Tethering of ANS Based Fluorescent Probes to Cyclodextrin Hosts with Potential Applications as a Fluorescent Sensor

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ABSTRACT

The ultimate goals of this Masters project were to synthesize a reusable, fluorescent molecular sensor and to evaluate its potential effectiveness. The molecular sensor was to be synthesized from an anilino naphthalenesulfonic acid (ANS) based fluorescent probe and a β-cyclodextrin (β-CD) host, either natural or modified. As a means of enhancing the effectiveness of the molecular sensor, it was determined that there should be a very large difference in intensity in the "on" and "off" positions, thus making it very easy to identify the sensors position.

Intermolecular studies were conducted involving several different ANS based fluorescent probes and dimethyl-β-CD and β-CD. It was identified that 2-toluidino 6-naphthalenesulfonic acid (2,6-TNS) would be the most effective probe to use due to its strong affinity to form inclusion complexes with β-CDs and the very large increase in fluorescence emission intensity upon inclusion.

Several synthetic techniques were investigated. Attempts were made to brominate 2,6-TNS and to protect the amine group of 2,6-TNS to be followed with further work to brominate the protected 2,6-TNS. These attempts were unsuccessful.

Attempts were also made to modify the CD before attaching the probe. These attempts were made with β-CD. Investigations were made into attaching an alkyl chain, which would act as the tether between the host and guest molecules, onto the larger secondary rim of β-CD, but attempts to do so were unsuccessful. Investigations were also made into modifying the primary rim of β-CD. β-CD was successfully mono-tosylated.

The mono-tosylated β-CD was reacted with 2,6-TNS and evidence suggests that the host and guest molecules are tethered via a single carbon alkyl chain.

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LIST OF ABBREVIATIONS

A	absorbance
AIBN	α,α'-azobis-(isobutyronitrile)
1,8-ANS	1-anlilino 8-naphthalenesulfonic acid
2,6-ANS	2-anlılino 6-naphthalenesulfonic acid
ATR	Attenuated Total Reflectance
BOC	*tert*-butoxycarbonyl
BOC_2O	di-*tert*-butyl dıcarbonate
CD	cyclodextrin
DMF	N,N-dimethyl formamide
DMSO	dımethyl sulfoxıde
DMSO-d_6	deuterated dimethyl sulfoxide
ΔE	energy gap
F	integrated area of fluorescence spectrum of probe in presence of CD
F/F_O	fluorescence enhancement
F_O	integrated area of fluorescence spectrum of probe
IC	internal conversion
ISC	intersystem crossing
K	association (binding) constant
Mesyl-Cl	methanesulfonyl chloride
NBS	N-bromo-succinimide
NMR	nuclear magnetic resonance

PSF	polarity sensitivity factor
TLC	thin layer chromatography
2,6-TNS	2-toluidino 6-naphthalenesulfonic acid
Tosyl-CD	tosylated β-cyclodextrin
Tosyl-Cl	*p*-toluene sulfonyl chloride
UV	ultraviolet
$\lambda_{F,max}$	wavelength of maximum fluorescence emission
τ_F	fluorescence lifetime
ϕ_F	fluorescence quantum yield

1.1 Introduction

The purpose of this project is to create a fluorescent molecular sensor with an obvious "on/off switch" with a very large difference between its "on" and "off" emission outputs, larger than has been previously accomplished. Principles of supramolecular chemistry will be employed to determine which host molecule and which fluorescent guest will best accomplish the desired goals. There are three major goals of this project. First is the formation of an appropriate inclusion complex which exhibits the required dramatic enhancement of fluorescence emission upon inclusion, and the study of the binding properties for this inclusion system. The second goal is to permanently link the host and guest molecules involved in the aforementioned inclusion complex, using organic synthesis, in order to create the aforementioned fluorescent molecular sensor. The third is to test the potential sensor properties of the resulting linked host-guest system.

