region around the (CGG)n repeat and two possible first exons with opposite orientations: exon G1Ex1 for the sense orientation and G2Ex1 for the antisense orientation (data not shown). To test whether these two predicted exons are indeed transcribed, RT-PCR with the PCR primers derived from G1Ex1 and G2Ex1 was performed. The extracted RNAs from skeletal muscle, brain tissue and skeletal muscle, lymphoblastoid cell lines were used in RT-PCR, after the RNA samples were treated by Dnase I (GIBCO BRL) to remove any possible contaminated genomic DNA. A fragment with the expected size was detected from all the samples tested for G1Ex1. No amplification for G2Ex1 was detected from the same samples. To test whether G1Ex1 represents the missing first exon of a gene containing EST 388929, an
Figure 4.23 FXGAG gene structure and its alternative spliced transcripts
Figure 4.24 Locations of the mapped genes in b393H10. b24O6 and b228D12 are BACs overlapping with b393H10. The CpG island and the (CGG)n repeat associated with FRAXG are indicated. The arrows indicate the orientations of the mapped genes in b393H10. The orientation of putative gene AK027409 cannot be determined currently.
RT-PCR with primers G1EST_1F and G1EST_1R derived from G1Ex1 and EST 388929 respectively was performed on the same four RNA samples. A major fragment with 1.3 kb in size was amplified from the lymphoblastoid cell line. The total PCR product was cloned into TA vector, and sequenced. Besides the 1.3 kb fragment, the clones with about 0.8 kb and 0.9 kb inserts were also present. Sequence aligning confirmed that the predicted G1Ex1 and EST 388929 were indeed transcribed together, and they represent the different exons for a same gene (illustrated in Fig. 4.23). This putative novel gene is name as FXGAG for fragile X G associated gene. The three different fragments: 0.8 kb, 0.9 kb and 1.3 kb, represent the differentially spliced products. Their complete sequencing and characterization of FXGAG is underway.

Whole BLAST sequence analysis of b393H10 identified two known genes EIFIAX and RSK2 and one unknown gene AK027409 (Chang et al., 1995; Lahn et al., 1997). Their orientations and relative locations were determined by PCR with primers from the 5' UTR and 3'UTR of the two genes (Fig. 4.24). The location and orientation of AK027409 could not be determined unequivocally. By using a probe from the 5'UTR of AK027409 in the Southern blot analysis of b393H10 with different restriction enzymes, AK027409 is not located within the over 20-kb flanking region of FRAXG CpG island and the (CGG)n repeat (data not shown).
4.4 DISCUSSION

To date, a total of five RHFFS have been characterized at the molecular level. They are all caused by the expansion of unstable (CCG)n/(CGG)n trinucleotide repeats. Three of them: FRAXA, FRAXE, and FRA11A have been linked with clinical phenotypes. Their molecular mechanisms are similar in that the expansion of (CCG)n/(CGG)n trinucleotide repeats is associated with the hypermethylation of the mutant repeats and adjacent CpG islands resulting in the downregulation of the transcription and/or translation of associated genes (Kremer et al., 1991a; Oberle et al., 1991; Verkerk et al., 1991; Yu et al., 1991; Knight et al., 1993; Parrish et al., 1994; Jones et al., 1995; Nancarrow et al., 1995).

Here we report the identification of a novel RHFFS FRAXG in Xp22.1, which is associated with short stature in the proband. This could be the fourth RHFFS associated with a clinical phenotype. Through positional cloning, we showed that FRAXG was probably contained in a single b393H10, from which a polymorphic (CGG)n trinucleotide repeat was isolated and shown to be expanded in FRAXG-expressing individuals in the studied family. The expansion of the (CGG)n triplets in FRAXG-expressing individuals is from around 500 to 1200 copies of the (CGG)n triplets. Both germline and somatic instabilities of the expanded repeats are present in the family.

Genotyping of the FRAXG (CGG)n locus in 283 individuals from the same geographic region with the family identified that normal individuals contain 9-21 copies of the (CGG)n triplets with almost half of them having 13 copies of the repeat.
and more than 85% of them having the repeats of 12-16 copies. It seems that the repeats within this range are overall stable from the inheritance study of the repeats in two normal CEPH families. These studies led us to believe that the expansions identified in FRAXG-expressing individuals in the family were not rare polymorphism. We conclude that expansion of the polymorphic (CGG)n trinucleotide repeat isolated from b393H10 is the molecular cause of FRAXG.

In normal populations, the FRAXG (CGG)n repeats vary very little, from 9-21 copies. (CCG)n/(CGG)n repeats within this range are usually stable from the studies mainly in FRAXA and FRAXE ( ). In the family studied, the (CGG)n repeats with over 500 copies are highly unstable with both germline and somatic instabilities present. Because this is the only reported family with FRAXG, it is yet unknown whether the FRAXG (CGG)n repeats are stable with the sizes intermediate between 9-21 repeat range found in the general population and the 500-1200 repeat range found in FRAXG-expressing individuals. It would be very interesting to trace back the expanded (CGG)n repeat identified in the proband’s mother. To identify whether this expansion is due to a single large jump or there is change of flanking sequences which may enhance the instability of the (CGG)n repeat during the transmission would be very useful in not only understanding how FRAXG occurs in the family, but also in the general understanding of the repeat instability and the mechanism of the repeat expansion.

