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ABSTRACT

Cyclodextrin compounds are widely known for their ability to bind organic molecules in aqueous solution and have therefore been extensively utilized in the design of both enzyme mimics and chemosensors. This thesis describes the synthesis and study of several new derivatives of β-cyclodextrin to expand the role of cyclodextrin research in these areas.

The synthesis of a new material containing active oxygen incorporated into β-cyclodextrin has been accomplished by the simple oxidation of β-cyclodextrin with hydrogen peroxide. The isolated material is believed to contain a hydroperoxy group located on C2 of the 2°- side of the cyclodextrin. The ability of this material to act as an enzyme mimic was examined in the transacylation of esters. The observation of only a modest increase in reactivity over native β-cyclodextrin, as well as inhibition studies, has lead to the conclusion that the hydroperoxy group is positioned away from the cyclodextrin cavity.

A second oxidation reaction, on the 1°- side of the cyclodextrin, has lead to the synthesis of 6-deoxy-6-formyl-β-cyclodextrin. This compound was obtained utilizing a simple two step process from native β-cyclodextrin involving the Nace DMSO oxidation. The aldehyde functionality has shown wide reactivity with various nucleophiles, including bisulfite, hydroxylamine and hydrazine, leading to the synthesis of many new cyclodextrin
derivatives. In addition, this compound is susceptible to reduction and to reductive amination with aromatic amines.

Finally, a new fluorescent chemosensor based on a bis-cyclodextrin anthracene compound was synthesized. This chemosensor was found to have the correct physical orientation for the effective binding of polynucleotides in aqueous solution, with specificity for purine based polynucleotides. The chemosensor, termed the duplex, utilized β-cyclodextrin units as binding sites for the nucleic acid bases for both polynucleotides. However, photoinduced electron transfer lead to an observed fluorescence enhancement in the presence of polyadenylic acid and an interaction between aromatic moieties lead to an observed fluorescence quenching in the presence of polyguanylic acid. It was also possible to detect 5'-monophosphate guanylic acid in this manner. The fluorescent detection of polyadenylic acid was reversible as evidenced by a return of fluorescence upon the addition of Mg$^{2+}$ or polyuridylic acid.
To
My Family and
Mark
ACKNOWLEDGMENTS

First and foremost, I must thank Dr. Anthony Czarnik. I hold Dr. Czarnik in the highest regard as a scientist, an educator, and a person. If I take with me only a small fraction of his insight and enthusiasm, I will be forever in his debt. I would also like to thank Dr. Matthew Platz who took me into his group and graciously acted as my co-advisor in Dr. Czarnik's absence. My acknowledgments would not be complete without mentioning my gratitude to Mr. Bryant, my high school chemistry teacher, who started me on this path many years ago.

The Czarnik research group, which was comprised of many friends, offered me a venue to learn and grow throughout my graduate work. In particular, I warmly acknowledge: Linda for all her support and understanding; Jude for his eternal optimism and love of chemistry; John and Mark for their generous advice and insight; Wade for his friendship.

I would also like to thank my parents for their constant encouragement in all of my endeavors and my sister, Mindy, for always believing in me. Finally, I would like to thank Mark for always helping me to see the light at the end of the tunnel and for cheering me on with each small step that I took in accomplishing this goal.
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CHAPTER 1
SYNTHESIS AND STUDY OF
2-DEOXY-2-HYDROPEROXY-Β-CYCLODEXTRIN

The field of cyclodextrin technology is an area of bioorganic chemistry that is rapidly growing and expanding. Cyclodextrins have been employed in a wide variety of applications spanning the areas of enzyme mimicry, separations technology and medicinal chemistry. Cyclodextrins have even been utilized as food additives. The appeal of cyclodextrin chemistry lies in the molecular shape and subsequent properties of these molecules.

CYCLODEXTRINS

Cyclodextrins are cyclic oligomers of glucose units connected by α (1 → 4) linkages. Although cyclodextrins of higher order are known, the most common cyclodextrins are composed of 6, 7 and 8 glucose units and are known as α, β and γ-cyclodextrin, respectively. Cyclodextrins were first discovered in 1891 but were not fully described until the early 1900's by Schardinger. In recent literature, the name “cyclodextrin” is almost exclusively utilized; however, older references refer to these compounds as Schardinger’s dextrins, cycloamyloses or cycloglucans. Today, cyclodextrins are inexpensive reagents and are made commercially available through the enzymatic degradation of starch by Bacillus macernas.
The cyclic arrangement of glucose units in cyclodextrin resembles a truncated cone (Figure 1). The interior of the cone is lined with glycosidic oxygens and C-H bonds making the cavity hydrophobic compared to water, whereas the rims of the cone are lined with hydroxyl groups. These hydroxyl groups impart a hydrophilic nature to the exterior of the molecules making them water soluble. There are two different types of hydroxyl groups in cyclodextrin, the primary hydroxyls (1°; located at C-6 of the glucose units) and the secondary hydroxyls (2°; located at C-2 and C-3). All of the 1° hydroxyls are positioned on one rim of the cyclodextrin, known as the 1°- side, and all of the 2° hydroxyls are positioned on the opposite rim, known as the 2°- side. Free rotation of the 1°- side hydroxyl groups decreases the rim diameter of the 1°- side compared to the 2°- side giving the cyclodextrin the overall cone shape.

The most important difference between the 1°- and 2°- side hydroxyl groups is their pKₐ’s. The 1°- side hydroxyls have a pKₐ of 16, typical of a primary alcohol. However, the pKₐ of the 2°- side hydroxyls (specifically the C-2 hydroxyls) is only 12.2. This increase in acidity is due to intramolecular hydrogen bonding between the hydroxyl groups on the secondary side. The hydrogen of the C-3' hydroxyl group is in a position to hydrogen bond to the oxygen of the C-2 hydroxyl group in an adjacent glucose unit (Figure 2). In β-cyclodextrin, this intramolecular H-bonding is uninterrupted around the entire 2°- side rim. This leads to β-cyclodextrin having the lowest water solubility of the three most common cyclodextrins. The pKₐ difference between the 1°- and 2°- side hydroxyl groups can be exploited in the preferential functionalization of the 1°- or 2°- side.
Figure 1. Representations of β-cyclodextrin.
Figure 2. Hydrogen bonding between the C-2 and C-3' hydroxyl groups on the 2°- side of cyclodextrin.
In general, cyclodextrins are soluble in polar solvents such as dimethylformamide, pyridine and water. They are insoluble in typical organic solvents such as ether, chloroform and acetone (Table 1). This distinction makes working with cyclodextrins a unique experience in organic chemistry.

**CYCLODEXTRINS AS HOST MOLECULES**

The hydrophobic interior of the cyclodextrin cavity provides a binding pocket for small molecules in aqueous solution. A variety of molecules can bind in the cyclodextrin cavities ranging from inorganic compounds such as iodine to aromatic organic compounds. The binding of molecules can sometimes be monitored by changes in the UV, NMR or fluorescence spectra of the included molecules, as well as by various other techniques. The diameter of the interior cavity of the cyclodextrin strongly influences the binding ability of any substrate (Table 2). Substrates that can occupy a maximum amount of space in the cavity, without steric interference, are tightly bound. For β-cyclodextrin, the cavity has the correct dimensions to easily accommodate derivatives of benzene. Some examples of substrates and their respective binding constants can be found in Table 3.

Many factors are involved in providing the driving force for the binding of substrate molecules into a cyclodextrin host. One factor is the interaction between the guest molecule and the cyclodextrin host. This interaction can take the form of van der Waal’s attraction between the substrate and the hydrophobic interior of the cavity or hydrogen bonding between the substrate and the hydroxyl groups of the cyclodextrin. In addition, substrate binding allows the release of high energy water molecules from the interior of the cavity into
Table 1. Solubility of α-, β- and γ-cyclodextrins, as indicated, in various solvents at 25°C.

<table>
<thead>
<tr>
<th>Cyclodextrin</th>
<th>Solvent</th>
<th>Solubility (wt %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α</td>
<td>H₂O</td>
<td>0.127</td>
</tr>
<tr>
<td>β</td>
<td>H₂O</td>
<td>0.0182</td>
</tr>
<tr>
<td>γ</td>
<td>H₂O</td>
<td>0.188</td>
</tr>
<tr>
<td>β</td>
<td>Dimethylsulfoxide</td>
<td>&gt;42.0</td>
</tr>
<tr>
<td>β</td>
<td>Dimethylformamide</td>
<td>28.3</td>
</tr>
<tr>
<td>β</td>
<td>Pyridine</td>
<td>36.7</td>
</tr>
<tr>
<td>β</td>
<td>Ethanol</td>
<td>0.0</td>
</tr>
<tr>
<td>β</td>
<td>Acetone</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Table 2. Physical characteristics of α-, β- and γ-cyclodextrins.

<table>
<thead>
<tr>
<th>No. Glucose Units</th>
<th>α</th>
<th>β</th>
<th>γ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular Weight</td>
<td>972</td>
<td>1135</td>
<td>1297</td>
</tr>
<tr>
<td>Height of Torus (Å)</td>
<td>7.3</td>
<td>7.8</td>
<td>7.8</td>
</tr>
<tr>
<td>1° Rim Diameter (Å)a</td>
<td>5.6</td>
<td>6.8</td>
<td>8.0</td>
</tr>
<tr>
<td>2° Rim Diameter (Å)b</td>
<td>8.8</td>
<td>10.8</td>
<td>12.0</td>
</tr>
<tr>
<td>Vol. of Cavity (Å³)</td>
<td>174</td>
<td>262</td>
<td>472</td>
</tr>
</tbody>
</table>

a Diameter measured at C-6 position.
b Diameter measured at O-2 position.
<table>
<thead>
<tr>
<th>Cyclodextrin Host</th>
<th>Substrate</th>
<th>$10^2 K_d$ (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\beta$</td>
<td>1-Adamantanecarboxylic acid</td>
<td>0.40</td>
</tr>
<tr>
<td>$\beta$</td>
<td>Methylpenicillin</td>
<td>3.3</td>
</tr>
<tr>
<td>$\beta$</td>
<td>Iodide Ion</td>
<td>5.5</td>
</tr>
<tr>
<td>$\alpha$</td>
<td>$m$-Butylphenyl acetate</td>
<td>0.20</td>
</tr>
<tr>
<td>$\beta$</td>
<td>$m$-Butylphenyl acetate</td>
<td>0.013</td>
</tr>
<tr>
<td>$\gamma$</td>
<td>$m$-Butylphenyl acetate</td>
<td>0.99</td>
</tr>
<tr>
<td>$\alpha$</td>
<td>$p$-Butylphenyl acetate</td>
<td>0.65</td>
</tr>
<tr>
<td>$\alpha$</td>
<td>$m$-Nitrophenyl acetate</td>
<td>1.9</td>
</tr>
<tr>
<td>$\beta$</td>
<td>$m$-Nitrophenyl acetate</td>
<td>0.80</td>
</tr>
<tr>
<td>$\alpha$</td>
<td>$p$-Nitrophenyl acetate</td>
<td>1.2</td>
</tr>
<tr>
<td>$\beta$</td>
<td>$p$-Nitrophenyl acetate</td>
<td>0.61</td>
</tr>
<tr>
<td>$\alpha$</td>
<td>$p$-Nitrophenolate</td>
<td>0.040</td>
</tr>
</tbody>
</table>

Table 3. Dissociation constants ($K_d$) for various substrate molecules and $\alpha$-, $\beta$- and $\gamma$-cyclodextrin hosts, as indicated.
the bulk solvent. Although there has been some debate as to which of these factors dominates, all of these forces play some role in binding. It is the cyclodextrins' ability to bind small molecules in aqueous solution that has made it so widely utilized as an enzyme mimic.⁹

**CYCLODEXTRINS AS ENZYME MIMICS**

The active site of an enzyme possesses two essential features, a binding cavity and a reactive moiety which together allow enzymes to function as extremely efficient catalysts. Enzymes achieve high catalytic activity by exploiting the thermodynamics of the reaction pathway. The first step of an enzymatic reaction is the binding of the substrate into the cavity of the active site. This preassociation of the substrate effectively transforms what would otherwise be an intermolecular (bimolecular) reaction into an intramolecular (unimolecular) reaction and brings the reactive moiety into close proximity to the bound substrate. Additionally, in the course of the reaction, the transition state is more tightly bound in the active site than either the substrate or the final product. These capabilities lead to a lowering of the energy of activation of the reaction and a corresponding increase in the reaction rate. Of course, for the enzyme to be a true catalyst and to exhibit turnover activity, the enzyme must be returned to its original state (unchanged) after the reaction is complete and the product is released. The kinetic process can be described as:

\[ E + S \rightleftharpoons ES \rightarrow EP = E + P \]

where \( E \) represents enzyme, \( S \) represents substrate, \( P \) represents product, and \( ES \) and \( EP \) are enzyme-substrate and enzyme-product complexes, respectively.
Cyclodextrins are excellent enzyme mimics for several reasons. Cyclodextrins, like biological enzymes, are water soluble. In addition, they possess an efficient binding pocket. The “active site” can be completed by attaching a reactive moiety to either of the cyclodextrin rims through derivatization of the hydroxyl groups or by utilizing the hydroxyl groups themselves as reactants. Thus, in aqueous solution, cyclodextrins can preassociate a substrate and subsequently react with the bound substrate leading to product formation.

The first extensive utilizations of cyclodextrins as enzyme mimics were reported in 1967. In Bender’s laboratory, the transacylation of phenyl acetates with different substituent groups in the presence of α-, β-, and γ-cyclodextrin at pH 10.6 was studied (Scheme 1). For \( m \)-tert-butylphenyl acetate, an acceleration of 250-fold was observed for the reaction with β-cyclodextrin (10 mM) over the uncatalyzed reaction. Several important observations likened the catalysis of the cyclodextrins to that of enzymes. It was shown that the catalysis followed saturation kinetics indicating preassociation of the phenyl acetate into the cyclodextrin cavity prior to transacylation. In addition, the substitution pattern of the phenyl acetate had a large effect on the rate of acceleration. For example, the \( p \)-tert-butylphenyl acetate showed only a 2-fold increase in the transacylation rate compared to the larger increase for the \( meta \) isomer. This observation, coupled with the fact that substituent effects did not follow a Hammett relationship, suggested that the binding of the substrate was crucial for catalysis. This was confirmed by the addition of organic compounds to the reaction mixture which can bind competitively with the phenyl acetate substrate in the cyclodextrin cavity. Competitive binding inhibited the reaction as observed in enzyme catalysis. However, the strength of the binding was not as important as the orientation of the
Scheme 1. Mechanism of transacylation of p-nitrophenyl acetate with cyclodextrin.
bound substrate. This was demonstrated in the comparison of the transacylation of \( p \)- and \( m \)-nitrophenyl acetate. Although \( \beta \)-cyclodextrin has a larger binding constant with the \textit{para} isomer than with the \textit{meta} isomer, the \textit{meta} isomer shows a larger rate acceleration (54-fold versus 7-fold for the \textit{para} isomer). Bender has proposed that in the binding of the \textit{meta} isomer, the carbonyl group is held in close proximity to the \( 2^\circ \)-side hydroxyl groups which have been shown to be the nucleophilic agents in the transacylation.

These results were compared to the transacylase chymotrypsin. Chymotrypsin at pH ~ 8 accelerates the transacylation of acetyl-tryptophan ethyl ester \( 10^6 \)-fold faster than hydroxide ion. For \( \beta \)-cyclodextrin, the rate acceleration of \( m \)-tert-butylphenyl acetate at pH ~ 13 was \( 2.6 \times 10^4 \)-fold over hydroxide ion. Therefore, the cyclodextrin is an efficient transacylation enzyme mimic; however, there are some limitations. The cyclodextrin hydroxyl groups are only reactive under basic conditions and still do not equal the capabilities of the actual enzyme. More importantly, the cyclodextrin does not act as a true catalyst. Deacylation of the cyclodextrin, the rate-limiting step, is slow and prevents the cyclodextrin from entering into a catalytic cycle and exhibiting turnover.

New derivatives of cyclodextrin have been synthesized by modification of either the \( 1^\circ \)- or \( 2^\circ \)-side in order to circumvent these limitations, as well as improve the catalytic ability of the cyclodextrins. As a result, catalysts have been designed for use in numerous, diverse reactions including, but not limited to, transamination, transphosphorylation, reduction and oxidation.

Ikeda \textit{et al.} designed a successful transacylation enzyme mimic by attaching a histamine moiety to the \( 1^\circ \)-side of \( \beta \)-cyclodextrin (1; Figure 3). The new derivative
demonstrated catalytic turnover with a rapid deacylation step and gave rate enhancements comparable to chymotrypsin. In a similar compound, D' Souza et al. synthesized a 2°- side derivative of β-cyclodextrin containing a catalytic triad similar to the catalytic triad found in serine proteases (2; Figure 3). This derivative also exhibited catalytic turnover. However, the accelerations were not larger than those observed with native β-cyclodextrin.

