Development of an Effective Therapeutic for Nerve Agent Inhibited and Aged Acetylcholinesterase

THESIS

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By

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Abstract

Organophosphorus nerve agents (OPs) are a very toxic class of compounds, and compounds for which there is a great need of effective therapeutics. OPs are phosphoryl-containing compounds that are typically used as pesticides and have been used as chemical warfare agents. The toxicity of OPs is derived from the inhibition of acetylcholinesterase (AChE), an enzyme found in the human body that regulates levels of the neurotransmitter acetylcholine. OPs inhibit AChE by undergoing nucleophilic attack by the catalytic Ser203 residue. However, while a water molecule is sufficiently nucleophilic to attack the carbonyl center and break the C–O bond between acetylcholine and Ser203 in the normal catalytic cycle, a water molecule is not sufficiently nucleophilic to attack the phosphoryl center. Addition of a stronger nucleophile, such as an oxime, is effective for attack at the phosphoryl center and cleavage of the P–O(Ser203) bond in order to reactivate AChE. However, a second reaction occurs in the inhibition process, called “aging,” in which the phosphoryl O-alkyl functionality is dealkylated creating an anionic phosphate or phosphonate in the active site. After aging has occurred, oximes are no longer able to attack the phosphoryl center to reactivate AChE.

Since the aging process is simply dealkylation of the O-alkyl group, the logical solution for reactivation of aged AChE would be to realkylate the OP to replace the departed alkyl group with a new alkyl group. Therefore, an efficient alkylating agent must be developed. One such alkylating agent, quinone methides (QMs), could possibly
be utilized as they have previously been shown to have highly tunable reactivity and can be administered in an unreactive prodrug form.

In this thesis, the relationship between structure and reactivity of quinone methides were investigated. The reaction mechanisms for alkylation of the QMs were modeled using computational methods. The electronics of the substituents on the ring, as well as the leaving group at the benzylic carbon, contribute to the reaction energetics, for both the concerted and stepwise mechanisms of the alkylation reaction. Electron-withdrawing substituents have been demonstrated to destabilize the electron-poor QM ring, thereby making QM formation less favorable. However, the same electron-withdrawing substituents make alkylation of the QM intermediate more favorable due to destabilization of the ring. The exact opposite is true of electron-donating groups, which stabilize the QM intermediate, favoring QM formation, but disfavoring the subsequent alkylation reaction. The leaving group at the benzylic carbon also contributes to the reaction energetics, mainly due to the relative nucleophilicity of the departed leaving group.

Based on our computational studies, we sought to establish an experimental protocol for monitoring both the quinone methide intermediate and the alkylation reaction in general by UV-vis and GC-MS techniques. Alkylation of the QM precursors with various nucleophiles at multiple temperatures was observed by GC-MS methods, while the QM intermediate of the di-\(t\)-butyl precursor could be monitored by time-resolved UV-vis spectroscopy. These data demonstrate that the reactivity of the QM
precursors can be greatly modulated and tuned, and thus may be ideal candidates for realkylation of aged AChE.
Acknowledgments

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Fields of Study

Major Field: Chemistry
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Chapter 1: Introduction to Acetylcholinesterase and Organophosphorus Nerve Agents and Design of an Effective Therapeutic Against Nerve Agent Inhibition of Acetylcholinesterase

1.1: INTRODUCTION TO ACETYLCHOLINESTERASE AND ORGANOPHOSPHORUS NERVE AGENTS

Organophosphorus nerve agents (OPs) are a very toxic class of compounds, and these are compounds for which there is a great need of efficient therapeutics. OPs are phosphoryl-containing compounds that are typically used as pesticides and have been used as chemical warfare agents. OPs have been used in wars and by terrorists against both military and civilians. They were originally designed by the German scientist Dr. Gerhard Schrader in 1936. Schrader and his coworkers accidentally discovered the lethality of OPs, becoming exposed to tabun while trying to synthesize effective insecticides.\(^1,2\) This discovery led to the development of other OPs, which were eventually weaponized. Iraq was the first nation to utilize organophosphates as a weapon when it used tabun and other chemical weapons were used multiple times in the Iran-Iraq war as well as during the Shi’a rebellion in 1991.\(^3\) Although nerve agents were not deployed during the first Gulf War, a small group of American soldiers were exposed to chemical agents in the demolition of the Khamisiyah Pit in May 1996.\(^4\) Nerve agents have also been used in terrorist attacks on civilians, such as in the 1995 Tokyo subway.
attack. The terrorist group Aum Shinrikyo released sarin in the Tokyo subway system killing 12 and injuring thousands. OP poisoning has also caused adverse effects in agricultural workers due to long-term exposure to pesticides.\textsuperscript{5,6}

The toxicity of OPs is derived from the inhibition of acetylcholinesterase (AChE), an enzyme found in the human body that regulates levels of the neurotransmitter acetylcholine. AChE is typically found in the central and peripheral nervous systems.\textsuperscript{7} AChE is a serine hydrolase containing an active site with a catalytic triad located at the bottom of deep 20 Å gorge.\textsuperscript{8} The catalytic triad for human AChE consists of Ser203, His447, and Glu334.\textsuperscript{9,10,11} Other subsites located in the active site help stabilize and orient substrates within the active site. These subsites include the cation-binding pocket, which consists of Trp86 and Glu202, and the oxyanion hole, which consists of the amide N–H bonds of the Gly121, Gly122, and Ala204 residues (Figure 1.1).\textsuperscript{10}

![Figure 1.1. The active site of human AChE (PDB id 1B41) containing the catalytic triad (Ser203, His447, and Glu334), the cation-binding pocket (Trp86 and Glu202), and the oxyanion hole (Gly121, Gly122, and Ala204).](image-url)
Scheme 1.1. The reaction cycles of AChE. A) The hydrolysis of acetylcholine, which cleaves off acetate and choline while regenerating functional AChE. B) The inhibition mechanism of AChE by an OP in which a stable product similar to the acetylcholine tetrahedral intermediate is formed preventing regeneration of functional AChE.

When acetylcholine reaches the active site of AChE, the carbonyl carbon undergoes nucleophilic attack by the hydroxyl moiety of Ser203, proceeding through a tetrahedral intermediate prior to loss of the choline group. After undergoing subsequent reactions with water, acetylcholine is released as acetate and choline, and functional AChE is regenerated (Scheme 1.1A). The toxicity of OPs lies in their ability to inhibit the normal function of AChE. In the normal catalytic cycle of AChE, once acetylcholine is hydrolyzed, functional AChE is regenerated. However, when an OP reacts with the active
site serine (Ser203), a tetrahedral phosphonate (or phosphate) is formed, which blocks any further binding or hydrolysis of acetylcholine (Scheme 1.1B). If untreated, this leads to a buildup of acetylcholine and overstimulation of the acetylcholine receptors in the nervous system, eventually causing paralysis and death.12

OP inhibition of AChE occurs in two different steps. The initial step, called inhibition, involves nucleophilic attack of the OP by Ser203 forming a covalent bond. Only at this initial step can strong nucleophiles be used to reverse inhibition by attacking the phosphorus and breaking the P–O(Ser) bond.13 Currently, pyridinium oximes, such as 2-pralidoxime (2-PAM), are used to remove the OP moiety from the active site and restore AChE functionality (Figure 1.2).12,14 This mechanism involves nucleophilic attack on the phosphoryl center by the oximate oxygen forming a penta-coordinate intermediate. This intermediate can then decompose via cleavage of the P–O(Ser) bond in order to regenerate functional AChE (Scheme 1.2).15
Figure 1.2. The structures of some common OPs that inhibit AChE (A) and oximes used for reactivation (B).

Scheme 1.2. The mechanism of reactivation of inhibited AChE by a model oxime.
The second inhibited state of AChE, called “aging,” is caused by dealkylation of the phosphonate ester bond. This process is believed to proceed through cleavage of the C–O bond of one of the alkoxy substituents on the OP, leaving an anionic phosphate that is resistant to nucleophilic attack (Scheme 1.3).\textsuperscript{9,15} The rate of aging depends on the OP but can vary from a half-life of 231 hours for butylsarin to 48 hours with VX and only 5 minutes with soman (Table 1.1).\textsuperscript{12,16,17} Currently, there is no treatment available to regenerate enzyme activity after the aging process has occurred, and it is considered unreactivatable.

Scheme 1.3. The aging process of inhibited AChE.

Table 1.1. The rates of inhibition and subsequent aging for various OPs with AChE.\textsuperscript{12}

<table>
<thead>
<tr>
<th>Nerve Agent</th>
<th>Inhibition Rate (M⁻¹ min⁻¹)</th>
<th>Aging Rate (h⁻¹)</th>
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<tbody>
<tr>
<td>Sarin</td>
<td>2.7 x 10⁷</td>
<td>0.228</td>
</tr>
<tr>
<td>Butylsarin</td>
<td>6.1 x 10⁸</td>
<td>0.003</td>
</tr>
<tr>
<td>Soman</td>
<td>9.2 x 10⁷</td>
<td>6.6</td>
</tr>
<tr>
<td>Cyclosarin</td>
<td>4.9 x 10⁸</td>
<td>0.099</td>
</tr>
<tr>
<td>VX</td>
<td>1.2 x 10⁸</td>
<td>0.019</td>
</tr>
<tr>
<td>VR</td>
<td>4.4 x 10⁸</td>
<td>0.005</td>
</tr>
<tr>
<td>Tabun</td>
<td>7.4 x 10⁶</td>
<td>0.036</td>
</tr>
</tbody>
</table>
1.2: DESIGN OF A THERAPEUTIC FOR REACTIVATION OF INHIBITED ACETYLCHOLINESTERASE

Due to the extreme adverse effects and possible exposure to OPs, design of an effective therapeutic is extremely important. There are three possible opportunities for protection against nerve agents (Figure 1.3). The first opportunity is when the nerve agent enters the bloodstream. In this treatment, prophylactic molecules called “bioscavengers” would hydrolyze OPs before AChE inhibition can occur. These bioscavengers are typically enzymes, such as mutated AChE or butyrylcholinesterase, that are designed to try to detoxify OPs.\textsuperscript{18,19} Unfortunately, current bioscavengers are either stoichiometric or have extremely low catalytic activity for nerve agent hydrolysis, making them non-ideal for treatment of OP exposure.\textsuperscript{20}
Figure 1.3. The various opportunities for treatment of OP inhibition of AChE. 1) Use of a bioscavenger to hydrolyze the OP before it can inhibit AChE, 2) Reactivation of AChE after inhibition by use of an oxime to remove the OP from the enzyme active site, and 3) Realkylation of aged AChE by replacing the departed \(O\)-alkyl group, so that a conventional oxime can be used to reactivate the enzyme.

The second opportunity for OP treatment is introduction of a strong nucleophile, such as an oxime, to reactivate AChE by cleaving the P–O(Ser) bond after inhibition, but for this strategy to be successful, the nucleophile must react with the inhibited AChE form, and prior to aging of AChE. Although oximes have been shown to be effective at reactivating inhibited AChE, their efficiency varies greatly depending on the OP. Hence, the nerve agent used must be identified before oxime treatment can be successful, which becomes a problem as some OPs have extremely rapid aging rates.\(^{12}\) Development of a broad-spectrum oxime for phosphorylated AChE is a prominent research goal in the cholinesterase community.
The third therapeutic approach against OP exposure focuses on reactivation of AChE after the aging process has occurred. This treatment option would realkylate the aged OP to replace the departed O-alkyl group, allowing a conventional oxime to reactivate the inhibited AChE. This route was briefly examined in the 1960s and 1970s with various phenacyl bromo alkylating agents, and those compounds were found to be unsuccessful at regenerating functional AChE, primarily due to a lack of knowledge about the AChE structure and inhibition mechanism. Specifically, the x-ray crystal structure of AChE was not published until 1991, and details of the active site of this very efficient enzyme were only hypothesized prior to that date. This treatment option has not been heavily investigated because current oxime treatments have been shown to be completely ineffective once aging has occurred. Also, the current thought is that conventional methods can be used for treatment against OP exposure. However, unless current treatments are rapidly administered following exposure, then some amount of the aged enzyme will accumulate, making this treatment strategy largely ineffective. This has led several groups to reinvestigate the feasibility of targeted alkylation of the aged OP in order to facilitate reactivation.