1.2 Supramolecular Chemistry

Supramolecular chemistry is a term used to describe molecular scale interactions. It was first defined in 1987 by J.-M. Lehn as "chemistry beyond the molecule."[1, 2, 3] Supramolecular chemistry is a term that may be used interchangeably with inclusion phenomena, host-guest chemistry or molecular recognition,[1] although inclusion phenomena is generally a broadly applied term describing any type of inclusion complex. Supramolecular chemistry is an area of study that encompasses chemistry, biochemistry, physics and material science.[3] It is a very diverse field which includes work in such areas as inclusion complexes, such as those involving

cyclodextrins,[3, 4] charge transfer complexes,[3, 5] liquid crystals,[3, 6] micelles,[3] mono- and polylayers,[3] vesicles,[3, 7] and co-crystals consisting of at least two different molecular species.[3, 8]

The origins of host-guest chemistry and molecular recognition are based on work proposed by E. Fischer in 1894 dealing with the lock and key mechanism of biological catalysis.[3, 9] The term "supermolecule" was introduced in the 1930's to describe molecular entities with higher organization resulting from the association of two (or more) co-ordinatively saturated molecules.[2, 3, 10, 11, 12] Lehn stated in his 1987 Nobel laureate address that supermolecules are to molecules and the intermolecular bond what molecules are to atoms and the covalent bond."[2] The terminology molecular receptor and substrate implies the similarity of inclusion complexes to biological receptors and substrates. The basic functions of a supermolecule include molecular recognition, translocation and transformation.[2] Receptor molecules may be very selective in what substrate molecules they will bind with, similar to a lock and key. As only one key can open a particular lock, some receptor molecules will only bind with a particular substrate molecule. A lipophilic, membrane-soluble host molecule may act as a carrier for an included guest molecule, effecting the translocation of said guest. If a receptor molecule possesses a reactive function, it may behave as a catalyst, inducing the reaction of a molecule on the bound substrate.[2]

Inclusion complexes usually consist of two molecules, a larger molecule with an internal cavity, referred to as the receptor or host molecule, and a smaller molecule that fits into the cavity, referred to as the substrate or guest molecule. The host and guest molecules are held together by intermolecular forces, such as hydrogen

bonding, van der Waals forces or electrostatic interactions. The guest molecule may be either entirely or partially included within the host cavity. As inclusion complexes consist of host and guest molecules, they are described using a ratio of host to guest molecules per supermolecule. Figure 1 illustrates the stoichiometry of some common host:guest ratios, the most common of which is 1:1.[13] An inclusion complex can be characterized by its geometric, thermodynamic or kinetic features, in other words its structure and conformation, its stability, enthalpy and entropy of formation and its rates of formation and dissociation.[3, 14]

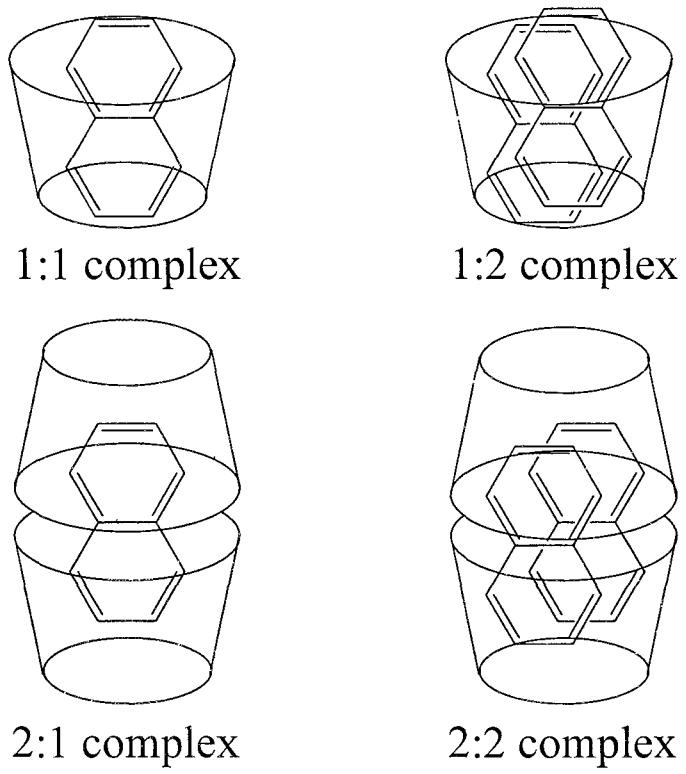


Figure 1. Illustration of the stoichiometry of inclusion complexes.