![Hydrazine](image1.png)  
![Hydroxylamine](image2.png)

**Figure 3.** Selected enzyme mimics based on β-cyclodextrin.

**α-NUCLEOPHILE DERIVATIVES OF CYCLODEXTRIN**

In the Czarnik research group, several new derivatives have been designed by attaching α-nucleophiles to both the 1°- and 2°- side of β-cyclodextrin. α-Nucleophiles contain an atom with unshared electron pairs adjacent to the nucleophilic center. Hydrazine, hydroxylamine and hydrogen peroxide are all examples of α-nucleophiles. α-Nucleophiles are recognized as exhibiting high reactivity compared to isosteric compounds and are more reactive than their relative basicities would suggest. The origin of this
reactivity has been rationalized by different theories of polarizability, transition state stabilization and lack of steric hindrance around the nucleophilic center. Although the actual basis for the enhanced reactivity is still unclear, the α effect is evident in the reactivity of these compounds toward substrates such as esters, nitriles and tetrahedral phosphorus.\(^{14}\)

In addition to showing enhanced reactivity, α-nucleophiles offer other advantages as reactive moieties attached to cyclodextrin. α-Nucleophiles are typically less basic than isosteric amines or alcohols and therefore often exist in an unprotonated state near neutral pH. Since the distance between the reactive group and the bound substrate is relevant to the reaction rate, α-nucleophiles also potentially have the advantage of being positioned proximal to the cyclodextrin binding cavity due to their small physical size. In the Czarnik research group, hydroxylamine has been attached to both the 1°- and 2°- sides of β-cyclodextrin (3\(^{15}\) and 4\(^{16}\), respectively; Figure 4). Oxidation of these derivatives has also allowed the isolation of the 1°- and 2°- side oximes of β-cyclodextrin (5 and 6, respectively).\(^{17}\) Finally, hydrazine has been attached to the 1°- side of β-cyclodextrin (7).\(^{15}\)

The transacylating ability of these derivatives was studied extensively. Compound 3 showed the largest rate acceleration for the transacylation of \(p\)-nitrophenyl acetate. At pH 6.5, a rate acceleration over β-cyclodextrin of 1900-fold and a rate acceleration of 4-fold over \(\text{CH}_3\text{NHOH}\) was observed demonstrating that preassociation of the \(p\)-nitrophenyl acetate is occurring. The 2°- side hydroxylamine derivative (4), even though less reactive (showing only a 43-fold rate increase over β-cyclodextrin at pH 6.5), exhibits a pH dependence not observed with \(\text{CH}_3\text{NHOH}\) or 3. This observation has been rationalized by hydrogen bonding between the NHOH moiety and the hydroxyl groups of the 2°- side of the cyclodextrin.
Figure 4. α-Nucleophiles of β-cyclodextrin designed in the Czarnik research group.
Therefore, cyclodextrin appears capable of modifying the reactivity of appendent groups much like an enzyme. The oxime and hydrazine derivatives did not exhibit exceptional reactivity.

**DESIGN OF A NEW α-NUCLEOPHILE OF CYCLODEXTRIN**

The hydroperoxide group, another α-nucleophile, has also been shown to have enhanced reactivity over its isosteric compound, hydroxide ion or water. In the reaction of hydrogen peroxide with p-nitrophenyl acetate, McIsaas et al. report the hydroperoxide ion to be approximately 80 times more reactive at pH ~7 than hydroxide ion at pH ~9.5. Jencks and Carriuolo, however, report that the hydroperoxide ion is approximately 300 times more reactive at pH 6.25 than hydroxide ion at pH ~11. Methyl hydroperoxide exhibits slightly lower reactivity with only a 120-fold acceleration at pH 6.7 compared to hydroxide ion at pH ~11.

In addition to being reactive toward esters, hydroperoxides can act as oxidizing agents. This can be observed, for example, in the epoxidation of olefins, the conversion of amines to nitroso compounds and the conversion of sulfides to sulfoxides.

Due to the hydroperoxides' reactivity and synthetic capability, the synthesis of a derivative of β-cyclodextrin with an appendant hydroperoxide group was desirable. This compound could potentially act as a preassociating α-nucleophile in the transacylation of esters and as a chiral oxidizing agent in various oxidation reactions. It was the initial goal of this project to synthesize a 1°-side hydroperoxide of β-cyclodextrin. However, in this pursuit, an unexpected reaction was discovered that has lead to the isolation of a material
containing a 2°-side hydroperoxide which has been designated 2-deoxy-2-hydroperoxy-β-cyclodextrin. The reactive properties of this material were examined with various substrates.

SYNTHESIS AND PURIFICATION OF 2-DEOXY-2-HYDROPEROXY-β-CYCLODEXTRIN

The initial attempts at the synthesis of the 1°-side hydroperoxide involved the displacement reaction of the 1°-side monotosylate with H₂O₂. This reaction was attempted under a wide variety of conditions: temperature ranges from room temperature to 60°C; solvents of H₂O, CH₃OH and 30% H₂O₂; base (KOH) concentrations ranging from zero to a large excess. Many of these reactions resulted in new species as evidenced by TLC and isolated solids exhibited changes in the ¹H NMR spectra from that of native β-cyclodextrin. Hydrogen peroxide oxidation of 6-deoxy-6-hydrazinyl-β-cyclodextrin was also briefly examined.²¹

At this point, control experiments in which hydrogen peroxide was reacted with native β-cyclodextrin also showed signs of reactivity. The reaction with native β-cyclodextrin was evident even at room temperature and occurred both in the presence and absence of base (KOH). Therefore, it was determined that even if the tosylate was reacting on the 1°-side, the competing reaction with β-cyclodextrin could cause significant interference. For this reason, concentration was placed on the reaction of β-cyclodextrin to determine its scope and versatility. It was discovered that the reaction between β-cyclodextrin (β; βCD) and 30% H₂O₂ (neat) when heated at 60°C for several days yielded a material that contained active oxygen and produced a new ¹H NMR signal (D₂O) at 5.2
ppm (Scheme 2). This material was later found to contain 2-deoxy-2-hydroperoxy-β-cyclodextrin (9; βCDOOH; stereochemistry presumed). The cyclodextrins could be isolated by precipitation from isopropanol.

The amount of active oxygen was determined utilizing the assaying procedure of Siggia. Although there are numerous procedures for this type of determination, the Siggia procedure was preferred due to its compatibility with aqueous soluble compounds. The assay involves the titration of a solution of the sample and As₂O₃ with a standardized solution of I₂. From the volume of I₂ required to reach the endpoint, the amount of active oxygen in the sample can be calculated.

Purification of the material would necessarily involve the removal of all physically entrained H₂O₂ from the isolated cyclodextrins. To determine the number of successive precipitations required to remove all of the free H₂O₂, a study was performed in which a portion of the isolated solid was tested for active oxygen content after each precipitation in a series of five (Figure 5). After the second precipitation, virtually all free H₂O₂ had been removed as evidenced by the attainment of a constant level of active oxygen beyond this point (approximately 25% upon heating for 3 days). Three precipitations were then performed on all subsequent samples to ensure any remaining active oxygen originated from the cyclodextrinyl hydroperoxide. A similar experiment was performed on a sample which had been reacted for only 15 min (Figure 6). After the second precipitation, the active oxygen content was 2.2%, confirming that the active oxygen was coming from a new compound and not from βCD itself or free H₂O₂.
Scheme 2. Synthesis of 2-deoxy-2-hydroperoxy-β-cyclodextrin (9; βCDOOH).
Figure 5. Percent active oxygen as a function of the number of times the sample of βCDOOH (isolated after 9 d at 60°C) was precipitated from isopropanol.

Figure 6. Percent active oxygen as a function of the number of times the sample of βCDOOH (isolated after 15 min at 60°C) was precipitated from isopropanol.
It was also desirable to determine the optimal reaction time to obtain a sample with the highest level of active oxygen content. A study of a single reaction was performed in which an aliquot (25 mL) was removed each day and assayed for active oxygen over a 15 day period (Figure 7). The active oxygen content leveled off after three days with a spike on day 7 and 8. This spike was found to be reproducible. $^1$H NMR spectra were also taken of each aliquot and featured an increase in the signal at $\delta$ 5.2 with increasing time. A similar study was performed in which the reactivity of the isolated sample with $p$-nitrophenyl acetate ($p$-NPA) was determined as a function of time over a 15 day period. The results (Figure 8) show the same trend as the active oxygen assay with a leveling of the reactivity after 3 days. From these studies, it was concluded that three days would be an optimal time for the reaction. This was based on three factors. First, the ease of isolation of the cyclodextrin material by precipitation was not consistent from day to day (ranging from 0.2 to 1.1 g isolated). However, the isolation was consistently easier with shorter reaction times. Secondly, the spike in reactivity was not believed to be an actual increase in the amount of hydroperoxide incorporation but a reflection of a change in the amount of $\beta$CDOOH in the isolated material. Finally, the shorter reaction time was more practical in a laboratory sense.

The $\beta$CDOOH was found to decompose with time and the proposed structure of the decomposition product is the $2^\circ$-side dialdehyde (10). It has been previously shown that $\beta$-hydroxyhydroperoxides can decompose to dialdehydes in this manner by a simple elimination process as shown in Scheme 3. 23 This $2^\circ$-side dialdehyde is also the known decomposition product of the oxidation of $\beta$-cyclodextrin by periodate oxidation 24 and cerium (IV) oxidation. 25 In this case, this structure was implied from a combination of
Figure 7. Percent active oxygen as a function of the number of days the isolated βCDOOH sample was stirred at 60°C.
Figure 8. Rate constants for the transacylation of $p$-nitrophenyl acetate as a function of the number of days the isolated $\beta$CDOOH sample was stirred at 60°C.
Scheme 3. Mechanism of the base catalyzed decomposition of 2-deoxy-2-hydroperoxy-\(\beta\)-cyclodextrin (\(\beta\)CDOOH) to the 2°- side dialdehyde (10).
various indicative tests and known reactivities. The product of decomposition was found to contain an aldehyde functionality by positive results with 2,4-dinitrophenylhydrazine and Tollen's reagent. (Attempts at quantification of the aldehyde with 2,4-dinitrophenylhydrazine were unsuccessful due to the partial water solubility of the hydrazone derivative.) By $^1$H NMR (D$_2$O), the decomposition product was shown to be reactive toward NH$_2$OH. The addition of NH$_2$OH produced a new signal at $\delta$ 7.38 that resembled the CH$_2$OH signal of the 1°- and 2°- side $\beta$-cyclodextrinyl oximes (5 and 6). However, the signal was a multiplet as opposed to doublets in the oxime cases. In addition, the decomposition product was also found to be reactive toward CH$_3$NH$_2$. This was evidenced by the appearance on two new $^1$H NMR signals at $\delta$ 7.55 and 8.22 corresponding to the cis and trans isomers of the hydrazone. All of these results strongly suggest that the decomposition product is an aldehyde. Comparison of the NMR spectra of these derivatives and the reactivity of this compound to that of the known 1°- side aldehyde of $\beta$-cyclodextrin$^{36}$ confirms that the aldehyde is located on the 2°- side. This, in turn, verifies the position of the hydroperoxide moiety as also on the 2°- side of the cyclodextrin.

Although it was not determined whether the hydroperoxide moiety is located on C-2 or C-3 of the glucose ring, two possible mechanisms for its formation are proposed in Scheme 4. Even though oxygen nucleophiles have been shown to be unreactive toward hydrogen peroxide,$^{14,27}$ the known internal H-bonding between the C-2 and C-3' hydroxyl groups may activate the C-2 hydroxyl toward such nucleophilic attack (A; Scheme 4). Alternatively, a backside S$_N$2 attack of hydrogen peroxide on the carbon at the C-2 position is also conceivable (B; Scheme 4) due to the electron deficient nature of the C-2 carbon and
Scheme 4. Proposed mechanisms for the formation of 2-deoxy-2-hydroperoxy-β-cyclodextrin (βCDOOH) via nucleophilic attack by the 2° hydroxyl group (A) or hydrogen peroxide (B).
the greater nucleophilicity of H₂O₂ as compared to a hydroxyl group. In either case, however, the hydroperoxide moiety would be preferentially formed at the C-2 position.

With this insight, the large scale synthesis of βCDOOH was performed by heating βCD at 60°C in 30% H₂O₂ for three days. Isolation of the cyclodextrin material was accomplished by first concentrating the solution *in vacuo* to approximately ¼ of the original volume, followed by three sequential precipitations from isopropanol. TLC of the resulting solid showed a mixture of βCD (Rf 0.12) and a new species (Rf 0.05). The ¹H NMR spectrum (Figure 9) of βCDOOH in D₂O revealed a new signal at δ 5.2. The ¹³C NMR spectrum (Figure 9) also exhibited signals different from βCD itself at δ 102, 80 and 72. Although FAB mass spectral analysis did not yield a molecular ion peak for βCDOOH, it did yield evidence for its formation with a mass peak at 1173 (βCDOOH + Na), as well as the βCD mass peaks at 1135 (βCD) and 1157 (βCD + Na). The mixture contains approximately 25% βCDOOH based on active oxygen assays and therefore, the ratio of βCD:βCDOOH is 3:1. Although it is possible that this sample contains other hydroperoxy derivatives, the material that is isolated is significant in the fact that, in one step, a hydroperoxide species of β-cyclodextrin can be formed.

2-DEOXY-2-HYDROPEROXY-β-CYCLODEXTRIN AS AN ENZYME MIMIC

With the isolation of a new α-nucleophile derivative of β-cyclodextrin completed, the evaluation of this material as an enzyme mimic was then undertaken. The first system to be studied was the transacylation of *p*-nitrophenyl acetate (*p*-NPA).
Figure 9. $^1$H NMR and $^{13}$C NMR spectra of 2-deoxy-2-hydroperoxy-β-cyclodextrin (9; βCDOOH) in D$_2$O.
Initially, a saturation curve was generated by measuring the rate of transacylation of p-NPA at pH 8.5 for varying concentrations of βCDOOH ranging from 10 to 40 mM (Figure 10). However, the plot of rate constant versus concentration remained linear within this entire range. The fact that βCDOOH did not show saturation could be attributed to the fact that the sample only contains approximately 25% of the reactive compound, βCDOOH. A concentration of 10 mM βCDOOH was chosen for all subsequent studies due to solubility factors and conservation of material.

Since the βCDOOH was known to decompose, the rate of decomposition of this material was of primary interest. This rate was determined by measuring the decrease in the rate constant for the transacylation of p-NPA with time. These rates were measured in a buffered solution (BTP) at pH 8.5 (Figure 11). Decomposition was complete after approximately 11 h with a corresponding half-life of 1 h. The decomposition study was repeated in the presence of ethylenediaminetetraacetic acid (EDTA; 0.2 mM) and the half-life increased to 3 h. It is well documented that metal ions are capable of breaking down hydroperoxide compounds in solution by way of radical mechanisms. Since EDTA is capable of complexing metal ions present in the solution, this destructive pathway is at least partially inhibited leading to the greater stability of the hydroperoxide.

In a similar study, the decomposition of the βCDOOH as a solid was examined. A smooth decay of reactivity was not observed over the measured period of 135 days. However, it is noteworthy that even after 135 days, the sample still showed significant reactivity. This indicates that the sample is relatively stable in the solid form, even though a precise half-life cannot be determined.
Figure 10. Rate constants for the transacylation of \( p \)-nitrophenyl acetate (50 \( \mu \)M) as a function of \( \beta \)CDOOH concentration at pH 8.5.
Figure 11. Percent activity of βCDOOH (10 mM) in the transacylation of p-nitrophenyl acetate (50 μM) at pH 8.5 in the absence of EDTA (●) and in the presence of 0.20 mM EDTA (●).
Next, the pH rate profile for the transacylation of p-NPA was examined. Since the profile ranges from pH 7 to 10, it was necessary to carry out the kinetic studies using three different buffers. For this reason, it was essential to generate buffer uncatalyzed rate constants for accurate comparison at varying pH's. At each pH, rate constants were measured at buffer concentrations of 0.1, 0.2 and 0.4 M. Linear plots of buffer concentration versus rate constants were generated and extrapolated to zero buffer concentration, an example of which is shown in Figure 12. Initially, kinetics were also attempted at pH 11. However, the rate of decomposition of \( \beta \text{CDOOH} \) at this high pH is extremely rapid and the rate of hydrolysis is also fast. Therefore it is was not possible to accurately determine the rate constants at this pH.

At pH 10, the rate constants were first determined in CAPS buffer. Surprisingly, it was found that the rate actually decreased with increasing buffer concentration. The fact that the buffer was inhibiting the rate with increasing concentrations could be attributed to binding of the buffer into the cyclodextrin cavity preventing preassociation of the substrate. Alternatively, the binding of the buffer could show inhibition by sterically blocking the reactive hydroperoxide group. The remaining studies were performed using NaHCO\(_3\) buffer at pH 10.

pH profiles for the hydrolysis catalyzed by native \( \beta \text{CD} \) and \( \text{t}-\text{butylhydroperoxide} \), a non-preassociating \( \alpha \)-nucleophile, were also measured for comparison to \( \beta \text{CDOOH} \). \( \text{t}-\text{Butylhydroperoxide} \) was chosen for this study due to its stability in aqueous solution and its relative accessibility. The buffer uncatalyzed rate constants for all three compounds are shown in Table 4 and Figure 13.
Figure 12. Extrapolation to zero buffer concentration in the transacylation of p-nitrophenyl acetate (50 μM) with βCDOOH (10 mM) at pH 8.0 in TRIZMA buffer.
Figure 13. Buffer uncatalyzed rate constants for the transacylation of $p$-nitrophenyl acetate (50 $\mu$M) with 10 mM $\beta$CDOOH (●), $\beta$CD (●) or $t$-butylhydroperoxide (▲).
βCDOOH shows its highest rate acceleration over βCD (2.5 fold) at pH's 7 and 8. At higher pH, it is likely that the decomposition of βCDOOH competes more effectively with the hydrolysis of p-NPA leading to the lower $k_{\text{obs}}$. The hydrolysis with $t$-butylhydroperoxide shows the same pH dependence as that with βCDOOH. This would indicate that the βCDOOH is not hydrogen bonding to the 2°- side hydroxyl groups of cyclodextrin in a manner that significantly changes the reactivity of the hydroperoxide moiety as seen in the case of the 2°- side hydroxylamine (4). The increase in rate over $t$-butylhydroperoxide is highest at pH 9, with a 12-fold increase. This is not a large rate increase and may be attributed to the steric hindrance of the tertiary $t$-butylhydroperoxide rather than preassociation of the substrate.

<table>
<thead>
<tr>
<th>pH</th>
<th>βCD</th>
<th>$t$-BuOOH</th>
<th>βCDOOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.0</td>
<td>0.13</td>
<td>0.14</td>
<td>0.32</td>
</tr>
<tr>
<td>8.0</td>
<td>1.6</td>
<td>0.40</td>
<td>4.0</td>
</tr>
<tr>
<td>9.0</td>
<td>20.5</td>
<td>3.9</td>
<td>47</td>
</tr>
<tr>
<td>10.0</td>
<td>170</td>
<td>45</td>
<td>250</td>
</tr>
</tbody>
</table>

Table 4. Buffer uncatalyzed rate constants for the transacylation of p-NPA (50 μM) at 23°C with various nucleophiles (all 10 mM) in BTP (pH 7 and 9), TRIZMA (pH 8) or NaHCO₃ (pH 10).
Even though the accelerations observed with \( p \)-NPA were not large, the hydrolysis of a less activated ester, \( m \)-nitrophenylacetate (\( m \)-NPA), was examined. It is conceivable that large rate accelerations were not observed with the \textit{para} compound because of its orientation in the cyclodextrin cavity. The \textit{para} compound may have been bound into the cavity such that the hydroperoxide moiety was not in close proximity to the carbonyl group. The \textit{meta} compound, on the other hand, could bind into the cyclodextrin bringing the two reactive groups closer together.