In order to reactivate aged AChE, an efficient alkylating agent must be developed. One such alkylating agent, quinone methides (QM), could possibly be utilized as they have previously been shown to have highly tunable reactivity and can be administered in an unreactive prodrug form. QMs are methylene cyclohexadienones with three possible isomers, ortho, para, and meta (Figure 1.4). The ortho and para isomers, both closed-shell species, are highly polarizable and therefore have both electrophilic and
nucleophilic characteristics. The ortho-quinone methides (o-QMs) have been shown to be more reactive than para-quinone methides (p-QMs), mainly due to the close proximity of the exocyclic oxygen to the electrophilic methylene. This close proximity is especially important when the nucleophile contains acidic protons, which can be better stabilized by o-QMs than p-QMs. However, the reactivity of both ortho- and para-QMs can be greatly modified by adjusting the substituents on the rings. An ideal feature of using a QM to realkylate aged AChE is that it could be administered as the QM precursor to get to the active site, and then the active QM could be formed in the active site for realkylation. The QM precursors are very close in structure to edrophonium, an AChE inhibitor that has been shown to bind in the active site (Figure 1.4), suggesting that the QM precursors could also bind in the AChE active site.

Figure 1.4. The structures of ortho, para, and meta QMs, the proposed QM precursors, and the AChE inhibitor edrophonium.

QMs are generally focused on alkylation of DNA, and therefore reactivity against major biological nucleophiles, including amino acids, nucleic acids, and phosphates, have been reported (Scheme 1.4A). Notably, a p-QM has been used to alkylate a phosphodiester at physiological temperature (Scheme 1.4B). As the alkylation of
phosphates under physiological conditions has been previously illustrated, it stands to reason that a QM or its precursor that binds to aged AChE could be used to realkylate the anionic oxygen of aged AChE so that an oxime could then be used to regenerate functional enzyme from its inhibited state (Scheme 1.5).

Scheme 1.4. Examples of quinone methides alkylating an amino acid (A) and a phosphodiester (B) near physiological temperatures.

Scheme 1.5. The proposed realkylation of aged AChE by a model QM and subsequent reactivation by an oxime.
1.3: THESIS SUMMARY

This thesis will cover the design and testing of a quinone methide alkylating agent for the purpose of realkylating aged AChE. Chapter 2 will investigate the relationship between structure and reactivity of quinone methides. Computational modeling on both the generation of quinone methides from precursors as well as the alkylation of various nucleophiles will be carried out in order to understand the effect of substituents on reactivity and specificity of QM alkylating agents. Chapter 3 will explore experimental efforts of establishing a protocol to observe the alkylation reaction using UV-vis spectroscopy to monitor the concentrations of reactant, alkylated product, and/or the possible QM intermediate. The UV-vis techniques used in Chapter 3 will be combined in Chapter 4 with a more quantitative measure for the alkylation of the various nucleophiles in GC-MS. Chapter 4 will use both spectroscopic techniques to probe the alkylation reaction to assist in the development of an efficient therapeutic for realkylation of aged AChE.

1.4: REFERENCES FOR CHAPTER 1


2.1: INTRODUCTION

OPs inhibit acetylcholinesterase (AChE) by undergoing nucleophilic attack at the catalytic Ser203 residue. However, while a water molecule is sufficiently nucleophilic to attack the carbonyl center and break the C–O bond between acetylcholine and Ser203 in the normal catalytic cycle, a water molecule is not sufficiently nucleophilic to attack the phosphoryl center. Addition of a stronger nucleophile, such as an oxime, has been shown to be effective for attack at the phosphoryl center and cleavage of the P–O(Ser203) bond in order to reactivate AChE. However, a second reaction occurs in the inhibition process, called “aging,” in which the phosphoryl O-alkyl functionality is dealkylated creating an anionic species.$^{1,2}$ After aging has occurred, oximes, due to their anionic character in their reactive oximate form, are no longer able to attack the phosphoryl center to reactivate AChE (Scheme 2.1).$^3$ The aging process can occur within minutes, depending on the structure of the OP,$^4$ so an efficient therapeutic is required to allow reactivation of AChE after aging.
Scheme 2.1. The aging process of inhibited AChE. The anionic aged species is no longer electrophilic and therefore will no longer undergo nucleophilic attack by an oxime. Instead, an alkylating agent, such as a quinone methide (QM) precursor, could be used to realkylate the aged enzyme to then be reactivated by an oxime.

Since the aging process is simply dealkylation of the $O$-alkyl group, the logical solution for reactivation of aged AChE would be to realkylate the OP to replace the departed alkyl group with a new alkyl group (Scheme 2.1). One benefit of designing a therapeutic to reactivate AChE after aging is that each of the common OPs, except for tabun, will generate one of two unique phosphate or phosphonate structures after aging has occurred. This fact is important, as the rate of aging depends on the branching of the alkyl chain. The more branched the alkyl chain on the alkoxy carbon becomes, the faster the aging process occurs in AChE. The alkyl chain also correlates to oxime efficacy for
reactivation of AChE. This means current treatments require that the OP must be identified after exposure and the correct oxime administered before the aging process has occurred. The benefit of designing a therapeutic to realkylate aged AChE is twofold; a single alkylation therapeutic will treat the identical aged structures, and the same oxime could be used for all realkylated OPs.

There are several chemical features to consider when designing an alkylation agent for aged AChE. The alkylation agent needs to be specific to the dealkylated phosphoryl O\(^-\) of aged AChE because alkylation of other biological targets, such as DNA and glutathione, can occur with undesired consequences. Since the rate of aging for OPs depends on the extent of branching of the alkyl chain, the alkylation agent should also be chosen such that it is sufficiently branched to prevent immediate re-aging of the inhibited enzyme before an effective oxime can be administered for reactivation.

In the late 1960s to 1970s, several groups attempted to realkylate aged AChE, but without success. The first study incorporated a series of compounds containing an alkylsulfonate group as well as a quaternary nitrogen. However, these compounds were never applied to aged AChE. A later study by Steinberg et al. explored using phenacyl bromides as the alkylation agents (Figure 2.1). These alkylation agents were tested against aged AChE, but no reactivation was observed. The authors pointed out that only a few of the studied compounds contained the “needed” positive charge for enzyme binding in the form of a pyridinium ring.
Steinberg postulated that one reason they did not observe reactivation of aged AChE was that the active site may undergo a conformational change during aging, thereby preventing alkylation by their model compounds, or that the alkylation does occur, but the enzyme active site is not a good leaving group.\textsuperscript{8} Since then, crystallographic structures have shown that very little structural rearrangement, if any, occurs following the aging process. Also, an inhibitor with the exact same structure as what Steinberg proposes is the alkylated product has been shown to undergo spontaneous reactivation following inhibition of AChE.\textsuperscript{9} This suggests that the phenacyl bromide compounds were either not alkylating at the phosphoryl O\textsuperscript{-}, or they were also alkylating at other positions within the active site gorge preventing any catalytic activity of AChE. These phenacyl bromides did show alkylation of model phosphonates in aqueous solutions,\textsuperscript{8} but obviously an improved alkylating agent is required in order to reactivate
aged AChE. Quinone methides (QMs) are alkylating agents that could possibly be utilized as they have previously been shown to have highly tunable reactivity. A p-QM has been shown to alkylate a phosphodiester at physiological temperature even though phosphates are generally poor nucleophiles (Scheme 2.2).\textsuperscript{10} The QM precursors are very close in structure to edrophonium, an AChE inhibitor known to bind in the active site cation binding pocket where the alkylating agent must reside for reactivity (Figure 2.2).\textsuperscript{11} Furthermore, QMs can be administered in a less reactive precursor form, minimizing undesired alkylation \textit{in vivo} if the QM precursor is only activated in the aged AChE active site. As the alkylation of phosphates under physiological conditions has been previously illustrated and the QM precursors are similar in structure to a compound known to bind in the AChE active site, it stands to reason that a QM or its precursor could be used to realkylate the anionic oxygen of aged AChE so that an oxime could then be used to regenerate the functional enzyme.

![Scheme 2.2. Turnbull and coworkers showed that a phosphonate can be alkylated by a quinone methide at physiological temperatures.\textsuperscript{10}](image-url)
The reactivity of QMs has been studied extensively, mainly due to their ability to reversibly alkylate DNA and block transcription. The substituents on the ring have been shown to greatly modulate the reactivity of QMs. Weinert et al. have shown that electron-donating groups, such as methoxy substituents, help stabilize the electron deficient QM ring and therefore greatly promote QM generation by photochemical methods. On the other hand, strong electron-withdrawing groups, such as ester substituents, destabilize the ring and therefore inhibit QM generation. However, these trends are reversed when looking at the alkylation by QMs; electron-donating groups inhibit the alkylation reaction because of the higher stability of the ring, while electron-withdrawing groups promote QM alkylation due to the destabilized ring. The steric effects of the substituents also play a role in the reactivity of QMs. The presence of two \textit{t}-butyl groups at the 2 and 2’ positions on a \textit{p}-QM has been shown to greatly increase the lifetime of the QM (Figure 2.4). Turnbull has postulated that this may be a result of the reactive QM species actually being protonated by a water molecule at the quinoidal oxygen, which is blocked by the \textit{t}-butyl groups.
Table 2.3. Effects of varying the 2 and 2' substituents on the p-QM half-life.

<table>
<thead>
<tr>
<th>R^1</th>
<th>R^2</th>
<th>Half-life (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H</td>
<td>OCH₃</td>
<td>1.3</td>
</tr>
<tr>
<td>CH₃</td>
<td>t-Bu</td>
<td>47</td>
</tr>
<tr>
<td>t-Bu</td>
<td>t-Bu</td>
<td>3060</td>
</tr>
</tbody>
</table>

Weinert _et al._ have also demonstrated that the leaving group on the QM precursor (which dissociates upon QM formation) can strongly affect its stability and reactivity in solution. Leaving groups such as acetate, hydroxyl, morpholine, and various amines were investigated, and the quaternary ammonium salt leaving groups were found to form more stable QM intermediates. Weinert postulated that this was due to the low nucleophilicity of the tertiary amines shifting the equilibrium from the precursor to the QM. These benzyl ammonium salts are also able to thermally generate QMs at temperatures as low as 38 °C with a slightly basic pH of 7.8 (Scheme 2.3), rendering them to be ideal candidates for alkylating agents at physiological conditions.
A small number of studies have also investigated the reactivity of QMs using computational methods.\textsuperscript{16,17,18} Di Valentin \textit{et al.} probed the mechanism of alkylation of QMs using a number of nucleophiles, including ammonia, water, and hydrogen sulfide.\textsuperscript{16} They extended the findings of the first study to examine QM alkylation of various pyrimidine bases.\textsuperscript{17} They discovered that the water-assisted mechanism was favored over the uncatalyzed reaction in the gas phase by 5.6 and 4.0 kcal/mol for the alkylation of ammonia and water respectively. However, in solvent, the uncatalyzed reaction becomes the favored pathway by 7.0 kcal/mol for ammonia and 4.0 kcal/mol for hydrogen sulfide, suggesting water does not play a direct role in the mechanism.\textsuperscript{16} Although these studies were quite thorough in the nature of the alkylation mechanism for QMs, no effort was spent investigating the formation of the QM intermediate, as chemical or photolytic
methods are used for the common *in vitro* applications of QMs. Deeper studies into substituent effects were also not performed.

This chapter will focus on understanding the reaction mechanism of alkylation by benzylammonium QM precursors that we have developed as possible alkylating agents (Table 2.1). Each of the QM precursors studied were synthesized by Dr. Chris Callam and Dr. Carolyn Reid. The effects of varying substituents on the benzene ring, as well as various leaving groups, will be explored. Two possible mechanisms of alkylation, from the QM intermediate (stepwise) or directly from the precursor (concerted), will be studied. These data will help in the identification of which chemical modifications will result in efficient realkylation of aged AChE.