The intermolecular bonding which occurs in supramolecular systems has lower energy than that involved in covalent bonding. For this reason some issues

may arise when dealing with supramolecular systems. When in solution, some supramolecular complexes, such as cyclodextrin inclusion complexes (see next section), may not exist simply as guest molecules bound within host molecules; they may exist as a mixture of rapidly converting complexed and free molecular species as depicted in Figure 2. For this reason, supramolecular systems may exist slightly differently in solution than in the solid state, where no rapid conversions are possible.

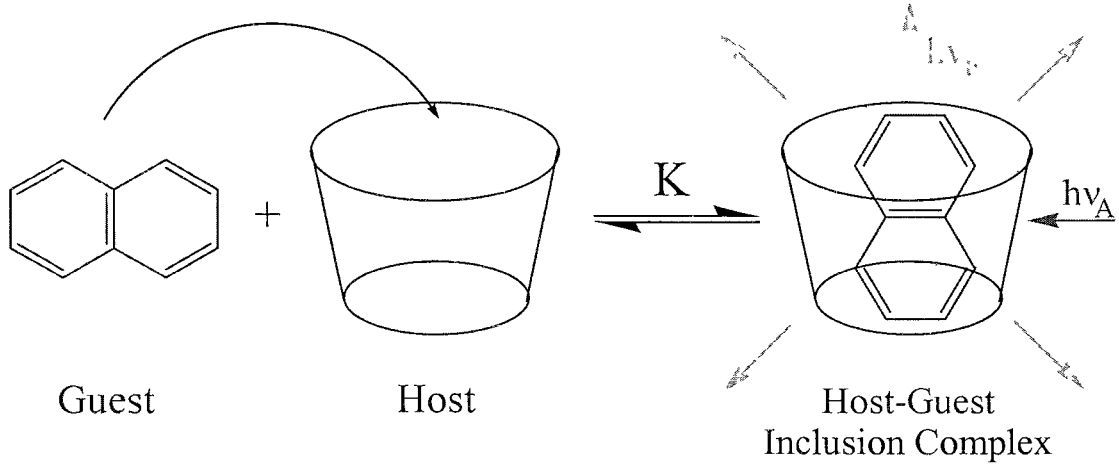


Figure 2. Illustration of a supramolecular host-guest inclusion complex in solution depicting the equilibrium between the complexed and free guest molecules.

By far, the most common family of host molecules used in supramolecular research and in industrial and commercial applications are the cyclodextrins; cyclodextrins will be the host molecule of choice in this research project, as the host component in the proposed fluorescent molecular sensor.

1.3 Cyclodextrins

Due to their general shape and their chemical composition, cyclodextrins (CDs) are ideal molecules for forming inclusion complexes. CDs are homochiral cyclic oligosaccharides.[15, 16] They are produced through the degradation of starch by the enzyme cyclodextrin glucosyltransferase[15]. The CD structure consists of glucose units in the 4C1 chair conformation joined via 1,4-glucosidic linkage,[15, 16, 17, 18] as shown in Figure 3 and 4. CDs are large structures that resemble a bottomless bucket, as depicted in Figure 5. Due to the orientation of the glucose units within a CD molecule, all secondary hydroxyl groups are located on one rim of the "bucket," the larger rim, and all primary hydroxyl groups are on the other smaller rim of the "bucket."[ref60,55] The rigid structures of CDs are stabilized by intermolecular H-bonding between the secondary hydroxyl groups of adjacent glucose units.[15, 16, 18, 19, 20] Due to their shape, other molecules, the guest molecules as described earlier, are able to enter the interior cavity of the CD molecule, as shown in Figure 2. The interior of the "bucket" is hydrophobic and lined with hydrogen and ether oxygen atoms.[15, 16, 18] These hydrogen and oxygen atoms allow for hydrogen bonding between the CD host and the guest. The primary hydroxyl groups of a CD are positioned on the narrow rim of the "bucket", while the secondary hydroxyl groups are positioned on the large rim of the "bucket". All inclusion complexes have an association constant, K. This value describes how tightly the guest and host molecules bind together. The larger the K value, the stronger the association of the molecules leading to the formation of the inclusion complex.