The buffer uncatalyzed rate constants for the hydrolysis of \( m \)-NPA were determined at pH 8 and 9 for reaction with \( \beta \)CDOOH and \( \beta \)CD. There were some complications with determining these hydrolysis rates. At pH 9, the decomposition of the \( \beta \)CDOOH in solution lead to a change in absorbance at 390 nm, the absorbance maximum for \( m \)-nitrophenol. Therefore, in this instance, reference solutions were prepared containing only buffer and \( \beta \)CDOOH and the change in absorbance subtracted from the change in absorbance of the solutions containing \( m \)-NPA. At pH 8, however, reference solutions showed no significant change in absorbance at 390 nm and were unnecessary. The hydrolysis rates are shown in Table 5 and Figure 14. No significant rate accelerations were observed for \( \beta \)CDOOH over native \( \beta \)CD.

In order to explain the lack of activity observed for \( \beta \)CDOOH, an inhibition study was performed to measure the rate of hydrolysis of \( m \)-NPA in the presence of increasing concentrations of \( n \)-butanol. \( n \)-Butanol has been shown to bind in the cyclodextrin cavity and consistently inhibit preassociation of \( m \)-NPA leading to rate decreases.\textsuperscript{30} The \( m \)-NPA isomer was chosen for this study due to the fact that inhibition studies with \( p \)-NPA did not
Figure 14. Buffer uncatalyzed rate constants for the transacylation of \textit{m}-nitrophenyl acetate (50 \(\mu\text{M}\)) with 10 mM \(\beta\text{CDOOH (○)}\) or \(\beta\text{CD (■)}\).
always lead to rate decreases upon addition of the inhibitor. Native βCD showed normal inhibition (Figure 15) exhibiting a decreasing rate of reactivity with increasing concentrations of inhibitor. βCDOOH, however, showed unusual activity (Figure 15). This suggests that the reaction between βCDOOH and the substrate is not strictly proceeding through a pathway involving precomplexation.

In view of the inhibition data, it seems likely that the hydroperoxide moiety is not oriented toward the cavity of the cyclodextrin properly to allow reaction between the hydroperoxide group and a bound substrate. This would explain the only marginal acceleration of transacylation for βCDOOH in the case of p-NPA and m-NPA over native βCD. A non-preassociating pathway is also consistent with the observation of nonsaturation kinetics. Although the CAPS buffer did appear to show an inhibition effect, perhaps this was due largely to a steric effect of the bound buffer in which the buffer shields the hydroperoxide group from approach of an unbound substrate.

<table>
<thead>
<tr>
<th>pH</th>
<th>βCD</th>
<th>βCDOOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.0</td>
<td>6.1</td>
<td>7.7</td>
</tr>
<tr>
<td>9.0</td>
<td>43.3</td>
<td>68.5</td>
</tr>
</tbody>
</table>

Table 5. Buffer uncatalyzed rate constants for the transacylation of m-NPA (50 μM) at 23°C with various nucleophiles (all 10 mM) in TRIZMA (pH 8) or BTP (pH 9).
Figure 15. Rate constants for the transacylation of \( m \)-nitrophenyl acetate (50 \( \mu \)M) with 10 mM \( \beta \)CDOOH (++) and 10 mM \( \beta \)CD (●) at pH 8.5 in 0.1 M BTP with varying concentrations of \( n \)-butanol.
Before the results of the inhibition studies were concluded, the potential oxidation ability of pCDOOH was explored. Even though the pCDOOH was not facile at the cleavage of esters, this material still possesses a chiral hydroperoxide moiety due to the inherent chirality of the cyclodextrin. For this reason, the enantiomeric epoxidation of cinnamaldehyde was attempted (Scheme 5). It has been shown that trans-cinnamaldehyde in the presence of cyclodextrin and \( \text{H}_2\text{O}_2 \) can be oxidized to the corresponding epoxide with some enantioselectivity. The highest enantioselectivity observed was 8\% ee with an equimolar amount of \( \alpha \)-cyclodextrin and \( \text{H}_2\text{O}_2 \).\(^\text{31}\) This epoxidation with pCDOOH was tried under various conditions. However, no product formation was observed. Epoxidation or oxidation of non-binding substrates may be more lucrative since it has been concluded that the hydroperoxide group is oriented away from the cyclodextrin cavity.

**CONCLUSION**

The synthesis of a new material containing active oxygen incorporated into \( \beta \)-cyclodextrin has been accomplished. This material has been shown to include a new derivative of \( \beta \)-cyclodextrin, 2-deoxy-2-hydroperoxy-\( \beta \)-cyclodextrin, which decomposes to a 2°-side dialdehyde. The reactivity of this material has been examined in the transacylation
of esters. Although this material shows only a modest acceleration over that of \( \beta \)-cyclodextrin, inhibition studies show that the reaction does not proceed through a pre-association pathway suggesting that the hydroperoxide moiety is positioned away from the cyclodextrin cavity. The lack of reactivity of this material in the epoxidation of cinnamaldehyde further supports this theory. Although the systems studied here did not exploit this new material, 2-deoxy-2-hydroperoxy-\( \beta \)-cyclodextrin, a chiral oxidizing agent, may have potential use in other systems owing to its inexpensive and simple synthesis.

**EXPERIMENTAL**

**General.** Mass spectra were obtained by use of a Kratos-30 mass spectrometer with fast atom bombardment (FAB). UV data were obtained on a Hewlett-Packard 8452A diode array spectrophotometer. Most of the chemicals used in this study were obtained from Aldrich Chemical (Milwaukee, WI). The biological buffers BTP (1,3-bis(tris(hydroxymethyl)methylamino)-propane), CAPS (3-cyclohexylamino-1-propanesulfonic acid) and TRIZMA (tris(hydroxymethyl)aminomethane) were obtained from Sigma Chemical (St. Louis, MO). Thin layer chromatography was performed on aluminum-backed silica gel plates and cyclodextrin compounds were visualized by charring after staining with \( p \)-anisaldehyde stain: \( p \)-anisaldehyde/methanol/acetic acid/sulfuric acid (1:200:20:10). Elution was accomplished with a solvent system of ethyl acetate/2-propanol/NH\(_4\)OH/H\(_2\)O (7:7:4:4; v/v).

2-Deoxy-2-hydroperoxy-\( \beta \)-cyclodextrin (9). \( \beta \)-Cyclodextrin (8; 10.0 g, 8.80 mmol) was dissolved in neat 30% H\(_2\)O\(_2\) (200 mL). The solution was stirred at 60°C for 3 d, then
concentrated in vacuo to approximately ¼ of the original volume. The solution was added dropwise to isopropanol (1 L) and the precipitate collected under vacuum. The precipitate was dissolved in warm water (20 mL) and added dropwise to isopropanol (1 L). Precipitation was then repeated to yield a colorless solid containing ca. 25% 9 and β-cyclodextrin (8.7 g, 86% yield): Rf 0.12 (8), 0.05 (9); $^1$H NMR (D$_2$O) δ 5.21 (m, 2, H-1'), 4.90 (d, 5, H-1), 4.03-3.08 (m, 42, CD); $^{13}$C NMR (D$_2$O) δ 104.7, 102.6, 102.5, 84.0, 79.8, 76.2, 76.0, 75.8, 74.9, 74.7, 74.4, 74.1, 72.2, 63.1; FAB mass spectrum m/z 1135.45 (M$^+$) for βCD, 1157.38 (M$^+$ + Na) for βCD, 1173.42 (M$^+$ + Na) for βCDOOH.

Active Oxygen Assay. A 0.1 N As$_2$O$_3$ solution was prepared by dissolving As$_2$O$_3$ (2.5 g) in water (100 mL). NaOH was added until the solution was homogeneous. The pH was adjusted to 7.0 by the addition of 1 N HCl. NaHCO$_3$ (6.25 g) was added and the solution brought to volume (250 mL) with water. Then to the As$_2$O$_3$ solution (20 mL) was added the mixture to be tested (0.6 g). H$_2$SO$_4$ (1 N, 25 drops) was added followed by NaHCO$_3$ (0.5 g). The solution was then titrated against a 0.1 N I$_2$ solution until a yellow endpoint was reached. From the titration volume, the amount of As$^{3+}$ remaining in the solution could be calculated and hence the amount of active oxygen determined. (Siggia reports active oxygen percentages to be within 0.5 % for active oxygen contents of 0.008 g.)

The active oxygen assay by Siggia is based on the oxidation of As (III) to As (V). The sample to be analyzed is reduced to an alcohol by reaction with an arsenous oxide solution according to equation 1.

$$\text{HAsO}_2 + \text{ROOH} + \text{H}_2\text{O} \rightarrow \text{H}_3\text{AsO}_4 + \text{ROH}$$  \[1\]
The resulting solution is then titrated with a 0.100 N I₂ solution to oxidize the remaining As (III) according to equation 2.

\[ \text{HAsO}_2 + \text{I}^- + 2 \text{H}_2\text{O} \rightarrow \text{H}_3\text{AsO}_4 + 3 \text{I}^- + 2 \text{H}^+ \]  \[2\]

The amount of active oxygen in the original sample can then be determined from simple stoichiometric relationships.

**Transacylation of p-Nitrophenyl Acetate.** The hydrolysis of p-nitrophenyl acetate (p-NPA) was carried out at the appropriate buffer concentration at room temperature. The buffers utilized were BTP (pH 8.0 and 8.5), TRIZMA (pH 9) and NaHCO₃ (pH 10). Hydrolysis was initiated by the addition of 10 μL of p-NPA solution (50 mM in CH₃CN) to 990 μL of the cyclodextrin solution (10 mM in buffer). For hydrolysis with tert-butylhydroperoxide, a solution of the hydroperoxide (10 mM) was prepared by diluting a 70% aqueous solution of tert-butylhydroperoxide (13.7 μL) in buffer to 10 mL. The formation of p-nitrophenol (p-nitrophenolate) was followed spectroscopically at 398 nm. Pseudo-first-order rate constants were calculated using the ENZFITTER program (Elsevier-BIOSOFT, Cambridge, UK).

**Transacylation of m-Nitrophenyl Acetate.** The hydrolysis of m-nitrophenyl acetate (m-NPA) was carried out in the appropriate buffer at room temperature. Hydrolysis was initiated by the addition of 10 μL of m-NPA solution (50 mM in CH₃CN) to 990 μL of the cyclodextrin solution (10 mM in buffer). The formation of m-nitrophenol (m-nitrophenolate) was followed spectroscopically at 390 nm. At pH 9 with βCDOOH, a reference solution was also monitored which did not contain m-NPA. The absorbance of the reference solution was then subtracted from the absorbance of the solution containing m-NPA, before calculation
of the rate constant. Pseudo-first-order rate constants were calculated using the ENZFITTER program (Elsevier-BIOSOFT, Cambridge, UK).

**Inhibition Kinetics.** Hydrolysis of \( m \)-nitrophenyl acetate was monitored at pH 8.5 in BTP buffer as stated previously with the appropriate amount of \( n \)-butanol added to the cyclodextrin solution to result in solutions containing 0, 55 and 110 mM \( n \)-butanol (0, 0.05, 0.10 mL respectively).
CHAPTER 2
SYNTHESIS OF 6-DEOXY-6-FORMYL-β-CYCLODEXTRIN
AND DERIVATIVES

The field of cyclodextrin technology has impacted many aspects of pure and applied chemistry. However, in order for cyclodextrin chemistry to continue to expand and develop, the design and synthesis of new cyclodextrin derivatives is essential. Although numerous derivatives have been synthesized, the majority of these compounds result from displacement reactions on the 1°- and 2°- side tosylates of cyclodextrin. Only a few derivatives are the result of reduction or oxidation reactions.

In the case of reduction reactions, the reduction of the 1°- hydroxyl groups of cyclodextrin has been accomplished to yield 6-deoxy-β-cyclodextrin with a range of substitution patterns. These reactions are generally performed via NaBH₄ reduction of the corresponding bromo or iodo derivative in dimethyl sulfoxide. Even though reductions of cyclodextrin are fairly uncommon, there are even fewer examples of oxidations.

OXIDATIONS OF CYCLODEXTRIN

The oxidation of native cyclodextrin by periodate ion or Ce(IV) occurs on the 2°- side of the cyclodextrin rim. In this process, the 2°- side diol is cleaved to form the 2°- side
dialdehyde. The dialdehyde prepared in this manner has been utilized extensively in biological applications. Owing to the dialdehyde’s ability to form Schiff bases with free amine groups in an enzyme, it has been utilized to inhibit the reactivity of several enzymes.\(^{15}\)

Oxidation of the \(1^\circ\)-side of cyclodextrin has been achieved by varied processes to yield oxidized derivatives of different substitution patterns. Complete oxidation of the \(1^\circ\)-side hydroxyl groups of \(\beta\)-cyclodextrin, yielding the hepta-substituted carboxy derivative, has been reported via oxidation by \(O_2 / Pt\) \(^{36}\) and \(N_2O_4\).\(^{36,37}\) However, only in the latter case was the product characterized. The hepta-substituted formyl derivative of \(\beta\)-cyclodextrin was also synthesized by photolysis of the corresponding hepta-azide derivative.\(^{37}\)

Selective oxidation of a single \(1^\circ\)-hydroxyl group has been achieved, in one case, by reaction with periodinane.\(^{38}\) However, this procedure was performed on a \(\beta\)-cyclodextrin derivative in which all of the \(2^\circ\)-hydroxyl groups and six of the \(1^\circ\)-hydroxyl groups were protected by methoxy moieties. Additionally, Weselake and Hill have reported the synthesis of \([{}^3H\] \(\beta\)-cyclodextrin by the oxidation of \(\beta\)-cyclodextrin at the C-6 position by Cr(VI) oxide followed by reduction with sodium borotritide.\(^{39}\) Although the intermediates were not isolated, the oxidation was found to proceed, at least partially, past the aldehyde stage to the carboxylic acid.

A more synthetically useful preparation of a monoformyl derivative of cyclodextrin, 6-deoxy-6-formyl-\(\alpha\)-cyclodextrin (11), was reported by Gibson \textit{et al.} via two pathways (Scheme 6).\(^{40}\) Both pathways involved conversion of \(\alpha\)-cyclodextrin to 6-deoxy-6-tosyl-\(\alpha\)-cyclodextrin followed by displacement of the tosyl group with \(\text{NaN}_3\). In the first pathway, the 6-deoxy-6-azido-\(\alpha\)-cyclodextrin was then photolyzed to yield an impure sample of the
Scheme 6. Synthesis of 6-deoxy-6-formyl-α-cyclodextrin (11) via two pathways from α-cyclodextrin (αCD).
corresponding monoaldehyde in an unreported yield. The second pathway involved
conversion of the 6-deoxy-6-azido-α-cyclodextrin to the corresponding amine by reduction
with palladium black. The amine was then oxidized by ninhydrin to the mono-aldehyde in
approximately a 60% yield from the amine. The disadvantage to each of these procedures
is the fact that there are three or more steps required in the synthetic route. This allows for
a large amount of product loss due to purification of intermediate cyclodextrin species.
Therefore a more facile synthetic route for the oxidation of cyclodextrin to the monoaldehyde
is desirable.

**DERIVATIZATION OF ALDEHYDES**

The aldehyde functionality opens the door to the synthesis of a wide variety of new
derivatives due to its versatile reactivity. Aldehydes are subject to the addition of numerous
nucleophiles to the carbonyl group.\(^{41}\) Bisulfites and cyanides are capable of addition to the
carbonyl group to form sulfoxides and products containing new carbon-carbon bonds.
Similarly, amines have the ability to add to aldehydes to yield simple imines. This reaction
can be exploited further by reductive amination of the imine to form new amine derivatives.
Additionally, \(\alpha\)-nucleophiles, such as hydroxylamine and hydrazine, react readily with
aldehydes to yield oximes and hydrazones, respectively. Aside from addition reactions,
further oxidation of the aldehyde itself can lead to the formation of a carboxylic acid. Thus,
the synthesis of the aldehyde can then be considered only the first step toward the synthesis
of a broad spectrum of new cyclodextrin derivatives.
ALDEHYDES AS ENZYME MIMICS

The aldehyde functionality, predominantly in the form of the hydrate, has been shown to be an efficient and promising catalyst in transacylation reactions. As a demonstration of the proficiency of an aldehyde in this process, Menger and Whitesell developed a micellar enzyme mimic with an aldehyde functionality on the surface of the micelle (Figure 16).\(^\text{42}\) It was shown that the acylation of \(p\)-nitrophenyl diphenyl phosphate was accelerated 1800-fold in the presence of the aldehydic micelle over the uncatalyzed reaction in buffer at pH 9. This ability of an aldehyde to act as a superior catalyst, in the form of the hydrate, compared to a typical alcohol is twofold. First the hydrate has a lower \(pK_a\) (ca. 10 to 11) than an isomeric alcohol. This allows for anion formation to occur to a significant extent at a lower pH compared to the alcohol. Secondly, and more importantly, the hydrate is capable of rapid deacylation assisted by the second hydroxyl group of the hydrate (Scheme 7). Reformation of the aldehyde functionality in this manner allows for turnover capabilities and true catalytic behavior. This exhibition of "true catalysis" is not shown by most enzyme mimics based on cyclodextrin. Therefore, an aldehyde functionality directly attached to the cyclodextrin cavity has the potential to lead to an enzyme mimic capable of performing transacylation reactions with catalytic turnover.

![Figure 16. Micellar enzyme mimic with aldehyde functionality.](image)

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Scheme 7. Catalytic cycle for the hydrolysis of an ester by an aldehyde in the form of its hydrate.
**MONOALDEHYDE OF β-CYCLODEXTRIN**

The goal of this project was to develop a simple synthesis of the monosubstituted, 1°-side aldehyde of β-cyclodextrin. After development of a successful synthetic route, the cyclodextrinyl aldehyde was studied for its potential to act as an enzyme mimic in transacylation reactions. Finally, the utility of the monoaldehyde of β-cyclodextrin as a stepping stone to a wide variety of new cyclodextrin derivatives via derivatization of the aldehyde functionality was explored.