### Table 2.1. The structures of the *p*-quinone methide (*p*-QM) precursors.

<table>
<thead>
<tr>
<th>QM Precursor</th>
<th>R&lt;sup&gt;1&lt;/sup&gt;</th>
<th>R&lt;sup&gt;2&lt;/sup&gt;</th>
<th>R&lt;sub&gt;A&lt;/sub&gt;</th>
<th>R&lt;sub&gt;B&lt;/sub&gt;</th>
<th>R&lt;sub&gt;C&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>QMP-1V1</td>
<td>OMe</td>
<td>H</td>
<td>Me</td>
<td>Me</td>
<td>Me</td>
</tr>
<tr>
<td>QMP-1V2</td>
<td>OMe</td>
<td>H</td>
<td>Me</td>
<td>Me</td>
<td>H</td>
</tr>
<tr>
<td>QMP-1V3</td>
<td>OMe</td>
<td>H</td>
<td>Me</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>QMP-1V4</td>
<td>OMe</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>QMP-1V5</td>
<td>OMe</td>
<td>H</td>
<td>Et</td>
<td>Et</td>
<td>Me</td>
</tr>
<tr>
<td>QMP-1V6</td>
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<td>H</td>
<td>Et</td>
<td>Et</td>
<td>H</td>
</tr>
<tr>
<td>QMP-1V7</td>
<td>OMe</td>
<td>H</td>
<td>Et</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>QMP-1V8</td>
<td>OMe</td>
<td>H</td>
<td><em>i</em>-Pr</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
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<td>H</td>
<td>H</td>
<td>Morpholine</td>
<td></td>
</tr>
<tr>
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<td>Me</td>
<td>Me</td>
</tr>
<tr>
<td>QMP-3</td>
<td>OH</td>
<td>H</td>
<td>Me</td>
<td>Me</td>
<td>Me</td>
</tr>
<tr>
<td>QMP-4</td>
<td>Me</td>
<td>H</td>
<td>Me</td>
<td>Me</td>
<td>Me</td>
</tr>
<tr>
<td>QMP-5</td>
<td>OMe</td>
<td>OMe</td>
<td>Me</td>
<td>Me</td>
<td>Me</td>
</tr>
<tr>
<td>QMP-6</td>
<td>Cl</td>
<td>Me</td>
<td>Me</td>
<td>Me</td>
<td>Me</td>
</tr>
<tr>
<td>QMP-7</td>
<td>Me</td>
<td>Me</td>
<td>Me</td>
<td>Me</td>
<td>Me</td>
</tr>
<tr>
<td>QMP-8</td>
<td>COOMe</td>
<td>H</td>
<td>Me</td>
<td>Me</td>
<td>Me</td>
</tr>
<tr>
<td>QMP-9</td>
<td><em>t</em>-Bu</td>
<td><em>t</em>-Bu</td>
<td>Me</td>
<td>Me</td>
<td>Me</td>
</tr>
</tbody>
</table>

*Refer to Figure 2.2 for a definition of the substituent nomenclature.*
2.2: COMPUTATIONAL METHODS

The geometries of all structures were optimized using a Becke three-parameter exchange functional with a Lee-Yang-Parr correlation functional (B3LYP).\textsuperscript{19,20} All atoms were treated with a 6-31G* basis set.\textsuperscript{21} This combination has been shown to perform well in calculating the gas-phase structures on the potential energy surface for QM alkylation.\textsuperscript{16} The role of the solvent was considered by using a Self Consistent Reaction Field (SCRF) method for implicit water with radii from the SMD model, which performs well in determining the contribution to free energy change of a reaction from bulk solvent.\textsuperscript{22} All calculations were performed using Gaussian09.\textsuperscript{23} Transition states were located by performing a relaxed potential energy surface scan by varying the C–N bond from a length of 2.0 Å to 4.5 Å, then this starting geometry was optimized to a transition state. A vibrational frequency analysis confirmed each stationary point to be a minimum (all real vibrational frequencies) or a transition state (one imaginary vibrational frequency) on the potential energy surface. Each transition state was connected to the corresponding minima by performing an intrinsic reaction coordinate (IRC)\textsuperscript{24} calculation, followed by an optimization to the bottom-of-the-well geometry. Single-point energy calculations were performed on the optimized B3LYP/6-31G* geometries using the 6-311+G** basis set.\textsuperscript{21} Thermal and entropic corrections to the free energy were taken from the B3LYP/6-31G* vibrational frequency analysis, and with unscaled vibrational frequencies. The zero-point vibrational energy for each stationary point was scaled by a factor of 0.9613.\textsuperscript{25} All energies presented herein are ΔG (310 K) at the B3LYP/6-
311+G**// B3LYP/6-31G* level of theory with thermal and entropic corrections to the free energy.

2.3: COMPUTATIONAL MODELS OF QUINONE METHIDE FORMATION

The formation of the quinone methide intermediate was investigated by altering the substituents on the ring as well as the leaving group (Table 2.1). As pH has been shown to affect QM generation, both the phenol and phenoxide precursors were investigated. However, the phenol precursors were found to follow an elimination pathway with an asymptotic energy barrier of 29.09 kcal/mol (QMP-1V1) at 37 °C. Meanwhile, the phenoxide precursor was found to have a much lower energy barrier of 10.74 kcal/mol, and the transition state could be calculated for the QM formation from the phenoxide precursor. As can be seen from the geometries in Figure 2.4, the shorter C–O bond in the phenoxide precursor (1.29 Å versus 1.37 Å for the phenol precursor) and a slightly longer C–N bond (1.56 Å versus 1.54 Å) indicate that the phenoxide reactant is already shifted slightly toward the QM intermediate. This correlates with the experimental studies of Modica et al. that the QM formation was possible at 38 °C under slightly basic conditions but required 80 °C under neutral conditions,15 suggesting that removal of the hydroxyl proton from the phenol precursor is required for QM formation. Given the large distance between the amine and hydroxyl proton in the p-QM precursor, removal of the proton could possibly be a solvent-mediated process.
Changing the amine substituents for the QM precursors had a significant impact on the energetics of QM formation (Table 2.2). The energy barrier was lower and QM formation was more favorable with an increase in the number of alkyl groups attached to the leaving group nitrogen. This correlates with how stable/nucleophilic the dissociated amine is in solution. The electronic density is much better spread out over three methyl groups than either two or one, and therefore NMe₃ is not as nucleophilic and more stable in solution than NHMe₂ or NH₂Me. This can be seen with the calculated energetics, as the barrier for QM formation was 10.74 kcal/mol for QMP-1V1 (NMe₃), 15.30 kcal/mol for QMP-1V2 (NHMe₂), and 15.36 kcal/mol with QMP-1V3 (NH₂Me).
Table 2.2. Reaction energetics for QM formation from the phenoxide precursor with various ammonium leaving groups.\(^{a}\)

<table>
<thead>
<tr>
<th>QM Precursor</th>
<th>(R_A)</th>
<th>(R_B)</th>
<th>(R_C)</th>
<th>(\Delta G^\ddagger)</th>
<th>(\Delta G_r)</th>
</tr>
</thead>
<tbody>
<tr>
<td>QMP-1V1</td>
<td>Me</td>
<td>Me</td>
<td>Me</td>
<td>10.74</td>
<td>4.15</td>
</tr>
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<td>QMP-1V2</td>
<td>Me</td>
<td>Me</td>
<td>H</td>
<td>15.30</td>
<td>8.55</td>
</tr>
<tr>
<td>QMP-1V3</td>
<td>Me</td>
<td>H</td>
<td>H</td>
<td>15.36</td>
<td>9.12</td>
</tr>
<tr>
<td>QMP-1V4</td>
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<td>H</td>
<td>H</td>
<td>12.89</td>
<td>4.85</td>
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<td>QMP-1V5</td>
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<td>Et</td>
<td>Me</td>
<td>10.04</td>
<td>3.44</td>
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<td>QMP-1V6</td>
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<td>Et</td>
<td>H</td>
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<td>5.90</td>
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<tr>
<td>QMP-1V7</td>
<td>Et</td>
<td>H</td>
<td>H</td>
<td>13.88</td>
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<td>H</td>
<td>H</td>
<td>14.86</td>
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<td></td>
<td>12.98</td>
<td>6.12</td>
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\(^{a}\) Reaction energetics determined at the B3LYP/6-311+G**//B3LYP/6-31G* level of theory with implicit (SMD) water solvation. All free energies are in kcal/mol relative to the \(\Delta G\) of the QM precursor.

Altering the substituents at the 2 and 2’ positions on the ring also greatly modulated the energetics of QM formation (Table 2.3). As can be seen in Table 2.3, electron-donating groups help favor QM formation while electron-withdrawing groups hinder QM formation. This trend is observed due to the stability of the QM intermediate.

Electron-donating groups help stabilize the electron deficient QM ring making QM formation much more favorable. The exact opposite occurs with electron-withdrawing groups, which destabilize the QM ring. Multiple electron-donating groups greatly enhance the favorability of QM formation, which can be seen in QMP-5 with two methoxy substituents, with a barrier of formation of 4.55 kcal/mol, while QMP-1V1 with one methoxy substituent had a 10.74 kcal/mol barrier. The same trend occurs when one or two methyl groups (QMP-4 and QMP-7) are present, with free energy of activation barriers of 11.71 kcal/mol and 8.47 kcal/mol respectively. Placing one electron-donating and one electron-withdrawing group on the same ring (QMP-6, with a chloro and methyl...
substituents and a 13.04 kcal/mol barrier) made QM formation more favorable than with just the electron-withdrawing group (QMP-2, with a chloro substituent and a 15.22 kcal/mol barrier), but less favorable than with just the electron-donating group (QMP-4, with a methyl substituent and a 11.71 kcal/mol barrier). As expected, the least favorable reaction occurred with a strong electron-withdrawing group in the form of an ester, while the QMP with two methoxy substituents (QMP-5) had the lowest barrier of formation. The second lowest barrier of QM formation, and the only reaction that was exoergic, occurred with QMP-9, which had two t-butyl groups on the ring. In addition to being electron-donating groups, the steric hindrance of the t-butyl groups could also help QMP-9 form a more stable quinone methide. Bolton et al. proposed that the QM formed from QMP-9 was stable with a long lifetime due to the steric hindrance of the alkyl groups preventing hydrogen bonding between the exo-cyclic oxygen and solvent and therefore suppressing charge separation, which could enhance the electrophilicity of other QMs (Figure 2.5).
Table 2.3. Reaction energetics for QM formation from the phenoxide precursor with various substituents at the 2 and 2’ positions.\(^a\)

<table>
<thead>
<tr>
<th>QM Precursor</th>
<th>R(^1)</th>
<th>R(^2)</th>
<th>(\Delta G^\ddagger)</th>
<th>(\Delta G_r)</th>
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<tbody>
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<td>QMP-1V1</td>
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<td>Me</td>
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<td>8.18</td>
</tr>
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<td>QMP-9</td>
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<td>t-Bu</td>
<td>6.69</td>
<td>–0.64</td>
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</tbody>
</table>

\(^a\) Reaction energetics determined at the B3LYP/6-311+G**//B3LYP/6-31G* level of theory with implicit (SMD) water solvation. All free energies are in kcal/mol relative to the \(\Delta G\) of the QM precursor.

Figure 2.5. The resonance structures of \(p\)-QMs. Bolton et al. proposed that hydrogen bonding between the exocyclic oxygen and the solvent helps stabilize the charge-separated resonance structure, which increases the positive charge density on the exocyclic carbon making it more susceptible to nucleophilic attack.\(^26\)

These results correlate with previous experimental studies of QM formation in which electron-donating groups help stabilize the QM intermediate while electron-withdrawing groups destabilize the QM intermediate.\(^{12,13,14}\) The steric hindrance of the \(t\)-butyl groups in QMP-9 also help stabilize the QM intermediate making QM formation more favorable. The leaving group was also found to play a role in QM formation based
largely on the stability and nucleophilicity of the departed amine in that the more stable/less nucleophilic the departed amine is, the more favorable the QM formation. Most importantly, these results suggest that the rate of QM formation from a QM precursor could be tuned in order to try to enhance selectivity for the active site of aged AChE.