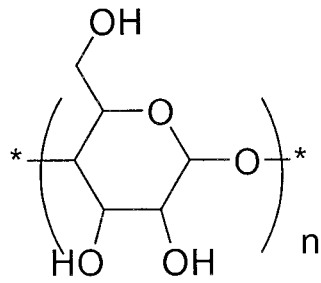


Figure 3. 1,4-Glucosidic linkage.

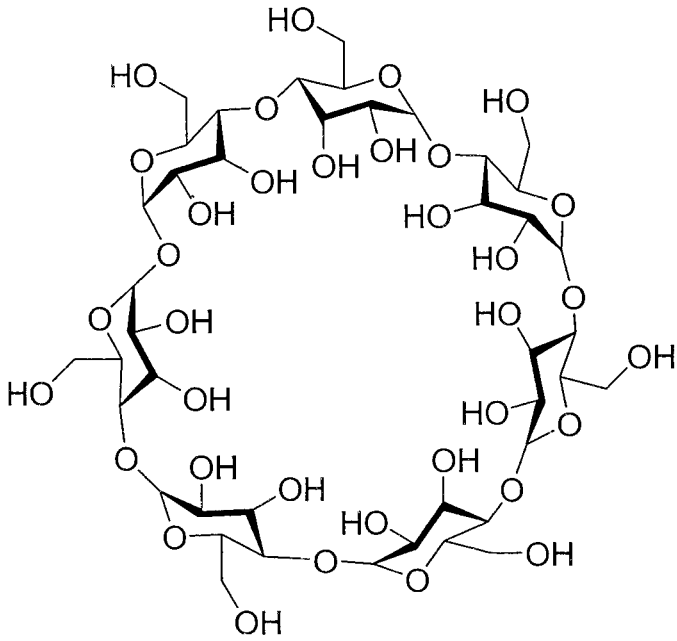


Figure 4. Structure of β-CD.

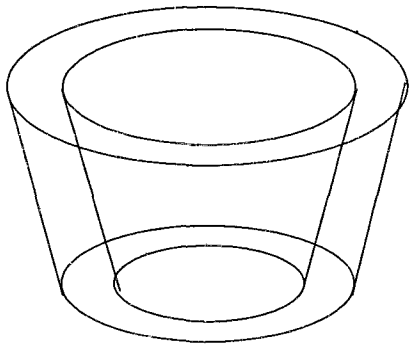


Figure 5. Depiction of the general shape of a CD molecule.

The first naturally occurring CDs were reported in 1891,[15, 16, 18, 21, 20] and by 1997 more than 15,000 articles had been published relating to CDs.[15, 18] The most common naturally occurring CDs are α-CD, β-CD and γ-CD, comprised of 6, 7 and 8 glucose monomer units, respectively.[15, 16, 17] The glucose monomer units that make up these naturally occurring CDs are unmodified, but it is possible to modify natural CDs. Furthermore, the dimensions of a CD can influence the ability of a guest molecule to form an inclusion complex. The physical dimensions of these common CDs are listed in Table 1.