**SYNTHESIS OF 6-DEOXY-6-FORMYL-β-CYCLODEXTRIN**

There are many known methods of synthesizing aldehydes from alcohols utilizing dimethyl sulfoxide (DMSO) oxidations. In some instances, an alcohol can be converted directly into an aldehyde in a single step process. Therefore, the initial attempt at synthesizing the cyclodextrinyl aldehyde was the direct oxidation of native β-cyclodextrin with DMSO; however no product formation was observed under these conditions.

The second attempt at formation of the aldehyde was via oxidation of the β-cyclodextrin monotosylate (12). DMSO oxidation of a tosylate occurs by the initial displacement of the tosyl group by DMSO. This is followed by the loss of dimethyl sulfide with simultaneous formation of the carbon-oxygen double bond of the aldehyde, often assisted by the addition of base (Scheme 8). This pathway was advantageous since the 1°-side tosylate of β-cyclodextrin can be readily prepared in one step and easily purified.
Scheme 8. Typical aldehyde synthesis by DMSO oxidation of the corresponding tosylate in the presence of base.
The direct conversion of the β-cyclodextrin monotosylate into an aldehyde was found to occur by heating a solution of the tosylate in neat DMSO at 135°C. This conversion was followed by $^1$H NMR over a 3 hour time period. The half-life of the reaction was approximately 15 minutes based on the disappearance of the tosylate doublets at 7.4 and 7.7 ppm. This disappearance was observed with the concomitant appearance of new doublets at 7.1 and 7.5 ppm corresponding to tosic acid and a singlet at 9.7 ppm corresponding to an aldehyde signal. After 70 minutes at 135°C, additional signals in the aldehyde region of the NMR spectrum began to appear, even though a small amount of the tosylate was still present. Since native β-cyclodextrin was inert under these reaction conditions, these additional aldehyde signals were attributed to the decomposition of the aldehyde initially formed in the reaction. This decomposition was believed to be catalyzed by the tosic acid formed as a by-product under the high temperatures of the reaction conditions.

In order to reduce the decomposition of the aldehyde, a non-nucleophilic base was added to the solution to neutralize the tosic acid formed during the reaction. In addition, the base was expected to aid in the elimination of CH$_3$SCH$_3$ from the intermediate. The non-nucleophilic base initially employed in this capacity was NaHCO$_3$. A $^1$H NMR study of the reaction in the presence of NaHCO$_3$ showed that the base did, in fact, inhibit the acid catalyzed decomposition, but did not have a large effect on the rate of the reaction. Although serving its intended purpose, there were some difficulties in utilizing NaHCO$_3$ as the base in this process. First, the solubility of NaHCO$_3$ in DMSO is limited. More importantly, since sodium salts are known to complex in the cyclodextrin cavity, there was some concern as to the complete removal of the sodium salts during the purification process. For these
reasons, collidine was thereafter employed as the non-nucleophilic base to avoid both of these limitations. The conversion of primary tosylates (or halides) utilizing DMSO and collidine is known as the Nace reaction. 

On a preparative scale, the β-cyclodextrin monotosylate (12) could be converted to the 1°-side aldehyde, 6-deoxy-6-formyl-β-cyclodextrin (13; βCDCHO), by heating the tosylate at 135°C for 1.5 hours in DMSO in the presence of a 10 molar excess of collidine (Scheme 9). The aldehyde can then be isolated in a 65% yield from 12 by precipitating the solution from acetone to remove the DMSO, followed by a second precipitation from ethanol. The aldehyde has resisted purification by recrystallization and appears to contain only β-cyclodextrin as a minor impurity as determined from the ¹H NMR integrations. Not surprisingly, the aldehyde has similar solubility characteristics as native β-cyclodextrin.

βCDCHO was fully characterized. Its ¹H NMR spectrum in DMSO-d₆ (Figure 17) shows relevant signals at 9.7 ppm (CHO), 4.2 ppm (CHCHO) and typical signals corresponding to β-cyclodextrin. The ¹³C NMR spectrum in DMSO-d₆ of βCDCHO (Figure 17) also displays an aldehyde signal at 198 ppm. Mass spectral analysis of the sample shows a molecular ion peak at m/z 1133. Characterization of βCDCHO was further confirmed by tests indicative of the aldehyde functionality including a positive test with Tollen’s reagent and 2,4-dinitrophenylhydrazine.

As added proof of structure, the aldehyde was reduced with NaBH₄ to native β-cyclodextrin. Since β-cyclodextrin is a non-reducing sugar, the framework of the cyclodextrin was not effected by this reduction process. βCDCHO was reduced by reaction with NaBH₄ for one day followed by purification with ion exchange chromatography to
Scheme 9. Synthesis of 6-deoxy-6-formyl-\(\beta\)-cyclodextrin (13; \(\beta\)CDCHO) from \(\beta\)-cyclodextrin (8; \(\beta\)CD) via the 1°-side tosylate (12) and DMSO oxidation.
Figure 17. $^1$H NMR and $^{13}$C NMR spectra of 6-deoxy-6-formyl-$\beta$-cyclodextrin (\textit{\textalpha}CDCHO) in DMSO-d$_6$.
remove borate salts. The resulting product was identical to an authentic sample of β-cyclodextrin by TLC, NMR and FAB mass spectrometry.

6-DEOXY-6-FORMYL-β-CYCLODEXTRIN AS A POTENTIAL ENZYME MIMIC

One of the intended goals of this project was to exploit βCDCHO as an enzyme mimic. For βCDCHO to function effectively in this capacity, the aldehyde should preferentially exist in the form of the hydrate in aqueous solution. It is known that aldehydes which are electron deficient exist preferentially in this form. This is likewise true in the case of carbohydrate aldehydes, due to the electron deficient nature of the glucose ring.

The preference for βCDCHO to exist in the form of the covalent hydrate or in the form of an aldehyde was studied by 1H NMR in both D2O and DMSO-d6 solutions. In D2O, the aldehyde exists exclusively as the covalent hydrate by our detection limits, indicated by a broad singlet at 5.2 ppm that corresponds to the proton on the covalent hydrate carbon (Figure 18). However, in DMSO-d6, the aldehyde can exist in either form depending on the amount of water present in the DMSO solution. With little or no water, the aldehydic proton can be observed at 9.7 ppm and the proton α to the aldehyde can also be distinguished from the typical cyclodextrin protons as a doublet at 4.2 ppm (Figure 17). In contrast, with a small amount of water added to the solution, the aldehyde converts back to the covalent hydrate form (Figure 19). This can be seen in the appearance of a triplet at 5.1 ppm that corresponds to the proton on the carbon of the covalent hydrate. The triplet splitting pattern can be explained by the coupling of this proton to the hydroxyl protons of the covalent hydrate that
Figure 18. $^1$H NMR spectrum of 6-deoxy-6-formyl-β-cyclodextrin ($\beta$CDCHO) in D$_2$O.
Figure 19. $^1$H NMR spectra of 6-deoxy-6-formyl-$\beta$-cyclodextrin (BCDCHO) in DMSO-$d_6$ with a small amount of water present and in DMSO-$d_6$ after $D_2O$ exchange.
appear as a set of doublets centered at 5.5 ppm. As expected, deuterium exchange of the hydroxyl groups causes the triplet to collapse into a singlet (Figure 19).

From these observations, it was concluded that the aldehyde exists in the form of the covalent hydrate in aqueous solution or in non-aqueous solvent in the presence of an adequate supply of water. In addition, the NMR study demonstrated that the covalent hydrate was formed by the attack of solvent water on the aldehyde functionality. This is in contrast to the possible formation of a covalent "hydrate" via internal hemiacetal formation with one of the various hydroxyl groups on the cyclodextrin rim. In the case of internal covalent hydrate formation, the presence of only a single doublet due to the hydroxyl group of the covalent hydrate would have been observed.

In addition to forming the covalent hydrate, βCDCHO must be stable in aqueous solution to function as an enzyme mimic. The stability of the aldehyde was studied by 1H NMR in D2O under acidic (pH 2), neutral (pH 7) and basic (pH 12) conditions. The aldehyde remained stable in both acidic and neutral solutions for a period of at least one week as evidenced by no change in the 1H NMR spectra of these solutions. In basic solution however, the aldehyde rapidly decomposed, with decomposition occurring even after only one hour. Upon decomposition, the solution acquired a yellow color suggestive of the formation of unsaturated functional groups. It is believed that this decomposition is caused by the base catalyzed deprotonation of the α-proton of the aldehyde. Upon anion formation, β-elimination can occur to form an α,β-unsaturated aldehyde with concomitant cleavage of the α (1→4) linkage between glucose units (Scheme 10). This would naturally lead to the degradation of the cyclodextrin cylindrical framework. This type of degradation is known
Scheme 10. Proposed decomposition pathway of 6-deoxy-6-formyl-β-cyclodextrin (βCDCHO) under basic conditions.
and has been shown to occur in other 6-deoxy-6-formyl carbohydrate derivatives. The fact that the aldehyde exhibits this decomposition in basic solution precludes its use as an enzyme mimic since the aldehyde group should only show significant reactivity at higher pH levels where deprotonation of the hydroxyl groups of the hydrate occurs.

**ADDICTION OF SIMPLE NUCLEOPHILES TO βCDCHO**

Although the aldehyde did not appear to be ideal for utilization as an enzyme mimic, the aldehyde still possessed the potential to function as an intermediate in the synthesis of many new cyclodextrin derivatives. The first reaction of βCDCHO to be examined in this capacity was the addition of sodium bisulfite to the aldehyde carbonyl to yield the 6-sulfo-β-cyclodextrin sodium salt (14). This conversion occurred rapidly simply on the addition of excess NaHSO₃ to an aqueous solution of βCDCHO (Scheme 11). Precipitation of the cyclodextrins by addition of the aqueous solution to 2-propanol led to the isolation of a sample which contained 14 and some inorganic salts. The salts were removed by gel filtration column chromatography to yield a sample which exhibited approximately 70% conversion of the βCDCHO to 14 by microanalysis. This coincided with the fact that residual βCDCHO could also be seen by TLC analysis. The ¹H NMR spectrum in D₂O of the isolated sample showed the loss of the hydrate signal of βCDCHO at 5.2 ppm and the appearance of a new doublet at 4.1 ppm corresponding to the CH(OH)(OSO₂) proton (Figure 20). The ¹³C NMR spectrum showed signals typical for a cyclodextrin derivative (Figure 20).
Scheme 11. Synthesis of 6-sulfo-6-cyclodextrin sodium salt (14) from 6-deoxy-6-formyl-6-cyclodextrin (βCDCHO) in aqueous solution.
Figure 20. $^1$H NMR and $^{13}$C NMR spectra of 6-sulfo-β-cyclodextrin sodium salt (14) in D$_2$O.
The water solubility of 14 was significantly enhanced compared to the solubilities of both βCDCHO and native βCD, presumably due to the inherent charge of the bisulfite adduct. ¹H NMR studies have shown 14 to be stable in aqueous solution under neutral and acidic conditions. The ¹H NMR spectrum of 14 exhibited no change in D₂O alone and in D₂O in the presence of CF₃CO₂H for a period of two weeks. For these reasons, 14 could be utilized to increase the concentration of cyclodextrin compounds in situations where the solubility of native βCD is a limiting factor.

The second attempt at derivatization of βCDCHO was via the addition of potassium cyanide to the aldehyde carbonyl toward the formation of a new carbon-carbon bond. However, each attempt at the addition of KCN to an aqueous solution of βCDCHO led to complete decomposition of the cyclodextrin similar to the decomposition seen in the presence of base. Therefore, the cyanide anion under these circumstances appeared to primarily function as a base rather than as a nucleophile. The addition of KCN to an aqueous solution of the bisulfite adduct also lead to decomposition.

ADDITION OF AMINES TO βCDCHO

Aldehydes are also commonly known to be reactive toward the addition of amines to yield imines. For this reason the reactivity of numerous amines with βCDCHO was examined under various pH conditions. The amines employed in this effort included methyl amine, dimethyl amine, isopropyl amine, benzyl amine and 3,6-diaminoacridine. In basic solution, there was evidence of imine formation due to the appearance of new signals in the downfield region of the ¹H NMR spectrum, between 6 and 8 ppm, possibly corresponding
to the imine proton (\(\text{CHN}R\)). However, the appearance of these new signals was always concurrent with decomposition of the cyclodextrin. Under neutral or acidic conditions, most amines were unreactive. However, the solutions in which a reaction did occur also demonstrated evidence of decomposition.

The observation of imine signals does not necessitate that the aldehyde is reacting directly with the added amines. There are several scenarios which could explain the observation of imine signals in light of the concurrent decomposition. First, \(\beta\text{CDCHO}\) could be susceptible to the addition of amines, but the basic nature inherent in the amines leads to competing decomposition. Alternatively, it is possible that \(\beta\text{CDCHO}\) reacts readily with amines. However, the product imine may be the species that is actually undergoing decomposition. Finally, \(\beta\text{CDCHO}\) could be relatively unreactive toward the addition of the amines. Decomposition of \(\beta\text{CDCHO}\) via a basic mechanism could lead to products containing new aldehyde functionalities that are subsequently reacting to yield imine signals.

It was believed that if \(\beta\text{CDCHO}\) was actually reacting with the amines to form a small amount of the imine then this imine could be trapped via reductive amination. This hypothesis was tested with the aromatic amine, aniline. This amine was chosen not only for its interesting structure, but also since the corresponding imine should exhibit increased stability due to resonance stabilization with the aromatic ring.

The reductive amination of \(\beta\text{CDCHO}\) with aniline in the presence of NaCNBH\(_3\) was attempted at pH 4.5 and 6.0. Product formation was observed at both pHs with no evidence of decomposition, although TLC analysis showed the reaction to occur more rapidly at pH 6.0. This reaction was then performed on a large scale to yield 6-deoxy-6-(N-phenylamino)-
β-cyclodextrin (15; Scheme 12). The isolated product was purified from residual βCDCHO by reversed phase MPLC eluting with an increasing methanol gradient in water. The 

$^1$H and $^{13}$C NMR spectra exhibited the expected aromatic and β-cyclodextrin signals (Figure 21). Mass spectral analysis showed a molecular ion peak with $m/z$ 1211. This molecular ion peak confirmed the isolated product to be the desired compound and not a simple inclusion complex between aniline and βCDCHO which would exhibit a molecular ion peak with $m/z$ 1226.

The placement of a large aromatic moiety on the rim of the cyclodextrin alters the solubility characteristics of the compound. Unlike native βCD, 15 was found to be soluble in methanol. The formation of 15 is significant since it demonstrates that amines are capable of addition to the aldehyde and that this process can be successfully exploited by reductive amination. This pathway could potentially be used with a wide range of amines to produce new, structurally significant enzyme mimics of β-cyclodextrin.

**ADDITION OF α-NUCLEOPHILES TO βCDCHO**

It was hypothesized that stronger nucleophiles, such as α-nucleophiles (which are also typically weak bases), might react with the aldehyde carbonyl without competing base-catalyzed decomposition as seen in the case of amines. Furthermore, it is known that aldehydes are highly susceptible to the addition of both hydroxylamine and hydrazine to form the corresponding oxime and hydrazone, respectively.

The addition of a 50% aqueous solution of NH$_2$OH to a solution of βCDCHO resulted in the formation of cis- and trans-6-deoxy-6-oxo-β-cyclodextrin oxime (16; Scheme
Scheme 12. Synthesis of 6-deoxy-6-(N-phenylamino)-β-cyclodextrin (15) by reductive amination of 6-deoxy-6-formyl-β-cyclodextrin (βCDCHO) and aniline.
Figure 21. $^1$H NMR and $^{13}$C NMR spectra of 6-deoxy-6-(N-phenylamino)-β-cyclodextrin (15) in DMSO-$d_6$. 

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13). This oxime is a known compound, previously synthesized by David Winn of the Czarnik research group by air oxidation of the 1°- side hydroxylamine. In order to show that the sample isolated by precipitation was free of residual hydroxylamine, acetone was added to a 1H NMR sample of 16 in D2O. Acetone is known to react with free hydroxylamine to form a hydrazone and similarly to react with the hydroxyl group of the hydroxylamine moiety. Upon addition of acetone, the absence of new 1H NMR signals, between 1 and 2 ppm corresponding to the formation of the hydrazone of acetone, confirmed that no free hydroxylamine was present in the sample.

The 1H NMR spectrum (DMSO-d6) of 16 showed two doublets at 7.2 and 6.7 ppm corresponding to the CHNOH proton signal of the trans and cis isomers of the oxime, respectively (Figure 22). It was observed that the isomers were not formed in equal amounts but that the trans isomer formed to a larger extent based on NMR integrations. This conclusion is based on the fact that in the 1H NMR spectrum of an oxime, the trans isomer generally resonates at a lower field than the cis isomer. This distinction between isomers was not observed in the 13C NMR spectrum, however. Only one carbon signal at 147 ppm was exhibited for the two isomers (Figure 22). The two carbon signals may in fact be unresolved or, more likely, the signal for the minor cis isomer may be lost in the noise.

Once it was confirmed that hydroxylamine was reactive toward βCDCHO, the addition of another α-nucleophile, hydrazine, to βCDCHO was explored. Hydrazine hydrate was added to an aqueous sample of βCDCHO to yield the corresponding hydrazone, 6-deoxy-β-cyclodextrin hydrazone (17; Scheme 14). The formation of 17 was evidenced by the appearance of a new signal in the 1H NMR spectrum at 7.1 ppm corresponding to the
Scheme 13. Synthesis of \textit{trans}- and \textit{cis}-6-deoxy-6-oxo-\(\beta\)-cyclodextrin oxime (16) from 6-deoxy-6-formyl-\(\beta\)-cyclodextrin (\(\beta\)CDCHO) in aqueous solution.
Figure 22. $^1$H NMR and $^{13}$C NMR spectra of 6-deoxy-6-oxo-β-cyclodextrin oxime (16) in DMSO-d$_6$. 
CHNNH$_2$ proton of the hydrazone and a signal in the $^{13}$C NMR spectrum at 138 ppm corresponding to the carbon of the hydrazone (Figure 23).

Once it was confirmed that the hydrazone was being formed, the purity of the sample was tested. The sample, isolated by precipitation from 2-propanol, was analyzed for contamination by free hydrazine by the addition of acetone to a $^1$H NMR sample of the compound in D$_2$O. Similar to the reaction with hydroxylamine, hydrazine will form an azine with acetone in D$_2$O solution. The absence of proton signals between 1.8 and 1.6 ppm signified that no free hydrazine was present in the sample.