2.4: COMPUTATIONAL MODELS OF QUINONE METHIDE ALKYLATION

After QM formation had been studied, the QM alkylation reaction was also investigated. The QM alkylation reaction was studied by using piperidine as the model nucleophile. Only the QM compounds with varying ring substituents were modeled, as the compounds with varying leaving groups studied in Section 2.3 form the same QM. Just as the substituents greatly influenced QM formation, the reactivity of QM alkylation was also incredibly affected by the various substituents (Table 2.4).
Table 2.4. Reaction energetics for QM alkylation using piperidine as the nucleophile and with various substituents at the 2 and 2’ positions of the QM.\(^a\)

<table>
<thead>
<tr>
<th>Quinone Methide</th>
<th>(\text{R}^1)</th>
<th>(\text{R}^2)</th>
<th>(\Delta G^\ddagger)</th>
<th>(\Delta G_r)</th>
</tr>
</thead>
<tbody>
<tr>
<td>QM-1</td>
<td>OMe</td>
<td>H</td>
<td>7.24</td>
<td>-7.75</td>
</tr>
<tr>
<td>QM-2</td>
<td>Cl</td>
<td>H</td>
<td>3.91</td>
<td>-13.46</td>
</tr>
<tr>
<td>QM-3</td>
<td>OH</td>
<td>H</td>
<td>6.55</td>
<td>-9.57</td>
</tr>
<tr>
<td>QM-4</td>
<td>Me</td>
<td>H</td>
<td>5.43</td>
<td>-8.78</td>
</tr>
<tr>
<td>QM-5</td>
<td>OMe</td>
<td>OMe</td>
<td>6.30</td>
<td>-7.58</td>
</tr>
<tr>
<td>QM-6</td>
<td>Cl</td>
<td>Me</td>
<td>5.66</td>
<td>-10.66</td>
</tr>
<tr>
<td>QM-7</td>
<td>Me</td>
<td>Me</td>
<td>6.00</td>
<td>-7.65</td>
</tr>
<tr>
<td>QM-8</td>
<td>COOMe</td>
<td>H</td>
<td>3.94</td>
<td>-14.66</td>
</tr>
<tr>
<td>QM-9</td>
<td>(t)-Bu</td>
<td>(t)-Bu</td>
<td>8.20</td>
<td>-3.33</td>
</tr>
</tbody>
</table>

\(^a\) Reaction energetics determined at the B3LYP/6-311+G**/B3LYP/6-31G* level of theory with implicit (SMD) water solvation. All free energies are in kcal/mol relative to the \(\Delta G\) of the QM precursor. See Figure 2.5 (left) for a representative structure of the substituted \(para\)-quinone methide.

The exact opposite trend for QM formation, where electron-donating groups had a lower energy barrier and more favorable product than QM precursors with electron-withdrawing groups, was observed with QM alkylation. However, both trends occur because of the same reason. Electron-donating groups help stabilize the QM intermediate, which helps promote QM formation from the precursor. While electron-donating groups promote QM formation, they also inhibit alkylation of the stabilized QM intermediate. Moreover, electron-withdrawing groups destabilize the QM intermediate, thereby inhibiting QM formation from the precursor, but also promoting QM alkylation. This can be observed for QM-1, which possesses a methoxy substituent and encounters an energy barrier of 7.24 kcal/mol for QM alkylation by piperidine, whereas QM-2 with a chloro-substituent encounters a barrier of 3.91 kcal/mol. The di-\(t\)-butyl QM (QM-9) encounters the highest barrier for alkylation at 8.20 kcal/mol, which again supports the hypothesis of Bolton et al. that the steric hindrance of the \(t\)-butyl groups inhibits hydrogen bonding.
with the solvent, destabilizing the charge separated resonance structure.\textsuperscript{26} The transition state structure of QM-9 shows that the exocyclic carbon was not as electrophilic as with other QMs as it required a shorter C–N bond (Figure 2.6). As can been seen in the transition state geometries, comparing QM-9 with both electron-donating (QM-4) and electron-withdrawing (QM-2) substituents, the bond lengths were essentially unaffected with the exception of the C–N bond in the transition states, which was 2.67 Å in QM-2, 2.49 Å in QM-4, and 2.41 Å in QM-9.
The calculated energies of QM alkylation of piperidine also correlated with previous studies that indicate that the electronic effect of the substituent greatly affects the stability of the quinone methide ring, altering the reactivity of the QM intermediate.\textsuperscript{12,13,14} A more electron-rich ring means that the QM intermediate is more stable, which makes QM formation more favorable, but alkylation of the QM by a nucleophile is also less favorable. Since the formation of QMs from a precursor and the
reactivity of the QMs can be greatly modulated by both substituents on the ring and the leaving group on the precursor, one can suggest that the QM precursor can be designed to target the AChE active site after aging has occurred.

2.5: COMPUTATIONAL MODELS OF CONCERTED VERSUS STEPWISE MECHANISM OF ALKYLATION OF QUINONE METHIDE PRECURSORS

Rather than forming a QM intermediate, alkylation of nucleophiles could occur via a concerted $S_{N2}$ mechanism, where the nucleophile attacks the benzylic carbon and displaces the amine leaving group (Scheme 2.4). The energetics of the concerted mechanism were studied and compared to the stepwise mechanism.

Scheme 2.4. The stepwise mechanism of alkylation (A) consisting of formation of the QM followed by alkylation versus the concerted mechanism of alkylation, and (B) consisting of direct displacement of the leaving group by the nucleophile.
The concerted mechanism for alkylation of the QM precursors with varying leaving groups was studied using both the phenol and phenoxide precursors (Table 2.5). Modifying the amine leaving groups resulted in a great deal of variation in the energetics of alkylation of the QM precursors. The energy barrier for alkylation of the phenol precursor ranged from 25.66 kcal/mol for QMP-1V1 (NMe$_3$) to 34.85 kcal/mol for QMP-1V8 (NH$_2$/Pr). The barrier for alkylation of the phenol precursors follows the relative nucleophilicity of the resulting amine, just as it did for QM formation. Alkylation of the phenoxide precursors does not seem to follow as close of a trend or be very consistent in alkylation barriers with the phenol precursors. However, each of the phenoxide precursors is destabilized when compared to the relative stability of the phenol precursor, and therefore has a higher overall energy barrier of about 4.5 to 6.5 kcal/mol (Table 2.6).

Table 2.5. Reaction energetics for the concerted mechanism of alkylation of the QM precursors using piperidine as the nucleophile and with varying leaving groups on the QM precursors.$^a$

<table>
<thead>
<tr>
<th>QM Precursor</th>
<th>R$_A$</th>
<th>R$_B$</th>
<th>R$_C$</th>
<th>Phenol $\Delta G^\circ$</th>
<th>Phenol $\Delta G_r$</th>
<th>Phenoxide $\Delta G^\circ$</th>
<th>Phenoxide $\Delta G_r$</th>
</tr>
</thead>
<tbody>
<tr>
<td>QMP-1V1</td>
<td>Me</td>
<td>Me</td>
<td>Me</td>
<td>25.66</td>
<td>-3.57</td>
<td>28.56</td>
<td>-2.76</td>
</tr>
<tr>
<td>QMP-1V2</td>
<td>Me</td>
<td>Me</td>
<td>H</td>
<td>30.28</td>
<td>1.56</td>
<td>26.75</td>
<td>1.90</td>
</tr>
<tr>
<td>QMP-1V3</td>
<td>Me</td>
<td>H</td>
<td>H</td>
<td>29.72</td>
<td>0.86</td>
<td></td>
<td></td>
</tr>
<tr>
<td>QMP-1V4</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>26.97</td>
<td>-4.07</td>
<td>24.86</td>
<td>-3.69</td>
</tr>
<tr>
<td>QMP-1V7</td>
<td>Et</td>
<td>H</td>
<td>H</td>
<td>28.94</td>
<td>-0.57</td>
<td>26.89</td>
<td>1.20</td>
</tr>
<tr>
<td>QMP-1V8</td>
<td>i-Pr</td>
<td>H</td>
<td>H</td>
<td>34.85</td>
<td>-0.65</td>
<td>26.82</td>
<td>-0.65</td>
</tr>
</tbody>
</table>

$^a$ Reaction energetics determined at the B3LYP/6-311+G**/B3LYP/6-31G* level of theory with implicit (SMD) water solvation. All free energies are in kcal/mol relative to the $\Delta G$ of the QM precursor. Refer to Figure 2.2 for a representative structure of the p-QM precursor.
Table 2.6. The energetics for piperidine alkylation of the QM precursors with various leaving groups for both the phenol and phenoxide precursors with each geometry compared to the respective phenol precursor.\(^{a}\)

<table>
<thead>
<tr>
<th>QM Precursor</th>
<th>Reactant</th>
<th>Transition State</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>QMP-1V1</td>
<td>Phenol</td>
<td>0.00</td>
<td>25.66</td>
</tr>
<tr>
<td></td>
<td>Phenoxide</td>
<td>4.68</td>
<td>33.24</td>
</tr>
<tr>
<td>QMP-1V2</td>
<td>Phenol</td>
<td>0.00</td>
<td>30.28</td>
</tr>
<tr>
<td></td>
<td>Phenoxide</td>
<td>6.47</td>
<td>33.22</td>
</tr>
<tr>
<td>QMP-1V4</td>
<td>Phenol</td>
<td>0.00</td>
<td>26.97</td>
</tr>
<tr>
<td></td>
<td>Phenoxide</td>
<td>4.47</td>
<td>29.33</td>
</tr>
<tr>
<td>QMP-1V7</td>
<td>Phenol</td>
<td>0.00</td>
<td>28.98</td>
</tr>
<tr>
<td></td>
<td>Phenoxide</td>
<td>4.59</td>
<td>31.47</td>
</tr>
<tr>
<td>QMP-1V8</td>
<td>Phenol</td>
<td>0.00</td>
<td>34.85</td>
</tr>
<tr>
<td></td>
<td>Phenoxide</td>
<td>5.20</td>
<td>32.03</td>
</tr>
</tbody>
</table>

\(^{a}\) The free energy of reaction determined at the B3LYP/6-311+G**//B3LYP/6-31G* level of theory with implicit (SMD) water solvation. The energies for each phenol compound were added to the energy for trimethylamine, while the phenoxide compound energies were added to the energy for trimethylammonium ion. Each of the energies was then set relative to the phenol QM precursor. All free energies are in kcal/mol. Refer to Figure 2.2 for a representative structure of the \(p\)-QM precursor.

The geometries along the potential energy surface of QM alkylation are fairly similar for both the phenol and phenoxide precursors (Figure 2.7). The C–N bonds with the alkylated amine in either reactant or product are consistent at roughly 1.54 Å for both precursors, while the unalkylated amines each have a C–N bond slightly longer than 4 Å. Each of the C–N bonds are around 2.2 Å in the respective transition states. However, the two precursors differ in the length of the C–O hydroxyl bond, which is 1.37 Å in alkylation of the phenol precursor, but a shorter 1.29 Å during the phenoxide alkylation, which helps contribute to the slightly destabilized QM precursor due to the anionic phenoxide oxygen pushing electron density into the ring.
Although modifying the leaving group greatly affected the energetics for the concerted mechanism of alkylation, adjusting the ring substituents did not cause nearly as much of a variation in energetics, except for QMP-9 (Table 2.7). The large energy barrier observed with the phenol precursor QMP-9 could be due to the steric issues between the \( t \)-butyl groups and the nucleophile. Other than the steric hindrance factor with QMP-9, the substituent effects do not make as much of a difference in the concerted mechanism as they do in the step-wise process. This is to be expected because the concerted mechanism does not form the QM intermediate, which has a much greater ability to
transfer electronic effects from the substituents to the benzylic carbon than the QM precursor.

Table 2.7. Reaction energetics for the concerted mechanism of alkylation of the QM precursors using piperidine as the nucleophile and with varying leaving groups on the QM precursors.\(^a\)

<table>
<thead>
<tr>
<th>QM Precursor</th>
<th>(R^1)</th>
<th>(R^2)</th>
<th>Phenol (\Delta G^\ddagger)</th>
<th>Phenol (\Delta G_r)</th>
<th>Phenoxide (\Delta G^\ddagger)</th>
<th>Phenoxide (\Delta G_r)</th>
</tr>
</thead>
<tbody>
<tr>
<td>QMP-1V1</td>
<td>OMe</td>
<td>H</td>
<td>25.66</td>
<td>–3.57</td>
<td>28.56</td>
<td>–2.76</td>
</tr>
<tr>
<td>QMP-2</td>
<td>Cl</td>
<td>H</td>
<td>26.14</td>
<td>–3.52</td>
<td>17.38</td>
<td>–2.73</td>
</tr>
<tr>
<td>QMP-3</td>
<td>OH</td>
<td>H</td>
<td>27.03</td>
<td>–2.57</td>
<td>23.31</td>
<td>–5.34</td>
</tr>
<tr>
<td>QMP-4</td>
<td>Me</td>
<td>H</td>
<td>24.75</td>
<td>–3.10</td>
<td>22.90</td>
<td>–2.91</td>
</tr>
<tr>
<td>QMP-5</td>
<td>OMe</td>
<td>OMe</td>
<td>25.35</td>
<td>–4.92</td>
<td>21.39</td>
<td>–3.27</td>
</tr>
<tr>
<td>QMP-6</td>
<td>Cl</td>
<td>Me</td>
<td>26.62</td>
<td>–2.95</td>
<td>23.00</td>
<td>–2.63</td>
</tr>
<tr>
<td>QMP-7</td>
<td>Me</td>
<td>Me</td>
<td>25.94</td>
<td>–2.52</td>
<td></td>
<td></td>
</tr>
<tr>
<td>QMP-9</td>
<td>t-Bu</td>
<td>t-Bu</td>
<td>40.71</td>
<td>4.31</td>
<td>21.49</td>
<td>–4.09</td>
</tr>
</tbody>
</table>

\(^a\) Reaction energetics determined at the B3LYP/6-311+G**//B3LYP/6-31G* level of theory with implicit (SMD) water solvation. All free energies are in kcal/mol relative to the \(\Delta G\) of the QM precursor. Refer to Figure 2.2 for a representative structure of the \(p\)-QM precursor.