Table 1. The approximate physical dimensions of α-CD, β-CD and γ-CD.[13, 17, 18, 23]

	α-CD	**β-CD**	**γ-CD**
Internal Diameter (top rim)	570 Å	780 Å	950 Å
External Diameter (top rim)	1370 Å	1530 Å	1690 Å
Height of Molecule	780 Å	780 Å	780 Å
Cavity Volume per molecule	174 $Å^3$	262 $Å^3$	427 $Å^3$
Cavity Volume per gram CD	0.10 mL	0.14 mL	0.20 mL
Cavity Volume per mole CD	104 mL	157 mL	256 mL

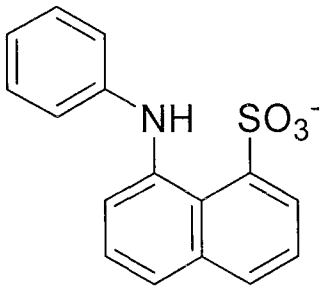
CDs are important among potential receptor molecules for several reasons. CDs are "semi-natural" compounds; they are synthesized from starch, a natural, renewable material. Thousands of tonnes of CDs are produced each year by a relatively simple enzymatic conversion. This method is very environmentally friendly, as there are no unusable byproducts produced and no pollutants are released from the reaction apparatus. CDs themselves are not harmful to the environment upon degradation; the main degradation product from CDs is glucose. Initially CDs were very expensive to produce, but as manufacturing technologies utilized to produce them have evolved, the cost to produce CDs has decreased, making them a cost effective option for industrial applications.

CDs can safely be used in cosmetics, pharmaceuticals and in food products for human consumption as any toxic effects from them are of secondary character and can be eliminated with the appropriate selection of CD, CD derivative, or mode of action.[13, 18] CDs have many different applications in various industries, including food processing, cosmetics, pharmaceuticals and pesticides.[13, 15] CDs became an accepted, common food additive in the 1980's; their use in food production and their consumption has steadily increased since that time.[24] They can aid in the delivery of desirable components, such as pleasant flavor or scent molecules, and encapsulate undesirable components, lessening the impact of such undesirables on a finished product.[13, 24, 25] In cosmetics, CDs have been used to help stabilize ingredients within product formulations by improving the solubility or slowing degradation of individual ingredients.[13, 25, 26] The fact that CDs are soluble in hydrophilic media while their

internal cavity is hydrophobic has been utilized in the pharmaceutical industry. When drug molecules with limited water solubility are included within the cavity of a CD, the solubility of the drug may be increased.[27, 28, 29, 30]

When a guest molecule is included in the internal cavity of a CD, differences between the internal and external environments can affect some of the physical and spectroscopic properties of the guest. One spectroscopic property that may be affected by the hydrophobic environment of the internal cavity of a CD is fluorescence. Fluorescent probes are substrate molecules which undergo changes in their fluorescent properties as a result of non-covalent interactions with a macromolecule. The fluorescence of some probes, such as 1-anilino 8-naphthalenesulfonic acid (1,8-ANS), 2-anilino 6-naphthalenesulfonic acid (2,6-ANS) and 2-toluidino 6-naphthalenesulfonic acid (2,6-TNS), see Figure 6, is greatly increased upon inclusion within a CD, and as a result, these probes are frequently used in inclusion studies.[31]

a)



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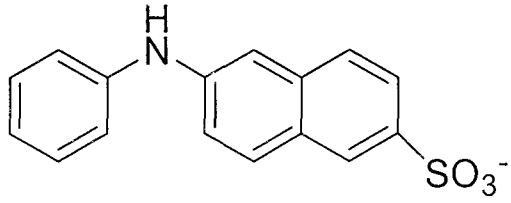


c)

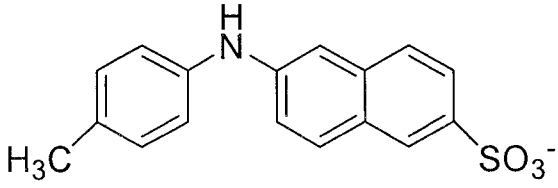


Figure 6. Fluorescent probes **a)** 1,8-ANS, **b)** 2,6-ANS and **c)** 2,6-TNS.