Although residual hydrazine had been removed, βCDCHO was observed to be persistent in the sample as evidenced by TLC analysis. Microanalysis subsequently confirmed this observation and suggested a 75% conversion of βCDCHO to 17. The incomplete conversion to the hydrazone was partially explained by a $^1$H NMR study of the isolated sample. A solution of the sample in D$_2$O demonstrated that the hydrazone was capable of reverting back into the aldehyde in aqueous solution. This was shown by concomitant disappearance of the proton signal at 7.1 ppm corresponding to 17 with an increase in the proton signal at 5.2 ppm corresponding to βCDCHO. Since 17 is in equilibrium with βCDCHO, the conversion to 17 in aqueous solution could be expected to be incomplete.

Since hydrazine consists of two reactive moieties, the possibility existed of multiple reactions with cyclodextrin to produce a bis-cyclodextrinyl azine (18; Figure 24). However, there was no evidence for the formation of 18 in this system. This was confirmed by the presence of a $^1$H NMR (DMSO-d$_6$) signal at 6.4 ppm (Figure 25). This D 9
Figure 23. $^1$H NMR spectrum in D$_2$O and $^{13}$C NMR spectrum in DMSO-d$_6$ of 6-deoxy-$\beta$-cyclodextrin hydrazone (17).
Figure 25. $^1$H NMR spectrum of 6-deoxy-β-cyclodextrin hydrazone (17) in DMSO-$d_6$. 
exchangeable signal was believed to correspond to the protons of the free \( \text{NH}_2 \) group of the hydrazone. Formation of 18 may be unfavorable as shown by an additional \(^1\text{H} \) NMR study. A \(^1\text{H} \) NMR sample of 17 and acetone in \( \text{D}_2\text{O} \) was studied over time. The azine formed by the reaction of 17 and acetone appeared to be significantly more unstable than 17 itself and demonstrated more rapid conversion back to \( \beta\text{CDCHO} \). Therefore, any 18 initially formed in solution would be highly subject to a breakdown of this fashion.

![Figure 24](image)

**Figure 24.** The hypothetical bis-cyclodextrinyl azine (18).

The reaction of \( \beta\text{CDCHO} \) was also attempted with a different hydrazine, 2,4-dinitrophenylhydrazine. A reaction between the two species was already known to occur from the qualitative tests initially performed on \( \beta\text{CDCHO} \) (Scheme 15). The reaction of \( \beta\text{CDCHO} \) and 2,4-dinitrophenylhydrazine in 2 N HCl solution was repeated on a large scale to yield the hydrazone, 6-deoxy-6-(2,4-dinitrophenyl)-\( \beta \)-cyclodextrin hydrazone (19).

The sample was isolated by precipitation from a large quantity of acetone. During isolation, excess 2,4-dinitrophenylhydrazine reacted with the acetone, subsequently dissolved in the acetone and was removed. The hydrazone was further purified from residual \( \beta\text{CDCHO} \) and other unidentified aromatic impurities by reversed phase MPLC eluting with
Scheme 15. Synthesis of 6-deoxy-6-(2,4-dinitrophenyl)-β-cyclodextrin hydrazone (19) from 6-deoxy-6-formyl-β-cyclodextrin (βCDCHO).
an increasing methanol gradient. It was necessary to perform the column purification twice in order to satisfactorily separate the desired product from all impurities.

The $^1$H NMR spectrum of 19 exhibited both aromatic signals and signals consistent with $\beta$-cyclodextrin (Figure 26). Although enough sample was not obtained to further characterize the compound by microanalysis, FAB mass spectral analysis displayed a signal at $m/z$ 1314 corresponding to the molecular ion of the hydrazone. Similar to the $1^\circ$-side tosylate of $\beta$-cyclodextrin, 19 was only partially water soluble due to the hydrophobic aromatic moiety. Also distinguishing this compound from typical cyclodextrin compounds was the hydrazone's bright yellow color. The formation of 17 and 19 has shown conclusively that $\beta$CDCHO is reactive toward the addition of hydrazine itself and derivatives of hydrazine.

**FORMATION OF A BIS-CYCLODEXTRINYL HYDRAZONE**

A further extension of the aldehyde and hydrazine reaction lead to the formation of a bis-cyclodextrin compound by reaction of $\beta$CDCHO with 6-deoxy-6-hydrazino-$\beta$-cyclodextrin, a known compound (20; Scheme 16). This reaction was performed under argon with freshly prepared 20 due to the tendency of 20 to decompose over time. The formation of the bis-cyclodextrin, 6-deoxy-6-(6-deoxy-$\beta$-cyclodextrinyl)-$\beta$-cyclodextrin hydrazone (21), was evidenced by $^1$H NMR from the appearance of a doublet at 6.9 ppm corresponding to the HCN proton (Figure 27). In addition, mass spectral analysis of the product gave the correct molecular ion peak with $m/z$ 2264.
Figure 26. $^1$H NMR spectrum of 6-deoxy-6-(2,4-dinitrophenyl)-β-cyclodextrin hydrazone (19) in D$_2$O.
Scheme 16. Synthesis of 6-deoxy-6-(6-deoxy-β-cyclodextrinyl)-β-cyclodextrin hydrazone (21) from 6-deoxy-6-formyl-β-cyclodextrin (βCDCHO) and 6-deoxy-6-hyrazino-β-cyclodextrin (20) in aqueous solution.
Figure 27. $^1$H NMR spectrum of 6-deoxy-6-(6-deoxy-β-cyclodextrinyl)-β-cyclodextrin hydrazone (21) in D$_2$O.
Bis-cyclodextrin 21 was found to be impure with formation of 21 occurring to approximately 50% by NMR integrations. Purification proved difficult due to the nature of the contaminates which consisted of excess βCDCHO, 20 and the decomposition product of 20. Attempts at purification by recrystallization and reversed phase column chromatography proved unsuccessful. Although this sample of 21 was not analytically pure, it was still worthwhile to study due to the fact that this compound contained two cyclodextrin binding sites in close proximity. Therefore this compound is potentially capable of binding molecules that contain multiple ligands.

The complexation between 21 and a water insoluble compound containing two ligand sites, 1,4-diphenyl-1,3-butadiyne (22) was initially examined (Figure 28). An aqueous solution of 21 in contact with 22 in the solid form was able to solubilize 22 in water. This was shown by monitoring the UV absorbance of 22 at 330 nm over time (Figure 29). Compound 22 alone exhibited no solubility in aqueous solution. In addition, solutions of βCDCHO and 20 exhibited no ability to increase the solubility of 22. Therefore the second binding site of the bis-cyclodextrin 21 was crucial for adequate complexation to occur.

![Figure 28. Complexation of 1,4-diphenyl-1,3-butadiyne 22 with 21.](image-url)
Figure 29. The absorbance over time of 1,4-diphenyl-1,3-butadiyne (22) in aqueous solution in the presence of no added host (+), βCDCHO (■), 20 (▲) and 21 (●).
Water soluble compounds such as dipeptides with multiple ligands were also examined for complexation with 21. Phe-Phe, Tyr-Tyr and Trp-Trp all showed $^1$H NMR peak shifts upon the addition of 21 to the solution that are indicative of a binding process.

It was hoped that the complexation between 21 and various ligands could be exploited in the actual formation of the bis-cyclodextrin compound. Utilizing these ligands as templates, the formation of 21 could possibly be enhanced in a similar fashion as antigens enhance the formation of antibodies. Enhancing the formation of 21 could then possibly lead to more successful purification processes and the design of an artificial antibody system.

In order to study the effectiveness of these ligands as templates, it was necessary to be able to accurately monitor the rate of formation of 21. Although numerous methods were explored (including UV, $^1$H NMR and reaction with ninhydrin), no method was found in which all complications could be avoided. One of the major obstacles in these studies was that the quantitative measurement of the concentration of 21 had to be performed in the presence of both aldehyde and amine functionalities. Although in some cases the templates qualitatively appeared to affect the formation of 21 to a small extent, no quantitative data could be obtained for these processes.

**CONCLUSION**

The synthesis of 6-deoxy-6-formyl-β-cyclodextrin has been accomplished using a simple two step process from β-cyclodextrin involving the Nace DMSO oxidation. This compound was shown to be stable under both neutral and acidic conditions. In basic solution, it was susceptible to rapid decomposition thereby precluding its use as an enzyme
mimic under these conditions. The aldehyde functionality has shown wide reactivity with various nucleophiles leading to the synthesis of many new cyclodextrin derivatives. In addition, Juyoung Yoon of the Czarnik research group has utilized the aldehyde for further oxidation to carboxylic acid derivatives of $\beta$-cyclodextrin. The synthesis of this derivative, along with the other new compounds, establishes synthetic procedures that can be utilized for the formation of specifically designed cyclodextrin derivatives in the future. It has been shown that 6-deoxy-6-formyl-$\beta$-cyclodextrin is indeed a stepping stone into the future of cyclodextrin chemistry.

**EXPERIMENTAL**

**General.** Mass spectra were obtained by use of a Kratos-30 mass spectrometer with fast atom bombardment (FAB). UV data were obtained on a Hewlett-Packard 8452A diode array spectrophotometer. Reversed phase MPLC column (Lobar Lichroprep RP-18, 310 mm $\times$ 25 mm, 40-63 $\mu$m) was obtained from EM Separations Technology (Gibbstown, NJ). Elemental analyses were carried out at Galbraith Laboratories, Inc. (Norcross, GA). Most of the chemicals used in this study were obtained from Aldrich Chemical (Milwaukee, WI). Thin layer chromatography was performed on aluminum-backed silica gel plates and cyclodextrin compounds were visualized by charring after staining with $p$-anisaldehyde stain: $p$-anisaldehyde/methanol/acetic acid/sulfuric acid (1:200:20:10). Elution was accomplished with a solvent system of $n$-butanol/ethanol/H$_2$O (5:4:3; v/v).

**6-O-(p-Toluenesulfonyl)-$\beta$-cyclodextrin (12).** $\beta$-Cyclodextrin (8; 20.0 g, 17.6 mmol) was dissolved in pyridine (350 mL) and the solution cooled in an ice bath. A solution
of p-toluenesulfonyl chloride (8.50 g, 44.6 mmol, 2.5-fold excess) in pyridine (150 mL) was added dropwise to the cooled solution. The solution was stirred at rt overnight. H₂O (25 mL) was added and the solution stirred for 1 h. Solvent was removed in vacuo with warming to yield a thick, yellow oil. The oil was suspended in H₂O (300 mL) and a colorless solid collected by vacuum filtration. The solid was recrystallized from hot water to yield the desired product (4.7 g, 21%): Rf 0.68; ¹H NMR (DMSO-d₆) δ 7.73 (d, 2, PhH), 7.41 (d, 2, PhH), 6.00-5.50 (m, 14, OH), 4.88 (d, 6, H-1), 4.77 (d, 1, H-1'), 4.61-4.07 (m, 7, 1° OH), 3.88-3.05 (m, CD, HDO), 2.41 (s, 3, CH₃).

6-Deoxy-6-formyl-β-cyclodextrin (13). 6-O-(p-Toluenesulfonyl)-β-cyclodextrin (12; 1.0 g, 0.78 mmol) was dissolved in DMSO (10 mL). Collidine (1 mL, 7.6 mmol, 10 molar excess) was added and the yellow solution heated at 135°C for 1.5 h. The resulting dark brown solution was added dropwise to acetone (125 mL) and the precipitate collected under vacuum. The solid was dissolved in H₂O (10 mL) with warming and the solution added dropwise to ethanol (95%, 125 mL). The colorless precipitate was collected under vacuum to afford 13 (570 mg, 65%): Rf 0.36; ¹H NMR (D₂O) δ 5.23 (s, 1, CH(OH)₂), 4.90 (s, 7, H-1), 4.00-3.54 (m, 28, CD), 3.54-3.30 (m, 14, CD); ¹H NMR (DMSO-d₆) (NB: This contains both the aldehyde and the covalent hydrate form of the compound) δ 9.70 (s, 1, CHO), 5.97-5.55 (m, 14, 2° OH), 5.52 (d, 1, CH(OH)₂), 5.48 (d, 1, CH(OH)₂), 5.11 (t, 1, CH(OH)₂), 4.99-4.90 (d, 1), 4.90-4.69 (br d, 6, H-1), 4.60-4.42 (m, 6, 1° OH), 4.18 (d, 1, CHCHO), 3.93-3.08 (m, CD, HDO); ¹³C NMR (DMSO-d₆) δ 198.3, 101.9, 87.5, 81.6, 73.0, 72.4, 72.0, 59.9; FAB mass spectrum m/z 1133.49 (M⁺).

**β-Cyclodextrin (8).** 6-Deoxy-6-formyl-β-cyclodextrin (13; 51 mg, 0.045 mmol) was dissolved in H₂O (10 mL). NaBH₄ (26 mg, 0.69 mmol) was added and the solution stirred at rt for 1 d. The pH of the solution was adjusted to ca. 4 with acetic acid. The solution was purified from borate salts by elution with water on a Dowex HCR-W2 resin (8.9 × 5.7 cm). The appropriate fractions were pooled and lyophilized to afford a colorless solid (37 mg, 72%). The sample was identical to an authentic sample of βCD as determined by TLC, ¹H NMR, ¹³C NMR and FAB mass spectrometry.

**6-Sulfo-β-cyclodextrin sodium salt (14).** 6-Deoxy-6-formyl-β-cyclodextrin (13; 150 mg, 0.132 mmol) was suspended in H₂O (15 mL). NaHSO₃ (19 mg, 0.18 mmol) was added and the solution was stirred at rt for 3 d. The volume was reduced to ca. 2 mL by *in vacuo* solvent removal. The solution was added dropwise to 2-propanol (200 mL) and refrigerated overnight. A colorless solid was collected under vacuum (113 mg, 69%). The sample was purified by gel filtration chromatography (Sephadex G-25, 6.0 × 2.2 cm) eluting with water. Appropriate fractions were pooled and lyophilized to yield a colorless solid (92% recovery) containing approximately 70% 14 as evidenced by microanalysis: Rₜ 0.29; ¹H NMR (D₂O) δ 4.89 (s, 7, H-1), 4.13 (d, 1, CHOH(OSO₂)), 3.97-3.53 (m, 28, CD), 3.53-3.22 (m, 14, CD); ¹³C NMR (D₂O) δ 105.3, 104.8, 104.7, 104.3, 84.9, 84.0, 83.9, 83.6, 83.3, 75.9, 75.7, 75.0, 74.9, 74.7, 74.5, 73.2, 63.2, 62.7; FAB mass spectrum *m/z* 1237.36 (M⁺).

Anal. Calcd for C₄₂H₉₆NaO₉₈S • 8 H₂O: C, 36.52; H, 6.20; Na, 1.66; S, 2.32. Found: C, 36.25; H, 6.04; Na, 1.46; S, 1.69.

**6-Deoxy-6-(N-phenylamino)-β-cyclodextrin (15).** 6-Deoxy-6-formyl-β-cyclodextrin (13; 203 mg, 0.179 mmol) was placed in H₂O (20 mL) and aniline (0.20 mL,
2.1 mmol) was added. The pH of the solution was adjusted to 6.0 with dilute HCl. NaCNBH₃ (1.0 M in THF, 0.50 mL, 0.50 mmol) was added and the solution stirred at rt for 6 d. The solution was added dropwise to acetone (250 mL). After refrigeration for 1 h, the colorless precipitate was collected under vacuum and rinsed with acetone (3 x 50 mL) to yield a colorless solid (191 mg, 88%). Purification was accomplished utilizing reversed phase MPLC, eluting with an increasing gradient of methanol to H₂O from 0 to 30% with a 34% recovery: Rf 0.43; ¹H NMR (DMSO-d₆) δ 7.00 (t, 2, PhH), 6.59 (d, 2, PhH), 6.49 (t, 1, PhH), 6.05-5.50 (m, 14, 2° OH), 5.21 (br t, 1, NH), 4.97-4.75 (m, 7, H-1), 4.72-4.30 (br m, 6, 1° OH), 3.90-3.05 (m, CD, HDO); ¹³C NMR (DMSO-d₆) δ 148.7, 128.8, 112.4, 102.1, 81.7, 72.6, 72.2, 59.9; FAB mass spectrum m/z 1210.54 (M⁺).

Anal. Calcd for C₆₈H₁₄₂N₅O₄⁸H₂O: C, 42.57; H, 6.77; N, 1.03. Found: C, 42.66; H, 6.72; N, 1.02.

6-Deoxy-6-oxo-β-cyclodextrin oxime (16). 6-Deoxy-6-formyl-β-cyclodextrin (13; 107 mg, 0.0944 mmol) was dissolved in a 50% aqueous solution of NH₂OH (10 mL) and stirred at rt for 4 h. The solution was added dropwise to ethanol (95%, 200 mL) and refrigerated for 5 h. The colorless precipitate was collected under vacuum to yield 16 (46 mg, 43%). The compound was compared to an authentic sample of the oxime as previously prepared in our laboratory: Rf 0.24; ¹H NMR (DMSO-d₆) δ 7.23 (d, 0.8, trans-CHNOH), 6.67 (d, 0.2, cis-CHNOH), 5.97-5.57 (m, 14, 2° OH), 5.54 (br s, 1, NOH), 5.02-4.73 (m, 7, H-1), 4.62-4.42 (m, 6, 1° OH), 4.10 (t, 1, CH₃CHNOH), 3.97-3.13 (m, CD, HDO); ¹³C NMR (DMSO-d₆) δ 146.6, 102.1, 101.8, 83.9, 81.4, 73.2, 72.9, 72.6, 72.2, 71.9, 69.3, 68.9, 59.9, 59.6, 58.8; FAB mass spectrum m/z 1148.50 (M⁺).
6-Deoxy-β-cyclodextrin hydrazone (17). 6-Deoxy-6-formyl-β-cyclodextrin (13; 100 mg, 0.0883 mmol) was dissolved in H$_2$O (10 mL). Hydrazine hydrate (3 mL, 0.1 mol) was added and the solution stirred at rt overnight. The solution was added dropwise to 2-propanol (200 mL) and refrigerated for 1 h. The precipitate was collected under vacuum, dissolved in H$_2$O (1.5 mL) and added dropwise to 2-propanol (60 mL). The solution was refrigerated for 1 h and the colorless solid, approximately 75% 17 as determined by microanalysis, collected under vacuum (60 mg, 59%): $R_f$ 0.32; $^1$H NMR (D$_2$O) δ 7.06 (d, 1, CH$_2$NNH$_2$), 5.21 (br s, 1, CH(OH)$_2$), 4.89 (d, 7, H-1), 4.11 (t, 1, CHCHNNH$_2$), 3.95-3.59 (m, 27, CD), 3.59-3.27 (m, 14, CD); $^{13}$C NMR (DMSO-d$_6$) δ 137.6, 102.1, 81.7, 81.4, 73.2, 72.9, 72.6, 72.2, 71.9, 60.0, 59.6; FAB mass spectrum $m/z$ 1147.52 (M$^+$).