When setting the energetics of the phenoxide compounds relative to the respective phenol precursor, the energy barriers for alkylation of the phenoxide precursors were found to be roughly the same as the barrier for alkylation of the phenol precursor, even though the phenoxide precursors were destabilized by about 4 kcal/mol over the phenol precursors for most of the compounds (Table 2.8). The products of the phenoxide alkylation reactions were also destabilized compared to the phenol reactions. These results were observed because the phenoxide precursors have more of a quinoidal geometry making the benzylic carbon more electrophilic, which stabilizes the transition state of the phenoxide reaction when compared to the phenol reaction. However, even
though a more electrophilic benzylic carbon stabilizes the transition state in which it is coordinated to two amines, it is more destabilized in both the reactant and product when it is bound to only one nitrogen.

Table 2.8. The energetics for piperidine alkylation of the QM precursors with varying substituents for both the phenol and phenoxide precursors with each geometry compared to the respective phenol precursor.

<table>
<thead>
<tr>
<th>QM Precursor</th>
<th>Reactant</th>
<th>Transition State</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>QMP-1V1</td>
<td>Phenol</td>
<td>0.00</td>
<td>25.66</td>
</tr>
<tr>
<td></td>
<td>Phenoxide</td>
<td>4.68</td>
<td>33.24</td>
</tr>
<tr>
<td>QMP-2</td>
<td>Phenol</td>
<td>0.00</td>
<td>26.14</td>
</tr>
<tr>
<td></td>
<td>Phenoxide</td>
<td>0.66</td>
<td>18.04</td>
</tr>
<tr>
<td>QMP-3</td>
<td>Phenol</td>
<td>0.00</td>
<td>27.03</td>
</tr>
<tr>
<td></td>
<td>Phenoxide</td>
<td>5.44</td>
<td>28.75</td>
</tr>
<tr>
<td>QMP-4</td>
<td>Phenol</td>
<td>0.00</td>
<td>24.75</td>
</tr>
<tr>
<td></td>
<td>Phenoxide</td>
<td>4.26</td>
<td>27.16</td>
</tr>
<tr>
<td>QMP-5</td>
<td>Phenol</td>
<td>0.00</td>
<td>25.35</td>
</tr>
<tr>
<td></td>
<td>Phenoxide</td>
<td>4.62</td>
<td>26.01</td>
</tr>
<tr>
<td>QMP-6</td>
<td>Phenol</td>
<td>0.00</td>
<td>26.62</td>
</tr>
<tr>
<td></td>
<td>Phenoxide</td>
<td>1.51</td>
<td>24.51</td>
</tr>
<tr>
<td>QMP-8</td>
<td>Phenol</td>
<td>0.00</td>
<td>24.86</td>
</tr>
<tr>
<td></td>
<td>Phenoxide</td>
<td>3.80</td>
<td>24.92</td>
</tr>
<tr>
<td>QMP-9</td>
<td>Phenol</td>
<td>0.00</td>
<td>40.71</td>
</tr>
<tr>
<td></td>
<td>Phenoxide</td>
<td>8.00</td>
<td>29.49</td>
</tr>
</tbody>
</table>

*a* The free energy of reaction determined at the B3LYP/6-311+G**//B3LYP/6-31G* level of theory with implicit (SMD) water solvation. The energies for each phenol compound were added to the energy for trimethylamine, while the phenoxide compound energies were added to the energy for trimethylammonium ion. Each of the energies was then set relative to the phenol QM precursor. All free energies are in kcal/mol. Refer to Figure 2.2 for a representative structure of the p-QM precursor.

Again, **QMP-9** with the two t-butyl groups does not follow the same trend as the other compounds, and as before, this is because of the steric hindrance of the substituents. However, in the phenoxide precursor, there is no hydroxyl H so the oxygen is free to
form a pseudo-double bond with the ring carbon, which in turn makes the exocyclic carbon more electrophilic and more susceptible to nucleophilic attack, which is seen in the reaction energetics.

The energetics for the concerted alkylation mechanism can also be compared to those for the stepwise mechanism (Figure 2.8). The results suggest that the likely mechanism follows the stepwise pathway with the QM intermediate as opposed to the direct displacement mechanism, especially under slightly basic conditions when the energy barrier is reduced for the stepwise pathway but either increased or about the same for the concerted mechanism. This correlates with a previous study by Modica et al. in which the alkylation results for both photochemical and thermal generation of the quinone methide were the same suggesting that the QM is the active intermediate in both reactions, and ruling out a direct displacement mechanism after thermal generation.¹⁵
Figure 2.8. The relative free energy surface ($\Delta G$, 310 K, kcal/mol, B3LYP/6-311+G**//B3LYP/6-31G* in implicit water) for both the stepwise and concerted mechanisms of alkylation by piperidine for QMP-1V1.

2.6: CONCLUSIONS

Alteration of the substituents and the leaving group of the QM precursors has shown to have a great effect on the reaction energetics. In the stepwise mechanism, electron-withdrawing substituents destabilize the electron-poor QM ring thereby making QM formation less favorable, but the same electron-withdrawing substituents make alkylation of the QM intermediate more favorable through destabilization of the ring. The exact opposite is true of electron-donating groups in that the QM intermediate is stabilized, so QM formation is favored, but alkylation of the QM after formation is
unfavorable. The leaving group at the benzylic carbon also contributes to the reaction energetics, mainly due to the relative nucleophilicity of the departed leaving group. The QM formation becomes more favorable as the nucleophilicity of the leaving group decreases.

For the concerted mechanism of alkylation, the ring substituents do not have nearly as large of an effect on the reaction energetics. As the quinone methide intermediate is never formed, the electronic effects of the ring’s substituents are not as readily transferred to the alkylation site on the benzylic carbon. However, the leaving group still plays a large role in alkylation for the direct displacement reaction. Again, the nucleophilicity of the departed group helps determine the reaction kinetics.

The stepwise mechanism, in which the QM intermediate is formed before alkylation occurs, appears to be the active pathway for alkylation, as the concerted mechanism has a larger energy barrier than the stepwise mechanism, especially under slightly basic conditions. However, strong electron-withdrawing groups might be able to shift the active pathway to the concerted mechanism, not because the substituent will affect the energetics for the direct displacement reaction, but because it will raise the energy barrier for QM formation, possibly enough to alter the active mechanism, such as in QMP-8 with almost a 7 kcal/mol higher energy barrier for QM formation than QMP-1V1. Either way, the energetics for alkylation of the QM precursors show that there is a great deal of tunability available, which is ideal for designing a therapeutic for realkylation of nerve agent inhibited and aged AChE that is specific to the active site after aging has occurred.
2.7: REFERENCES FOR CHAPTER 2


Yazyev, O.; Austin, A. J.; Cammi, R.; Pomelli, C.; Ochterski, J. W.; Martin, R. L.;
Dapprich, S.; Daniels, A. D.; Farkas, Ö.; Foresman, J. B.; Ortiz, J. V.; Cioslowski, J.;
Fox, D. J. Gaussian, Inc., Wallingford CT, 2009.
3.1: INTRODUCTION

Quinone methides (QMs) are alkylating agents that could possibly be utilized for realkylation of the aged form of AChE as QMs have previously been shown to alkylate phosphoesters at physiological temperatures. QMs have also been shown to have highly tunable reactivity, and when coupled with the fact that a QM could be administered in a less reactive precursor form, a quinone methide may be an ideal candidate for realkylation of aged AChE. We have previously investigated the reactivity of various QM precursors and their QM intermediates via computational methods. Both mechanisms of alkylation of piperidine, stepwise and concerted, were studied (Scheme 3.1).
Scheme 3.1. The stepwise mechanism of alkylation (A) consisting of formation of the QM followed by alkylation versus the concerted mechanism of alkylation, and (B) consisting of direct displacement of the leaving group by the nucleophile.

The calculations showed that alteration of the substituents and the leaving group of the QM precursors had a great effect on the reaction energetics. In the stepwise mechanism, electron-withdrawing substituents destabilized the electron-poor QM ring thereby making QM formation less favorable, but the same electron-withdrawing substituents made alkylation of the QM intermediate more favorable through destabilization of the ring. The exact opposite was true of electron-donating groups in that the QM intermediate was stabilized, so QM formation was favored, but alkylation of the QM after formation was unfavorable. The leaving group at the benzylic carbon also contributed to the reaction energetics, mainly due to the relative nucleophilicity of the
departed leaving group. The QM formation became more favorable as the nucleophilicity of the leaving group decreased. The leaving group nucleophilicity also played a large role in alkylation following the same trend for the direct displacement reaction. For the concerted mechanism of alkylation, the ring substituents did not have nearly as large of an effect on the reaction energetics. This was due to the fact that the quinone methide intermediate was never formed, so the electronic effects of the ring’s substituents were not as readily transferred to the alkylation site on the benzylic carbon. The calculations suggested that the stepwise mechanism, in which the QM intermediate is formed before alkylation occurs, is the active pathway for alkylation, as the concerted mechanism had a larger energy barrier than the stepwise mechanism, especially under slightly basic conditions. However, these results should be experimentally corroborated, perhaps by monitoring the direct alkylation of the QM precursors by experimental methods.

Quinone methides have been shown to form via photochemical and thermal generation.\textsuperscript{2,3,4,5,6} QMs have been generated by photochemical methods using a light source between 254 to 300 nm.\textsuperscript{2,3,4,5} Meanwhile, the temperature required for thermal generation can be as low as 38 °C under slightly basic conditions to 80 °C under neutral conditions.\textsuperscript{2,3,6} Wan discovered that various QMs showed absorption between 310 nm and 450 nm after photochemical generation at 266 nm.\textsuperscript{7} Modica \textit{et al.} have also monitored the formation of QMs by photochemical and thermal pathways, observing the QM intermediate at 400 nm.\textsuperscript{3}

In a recent study by Modica \textit{et al.},\textsuperscript{3} the alkylation conditions and rates for a series of quinone methides with several nucleophiles were investigated. After heating the QM
precursors to 80 °C or 38 °C, for neutral and basic conditions respectively, the QM intermediates were reacted with various nitrogen-, sulfur-, and oxygen-based nucleophiles including piperidine, morpholine, 2-mercaptoethanol, and various amino acids. The alkylation kinetics of thermally and photochemically generated QMs were also compared. Modica argued that similar product ratios between photochemical and thermal conditions support the presence of a QM intermediate, rather than direct displacement on the precursor.\(^3\)

The quinone methide intermediate has been illustrated to be highly reactive, and typically possesses a lifetime on the order of 10 seconds or less in aqueous solutions. However, the reactivity can be altered through variations of the substituents on the ring system. Zhou and Turnbull have shown that placing bulky functional groups, such as \(t\)-butyl, at the 2 and 2’ positions of \(para\)-quinone methide precursors increases the lifetime sufficiently so that the QM intermediate could be observed directly (Figure 3.1).\(^8\) They postulated that the increased lifetime of the \(di-t\)-butyl QM is caused by the steric bulk blocking solvent interactions with the exocyclic oxygen. By disrupting the hydrogen bonding that typically occurs with the exocyclic oxygen in solution, the partially negative charge of the oxygen in the zwitterionic QM resonance form is no longer stabilized by the solvent (Figure 3.2A).\(^9\) As a result, the exocyclic carbon is not as electrophilic making QM alkylation less favorable. Bolton \textit{et al.} confirmed that hydrogen bonding played a role in QM lifetime by also studying a compound with the same steric hindrance of the \(di-t\)-butyl compound but with the capability of intramolecular hydrogen bonding (Figure 3.2B). This QM had a half-life of 400 seconds, showing that the hydrogen
bonding stabilized the charge-separated resonance structure, although the alkylation rate was still depressed relative to the unsubstituted QM.⁹

![Chemical structure](image)

<table>
<thead>
<tr>
<th>R¹</th>
<th>R²</th>
<th>Half-life (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H</td>
<td>OCH₃</td>
<td>1.3</td>
</tr>
<tr>
<td>CH₃</td>
<td>t-Bu</td>
<td>47</td>
</tr>
<tr>
<td>t-Bu</td>
<td>t-Bu</td>
<td>3060</td>
</tr>
</tbody>
</table>

Figure 3.1. The effects of varying the 2 and 2’ substituents to increase the p-QM half-life.⁸

![Chemical structures](image)

Figure 3.2. The resonance structures of p-QMs (A). Bolton et al. proposed that hydrogen bonding between the exocyclic oxygen and the solvent helps stabilize the charge-separated resonance structure, which increases the positive charge density on the exocyclic carbon making it more susceptible to nucleophilic attack. To confirm this hypothesis, a QM that was capable of intramolecular hydrogen bonding (B) was studied and found to have a much shorter half-life.⁹

As quinone methides are short-lived reaction intermediates, direct observation of the QM intermediate can be quite difficult. This chapter will focus on developing a UV-
vis spectroscopic protocol for monitoring the QM intermediate and overall alkylation of the various QM precursors, which were introduced in Chapter 2. The concentrations of reactants and products will be monitored, as well as the presence of the QM intermediate. The alkylation of piperidine, \textit{para}-toluenethiol, and sodium hydroxide will be investigated at temperatures ranging from 35 to 100 °C for a series of QM precursors in order to experimentally correlate leaving groups and ring substituents with alkylation potency. These data will assist in the design of an efficient therapeutic to realkylate aged AChE for reactivation by a conventional oxime.