The FRAXG (CGG)n repeat is located within a CpG island. The CpG island is hypermethylated in the chromosomes with the expanded (CGG)n repeat. Based on the observations in FRAXA, FRAXE, and FRA11B that hypermethylation of the CpG
island downregulates the transcription and/or translation of the associated gene, we predict that the hypermethylation of the CpG island in the FRAXG-expressing individuals will similarly downregulate the expression of its associated gene. We have identified a novel gene FXGAG associated with this CpG island transcribed in several tissues, including brain, skeletal muscle, and lymphoblastoid cell lines. At least three exons of FXGAG have been identified. Whether the expression of FXGAG is affected in the FRAXG-expressing individuals is currently being investigated.

FRAXG was initially identified in the proband with idiopathic short stature. In the past two years, she has been given growth hormone treatment with a good response. As gene(s) for idiopathic short stature have been suggested to be located in the short arm of the X chromosome (Ballabio et al., 1989; Ogata et al., 1993; 1995a; Zinn et al., 1998b; Wandstrat et al., 2000), we hypothesized that FRAXG, located in Xp22.1, might be functionally associated with the short stature in the proband, and the FRAXG expression might be linked to the downregulation of the adjacent gene, resulting in short stature in the proband. Based on its association with the FRAXG (CGG)n repeat and the CpG island, FXGAG is a strong positional candidate. We postulate that the hypermethylation of the CpG island in the proband downregulates the transcription of FXGAG, resulting in short stature in the proband.

However, FRAXG is also present in the proband's mother and one of her sisters who have normal height. To this apparent contradiction, we proposed the following interpretations. Though we cannot exclude that FRAXG could be incidentally linked to short stature in the proband, we believe that the absence of short stature in the proband’s mother and one of her sisters could be due to the following factors. Firstly,
as shown in Fig. 4.19, the expansion of the (CGG)n repeat is much larger than those in the proband’s mother and one of her sisters. A much smaller expansion (equivalent to premutation in fragile X syndrome) was also detected in the mother and the normal sister, which indicates the chimeric status of the expansion in these two individuals. No such smaller expansion was detected in the proband. Whether this chimeric status may result in different levels of expression of the adjacent gene, thus protecting these two individuals from the phenotype is being investigated. Secondly, the three \textit{FRAXG} positive individuals are all heterozygous. Thus one allele of the candidate gene, like \textit{FXGAG}, may be downregulated due to the hypermethylation of its CpG island in the X chromosome expressing \textit{FRAXG}, another allele of the gene may be active. However, due to the random X chromosome inactivation, the expression of the gene from the allele containing normal (CGG)n repeat may be different among the three individuals and also could be different in different tissues in an individual, as have been reported in fragile X syndrome patients (Taylor \textit{et al.}, 1994; Dobkin \textit{et al.}, 1996; Maddalena \textit{et al.}, 1996). Though no major difference of this random inactivation of the active allele in lymphoblastoid cell line was detected, the X chromosome inactivation in other tissues, like skeletal muscle etc., may be different. The quantitation of the expression of the adjacent gene in different tissues may be necessary to address this possibility.

Short stature has been consistently linked with Turner syndrome (Ullrich, 1930; Turner, 1938). It is generally believed that genes underlying Turner syndrome may escape the X chromosome inactivation in female, and may have functional homologues in Y chromosome in male (Zinn \textit{et al.}, 1998a). Based on the
genotype/phenotype correlation study and the breakpoints mapping study in Turner syndrome patients with partial Xp deletions, a region of about 2 Mb, which is close to gene PDHA1 centromerically in Xp22.1, may contain gene(s) for short stature (Zinn et al., 1998b; Wandstrat et al., 2000). The physical location of FXGAG within this region constitutes it a positional candidate gene for TS. More suggestive of this, FXGAG, EIF1AX and RSK2 are mapped to a common region of less than 100 kb. EIF1AX has been shown to escape X chromosome inactivation and it has a functional homologue in the Y chromosome (Lahn et al., 1997). RSK2 has been shown to partially escape X chromosome inactivation (Carrel et al., 1999). Based on the observation that genes escaping the X chromosome inactivation appear to cluster together (Carrel et al., 1999), we hypothesize that FXGAG may escape the X chromosome inactivation as well. Whether there is a functional homologue of FXGAG in the Y chromosome remains to be investigated.