Anal. Calcd for C$_{42}$H$_{65}$NO$_{35}$·8H$_2$O: C, 39.04; H, 6.63; N, 1.08. Found: C, 38.92; H, 6.68; N, 0.96.

6-Deoxy-6-(2,4-dinitrophenyl)-β-cyclodextrin hydrazone (19). A saturated solution of 2,4-dinitrophenylhydrazine was prepared by dissolving 2,4-dinitrophenylhydrazine (250 mg) in 2 N HCl (60 mL) and gravity filtered to remove any solid. 6-Deoxy-6-formyl-β-cyclodextrin (13; 200 mg, 0.177 mmol) was dissolved in the 2,4-dinitrophenylhydrazine solution (50 mL). The solution was stirred for 2 h at rt and added dropwise to acetone (450 mL). After refrigeration for 1 h, a yellow solid was collected by vacuum filtration. The solid was rinsed repeatedly with acetone (30 mL) until the filtrate was colorless to yield a bright yellow solid (144 mg; 62%). Purification was accomplished
utilizing two reversed phase MPLC columns, eluting with an increasing gradient of methanol to 
H₂O from 0 to 40% with a combined recovery of 11%: Rₚ 0.51; ¹H NMR (D₂O)  δ  8.84 (d, 1, PhH), 8.00 (dd, 1, PhH), 7.67-7.57 (m, 2, PhH), 5.02-4.85 (m, 7, H-1), 4.36 (dd, 1, CHCHN), 3.90-3.27 (m, 42, CD); FAB mass spectrum m/z 1313.68 (M⁺).

6-Deoxy-6-(6-deoxy-β-cyclodextrinyl)-β-cyclodextrin hydrazone (21). To a 10 mL solution of 6-deoxy-6-formyl-β-cyclodextrin (13; 303 mg, 0.267 mmol) was added 6-deoxy-6-hydrazino-β-cyclodextrin (20; 295 mg, 0.257 mmol). The solution was stirred for 5 d under Ar. The solution was added dropwise to ethanol (95%, 200 mL) and the yellow and white precipitate was collected under vacuum to yield a mixture of 21, 13 and 20 (374 mg, 62%): ¹H NMR (D₂O)  δ  6.88 (d, 1, CHNNH), 5.20 (br s, 1), 5.02-4.77 (m, 14, H-1), 4.08 (t, 1, CHCHNNH), 3.98-3.17 (m, 84, CD); FAB mass spectrum m/z 2264.34 (M⁺ for 21).

Complexation of 1,4-Diphenyl-1,3-butadiyne (22) with 6-Deoxy-6-(6-deoxy-β-cyclodextrinyl)-β-cyclodextrin hydrazone (21). Three aqueous stock solutions of the cyclodextrin compounds of interest (13, 20 and 21) were prepared by dissolving 3 mg of the appropriate cyclodextrin compound in 10 mL solution. These solutions contained 0.3 mM cavity equivalents of the cyclodextrin compound. To 2 mL of stock solution was added solid 1,4-diphenyl-1,3-butadiyne (5 mg). As a control, the 1,4-diphenyl-1,3-butadiyne was also added to 2 mL of distilled water. The insoluble solid remained suspended on the surface of the solutions. The solubilization of 22 into the aqueous solution was followed by monitoring the UV absorbance of 22 at 330 nm over time.
CHAPTER 3
DESIGN AND STUDY OF A FLUORESCENT CHEMOSENSOR FOR
ADENINE NUCLEOTIDES BASED ON β-CYCLODEXTRIN

The design and study of new sensors for biological molecules and ions has become an increasingly important goal in the field of chemosensing. An effective chemosensor must contain a binding site for the species of interest and a method of communication between the sensor and the bound species. Many recent chemosensors incorporate fluorophores in conjunction with a binding site and therefore utilize changes in fluorescence as the method of signal transduction.51

Fluorescence has become such a valuable technique in this area because of its extreme sensitivity.52 Since fluorescence is typically measured against a zero background, fluorescent molecules can often be detected in nanomolar concentrations allowing for the design of very sensitive chemosensors. Fluorescence also allows for specificity in detection considering the fact that only about 10% of all molecules fluoresce. In addition, fluorescence involves the use of two wavelengths, excitation and emission, allowing for the detection of fluorescent molecules even in the presence of other fluorescing species. This can be achieved since molecules which may absorb at the same excitation wavelength could have different emission spectra.
Fluorescent chemosensors have been developed for numerous types of species including ions and neutral molecules. Chemosensors for cations have been the most extensively studied due to the existence of well established methods for the binding of metal ions. However, chemosensors for neutral molecules and anions are more rare. It was the goal of this project to further the area of anion chemosensors by developing a chemosensor for a dinucleotide, adenyl-3',5'-adenosine. This particular anion was chosen as the species of interest as a representative member of the class of compounds known as dinucleotide monophosphates.

In order to create a truly effective chemosensor, several considerations must be taken into account. The chemosensor should bind a particular analyte in the presence of many possible interfering substances. In addition, this binding process should be reversible in order to establish a real-time monitoring assay. It is also desirable that the binding process occur in aqueous solution due to the predominance of aqueous solutions, not only in biological applications, but environmental applications as well. For these specifications, a binding site based on cyclodextrin is optimal. Cyclodextrins are capable of binding a variety of species in their hydrophobic cavity and are known for their water solubility.

CHEMOSENSORS BASED ON CYCLODEXTRIN

Several fluorescent chemosensors have been designed based on the binding ability of cyclodextrin. In the simplest cases, native cyclodextrins have been utilized to change the photolytic properties of a bound substrate. For example, de Silva and Bissell utilized native β-cyclodextrin to protect a derivative of naphthalene from quenching by inclusion of the
naphthalene in the hydrophobic cavity (23, Figure 30).\textsuperscript{53} The naphthalene derivative, which was utilized as a phosphorescent receptor for cations by complexation through the amine functionality, exhibited an increase in phosphorescence in the presence of \( \beta \)-cyclodextrin leading to a more effective chemosensor.

More sophisticated chemosensors involve the modification of cyclodextrin with a pendant fluorophore. Ueno and coworkers developed a fluorescent sensor for steroidal compounds by modifying \( \beta \)-cyclodextrin with a dansyl derivative (24; Figure 30).\textsuperscript{54} In aqueous solution, the dansyl moiety was intramolecularly included in the cyclodextrin cavity. With the addition of the steroidal compounds, which are also capable of binding in the cyclodextrin cavity, a competition for the cavity occurs and the dansyl moiety is excluded into the aqueous solution. This causes a change in the fluorescence of the dansyl moiety and, therefore, the steroidal compounds could be detected by monitoring the fluorescence change of the pendant dansyl unit.

A more complicated system was designed for the detection of organic molecules by the modification of \( \gamma \)-cyclodextrin with a pyrene unit (25; Scheme 17).\textsuperscript{55} This compound dimerized in solution allowing the pyrene units to form an excimer. Upon addition of organic molecules such as \( n \)-butanol, the organic guest competes for the cyclodextrin binding cavity and the dimer dissociates leading to a decrease in the fluorescence of the pyrene excimer. The observation of a fluorescence change was used to detect organic molecules in solution and the magnitude of this change was related to the strength of the guest binding.

A similar process for the detection of organic molecules was utilized with a bisnaphthalene modified \( \beta \)-cyclodextrin (26; Figure 30).\textsuperscript{56} Due to the smaller size of the \( \beta \)-
Figure 30. Fluorescent chemosensors based on β-cyclodextrin.
Scheme 17. Mechanism of fluorescence signaling of organic guest molecules by a chemosensor based on \(\gamma\)-cyclodextrin modified with a pendant pyrene unit (25).
cyclodextrin cavity, only a single naphthalene can be included at any one time. Upon the addition of an organic guest molecule, the single naphthalene becomes excluded and is capable of interacting with the second naphthalene moiety. This leads to a change in fluorescence, signaling the presence of the organic molecule.

These examples have shown that the inclusion properties of cyclodextrin can be exploited in the sensing of neutral molecules by the binding of these substrates into the cyclodextrin cavity. Therefore, in the design of a chemosensor for adenine dinucleotides, the β-cyclodextrin cavity could be utilized to bind the neutral adenine base moieties of the dinucleotide.

**NUCLEOTIDE BINDING TO β-CYCLODEXTRIN**

In the design of a chemosensor, molecular recognition between the chemosensor and the compound of interest is essential. In the case of nucleotides, it has long been established that β-cyclodextrin is capable of binding mononucleotides in aqueous solution. Of all of the mononucleotides, adenine derivatives have been found to form the strongest complexes to β-cyclodextrin presumably due to size considerations.

This complexation has been demonstrated and even exploited in the selective 3'-cleavage of 2',3'-cyclic monophosphates (Scheme 18). From the observation of NMR shifts and circular dichroism properties, it was concluded that this catalysis occurred via inclusion of the nucleotide base of the monophosphate in the cyclodextrin cavity (27; Scheme 18). Complexation in this manner thus allowed for interaction between the cyclodextrin hydroxyl...
Scheme 18. Selective 3'-cleavage of 2',3'-cyclic monophosphates catalyzed by β-cyclodextrin and the proposed complexation structure (27) of the 2',3'-monophosphate of adenosine in the β-cyclodextrin cavity which is responsible for the selective cleavage.
groups and the phosphate unit leading to hydrolysis. As expected, the hydrolysis of the adenine cyclic monophosphate exhibited the greatest effect compared to other nucleotides.

Schneider and coworkers have proposed an alternative binding complex for adenosine monophosphate with amino-substituted β-cyclodextrins (Figure 31). Based on NOE experiments, Schneider concluded that, in this case, it was actually the ribose moiety that was included in the cyclodextrin cavity. This conformation brings the phosphate anion in close proximity to the amino groups enabling the formation of salt bridges which enhance the binding of the nucleotide.

This work has shown that β-cyclodextrin is capable of binding mononucleotides and that pendant amino groups on the rim of the cyclodextrin can hydrogen bond to the phosphate group of the substrate, thereby increasing the binding strength. These studies have also shown β-cyclodextrin to be selective in the binding of adenine derivatives preferentially over other mononucleotides. However, dinucleotides such as adeny1-3',5'-adenosine contain two hydrophobic segments. In order to bind both of these segments simultaneously, it would be necessary to utilize bis-cyclodextrin compounds.

Figure 31. Proposed binding of adenosine monophosphate in a hepta-substituted amino-β-cyclodextrin.
Many bis-cyclodextrin compounds have been synthesized and their binding capabilities investigated. Most mono-cyclodextrin compounds have binding constants on the order of $10^4 \text{ M}^{-1}$ for ideal substrates. However, bis-cyclodextrins have been found to bind substrates containing two hydrophobic segments with binding constants exceeding $10^8 \text{ M}^{-1}$. Therefore, the addition of a second binding site allows for the complexation of larger molecules and can significantly increase the strength of the binding.

For optimal binding, the two cyclodextrin units must be able to act in unison. Therefore, the proper spacing between the cyclodextrin units is essential for binding a desired substrate. Sikorski and Petter showed this effect by changing the tether length between two cyclodextrin units and observing the corresponding changes in the binding ability of a bis-cyclodextrin. In addition, the proper orientation of the two cyclodextrin functionalities can also influence the binding ability. Breslow and coworkers elegantly demonstrated this principle in the synthesis of a bis-cyclodextrin which was attached at two points on the cyclodextrin rims to form a "clamshell" structure (Figure 32). This double attachment kept the cyclodextrin units in the proper orientation to cooperatively bind suitable substrates. The two tethers, which were different in length, gave the bis-cyclodextrin a bent structure which was capable of actually closing around a substrate. Due to this inventive design, binding constants on the order of $10^{10} \text{ M}^{-1}$ were achieved for appropriate substrates.

In addition, Breslow and Zhang have synthesized a bis-cyclodextrin linked by a catalytic spacer. This bis-cyclodextrin was utilized in the Cu$^{2+}$ catalyzed hydrolysis of an ester (Figure 32). The metal ion was bound to the bis-cyclodextrin via the nitrogens of the bipyridine spacer. Binding of the two hydrophobic segments of the ester simultaneously
Figure 32. Bis-cyclodextrin sensors designed by Breslow and coworkers including a "clamshell" structure (28) and a catalytic bis-cyclodextrin (29).
into the two cyclodextrin cavities brings the carbonyl functionality into close proximity to the metal ion. This leads to an enhanced hydrolysis of the ester by 220,000-fold over the uncatalyzed reaction at pH 7. This clearly demonstrates that a bis-cyclodextrin compound can not only bind a specific substrate, but can be utilized to position or orient that substrate into close contact with the spacer unit.

CHEMOSENSORS FOR ANIONS

Research in the development of chemosensors for anions has been limited. Methods for binding anions are not nearly as established as those for binding metal cations. However, studies involving benzylic amines attached to an anthracene unit have allowed the development of a method for the binding and, more importantly, the subsequent fluorescence signaling of some phosphate anions. Since the cyclodextrin cavity was envisioned as a binding domain for the dinucleotide base units, the fluorescence change of an anthracene moiety was examined as a potential signaling method for this chemosensor.

The utilization of anthracene in this capacity was originally inspired by the observation that free benzylic amines attached to an anthracene moiety will quench the fluorescence of the anthracene due to photoinduced electron transfer (PET)\(^{65,66}\). Photoinduced electron transfer involves the movement of an electron from the amine lone pair in the ground state to the anthracene in its excited state. This transfer leads to an excited state species which is capable of a loss of energy via a non-fluorescent pathway (Scheme 19). The ability of the amine lone pair to quench fluorescence can be altered by changing the oxidizing capability of the amine. This can be accomplished by protonation of the amine.
Scheme 19. Mechanism of photoinduced electron transfer (PET) between benzylic amines and anthracene resulting in fluorescence quenching.
nitrogen or by a binding event involving the amine lone pair. These processes essentially tie up the amine lone pair, decreasing its oxidizability and preventing electron transfer from the amine to the anthracene moiety. This results in an increase in the fluorescence of the anthracene and serves as a fluorescent signal.

This PET phenomena has been utilized in the design of many fluorescent chemosensors in the Czarnik research group. Initially, this phenomenon was observed in the case of compound 30 (Figure 33) which is non-fluorescent due to the PET between the amines and the anthracene. However, upon chelation of ZnCl₂ to form compound 31, the fluorescence increased by a factor of over one thousand due to binding of the amine lone pair electrons.

This method of fluorescence quenching was then utilized in the design of a chemosensor for anions by attaching a polyamine directly to an anthracene unit (32; Scheme 20). The trication form of this molecule exhibits low fluorescence due to the benzylic amine lone pairs and is capable of binding phosphate by electrostatic interactions. Upon binding of inorganic phosphate, the hydroxyl group of the phosphate is placed in close proximity to the benzylic amine leading to intracomplex proton transfer to the amine lone pair. Subsequently, the fluorescence of the anthracene is enhanced by inhibiting the PET allowing for the fluorescence detection of inorganic phosphate. Although this chemosensor was capable of detecting the phosphate anion, the detection occurred in the millimolar concentration range. A more effective chemosensor, capable of detecting anions in the micromolar range, was later developed.
Figure 33. Fluorescent chemosensors based on PET designed in the Czarnik research group.
Scheme 20. Photoinduced electron transfer (PET) mechanism for the fluorescence signaling of inorganic phosphate by chemosensor 32.
An increase in sensitivity along with a selectivity differentiation between phosphate and pyrophosphate was achieved with chemosensor 33 (Figure 33). The increased effectiveness of this chemosensor is attributed to the fact that the sensor contains two amine groups which are both capable of interacting with the anthracene functionality. Upon binding of pyrophosphate, both amines become partially protonated leading to a fluorescence increase. Due to the fact that the chemosensor is capable of binding both sides of the pyrophosphate simultaneously, pyrophosphate binds significantly more tightly than inorganic phosphate. This discrimination allowed for the utilization of 33 in the real-time assay of pyrophosphate hydrolysis.

A similar chemosensor (34; Figure 33) was designed for the selective detection of adenosine triphosphate. The tetracation form of this compound exhibits fluorescence quenching upon the binding of adenosine triphosphate through numerous electrostatic interactions; however, the binding of adenosine monophosphate with only a single phosphate moiety showed no fluorescence signal. Therefore, this chemosensor was capable of being utilized to monitor the action of aoyrase, an enzyme which hydrolyzes adenosine triphosphate to adenosine monophosphate.

Although the chemosensors described thus far were effective, the presence of the free amine groups allowed for the possible complexation of interfering metal cations. For this reason, chemosensor 35 was designed which was insensitive to metal cations owing to quaternization of the amine groups (Figure 33). This chemosensor was utilized to detect single stranded and double stranded DNA in real-time hydrolysis assays catalyzed by metal ions. Fluorescence DNA detection by 35 was found to rely not only upon the electrostatic
interaction between the polyamine and the phosphate groups of DNA, but also upon template directed excimer formation. It was suggested that the presence of the DNA effected the ability of the chemosensor to aggregate in solution and correspondingly effected the fluorescence signal.

For the detection of simple anions, an electrostatic interaction is sufficient for binding. However, more complex anions such as adenosine triphosphate contain not only an anionic portion but an additional aromatic portion in the molecule. The ability to design a chemosensor to effectively compliment both portions of the molecule simultaneously was elegantly demonstrated by Lehn and coworkers. Lehn utilized anion binding in conjunction with π stacking to design a fluorescent chemosensor for adenosine triphosphate (36; Figure 34). Chemosensor 36 contains a macrocyclic polyamine which, when protonated, is capable of binding the phosphate functionality of adenosine triphosphate. Simultaneous π stacking interactions between the adenine base of the adenosine triphosphate and the acridine

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\text{Figure 34. Fluorescent chemosensor 36 for adenosine triphosphate designed by Lehn and coworkers.}
\]
A fluorescence enhancement is observed upon binding of adenosine triphosphate due to excimer formation between the two aromatic units.