3.2: EXPERIMENTAL METHODS

Each of the QM precursors studied were synthesized by Dr. Chris Callam and Dr. Carolyn Reid. All reactions were conducted in a 1:1 mixture of methanol:water with 0.10 to 0.15 M solutions for each QM precursor. The precursors were heated for thirty minutes with each nucleophile, piperidine or \textit{p}-toluenethiol, in a 1:10 ratio with the nucleophile in excess, at varying temperatures. The UV-vis spectra were obtained with a Hewlett-Packard 8452 Diode Array Spectrophotometer with a 1 cm path length, and analyzed by the instrument’s software. For the reactions carried out under an inert atmosphere, \textit{N}_2 was bubbled through the solution for 15 minutes, then the solution was placed under a constant positive pressure of \textit{N}_2 throughout the course of the reaction. The basic solutions of pH from 10 to 11 were prepared from NaOH and distilled water.
3.3: OBSERVATION OF ALKYLATION BY UV-VIS SPECTRA

The initial studies included two different QM precursors (Precursor A and Precursor B) in an attempt to monitor the alkylation with p-toluenethiol or piperidine by UV-vis (Scheme 3.2). Each precursor was heated for thirty minutes with each nucleophile at 35, 41, 56, 63, and 78 °C. No change was observed in either precursor when reacted with p-toluenethiol. Precursor B also did not show any change when reacted with piperidine. A new peak at 350 nm was observed in the UV-vis spectrum for precursor A with piperidine at each temperature, but not with Precursor B (Figure 3.3). The peak at 350 nm does not correspond to an alkylated product as the piperidine alkylated product of Precursor A exhibits only a single peak at 280 nm (Figure 3.3G).

Scheme 3.2. The quinone methide precursors that were heated at various temperatures with p-toluenethiol or piperidine to observe alkylation by UV-vis spectroscopy.
Figure 3.3. The UV-vis spectra of Precursor A by itself in solution at room temperature (A), with $p$-toluenethiol at varying temperatures (B), with piperidine at varying temperatures (C), the purified product from the piperidine alkylation of Precursor A (D), and Precursor B by itself in solution at room temperature (E), with $p$-toluenethiol at varying temperatures (F), with piperidine at varying temperatures (G).
Figure 3.3 continued

**Precursor A with Piperidine**

**Piperidine Alkylated Product of Precursor A**

**Absorbance**

**Wavelength (nm)**

- 250
- 300
- 350
- 400
- 450
- 500

**Precursor A**

**OH**

**O**

**N**

**CF₃**

**OH**

**O**

**N**
Figure 3.3 continued

**Precursor B**

![Graph of Precursor B absorbance vs. wavelength (nm)]

**Precursor B with p-toluenethiol**

![Graph of Precursor B with p-toluenethiol absorbance vs. wavelength (nm)]
As our experiments confirmed that the observed peak at 350 nm did not correspond to alkylated product, additional experiments were carried out to discern its origin. Precursor A was heated for thirty minutes at 43, 61, and 77 °C by itself in solution, with piperidine, and with NaOH. When Precursor A was heated by itself in solution, the 350 nm peak was only present when heated to 77 °C (Figure 3.4). However, the 350 nm peak was observed in the solutions containing piperidine at every temperature, as well as under basic conditions.
Figure 3.4. The UV-vis spectra of Precursor A by itself in solution (A), with piperidine (B), and with NaOH (C).
The time required for the formation of the 350 nm peak was analyzed. Precursor A was reacted with piperidine and NaOH at 40 °C. A solution of Precursor A was also heated to 80 °C because the 350 nm peak was not previously observed at lower temperatures under neutral conditions in the absence of a nucleophile. With both the piperidine and NaOH samples, the 350 nm peak was present after heating for 1 minute and remained over the course of the reaction (Figure 3.5). The 350 nm peak remained overnight, suggesting the peak is the result of a stable compound, not a transient intermediate. This rules out the possibility of the peak corresponding to the quinone methide intermediate as quinone methides have been shown to be a short-lived species under these conditions. The intensity of the 350 nm peak decreased slightly over time in
the samples containing piperidine or NaOH. However, in solutions containing only Precursor A, the intensity of the peak was low after 1 minute and increased slowly with heating. With the introduction of base to solutions containing Precursor A at room temperature, the 350 nm peak appeared instantly (Figure 3.6).

Figure 3.5. The time-resolved UV-vis spectra of (A) Precursor A heated at 80 °C by itself, (B) with piperidine at 40 °C, and (C) with NaOH at 40 °C. The intensity of the 350 nm peak was studied during the course of the reaction in each solution (D).
Figure 3.5 continued

Precursor A with Piperidine at 40 °C

Precursor A with NaOH at 40 °C
Figure 3.5 continued

![Intensity of 350 nm Peak](image)

**Intensity of 350 nm Peak**

- **Precursor A**
- **Prec. A with Piperidine**
- **Prec. A with NaOH**

![Precursor A at Room Temperature](image)

**Precursor A at Room Temperature**

- **Precursor A**
- **Prec. A with NaOH**

Figure 3.6. The UV-vis spectra of Precursor A by itself in solution and immediately after addition of NaOH at room temperature.
Numerous other QM precursors (Scheme 3.3) were tested under similar conditions at room temperature to determine which QM precursors exhibit an absorption at 350 nm. Out of the eleven compounds tested, only three compounds resulted in a peak at 350 nm upon addition of NaOH. There does not seem to be a trend between the compounds that did and did not display the 350 nm peak. Two of the compounds were cationic salts while the third was a neutral compound. Precursor A showed the 350 nm peak, but when the methyl group on the amine was changed to a proton, as in Precursor K, the 350 nm peak was absent. The opposite trend was seen with Precursors H and I, as the methylated compound did not display the 350 nm peak while the protonated analog did have the peak. Precursor D also showed the peak, but when the ethyl groups were changed to methyl groups, as in Precursor C, the peak again disappeared (Figure 3.7). The only consistent trend appears to be that each compound has one methoxy substituent on the ring, although not all methoxy compounds exhibited absorbance at 350 nm.
Scheme 3.3. Each of the QM precursors studied. The highlighted compounds showed absorbance at 350 nm upon addition of base.

Figure 3.7. The UV-vis spectra of the entire series of QM Precursors with NaOH at room temperature. Precursors A, D, and H are the only compounds that showed UV-vis absorbance at 350 nm.
Since the 350 nm peak did not correspond to the alkylated product or the quinone methide intermediate, and the peak was only present in the mono-methoxy substituted compounds, one possible explanation could be the formation of the enolate of vanillin under basic conditions. Vanillin was the starting material in the synthetic route for each of the mono-methoxy quinone methide precursors (Scheme 3.4). Vanillin has been shown to be completely deprotonated to form the enolate under basic conditions (pH >10). This enolate has been shown to give a strong absorption at 350 nm (Figure 3.7) and possesses a high quantum yield, allowing miniscule amounts of a vanillin impurity that are undetectable via NMR or MS methods, to appear in the UV-vis spectra.\textsuperscript{10,11}

Scheme 3.4. The synthetic route for vanillylamine (A) and formation of the vanillin enolate under basic conditions (B). Vanillylamine is the same compound as many of the QM precursors studied only with a different amine leaving group. Each of the mono-methoxy substituted quinone methide precursors followed this general synthetic scheme.
Precursor A and vanillin were each studied under similar conditions. The vanillin enolate was found to give a very strong absorption at 350 nm, identical to the peak observed with Precursor A. This peak had a long lifetime under basic conditions for both compounds. Under neutral or acidic conditions, there was no 350 nm peak in either vanillin or Precursor A (Figure 3.8).
Further exploration of the 350 nm peak was done to determine if it was due to the presence of vanillin and the strong absorptivity of the enolate under basic conditions, and thus purely dependent on the pH of the solution, or if it was due to oxidation of vanillin present in solution or oxidation of the QM precursor. Under basic conditions in the presence of O₂, vanillin has been shown to undergo auto-oxidation to various degradation products (Scheme 3.5). The solutions were studied under an inert atmosphere and purged with N₂ to test whether or not oxidation was causing the 350 nm peak. After NaOH was added, the solution was left open to the air for three hours and another UV-vis
spectrum was taken, but no change was observed (Figure 3.9). This suggests that the 350 nm peak was not due to auto-oxidation.

![Scheme 3.5. The proposed mechanism of auto-oxidation of vanillin.](image)

Figure 3.9. The UV-vis spectra of vanillin and Precursor A purged with N₂ and under a N₂ atmosphere both before and after addition of NaOH, as well as each solution open to the air for 3 hours after adding NaOH.
According to Englis *et al.*, if the 350 nm absorbance is not due to oxidation, but instead due to the vanillin enolate under basic conditions, then it should be entirely dependent upon the pH of the solution.\textsuperscript{11} Hydrochloric acid was added to the vanillin and Precursor A solutions that were previously placed under basic conditions. Once these solutions were returned to neutral conditions, their spectra were recorded (Figure 3.10). After adding HCl to return the pH to neutral, the 350 nm peaks disappeared and the peaks before addition of base returned, suggesting that the 350 nm peak is due to the presence of vanillin in the QM precursor solutions and purely due to the basicity of the solution. Thus, the 350 nm absorbance was not suggestive of any significant chemical transformation, but the result of an acid-base equilibrium of the starting material vanillin.
The UV-vis spectra of both vanillin and Precursor A at neutral conditions (before adding NaOH), and pH 11 (after adding NaOH), and returned to neutral pH by adding acid (after adding HCl). The lower intensities of the peaks after returning to a neutral pH are purely due to dilution of the solutions when adding HCl.

The molar absorptivity of the 350 nm peak of the vanillin enolate was calculated in order to determine the amount of vanillin in the precursor solution. The molar absorptivity of the 350 nm peak was determined to be $2.44 \times 10^{-4} \text{ M}^{-1}\text{cm}^{-1}$. From this value, the concentration of vanillin in the Precursor A solution was determined to be $1.13 \times 10^{-5} \text{ M}$. The concentration of Precursor A was $6.23 \times 10^{-4} \text{ M}$, therefore the percentage of vanillin in the solution was found to be 1.8%. Thus, such a small impurity could not be detected by other methods.
Since neither the alkylation product nor the QM intermediate could be observed by UV-vis with the selected compounds, a QM precursor known to have a longer QM lifetime was chosen for investigation (Scheme 3.6). Bolton et al. discovered that placing \( t \)-butyl substituents at the 2 and 2’ positions on the QM greatly increased the lifetime of the QM intermediate in solution.\(^9\) Therefore, the QM from Precursor T should be able to be observed in solution.

\[ \text{Precursor T} \]

Scheme 3.6. The investigated di-\( t \)-tert-butyl QM precursor that should form the same QM intermediate that has been shown to have a long lifetime in solution.\(^9\)

Precursor T was heated at 80 °C for 30 minutes with NaOH in a 1:1 mixture of acetonitrile:water. Multiple peaks appeared at 258 nm, 364 nm, 424 nm, and a shoulder at 296 nm after heating (Figure 3.11). A UV-vis spectrum of the solution was collected after the solution was left overnight at room temperature, and the peak at 424 nm disappeared (Figure 3.11B). 3,5-Di-\( t \)-butyl-4-hydroxybenzaldehyde, the starting material in the synthetic scheme for Precursor T, was also analyzed under basic conditions, and the 364
nm peak with Precursor T was determined to be due to the enolate absorption (Figure 3.11C).