A molecule with a larger association constant than that of an included molecule in a CD complex would be expected to displace the guest out of the CD cavity in order to enter the cavity itself, and form a new CD complex. This fact is the basis for some molecular sensors, those that detect the presence of other molecules or ions.

1.4 Fluorescence Spectroscopy

Luminescence is the emission of light from a substance; it occurs as molecules relax from electronically excited states. Luminescence can be divided into two types depending on the nature of the excited and final states, namely phosphorescence and fluorescence.[32]

Phosphorescence is the emission of light resulting from relaxation between electronic states of different multiplicity. This most commonly involves relaxation from a triplet excited state T_1 to a ground singlet state S_0.[32] This requires an electron to flip its spin, which is a spin-forbidden process. Thus phosphorescence emission rates are slow; phosphorescence lifetimes, which are a measure of the time between the excitation of molecule to an excited triplet state and its return to the ground state, can typically be measured in the range of milliseconds to seconds or even longer. An example of phosphorescence can be seen in "glow in the dark" toys. The phosphorescent substances these toys are constructed from can glow for several minutes; upon exposure to light, the excited triplet states formed relax very slowly to the ground state, resulting in an extended "glowing period." It should be noted that phosphorescence is a phenomena not usually seen in room temperature solutions as there are many deactivation processes which compete with light emission, such as non-radiative decay and quenching processes.[32]

Fluorescence is the light energy emitted by an excited molecule when it relaxes from an electronically excited state to a lower energy state of the same multiplicity. Aromatic molecules are good candidates for this process because of the π-bonds they contain. When the electrons of an aromatic molecule are excited, they

are promoted from a π-energy level up to a π*-energy level. When the energy source is removed from the molecule, the excited electrons rapidly relax back to their original π-energy level. In relaxing, the electrons release the energy they had accumulated in the form of light photons. These released photons of light make up the fluorescence of the molecule. Since this is a spin-allowed process, typical fluorescence lifetimes can be measured in nanoseconds.[32] This is why in contrast to the case of phosphorescence, with fluorescence once the light, or excitation, source has been removed, it appears that the light emission from the molecules stops immediately (*i e.* within nanoseconds).

There are also non-radiative transitions, including internal conversion (IC) and intersystem crossing (ISC), which result in the relaxation of excited electronic states to lower energy states, such as the ground state. These occur through relaxation of electrons from the excited state without the emission of a photon, but rather via the release of the excess electronic energy as heat, and thus compete with fluorescence and phosphorescence. If the non-radiative transitions are very strong, then the fluorescence (or phosphorescence) will be very weak. These various radiative and non-radiative transitions are shown in Figure 7

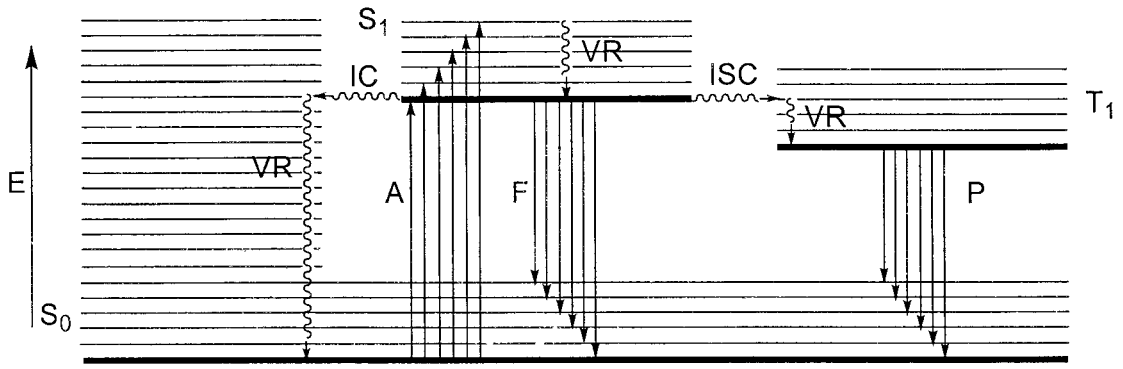


Figure 7. The Jablonski diagram.