**DESIGN OF A FLUORESCENT CHEMSENSOR FOR ApA**

It was the goal of this project to design a fluorescent chemosensor for the selective detection of adenyl-3',5'-adenosine (ApA) based on a bis-cyclodextrin compound. The basic design of the chemosensor included two cyclodextrin functionalities linked by a fluorescent spacer. The observation that adenine binds to cyclodextrin preferentially over the other nucleic acid bases should lead to selectivity in the binding event. The presence of two cyclodextrin units should allow for the binding of both adenine bases in ApA simultaneously. Therefore upon complexation, the cyclodextrin moieties should position the ApA in the correct orientation to interact with the fluorescent spacer. The spacer was envisioned to contain aminomethyl groups directly attached to an anthracene unit. The amines should increase the binding strength of the ApA by the formation of salt bridges. More importantly, the amines should act as a communication device. Upon complexation, the interaction between the phosphate of the substrate and the amines should influence the fluorescence intensity of the anthracene signal leading to the fluorescence detection of ApA in aqueous solution.

The spacer first envisioned to link the cyclodextrin units was a TRPN-methyl-anthracene unit to yield the potential chemosensor 37 (Figure 35). Upon the binding of both adenine bases in the cyclodextrin cavities, the phosphate group of ApA should be placed in close proximity to the amine functionalities. Upon complexation, the phosphate anion
Figure 35. Initial design for a fluorescent chemosensor (37) for the detection of ApA.

should then be capable of hydrogen bonding to the protonated amine. This should lead to the PET fluorescence quenching of the anthracene unit by making the amine lone pairs partially available to interact with the anthracene unit.

The synthesis of this chemosensor was attempted by John Beeson of the Czarnik research group. During the investigation, however, the $^1$H NMR spectrum of the impure compound suggested that the spacer in this design allowed for the intramolecular inclusion of the anthracene moiety into the cyclodextrin cavities. Therefore, the polyamine spacer was modified to a simple aminomethyl-anthracene. The shorter tether between the cyclodextrin units and the fluorophore should provide a more organized and controlled environment for complexation of the substrate dinucleotide. Chemosensor 38, termed the “duplex,” was then envisioned as an improved chemosensor designed for the fluorescence detection of ApA (Scheme 21).

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Scheme 21. Proposed mechanism of fluorescence quenching of the duplex (38) by PET upon binding of adeny1-3',5'-adenosine.
SYNTHESIS AND PRELIMINARY PURIFICATION OF THE DUPLEX

The first attempt at synthesis of the duplex by John Beeson of the Czarnik group involved the displacement reaction of 1,8-bis(aminomethyl)anthracene (39) with the monotosylate of β-cyclodextrin (12; Scheme 22). This reaction was performed in dimethylformamide (DMF) for 1 day at 85°C. Collidine was also added as a non-nucleophilic base. The major product of this reaction was isolated by two sequential purifications by ion exchange chromatography. Based on mass spectral analysis, the product was believed to be a monosubstituted cyclodextrin derivative with formylation from the solvent occurring at the second amine group (40).

A second synthetic route was then explored (Scheme 23) which involved nucleophilic displacement of bromine from 1,8-bis(bromomethyl)anthracene (41) with 6-deoxy-6-amino-β-cyclodextrin (42). Reactions were performed in DMF with a three to five molar excess of 42 in the presence of collidine. Due to the ease of decomposition of 41, the reactions were performed at room temperature. TLC analysis of the reaction products was encouraging, showing evidence for cyclodextrin-anthracene compounds. Purification of the reaction products was achieved by a gel filtration column followed by two ion exchange chromatography columns leading to the isolation of an impure sample of the duplex.

Mark Mortellaro of the Czarnik group continued the development of the synthetic pathway. Optimizing reaction conditions for the highest yield of the duplex, Mortellaro utilized reaction conditions containing a 2.5 molar excess of 42. The reaction was performed under argon with flame-dried glassware. In addition, the DMF utilized was anhydrous and the collidine was distilled. These precautions were exercised due to the fact that 41 was
Scheme 22. Initial proposed route for the synthesis of the duplex which resulted in the formation of a formylation product (40).
Scheme 23. Synthetic route for the formation of the duplex (38) and a monosubstituted by-product (43).
susceptible to the water displacement of bromine. The reaction flask was also wrapped in aluminum foil to prevent photolytic decomposition of 41 and any anthracene products obtained during the reaction. After stirring at room temperature for three days, the reaction solution was reduced in volume under vacuum and then precipitated from 2-propanol to yield a yellow solid.

Purification of the reaction products necessitated the separation of the duplex from an assortment of impurities including numerous unidentified anthracene compounds. The major impurity, however, was residual 42. Many methods of purification were attempted by Mortellaro without success, including recrystallization, selective precipitation and chromatography on charcoal. Preparative TLC afforded some purification of the sample but did not remove residual 42 and yielded a sample containing silica contaminants. Gel filtration utilizing a Sephadex column was also unsuccessful with all of the cyclodextrin compounds passing through the column in a single band.

Ion exchange chromatography on Sephadex-CM 25 eluting with ammonium bicarbonate produced the most promising results. A single column eluted with 0.05 M NH₄HCO₃ allowed the separation of 42 from the desired product due to the adherence of the amino-cyclodextrin to the column. Mortellaro utilized a second ion exchange column eluting with an increasing gradient of NH₄HCO₃ from 0.02 M to 0.2 M in order to remove another unidentified anthracene impurity.

Finally reversed phase MPLC was employed with the sample. Utilizing an increasing gradient of acetonitrile in water, Mortellaro was able to isolate two compounds. The first compound was believed to be a pure sample of the duplex by NMR and mass spectral
analysis, however complete characterization of the compound was not performed before decomposition of the sample had occurred. The second isolated compound was believed to be the monosubstituted derivative (43) with water displacement occurring on the amine group to yield a hydroxyl moiety. This compound was of secondary interest as a comparison compound to the duplex. The NMR spectrum of this compound proved to be extraordinarily complicated. It was suggested that the complication arose from dimer formation of 43 in solution by inclusion of the anthracene portion of one molecule into the cyclodextrin cavity of a second molecule.

FINAL PURIFICATION OF THE DUPLEX

Continuing on with the work that Mortellaro had accomplished, it was determined that two ion exchange columns were not necessary for purification of the duplex and an optimized purification scheme was finalized. The purification consisted of ion exchange chromatography followed by reversed phase MPLC.

The initial cation exchange column (Sephadex CM-25) was performed while wrapped in aluminum foil to prevent any possible anthracene decomposition during elution. The column was eluted with 0.05 M NH₄HCO₃ solution. Fractions were monitored by both TLC and UV spectrometry. The TLC plates were first viewed with a UV lamp to observe any anthracene derivatives by fluorescence and then charred with p-anisaldehyde stain to observe cyclodextrin compounds. In this way, compounds containing both anthracene and cyclodextrin moieties were identified. The fractions were also monitored by UV absorbance at 254 and 368 nm for relative concentrations of all anthracene derivatives (Figure 36). The
Figure 36. Elution profile of the Sephadex CM-25 ion exchange column monitored by UV spectroscopy at 254 nm (●) and 368 nm (■).
duplex and the monosubstituted derivative (43) eluted from the column immediately along with other impurities as evidence by TLC. The major impurity, the amino-cyclodextrin 42, was retained on the cation exchange column and was thereby removed from the mixture.

The isolated samples were then subjected to reversed phase MPLC to remove the remaining impurities. Due to the small capacity of the column, only a maximum of 50 mg could be eluted at a single time. The column was wrapped in aluminum foil and elution was accomplished with an increasing gradient of acetonitrile in water. The fractions were again analyzed by both TLC and UV spectrometry at 254 and 368 nm (Figure 37). The first band to elute from the column contained an unidentified material with a pink hue. This material was thought to be an oxidized form of the anthracene derivative due to its unusual color. The second band to elute contained the duplex. The sample isolated from this band appeared to be a single compound by TLC containing both an anthracene and a cyclodextrin moiety. In some cases, the isolated sample also contained a slight pink tint although no other compound could be detected in the sample. The last major band to elute contained the monosubstituted derivative 43 along with other unidentified impurities. It was not possible to isolate the monosubstituted product as a pure compound utilizing this purification scheme.

Due to the synthesis and the extensive purification process, the overall yield of the duplex was small, leading to only minor amounts of pure compound actually obtained. The purity of the compound was evidenced by TLC which showed only a single compound that was fluorescent and stained for cyclodextrin. Due to the small quantities of material, characterization of the compound rested on high resolution mass spectrometry by
Figure 37. Elution profile of the reversed phase MPLC column monitored by UV spectroscopy at 254 nm. Elution was accomplished with an acetonitrile in water gradient of 20% (●), 25% (■) and 30% (▲).
electrospray ionization. The compound exhibited a molecular ion peak with \( m/z \) 2468.84567 with a theoretical peak of \( m/z \) 2468.84964.

The \(^1\)H NMR spectrum of the duplex in D\(_2\)O (Figure 38) was complicated, containing signals consistent with cyclodextrin and signals in the downfield anthracene region. The anthracene signals, however, were broad and non-distinct. The addition of a trace amount of trifluoroacetic acid, CF\(_3\)CO\(_2\)H, to the NMR sample led to the resolution of the anthracene signals (Figure 38) and yielded the predicted anthracene pattern. The initial distorted anthracene pattern has been suggested to be due to the presence of trace amounts of an anthracene radical species as was observed in other anthracene compounds synthesized in the Czarnik group.\(^78\) The addition of acid to the sample inhibits the pathway for formation of this radical and thus eliminates the pattern distortion.

**DUPLEX STABILITY**

Before performing fluorescence binding studies with the duplex, it was necessary to determine the stability of the duplex in aqueous solution under various conditions. The stability of the duplex in neutral solution (pH 7) was examined in 0.1 M HEPES buffer varying the conditions of temperature and exposure to light. Stability was determined by observing changes in the TLC and UV absorbance of the samples. The duplex appeared to be stable in solution for a period of at least two months under conditions of either room temperature or refrigeration and exposed to either light or dark surroundings.

In addition, the stability of the duplex in basic and acidic solution was monitored under refrigeration and in dark surroundings. The duplex was stable in basic solution (pH
Figure 38. $^1$H NMR spectra in D$_2$O of the duplex (38) alone and in the presence of CF$_3$CO$_2$H.
10, 0.1 M CAPS buffer) for a period of at least two months. However, in acidic solution (pH 3, 0.1 M citric acid buffer), the duplex began to decompose slightly at the end of the two month period as evidenced by changes in the TLC properties of the sample.

As a result of the stability findings, the fluorescence binding studies could be performed under the conditions of normal room lighting and the stock solutions of the duplex did not have to be used immediately upon preparation. However, as a long-term safety precaution, all solid samples of the duplex were stored in the freezer wrapped in aluminum foil.

**FLUORESCENCE BEHAVIOR OF THE DUPLEX**

The excitation wavelength chosen for the fluorescence studies was 368 nm, one of the maximum wavelengths in the absorption spectrum of anthracene. The emission profile of the duplex exhibited a maximum at 416 nm. Therefore, all fluorescence studies were performed by observing the change in fluorescence of the duplex at 416 nm.

The concentration of the duplex to be utilized in the binding studies was determined by first confirming the concentration range in which the fluorescence of the duplex was linear. By measuring the fluorescence intensity at 416 nm over a range of duplex concentrations (Figure 39), it was determined that any concentration below 10 µM would give a linear fluorescence response. Taking this into consideration, the concentration of the duplex utilized in the binding studies was between 7 and 8 µM to maximize the fluorescence signal while remaining safely within the linear response region.
Figure 39. Fluorescence intensity of the duplex at 416 nm over a range of duplex concentrations.
In order for the duplex to signal the binding of dinucleotides, it was necessary for the duplex to exhibit a reversible change in fluorescence upon protonation and deprotonation of the benzylic amines. This expected change in fluorescence was shown by monitoring the fluorescence intensity of a duplex solution over a pH range of approximately 2 to 12 (Figure 40). By back titration, this fluorescence change was shown to be a completely reversible process. Given the shape of this pH profile, it was not possible to determine accurate pKₐ values; however, computer fitting of the data estimated the pKₐ of the duplex as 6.0 for the multiprotonation event.

The pH profile exhibited the expected trends. The fluorescence intensity was highest at lower pH and decreased as the pH increased. At lower pH, the benzylic amines were protonated and no electron transfer between the amine lone pairs and the anthracene unit was possible. Therefore, the anthracene fluoresced strongly upon excitation. As the pH increased and deprotonation occurred, there was increased interaction between the amine lone pairs and the anthracene unit allowing for PET quenching of the anthracene fluorescence. There was, however, an observed fluorescence minimum in the pH profile at a pH of approximately 7. Based on this pH profile, the duplex should be capable of signaling a binding event that leads to a change in the protonation state of the amines.

**FLUORESCENCE BINDING STUDIES**

The binding of the duplex with several adenosine derivatives was examined to determine the potential of the duplex to function as a fluorescent chemosensor. Binding studies were generally performed in 0.05 M HEPES buffer at pH 7. Neutral pH was chosen
Figure 40. The fluorescence intensity of the duplex at 416 nm over a pH range of approximately 2 to 12.
due to its biological significance. The pH of the solutions were measured both before and after the fluorescence studies to insure that there was no change in the pH of the solutions during the experiment, which could effect the fluorescence intensity of the duplex. In addition, ethylenediaminetetraacetic acid (EDTA) was utilized in the fluorescence studies in order to complex any trace metal ions present in solution which could complex to the duplex amines.

The binding of adenosine derivatives with only a single base was initially examined at pH 7. The three adenosine compounds that were studied, 5'-adenosine monophosphate (5'-AMP), 5'-adenosine diphosphate (5'-ADP) and 5'-adenosine triphosphate (5'-ATP), all contain a different number of phosphate units (Figure 41). These compounds, with only a single hydrophobic binding segment, were presumably bound to only one of the cyclodextrin cavities of the duplex. Upon binding, no significant change in fluorescence was observed. This was expected since these substrates can bind in the cyclodextrin cavity in a variety of orientations which do not necessitate the positioning of the phosphate moiety in close proximity to the duplex amines. Hence these substrates did not effect the fluorescence properties of the duplex. Binding of adenosine derivatives containing multiple base units should, however, allow for the positioning of the substrate in the desired orientation.

Duplex binding to adeny1-3',5'-adenosine (ApA; Figure 42) containing two hydrophobic segments was examined at pH 6.5, pH 7.0 and pH 7.4. Surprisingly, no change in fluorescence was observed upon the addition of ApA to a solution of the duplex at any of the pHs examined. Due to the known affinity of the adenosine base for the cyclodextrin cavity, it was strongly expected that the adenosine base would bind to the duplex. However,
Figure 41. Structures of adenosine mononucleotides including 5'-AMP, 5'-ADP and 5'-ATP.
Figure 42. Structures of adenosine nucleotides ApA and poly-A.
it is possible that the binding could be occurring via the secondary side of the cyclodextrin rim leading to a complex in which cooperative binding of both hydrophobic segments can not occur within the same duplex molecule (Figure 43). If preferential binding occurred in this fashion, the phosphate moiety would not be in close proximity to the duplex amine accounting for the lack of a fluorescence signal.

To further explore the binding of adenosine derivatives, the effect on the fluorescence of the duplex in the presence of polyadenylic acid (poly-A; Figure 42) was examined. This polymeric derivative of adenosine did increase the fluorescence of the duplex at pH 7. This increase can be seen in Figure 44 as compared to the lack of signal observed with 5'-AMP. The best dissociation constant for poly-A was 67 µM as determined by computer fitting at 7.3 µM duplex. The complexation between the polymer and the duplex was envisioned to occur from the secondary side of the duplex with the two adenine bases binding cooperatively (Figure 45). Although it was undetermined how many free adenosine units separate the bound adenosine units, CPK models suggested that one adenine base was a sufficient spacer for binding to occur in this fashion. This binding model would allow the phosphate moiety to interact with the protonated amine of the chemosensor and fluorescence signaling to occur.

Although it was originally expected that the binding event would lead to a quenching of the anthracene fluorescence, the observation of a fluorescence enhancement can be reasoned from the pH profile of the duplex (Figure 40). At pH 7, the duplex exhibits its minimum fluorescence intensity, therefore upon either an increase or decrease in pH, an increase in the fluorescence is observed. Consequently any change in the amine protonation
Figure 43. Proposed binding of ApA to two duplex molecules simultaneously via the secondary side of the cyclodextrin cavities.
Figure 44. Changes in the relative fluorescence intensity of the duplex (7.3 μM) at 416 nm with the addition of poly-A (●) and 5'-AMP (▲) in 0.05 M HEPES at pH 7.
Figure 45. Proposed binding complex of poly-A with the duplex.
state should lead to an increase in fluorescence. Based on this observation, it is reasonable to assume that the phosphate group of the polymer was causing a change in the protonation state of the amines and thereby changing the fluorescence of the anthracene.

To confirm the role of the phosphate group in the fluorescence change of the duplex, the binding of poly-A was studied in the presence of a metal ion, Mg$^{2+}$. The metal ion alone, in the form of the chloride salt, caused only a slight decrease in the fluorescence of the duplex. However, the addition of Mg$^{2+}$ to a solution containing the duplex and poly-A (Figure 46) caused a reversal of the fluorescence change previously observed with the poly-A alone (Figure 44). Therefore, upon complexation of the phosphate anion with a metal cation, the phosphate can no longer effect the protonation state of the duplex amine, thus the fluorescence of the duplex returns. This study showed that the duplex was capable of detecting poly-A via a reversible pathway intimately involving the phosphate group of the polymer.

The fluorescence signaling of poly-A by the duplex was also found to be pH dependent. Signaling of the polymer occurred at pH 7.0; however, no fluorescence change was observed at pH 7.4 (Figure 47). Although this observation cannot fully be explained, this effect could be due to subtle changes in the conformation of the complex upon further protonation of the amine groups or subtle changes in the interaction of the phosphate moiety with the amines upon changes in pH.