Figure 3.11. The UV-vis spectra of Precursor T by itself in solution at room temperature (A), with NaOH after heating at 80 °C for 30 minutes and then left at room temperature overnight (B), and 3,5-di-t-butyl-4-hydroxybenzaldehyde, the starting material in the synthetic scheme, under neutral and basic (pH 10) conditions (C).
Figure 3.11 continued

**Precursor T with NaOH**

![Graph showing absorbance vs. wavelength for precursor T with NaOH before and after heating.]

**3,5-di-t-butyl-4-hydroxybenzaldehyde at Varying pH**

![Graph showing absorbance vs. wavelength for 3,5-di-t-butyl-4-hydroxybenzaldehyde at neutral and basic pH levels.]

---

B

C
The decay of the 424 nm peak was analyzed because it could be the absorbance of the QM intermediate as it occurs in the range of previously observed p-QMs\(^7\) (Figure 3.12). The 424 nm peak was found to have a half-life of 37 ± 6 minutes, which corresponds to previous results of a half-life of 51 minutes under neutral conditions by Bolton et al.\(^9\) This suggests that the 424 nm peak corresponds to the QM intermediate, and is therefore a direct observation of the proposed intermediate for the alkylation reaction generated thermally.

![Precursor T with NaOH](image)

continued

Figure 3.12. The time-resolved UV-vis spectra of Precursor T and NaOH after heating at 80 °C for 30 minutes then leaving the solution at room temperature (A), and the decay of the 424 nm peak believed to correspond to the QM intermediate with a half-life of 37 minutes (B).
3.4: CONCLUSIONS

Observation of the alkylation reaction of various QM precursors was attempted by UV-vis spectroscopy. However, the alkylated product of each precursor was found to absorb at the same wavelength as the precursor, as the chromophore is a single aromatic ring with identical substitution for both products and reactants, and addition of NaOH, piperidine, or p-toluenethiol to the benzylic carbon do not exhibit any appreciable spectroscopic shift in the UV-vis spectra. With Precursor A, a spurious peak was observed at 350 nm with both piperidine and NaOH nucleophiles, but was ultimately
assigned to impurities of vanillin in its enolate form under alkaline conditions. It was determined that a small amount of vanillin (1.8% of the solution) was present in the solutions, which accounted for the observed 350 nm peaks, but would be unobservable using NMR or MS characterization methods. However, the quinone methide intermediate could not be observed for any of the precursors studied.

In order to try to observe the QM intermediate, our focus shifted to a precursor that is known to have a QM with a long lifetime. Precursor T, with two t-butyl substituents, was therefore also studied under basic conditions. After heating at 80 °C for 30 minutes, two new peaks at 364 and 424 nm were observed. The 364 nm peak was persistent and determined to correspond to the enolate of 3,5-di-t-butyl-4-hydroxybenzaldehyde, which was the starting material in the synthetic scheme. However, the 424 nm peak decayed over time and is believed to correspond to the QM intermediate with a half-life of 37 ± 6 minutes. The lifetime of this peak with various nucleophiles will be investigated further in Chapter 4.

3.5: REFERENCES FOR CHAPTER 3


Chapter 4: Monitoring Quinone Methide Alkylation of the Di-tert-butyl Precursor

4.1: INTRODUCTION

In the previous chapters of this thesis, quinone methides (QMs) have been shown to be effective alkylating agents.\textsuperscript{1,2} They have been observed to alkylate a wide range of nucleophiles, such as DNA, amino acids, and phosphates.\textsuperscript{3,4,5,6} QMs have also been shown to have highly tunable reactivity, which, coupled with the fact that QMs can be administered in a less reactive precursor form, makes a quinone methide an ideal candidate for a therapeutic to realkylate the aged form of AChE. Modica et al. have investigated the rate of QM formation from a number of \textit{o}-QM precursors and the subsequent alkylation of the QM intermediates. They discovered that QMs could be generated thermally at 38 °C under slightly basic conditions, but required 80 °C under neutral conditions. However, once the QM intermediates were formed, most nitrogen nucleophiles were observed to undergo appreciable alkylation in as little as one hour.\textsuperscript{4}

Turnbull has investigated \textit{p}-QMs for alkylation of various phosphates (Scheme 4.1).\textsuperscript{5,6} Even though these studies were carried out between 20 and 35 °C, each of the QMs studied were formed through oxidation of the QM precursors. This suggests that quinone methides can alkylate phosphoryl compounds under physiological conditions, and that generation of an active QM from a precursor will control alkylation rates. As
generation of the quinone methide intermediate at physiological temperature and pH is a slow process, administering an inactive precursor would potentially allow binding in the aged AChE active site prior to QM generation, decreasing the likelihood of unwanted alkylation of other biological species \textit{in vivo}.

Scheme 4.1. The alkylation of various phosphodiesters by $p$-QMs as investigated by Turnbull (A). These alkylation reactions were shown to proceed at 25 °C after the quinone methide was generated by oxidation from the precursor (B).

In order to select the most promising candidates for alkylation of aged AChE, the reaction rates of QM generation and alkylation of model nucleophiles must be quantified. In Chapter 3 of this thesis, we demonstrated that UV-vis spectroscopy was unable to track the concentration of reactant and product, as both species possess the same chromophore, and therefore the two peaks overlapped in the spectra. The short-lived QM intermediate was also not observed by UV-vis techniques, as the aqueous lifetime of the
reactive intermediate is generally less than 10 seconds in the absence of a nucleophile. The exception to this is with QM Precursor T (Scheme 4.2), which Bolton et al. have previously demonstrated to posses a lifetime of one hour in solution. They postulate that the persistence of the QM in the di-t-butyl compound is due to the steric bulk of the substituents disrupting hydrogen bonding between the solvent and the exocyclic oxygen in the zwitterionic resonance form of the QM. In our UV-vis studies utilizing Precursor T (Scheme 4.2), we were able to observe a peak at 424 nm under basic conditions that decayed over time. We found that this peak had a half-life of 37 ± 6 minutes, which correlates with the previous results of Bolton et al. of a 51-minute half-life under neutral conditions in the absence of a nucleophile.

Scheme 4.2. The formation of the quinone methide from Precursor T. Bolton et al. have shown that this QM has a long lifetime in solution due to the steric bulk of the t-butyl groups.

In this chapter, we will investigate the lifetime of the 424 nm peak for Precursor T with various nucleophiles in solution. In addition to UV-vis spectroscopic methods of detection, the alkylation of Precursor T will also be monitored by GC-MS, which will
allow a more accurate quantification of the extent of alkylation. The temperature range required for alkylation with various nucleophiles will be explored.

4.2: EXPERIMENTAL METHODS

Each of the QM precursors studied were synthesized by Dr. Chris Callam and Dr. Carolyn Reid. All reactions were conducted in pure acetonitrile or 1:1 mixtures of either methanol:water or acetonitrile:water. All GC-MS spectra were obtained from an Agilent 5975 Gas Chromatograph Mass Spectrometer, and analyzed with the instrument’s software. The GC system was operated at the following temperature settings $T$ (initial): 50 °C, $T$ (ramping): 20 °C/min, and $T$ (final): 250 °C for 6 min with a total run time of 16 min. Chromatographic separation was done with an Agilent J&W Capillary GC Column (30 m x 0.250 mm x 0.25 µm) at a column flow rate of 1.0 mL/sec (99.999% pure hydrogen as the carrier gas). The UV-vis spectra were obtained from an Agilent 8453 UV-visible Spectrophotometer with a 1 cm path length, and analyzed by the instrument’s software.

For the reactions under an inert atmosphere, the solution was purged with N$_2$ for 15 minutes, and then placed under a constant positive pressure of N$_2$ throughout the course of the reaction. Each of the reactions analyzed by GC-MS methods were run at approximately $4 \times 10^{-3}$ M for each reactant ($8 \times 10^{-3}$ M in morpholine for the 2:1 reaction of morpholine and Precursor T). The calibration curves were obtained by serial dilutions of a starting concentration of approximately $1.5 \times 10^{-2}$ M and final concentration of
approximately $9 \times 10^{-4}$ M for either Precursor T or the morpholine alkylated product and a constant concentration of approximately $6.5 \times 10^{-3}$ M for the internal standard biphenyl. The reactions analyzed by UV-vis methods were performed with concentrations of approximately $4 \times 10^{-3}$ M in Precursor T and $4 \times 10^{-1}$ M in morpholine.

4.3: EXPLORATION OF ALKYLATION CONDITIONS BY GC-MS

Precursor T was utilized for alkylation studies due to the relatively long lifetime of the corresponding quinone methide. This feature allows the QM intermediate to be observed in solution after thermal generation using steady-state spectrophotometric methods. We previously observed the QM in solution by UV-vis techniques via absorbance at 424 nm under basic conditions. However, since both the precursor and the alkylated product always possess the same chromophore and therefore cannot be differentiated by UV-vis, the alkylation conditions must first be investigated by other methods. Numerous nitrogen-, sulfur-, and oxygen-based nucleophiles, as well as a phosphate, were investigated for alkylation of Precursor T at varying temperatures (Figure 4.1).
Each nucleophile was tested with Precursor T in a 100:1 ratio for one hour at either 40, 60, or 80 °C. Piperidine, morpholine, imidazole, and p-toluenethiol showed alkylation at each temperature. Although p-toluenethiol showed alkylation at each temperature, the peak for the alkylated product was very small in each case. The thiol dimerized in a much greater quantity than alkylation with Precursor T. The presence of the alkylated product with benzyl alcohol or pyridine was not observed at any temperature. Meanwhile, the presence of the alkylated product could not be determined by GC-MS when using dibenzyl phosphate as the nucleophile because the phosphorylated product fragmented in the injector. However, the alkylated product with dibenzyl phosphate was observed by Dr. Carolyn Reid by NMR.
In order to quantify the degree of alkylation at the different temperatures, a calibration curve was obtained for both Precursor T and the morpholine alkylated product using biphenyl as the internal standard (Figure 4.2). By generating a calibration curve for each compound, the concentration of both reactant and product could be monitored.

Figure 4.2. The reaction scheme for the alkylation of morpholine with Precursor T (A). The GC-MS chromatogram for the alkylation of morpholine with Precursor T using biphenyl as an internal standard (B). Peak numbers 1–3 correspond to biphenyl, Precursor T, and the morpholine alkylated product, respectively. The calibration curves for Precursor T (C) and the alkylated product with morpholine (D) using biphenyl as the internal standard. (A_{area} corresponds to the area under the peak for the compound studied, while S_{area} and S_{conc} refer to the area under the peak and the concentration of biphenyl respectively.)
Figure 4.2 continued

**Figure B**

Retention Time (min)

**Figure C**

Precursor T Calibration Curve

$y = 1.7768x$

$R^2 = 0.99542$
Precursor T was reacted with morpholine at 80 °C in both 1:1 and 2:1 ratios of morpholine to Precursor T. After reacting for 1 hour in acetonitrile, the equimolar reaction had proceeded to roughly 58% alkylation, while the 2:1 reaction had progressed to 74% alkylation (Table 4.1). The reaction between Precursor T and morpholine at 80 °C in a 1:1 ratio was also monitored every 30 minutes for 4 hours (Figure 4.3). The reaction was found to have gone to equilibrium with no further alkylation after 30 minutes under these conditions. Tracking the reaction on a shorter timescale is difficult due to the retention time of 10.5 minutes for the product under the current GC conditions.
Table 4.1. The concentrations (M) of the reactants and products after heating at 80 °C for 1 hour in acetonitrile at 1:1 and 2:1 ratios of morpholine to Precursor T.