The first known fluorescent substance, or fluorophore, was reported in 1845 by Sir J. F. W. Herschel.[32,33] He described dissolving equal masses of sulphate of quinine and tartaric acid in water. This very dilute solution was completely colourless, but when Sir Herschel observed his solution in bright sunlight, it exhibited "an extremely vivid and beautiful celestial blue colour." We now know that this blue colour is the fluorescent emission of quinine when excited by UV light. Quinine and its fluorescent properties have been credited with stimulating the development of the first spectrofluorometers in the 1950's.[32] During World War II, the US Department of Defense was interested in monitoring antimalaria drugs, one such drug being quinine. As a result, a program was initiated at the US National Institute of Health to develop the first practical spectrofluorometer.[32 34]

Fluorescence spectral data is generally presented as an emission spectrum, a plot of fluorescence intensity versus wavelength or wavenumber (expressed in nm or cm^{-1}, respectively) and tend to be broad peaks, with a single maximum. The three main physical parameters that describe the fluorescence of a molecule are the

wavelength(s) of maximum emission ($\lambda_{F,max}$), the quantum yield (ϕ_F) and the fluorescence lifetime (τ_F).[16, 17]

Fluorescence lifetime is the time required for molecules excited to S_1 to decay by all pathways, fluorescence, internal conversion and intersystem crossing. It can be calculated by Equation 1, where k_F, k_{IC} and k_{ISC} are the first order rate constants for fluorescence, internal conversion and intersystem crossing.[16, 17] k_{IC} and k_{ISC} are often combined into a single rate constant, k_{nr}, representing non-radiative decay.[16, 17]

$$\tau_F = 1 / (k_F + k_{IC} + k_{ISC}) \qquad \text{Equation 1}$$

Fluorescence quantum yield is a way in which to describe the efficiency of fluorescence.[16, 17] It is the fraction of excited molecules which decay by fluorescence. It can be calculated by Equation 2.[16, 17] It can be determined experimentally by integrating the area under a fluorescence spectrum expressed as a function of frequency (or wavenumber). This area is then compared to the area of a reference fluorescent probe that has been measured under the exact same experimental conditions.[16, 17]

$$\phi_F = k_F / (k_F + k_{IC} + k_{ISC}) \qquad \text{Equation 2}$$

The physical parameters $\lambda_{F,max}$, ϕ_F and τ_F of a fluorescent probe can all be changed upon inclusion within the cavity of a host molecule. Changes in the parameters describing the fluorescence of a probe upon inclusion can be the result of one or more of the following five factors.[16, 17] First, upon inclusion, the probe may be protected from fluorescence quenchers. Second, the intramolecular rotational

mobility of the probe may be restricted, and therefore reduced, upon inclusion within the cavity of a host molecule. Third, the polarity of the environment inside the host cavity may be different than the polarity of the environment around the host molecule. Fourth, probe-water interactions may be prevented upon inclusion within a host cavity. Fifth, if inclusion occurs as dimers, excimer emission or self-quenching may occur.[16, 17]

The most prominent effect is generally that caused by the difference in polarity inside and outside of the host cavity.[16, 17] When a fluorescent guest in aqueous solution becomes included within the cavity of a CD, it experiences a significant decrease in polarity. This decrease in polarity results in a small destabilization of the S_0 ground state and a much larger destabilization of the more polar S_1 excited state.[16, 17] As shown in Figure 8, the difference in the destabilization results in a significantly larger energy gap, $\Delta E(S_1\text{-}S_0)$, in the less polar environment of the CD cavity as compared to the aqueous environment.[16, 17] The increase in the energy gap results in a decrease in the rate of IC from S_1 to S_0. According to the energy gap law, k_{IC} decreases exponentially with increasing energy gap: $k_{IC} \propto e^{-\Delta E(S1\text{-}S0)}$. As $\Delta E(S_1\text{-}S_0)$ is larger inside the CD cavity, k_{IC} is smaller.[16, 17] With a smaller rate constant, IC does not complete as well with fluorescence inside the CD cavity, and as a result, the fluorescence quantum yield of the guest is increased upon inclusion within the CD cavity, see Equation 2, resulting in enhanced fluorescence emission.[16, 17]