The successful fluorescence detection of poly-A with the duplex led to the investigation of the fluorescence change effected by other polynucleotides to determine the selectivity of the duplex for poly-A. The pyrimidine bases (Figure 48), polycytidylic acid
Figure 46. Changes in the relative fluorescence intensity of the duplex at 416 nm with the addition of Mg$^{2+}$ (▲) and changes in the relative fluorescence intensity of the duplex at 416 nm in the presence of 240 AMP equivalents of poly-A with the addition of Mg$^{2+}$ (●).
Figure 47. Changes in the relative fluorescence intensity of the duplex at 416 nm with the addition of poly-A in 0.05 M HEPES at pH 7.0 (●) and pH 7.4 (▲).
Figure 48. Structures of polynucleotides including poly-C, poly-U and poly-G.
(poly-C) and polyuridylic acid (poly-U), had no effect on the fluorescence of the duplex presumably due the ineffective binding of these bases in the cyclodextrin cavities (Figure 49).

In the presence of polyguanylic acid (poly-G; Figure 48), the duplex exhibited fluorescence quenching with a dissociation constant of 1.8 mM (Figure 49). This fluorescence change was presumably due to an alternative mechanism different from that proposed for the fluorescence increase observed with poly-A. With poly-G, quenching was attributed to the interaction of two aromatic units. Upon complexation of the guanosine base, energy transfer between the excited state anthracene and the base unit can occur leading to a return to the ground state via a non-fluorescent pathway, thus quenching the fluorescence of the duplex. The observed quenching was presumably due to an actual binding event with the cyclodextrin cavities since poly-G did not cause a change in the fluorescence of a non-binding anthracene compound, 1,8-bis(carboxymethyl)anthracene.

Opposite to the case of poly-A, the quenching effect of poly-G was not exclusive to the polymeric nucleotide; the same fluorescence quenching was observed with 5'-guanosine monophosphate (5'-GMP). Although the change in fluorescence was more dramatic with poly-G, a similar dissociation constant of 1.9 mM was calculated for 5'-GMP with the duplex (Figure 50). The observation that the change in fluorescence occurred with a mononucleotide indicated that the observed quenching was not dependent on the binding of more than one base unit simultaneously. However, the poly-G exhibited a larger change in fluorescence then 5'-GMP over the same range of equivalents. This suggested that poly-G
Figure 49. Changes in the relative fluorescence intensity of the duplex at 416 nm with the addition of poly-A (●), poly-C (◆), poly-U (▲) and poly-G (■) in 0.05 M HEPES at pH 7.
Figure 50. Changes in the relative fluorescence intensity of the duplex at 416 nm with the addition of poly-G (●) and 5'-GMP (▲) in 0.05 M HEPES at pH 7.
contained a larger concentration of the bound nucleotide positioned to interact with the anthracene of the duplex.

It has been shown that the duplex is capable of selectively detecting the purine based polynucleotides. These polynucleotides can be distinguished due to their opposite effects on the duplex fluorescence with poly-A leading to fluorescence enhancement and poly-G leading to fluorescence quenching. These binding events leading to changes in fluorescence should be reversible processes. In order to explore this reversibility, the competitive binding of these polynucleotides with their corresponding polynucleotide base pairs was examined in the presence of the duplex.

The first study involved the addition of poly-U to a solution containing poly-A bound to the duplex. As previously demonstrated, the poly-A alone caused an increase in the fluorescence of the duplex. As the poly-U was added, it effectively competed with the duplex for the binding sites of the poly-A, decomplexing the poly-A and the duplex. This caused a reversal of the fluorescence increase and the subsequent return of the fluorescence intensity to approximately its original value before the addition of the poly-A (Figure 51). This experiment demonstrated that the binding of the poly-A to the duplex was reversible and that the fluorescence change associated with this binding event was also reversible. In addition, this experiment offered a way of detecting poly-U in aqueous solution by competitive binding for poly-A with the duplex.

A similar experiment was performed utilizing poly-G and poly-C. However, in this case, the fluorescence quenching of the duplex by poly-G was not reversed upon the addition of poly-C to the solution. Since it only requires a single nucleotide base of the guanosine to
Figure 51. Changes in the relative fluorescence intensity of the duplex at 416 nm with the addition of poly-A (●) up to 150 equivalents of AMP, followed by the addition of poly-U (○) in 0.05 M HEPES at pH 7.
effect the fluorescence of the duplex, a greater proportion of the poly-G must become bound by the poly-C in order for a reversal of the fluorescence change to occur. Therefore, competitive binding of the poly-G with the poly-C may not be expected to completely reverse the quenching observed.

Finally it should be mentioned that the binding of other adenine containing molecules was also examined, including flavin adenine dinucleotide (FAD), β-nicotinamide adenine dinucleotide (NAD) and β-nicotinamide adenine dinucleotide, reduced form (NADH). All of these compounds exhibited fluorescence quenching with the duplex; however in all cases, the quenching effect was attributed to the UV absorbance of these compounds in the region of the duplex excitation wavelength. Therefore, any possible binding events with these compounds could not be detected over this interference.

CONCLUSION

In conclusion, a new fluorescent chemosensor termed the duplex was successfully synthesized and purified. This chemosensor was found to be physically appropriate for the effective binding of polynucleotides, with specificity for purine based polynucleotides. The binding of these polynucleotides yielded a fluorescence enhancement of the duplex with polyadenylic acid and a fluorescence quenching of the duplex was achieved with polyguanylic acid.

The duplex chemosensor utilized β-cyclodextrin units as binding sites for the nucleic acid bases for both polynucleotides. However, the fluorescence signaling device differed for the two different substrates. In the case of polyadenylic acid, the fluorescence signaling
consisted of an interaction between the benzylic amines of the anthracene spacer and the bound phosphate moiety. This interaction effected the photoinduced electron transfer between the amine lone pairs and the anthracene unit leading to an increase in the fluorescence of the anthracene. With polyguanylic acid, the fluorescence signaling consisted of an interaction between the aromatic moieties of the bound substrate and the anthracene of the spacer unit leading to a decrease in the duplex fluorescence. This alternative fluorescence signaling mechanism allowed the additional detection of 5'-guanosine monophosphate with the duplex by fluorescence quenching.

The reversibility of the binding of polyadenylic acid to the duplex was exhibited in several ways. The fluorescence of the original duplex was returned upon the addition of Mg$^{2+}$ or polyuridylic acid to a mixture of the duplex and polyadenylic acid. The latter experiment resulting in an indirect method for the detection of polyuridylic acid utilizing the duplex.

In summary, a new chemosensor for the selective detection of polyadenylic acid has been synthesized. It may be possible to alter the design of this chemosensor for the detection of dinucleotides by attaching the anthracene spacer to the secondary rims of the cyclodextrin cavities. Chemosensors of this type, capable of detecting biological molecules via fluorescence, may be utilized in biological assays or may eventually be incorporated into the development of fiber optic remote sensing devices for both biological and environmental applications.
EXPERIMENTAL

General. The high resolution mass spectrum utilizing electrospray ionization was graciously performed at Parke-Davis Pharmaceutical Research (Ann Arbor, MI) on a Finnigan MAT 900Q. Fluorescence measurements were obtained on a Perkin-Elmer LS-5 luminescence spectrometer. UV data were obtained on a Hewlett-Packard 8452A diode array spectrophotometer. Reversed phase MPLC column (Lobar Lichroprep RP-18, 310 mm × 25 mm, 40-63 μm) was obtained from EM Separations Technology (Gibbstown, NJ). Most of the chemicals used in this study were obtained from Sigma Chemical (St. Louis, MO) including the biological buffers HEPES (4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid and BIS-TRIS (bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane). Thin layer chromatography was performed on aluminum-backed silica gel plates and cyclodextrin compounds were visualized by charring after staining with p-anisaldehyde stain: p-anisaldehyde/methanol/acetic acid/sulfuric acid (1:200:20:10). Elution was accomplished with a solvent system of n-butanol/methanol/water/NH₄OH (4:3:2:4). The fluorimetric pKₐ of the duplex was determined by computer fitting with the program ENZFITTER (Elsevier-BIOSOFT). Reported dissociation constants were also determined with ENZFITTER utilizing one site ligand binding fitting of the data.

1,8-Bis((6-deoxy-6-amino-β-cyclodextrin)methyl)anthracene (Duplex, 38).

Glassware was flame-dried prior to use. To a solution of 6-deoxy-6-amino-β-cyclodextrin (42; 1.0 g, 0.88 mmol) and 1,8-bis(bromomethyl)anthracene (41; 0.13 g, 0.36 mmol) in anhydrous dimethylformamide (DMF; 20 mL) was added distilled collidine (0.2 mL, 1.5 mmol). The flask was wrapped in aluminum foil and stirred under Ar for 5 d. Solvent was
removed from the yellow solution *in vacuo* to yield a thick yellow oil. Water (3 mL) was added to the oil and the solution added dropwise to 2-propanol (150 mL). The precipitate was collected by vacuum filtration to yield an off white solid (1.0 g) which included several fluorescent and cyclodextrinyl compounds by TLC.

The impure sample (1.0 g) was dissolved in water (15 mL) and passed through a Sephadex CM-25 column (23 x 6 cm) eluting with 0.05 M NH₄HCO₃. Eluting fractions were analyzed by UV spectroscopy at 254 and 368 nm, as well as by TLC. The fractions containing the initial band were pooled into two separate portions. As evidenced by TLC, the first portion contained a higher concentration of the duplex than the second portion. For each portion, the solvent volume was reduced *in vacuo* to approximately 5 mL. The remaining solution was lyophilized to yield an off white solid (106 mg in portion one; 41 mg in portion two).

A portion of the sample (36 mg) was dissolved in water (1.5 mL). The brown solution was then passed through a reversed phase MPLC column. Elution was accomplished with an increasing CH₃CN/H₂O gradient with the duplex eluting at 20% CH₃CN in H₂O. The eluting fractions were analyzed by UV spectroscopy at 254 and 368 nm, as well as by TLC. The appropriate fractions were pooled, the solvent volume reduced *in vacuo* and the remaining solution lyophilized to yield a white solid (10 mg). In some cases, the final solid contained a slight pink tint: Rf 0.35; ¹H NMR (D₂O with CF₃CO₂H) δ 8.64 (d, 2, Ar), 8.17 (d, 2, Ar), 7.62 (d, 2, Ar), 7.50 (t, 2, Ar), 5.32 (m, 2), 4.92 (d, 4), 4.88 (s, 6), 4.37 (m, 2), 4.00-2.90 (m, 83), 2.78 (m, 2), 1.85 (m, 2).
High Resolution Mass Spectrum Calcd. for C\textsubscript{100}H\textsubscript{152}N\textsubscript{8}O\textsubscript{56}: m/z 2468.84964. Found: m/z 2468.84567.

**Duplex Stability Study.** Aqueous solutions of the duplex (50 \(\mu\)M) were prepared by dissolving approximately 0.5 mg of the duplex in 2 mL of the appropriate buffer solution, followed by a two-fold dilution of the solution with buffer. The solutions were stored in plastic cuvettes and dark conditions were simulated by wrapping the cuvettes in aluminum foil. Several solutions were prepared and stored under the following conditions: pH 7, 0.1 M HEPES, refrigerated, dark; pH 7, 0.1 M HEPES, rt, dark; pH 7, 0.1 M HEPES, rt; pH 10, 0.1 M CAPS, refrigerated, dark; pH 3, 0.1 M citric acid, refrigerated, dark. Periodically, the solutions were monitored for decomposition by observing changes in the UV spectrum and TLC analysis.

**Linearity of Duplex Fluorescence.** A 10 mL stock solution of the duplex (45 \(\mu\)M) was prepared in 0.2 M HEPES at pH 7.4 with EDTA (50 \(\mu\)M). Dilutions of the stock solution were prepared with total volumes of 3 mL to give a range of duplex concentrations. The fluorescence intensity of each solution was measured at 416 nm with an excitation wavelength of 368 nm.

**Fluorescence pH Profile.** A 25 mL solution of the duplex (8.1 \(\mu\)M) was prepared in 0.05 M HEPES (pH 7.4) with 10 \(\mu\)M EDTA. The pH of the solution was adjusted by the addition of small aliquots of concentrated HCl or NaOH. After each addition, the fluorescence intensity was measured at 416 nm with an excitation wavelength of 368 nm. The fluorescence change was monitored during back titration as well to insure the reversibility of the fluorescence change. The fluorimetric \(pK_a\) of the duplex, determined by
computer fitting with ENZFITTER, was 6.0, although this presumably represents more than one protonation event.

**Fluorescence Binding Studies.** Solutions of the duplex and the various substrates were prepared in 0.05 M HEPES at pH 7.0 with 10 μM EDTA unless otherwise specified. Binding studies were performed by adding aliquots of the substrate stock solution to the duplex solution. After the addition of each aliquot of the substrate, the solution was mixed and the fluorescence was monitored utilizing an excitation wavelength of 368 nm. The duplex emission was scanned from 400 to 450 nm with the recorded values taken at the maximum wavelength of 416 nm. Generally, intensity values were recorded as an average of 5 or 10 scans.

Fluorescence control experiments were performed on all of the substrates by the addition of aliquots of the substrate solution to water. These experiments, in the absence of the duplex, insured that the substrate molecules did not exhibit an interfering fluorescence signal at the concentration of substrate utilized. Additionally, the pH of the initial and final solutions were measured to insure that no change in pH occurred during the binding studies.

**Binding of Mononucleotides.** All of the mononucleotides were purchased as the corresponding sodium salts. Substrate stock solutions were prepared based on the solubility of the substrates. The binding of the mononucleotides was monitored as described by adding aliquots of the substrate stock solutions of the following concentrations to 2.5 mL of duplex solution (7.3μM): 5'-AMP (0.10 M), 5'-ADP (0.02 M; 50μM EDTA; 0.2 M HEPES), 5'-ATP (0.02 M; 50 μM EDTA; 0.2 M HEPES). In the case of 5'-GMP (0.04 M), the substrate
solution was added to 2.5 mL of duplex solution (8.1 μM). The dissociation constant calculated for 5'-GMP and the duplex was 1.9 mM.

**Binding of Adenyl-3',5'-Adenosine.** Binding of adenyl-3',5'-adenosine (ApA) was monitored at pH 6.5, 7.0 and 7.4. A solution of ApA (0.02 M) was prepared in 0.20 M HEPES at pH 7.0 with EDTA (50 μM). Aliquots of the ApA stock solution were added to 2.5 mL of the duplex solution (7.3 μM duplex; 10 μM EDTA; 0.05 M HEPES; pH 7.0) and the fluorescence monitored as described. Similarly, aliquots of the ApA solution were then added to 10 mL of the duplex solution (8.1 μM duplex; 10 μM EDTA; 0.05 M HEPES; pH 7.4) and the fluorescence monitored as described. Finally, aliquots of the ApA solution were added to 10 mL of the duplex solution (8.1 μM duplex; 10 μM EDTA; 0.05 M BIS-TRIS; pH 6.5) and the fluorescence monitored as described.

**Binding of Polyadenylic Acid.** Polyadenylic acid was purchased as the corresponding potassium salt. A solution of the polynucleotide was prepared containing 0.02 M monoadenosine units. Aliquots of the substrate stock solution were added to 10 mL of duplex solution (7.3 μM) at pH 7.0 and the fluorescence monitored as described. A dissociation constant of 67 μM was obtained from computer fitting. Additional binding studies were performed in a similar fashion with 8.1 μM duplex to yield an average dissociation constant of 730 μM.

A second solution of the polynucleotide was prepared containing 0.04 M monoadenosine units. Aliquots of the substrate stock solution were added to 10 mL of duplex solution (8.1 μM) at pH 7.4 and the fluorescence monitored as described.
**Binding of Mg$^{2+}$ with Polyadenylic Acid.** As a control experiment, aliquots of a MgCl$_2$ solution (0.10 M) were added to 2.5 mL of duplex solution (8.1 $\mu$M) and the fluorescence monitored as described.

To study the effect of the addition of Mg$^{2+}$ on the binding ability of polyadenylic acid, 250 equivalents of monoadenosine units of polyadenylic acid (0.02 M in monoadenosine units) were added to a 2.5 mL solution of the duplex (8.1 $\mu$M). To this mixture, aliquots of the Mg$^{2+}$ solution (0.10 M) were added and the fluorescence monitored as described.

**Binding of Polynucleotides.** All polynucleotides were purchased as the corresponding potassium salts and the fluorescence studies were performed utilizing the following procedure. A solution of the polynucleotide (0.02 M in mononucleotide units) was prepared and aliquots of the polynucleotide stock solution were added to 2.5 mL of the duplex solution (8.1 $\mu$M) and the fluorescence monitored as described. The dissociation constant calculated for polyguanylic acid was 1.8 mM.

**Competitive Binding of Polynucleotides.** To study the effect of the addition of polycytidylic acid on the binding ability of polyguanylic acid, 300 equivalents of monoguanosine units of polyguanylic acid (0.04 M in monoguanosine units) was added to a 2.5 mL solution of the duplex (8.1 $\mu$M). To this mixture, aliquots of a polycytidylic acid solution (0.02 M in monocytosine units) were added and the fluorescence monitored as described.

A similar procedure was utilized to study the effect of the addition of polyuridylic acid on the binding ability of polyadenylic acid. Polyadenylic acid (0.02 M in monoadenosine units) was added to 2.5 mL of duplex solution (8.1 $\mu$M) to yield 150
equivalents of monoadenosine units. To this mixture, aliquots of a polyuridylic acid solution (0.03 M in monouridine units) were added and the fluorescence monitored as described.
REFERENCES AND NOTES


8. (a) Dissociation constant for 1-adamantanecarboxylic acid was taken from: Tabushi, I.; Shimokawa, K.; Shimizu, N.; Shirakata, H.; Fujita, K. *J. Am. Chem. Soc.* 1976, 98, 7855; (b) Values for iodide ion, methylpenicillin and p-nitrophenolate were taken from ref 1 (a); (c) All other values were taken from: VanEtten, R. L.; Sebastian, J. F.; Clowes, G. A.; Bender, M. L. *J. Am. Chem. Soc.* 1967, 89, 3242.


76. The synthesis of this compound was perfected by John Beeson of the Czarnik group. For complete synthetic details see: Beeson, J. C.; Czarnik, A. W. *Bioorg. Med. Chem.* **1994**, *2*, 297.