In summary, we report the identification of a novel rare, heritable folate-sensitive fragile site FRAXG in Xp22.1 in the proband with short stature. Positional cloning of FRAXG identified that FRAXG was caused by the expansion of a normally polymorphic (CGG)n trinucleotide repeat, which is associated with the hypermethylation of the adjacent CpG island. A novel gene FXGAG associated with the (CGG)n repeat and the CpG island has been identified, and it is proposed as a strong candidate gene for short stature in general and in TS.
CHAPTER 5

CONCLUSION

Fragile sites are nonrandom chromosomal gaps or breaks that become apparent when cells are cultured under special inducing conditions (Sutherland, 1979a). Rare heritable, folate-sensitive fragile sites (RHFFS) induced by low folic acid and/or thymidine in culture medium have been clearly linked to several clinical phenotypes, including fragile X syndrome, the most common form of inherited mental retardation in children, and Jacobsen's syndrome (Sutherland et al., 2000). Molecular characterization of a total of five RHFFS to date has indicated a common underlying mutation and pathophysiological mechanism: the expansion of an unstable (CCG)n/(CGG)n trinucleotide repeat in the 5' promoter region and the hypermethylation of an adjacent CpG island, which together result in the transcriptional silencing of a downstream gene and the clinical phenotype in patients (Kremer et al., 1991a; Oberle et al., 1991; Verkerk et al., 1991; Yu et al., 1991; Knight et al., 1993; Nancarrow et al., 1994; Parrish et al., 1994; Jones et al., 1995).

The genetic basis for short stature (SS) in general and in Turner syndrome (TS) in particular has been investigated for at least five decades (Ullrich, 1930; Turner, 1938). One or multiple genes in the short arm, particularly p22, of the X chromosome
have been implicated (Rao et al., 1997; Zinn et al., 1998b; Wandstrat et al., 2000). However, the underlying gene(s) has been elusive, as most of the studies were based on the observed cytogenetic deletions that encompass several genes. Here, we reported the association of a novel RHFFS (FRAXG) in Xp22.1 with the SS phenotype in a Finnish proband. Based on its localization in Xp22.1 and its association with the SS phenotype, we hypothesize that FRAXG is functionally associated with the SS phenotype. Following this hypothesis, we set the research goals as to characterize FRAXG at the molecular level and to characterize any FRAXG-associated gene(s) and its involvement in the proband's SS phenotype.

A positional cloning approach was used to identify the molecular basis of FRAXG. FRAXG was initially mapped to a 1-Mb critical region in Xp22.1 by FISH using six contiged YACs in Xp22.1. Subsequently a complete genomic clone contig of 54 BACs and one cosmid covering a 2-Mb region including the mapped FRAXG critical region was assembled. The minimum tiling path consists of 23 BACs and one cosmid. Onto this contig, 30 new STSs derived from the unique end-sequences of the BACs, three ESTs, five genes, and seven CpG islands were mapped. Using the 23 BACs, FRAXG was further mapped to a region of less than 200 kb, covered by a single BAC (b393H10). From b393H10, a (CGG)$_{17}$ triplet repeat was cloned. As demonstrated by Southern blot, genomic DNA fragments containing the (CGG)$_{17}$ repeat are expanded by up to 3.6 kb in the FRAXG-expressing individuals in the Finnish family. The expansion was further mapped to a 1.6 kb EcoRI-NotI fragment containing the (CGG)$_{17}$ repeat. The expansion is associated with the hypermethylation of an adjacent CpG island. A population-based study indicated that the (CGG)$_n$ triplet
repeat is normally polymorphic. Together these data suggest that the expansion of (CGG)n triplet repeats underlies \textit{FRA\textsubscript{XG}}. the \textit{(CGG)}\textsubscript{17} repeat and the associated CpG island To identify \textit{FRA\textsubscript{XG}}-associated gene(s), \textit{b393H10} was shotgun sequenced. A 7-kb genomic DNA contig containing was assembled. A single EST was identified and confirmed, together with two alternative transcripts by RT-PCR. The identified EST and its alternative transcripts consist of two exons. The presence of the conserved polyadenylation site in the 3’ end of the EST suggests that the EST may contain the last exon and/or 3’UTR. The putative first exon, which is downstream of the \textit{(CGG)}\textsubscript{17} repeat and the associated CpG island, was predicted by the program FirstEF (Ramana V. Davuluri, ) and verified by RT-PCR. Thus, a novel gene, \textit{FXGAG}, associated with \textit{FRA\textsubscript{XG}} was identified. The largest identified \textit{FXGAG} transcript, composed of two exons, is close to 1.5 kb in size. Five alternative transcripts were identified from lymphoblastoid cell lines. Analysis of preliminary multiple tissue Northern blots indicates the wide expression of \textit{FXGAG}. The lack of an extended open reading frame for \textit{FXGAG} suggests that \textit{FXGAG} may be a functional RNA, or an endogenous antisense RNA. The association of \textit{FXGAG} with \textit{FRA\textsubscript{XG}}, and \textit{FRA\textsubscript{XG}} with the SS phenotype in the proband, suggests that \textit{FXGAG} is a positional candidate gene for SS.
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