<table>
<thead>
<tr>
<th></th>
<th>1:1 Ratio Reaction</th>
<th>2:1 Ratio Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Reactants</td>
<td>Products</td>
</tr>
<tr>
<td>Precursor T</td>
<td>$4.18 \times 10^{-3}$</td>
<td>$1.47 \times 10^{-3}$</td>
</tr>
<tr>
<td>Morpholine</td>
<td>$4.01 \times 10^{-3}$</td>
<td>$8.01 \times 10^{-3}$</td>
</tr>
<tr>
<td>Alkylated Product</td>
<td>N/A</td>
<td>$2.34 \times 10^{-3}$</td>
</tr>
</tbody>
</table>

Figure 4.3. The concentrations of Precursor T and the alkylated product with morpholine during the course of the reaction.
4.4: MONITORING QM INTERMEDIATE BY UV-VIS SPECTROSCOPY

We previously observed a UV-vis peak at 424 nm after heating Precursor T with NaOH at 80 °C. This peak had a half-life of 37 ± 6 minutes, correlating with results by Bolton et al. for the QM intermediate formed from Precursor T. The 424 nm UV-vis peak also had the same lifetime as a peak with a mass of 218 on the GC-MS, which is the same mass as the QM intermediate (Scheme 4.2), supporting our hypothesis that the 424 nm peak corresponds to the UV-vis absorption of the quinone methide intermediate. We have determined that Precursor T can alkylate piperidine, morpholine, imidazole, and p-toluenethiol. As a test study, morpholine was monitored with Precursor T by UV-vis to complement the GC-MS results of the morpholine alkylation of Precursor T. Specifically, Precursor T was heated at 80 °C for 30 minutes in 1:1 acetonitrile:water to thermally generate the quinone methide intermediate, then morpholine, being 100 times more concentrated than Precursor T, was added after heating, and then the QM peak was monitored at room temperature (Figure 4.4). The decay of the absorbance at 424 nm was found to have a half-life of 4.30 ± 0.57 minutes, correlating with the extreme reactivity of the QM intermediates (Figure 4.4B).

The previous results with NaOH gave a half-life of just over 30 minutes for the QM intermediate. However, the GC-MS results show that the alkylation of morpholine has reached equilibrium by the first data point at 30 minutes when there is a 1:1 ratio between morpholine and Precursor T. When the ratio is increased to 2:1 with morpholine in excess, the percent alkylation also increases. So having morpholine in excess by 100:1, the reaction rate and nucleophilic trapping of the QM intermediate should increase.
dramatically, which in turn decreases the QM intermediate lifetime dramatically. Another contribution to the significantly longer QM lifetime with NaOH than with morpholine could be that basic conditions have been shown to facilitate QM formation, both by our calculations as well as experimentally by other groups. Therefore, when Precursor T is heated with NaOH, the rate of QM intermediate generation would be significantly higher, while the equilibrium back to the precursor is limited by the concentration of the dimethylamine leaving group.

Figure 4.4. The UV-vis spectra of Precursor T, after heating at 80 °C for 30 minutes, with morpholine (A), and the decay of the absorbance at 422 nm, which is believed to correspond to the QM intermediate (B).
4.5: CONCLUSIONS

After observation of the QM intermediate for Precursor T by UV-vis spectroscopy, we expanded our methods to monitor the alkylation reaction by GC-MS methods. Precursor T was reacted with piperidine, morpholine, imidazole, pyridine, \( p \)-toluenethiol, benzyl alcohol, and dibenzyl phosphate. Alkylated product was observed by GC-MS with piperidine, morpholine, imidazole, and \( p \)-toluenethiol. The alkylated product could not be observed with using either benzyl alcohol or pyridine as the
nucleophile, although both the nucleophile as well as Precursor T could be observed by GC-MS. Presence of the alkylated product when using dibenzyl phosphate as the nucleophile could not be determined due to degradation of the phosphate in the injector.

Once alkylation of Precursor T by numerous nucleophiles was confirmed, a calibration curve was obtained for Precursor T and the morpholine alkylated product using GC-MS with biphenyl as an internal standard. This technique allowed for concentrations of both Precursor T and the alkylated product to be quantified throughout the reaction. Thus, reacting Precursor T with morpholine in acetonitrile at 80 °C for 1 hour yielded 58% and 74% alkylation for the reactions in a 1:1 and 2:1 ratio of morpholine to Precursor T respectively. The reaction reached equilibrium prior to 30 minutes with a 1:1 ratio of morpholine to Precursor T.

The alkylation of morpholine with Precursor T was also monitored by UV-vis techniques. As the QM intermediate of Precursor T was previously shown to absorb at 424 nm, the decay of this peak throughout the alkylation reaction was monitored. The QM intermediate was thermally generated, and then upon addition of morpholine (in a 100:1 ratio with Precursor T), an exponential decay of the 424 nm peak was observed. This decay possessed a half-life of $4.30 \pm 0.57$ minutes, which is much faster than the half-life of $37 \pm 6$ minutes with NaOH. The GC-MS results show that the reaction between morpholine and Precursor T is completed within 30 minutes with a 1:1 ratio between morpholine and Precursor T. By having morpholine in excess at 100:1, the trapping of the quinone methide intermediate should increase dramatically. The QM may also have a significantly longer lifetime with NaOH than with morpholine due to the
basic conditions, which have been shown to facilitate QM formation. Thus, when Precursor T is heated with NaOH, the QM intermediate could be in equilibrium with both the QM precursor and the alkylated product, and the QM could be regenerated from the product in solution. Regeneration of the reactant in this case would also be dependent upon a reaction between the QM intermediate and the leaving group amine. Since, the concentration of the amine is very small in solution, it is not as likely to react with the QM, thus contributing to the longer lifetime of the intermediate observed under basic conditions.

4.6: REFERENCES FOR CHAPTER 4

5.1: THESIS CONCLUSIONS

We have computationally investigated two mechanistic pathways, stepwise and concerted, for alkylation of model nucleophiles by quinone methide precursors that we have selected as possible therapeutics for realkylation of aged AChE. Alteration of the substituents and the leaving group of the QM precursors was shown to greatly modulate the reaction energetics in previous experimental studies, and is supported in a general sense by our computational work as well as by UV-vis and GC-MS studies. Electron-withdrawing substituents have been demonstrated to destabilize the electron-poor QM ring in the stepwise mechanism, thereby making QM formation less favorable. However, the same electron-withdrawing substituents make alkylation of the QM intermediate more favorable due to destabilization of the ring. The exact opposite is true of electron-donating groups, which stabilize the QM intermediate, favoring QM formation, but disfavoring the subsequent alkylation reaction. The leaving group at the benzylic carbon also contributes to the reaction energetics, mainly due to the relative nucleophilicity of the departed leaving group. The QM formation becomes more favorable as the nucleophilicity of the leaving group decreases.

Meanwhile, the ring substituents do not have nearly as large of an effect on the reaction energetics for the concerted mechanism of alkylation. As the quinone methide
intermediate is never formed, the electronic effects of the ring’s substituents are not as readily transferred to the alkylation site on the benzylic carbon. However, the leaving group still plays a large role in alkylation for the direct displacement reaction. Again, the nucleophilicity of the departed group helps determine the reaction kinetics.

The computational results suggest that the stepwise mechanism, in which the QM intermediate is formed before alkylation occurs, appears to be the active pathway for alkylation, as the concerted mechanism has a larger energy barrier than the stepwise mechanism, especially under slightly basic conditions. However, strong electron-withdrawing groups might be able to shift the active pathway to the concerted mechanism, not because the substituent will affect the energetics for the direct displacement reaction, but because it will raise the energy barrier for QM formation, possibly enough to alter the active mechanism. Regardless, the energetics for alkylation of the QM precursors show that there is a great deal of tunability available, which is ideal for designing a therapeutic that specifically targets realkylation of nerve agent inhibited and aged AChE that is specific to the active site after aging has occurred.

Based on our computational studies, we sought to establish an experimental protocol for monitoring both the quinone methide intermediate and the alkylation reaction in general. Our first attempts for monitoring the reaction were by UV-vis spectroscopic methods. The alkylated product of each precursor was found to absorb at the same wavelength as the precursor, as the chromophore is a single aromatic ring with identical substitution for both products and reactants. Therefore addition of various nucleophiles did not exhibit any appreciable spectroscopic shifts in the UV-vis studies.
We were also not able to observe the quinone methide from any of the initial precursors studied due to the QM being a reactive intermediate with a very short lifetime in solution. Therefore, we shifted our focus to a precursor that yields a QM intermediate known to possess a much longer lifetime.\textsuperscript{1,2} After heating this QM precursor (Precursor T) at 80 °C under basic conditions, a peak at 424 nm was observed in the UV-vis spectrum, which exhibited a half-life of 37 minutes. This half-life, coupled with a GC-MS peak at a mass of 218 (the same mass as the QM intermediate) and a similar lifetime, suggests that the 424 nm absorbance corresponds to the QM intermediate.

We expanded our methods for observation of the alkylation reaction to GC-MS methods. After testing Precursor T with piperidine, morpholine, imidazole, pyridine, \textit{p}-toluenethiol, benzyl alcohol, and dibenzyl phosphate, the alkylated product was observed by GC-MS with piperidine, morpholine, imidazole, and \textit{p}-toluenethiol. The alkylated product could not be observed with using either benzyl alcohol or pyridine as the nucleophile, although both the nucleophile as well as Precursor T could be observed by GC-MS. Presence of the alkylated product when using dibenzyl phosphate as the nucleophile could not be determined due to fragmentation of the phosphate in the injector.

After confirming alkylation of Precursor T by numerous nucleophiles, a calibration curve was obtained for Precursor T and the morpholine alkylated product using GC-MS with biphenyl as an internal standard. By using an internal standard, the concentrations of both Precursor T and the alkylated product could be quantified throughout the reaction. Thus, reacting Precursor T with morpholine in acetonitrile at 80
°C for 1 hour yielded 58% alkylation for an equimolar reaction and 74% alkylation for a reaction with twice the concentration of morpholine. The reaction reached equilibrium prior to 30 minutes with a 1:1 ratio of morpholine to Precursor T.

The alkylation reaction between Precursor T and morpholine was also monitored by UV-vis techniques. As we previously showed that the QM intermediate of Precursor T absorbs at 424 nm, we monitored the decay of this peak throughout the alkylation reaction. We observed an exponential decay of this absorbance upon addition of morpholine (in a 100:1 ratio with Precursor T) after thermal generation of the QM intermediate. This decay possessed a half-life of 4.30 ± 0.57 minutes, which is much faster than the half-life of 37 ± 6 minutes with NaOH. The GC-MS results show that the reaction between morpholine and Precursor T is completed within 30 minutes with a 1:1 ratio between morpholine and Precursor T. By having morpholine in excess at 100:1, the trapping of the quinone methide intermediate should increase dramatically. The QM may also have a significantly longer lifetime with NaOH than with morpholine due to the basic conditions, which have been shown to facilitate QM formation. Thus, when Precursor T is heated with NaOH, the QM intermediate could be in equilibrium with both the QM precursor and the alkylated product, and the QM could be regenerated from the product in solution.
5.2: FUTURE WORK

Other members of our group have carried out docking calculations with the QM precursors to probe their binding orientations in AChE, and molecular dynamics simulations of those binding poses to investigate the stability of the unique binding poses. However, these calculations were performed using the aged AChE enzyme. The same computations could also be carried out using apo-AChE to compare to the results with the aged enzyme. Ideally, the QM precursors will show a preference of binding with the aged enzyme, and thus, their binding constants with the apo-enzyme would be lower.

Also, our group has developed a family of ortho-QM precursors that is very similar to the para-QM precursors used in this study as possible realkylating agents. Similar investigations of the o-QMs could be performed as a comparison to the para family. Preliminary calculations show these ortho reagents are more reactive than their para counterparts, which correlates with previous experimental studies.

A protocol has been established to monitor the alkylation reaction by both UV-vis and GC-MS techniques. Further investigation into the alkylation of various QM precursors, including the o-QM precursors, could be performed to expand upon the results obtained with Precursor T. We have also started experiments to monitor the alkylation of a model phosphonate with a series of our QM precursors (Scheme 5.1). This model phosphonate contains a p-nitrophenoxy substituent, which upon alkylation and the subsequent addition of base, should undergo hydrolysis and show a strong absorption at 402 nm. Demonstrating alkylation of a phosphonate by our QM precursors will
greatly supplement our hypothesis that the precursors could be used to realkylate aged AChE.

Scheme 5.1. The alkylation of a model phosphonate by a QM precursor and subsequent hydrolysis of the \( p \)-nitrophenoxide substituent upon addition of base.

Another area of investigation into the possible realkylation of AChE by the QM precursors that we have developed is monitoring the binding of precursors with AChE. The various precursors can be tested with both native and aged enzyme to determine binding constants as well as to track alkylation of the aged phosphonate. Ideally, no alkylation will be observed with the native enzyme, either in the active site or on the enzyme surface, but alkylation will be observed with the aged enzyme. However, even if the ideal circumstances are not observed, we have shown that the reactivity of the QM precursors can be greatly modulated and tuned.

5.3: REFERENCES FOR CHAPTER 5


Chapter 1


Chapter 2


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**Chapter 3**


Chapter 4


**Chapter 5**