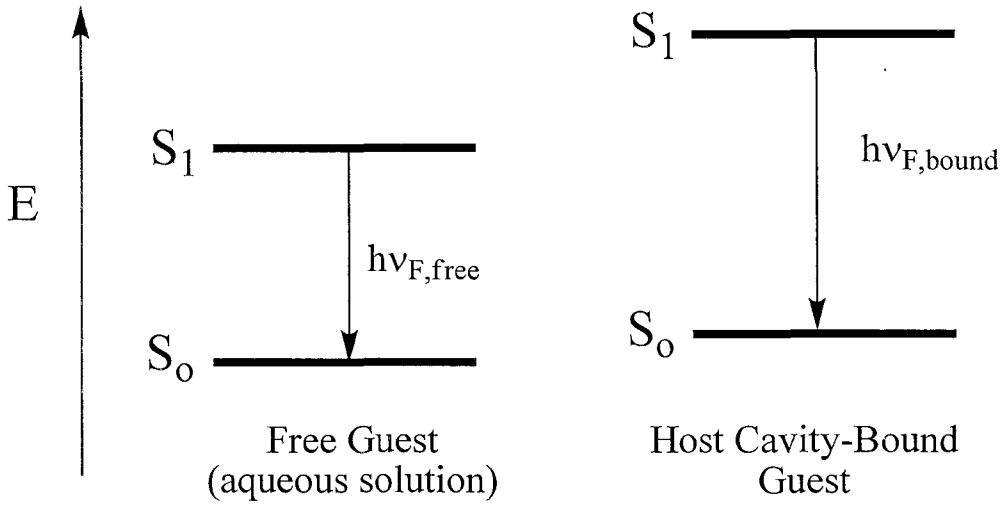


Figure 8. Relative S_1-S_0 energy gap of a polarity sensitive fluorescence guest in aqueous solution and included within a CD cavity.

1.5 Fluorescent Sensors

A fluorescent sensor is a type of molecule, or supramolecular inclusion complex, which experiences a substantial increase, or decrease, in fluorescence emission when exposed to particular conditions, such as pH or temperature.

There are two general types of fluorescent probes or fluorophores: intrinsic and extrinsic.[32] Intrinsic fluorophores are naturally occurring in the system, some examples include amino acids in proteins, flavins and some chlorophyll derivatives.[32] This type of fluorophore naturally possesses fluorescent properties that can be enhanced or quenched depending on their environment. These are frequently used when dealing with protein identification and monitoring.[32] Extrinsic fluorophores are added to a sample when no fluorophore exists or to change the fluorescent emission properties. Extrinsic fluorophores can be used to tag a non-fluorescent molecule of interest. A tagged molecule can then be studied in the presence of other non-

fluorescent molecules, as it is easily distinguishable. Examples of extrinsic fluorophores include dansyl chloride, fluorescein , rhodamine and the anilinonaphthalene sulfonates.[32]

A fluorescent sensor responds to fundamental supramolecular phenomena, a dramatic change in fluorescence emission can result in response to an external condition. Some fluorescent probes can act as fluorescent sensors by themselves, reacting to their environment; others will only act as effective fluorescent sensors when in the presence of a receptor molecule, the inclusion complex acting as the sensor, with the environment dictating whether or not the probe will be included. Fluorescent sensors can have several different modes of operation, but all modes of operation have one thing in common, the specific receptor-substrate binding induces an observable change in the molecule: no or weak fluorescence to highly fluorescent ("switch-on") or vice versa ("switch-off"). The fluorescence of the probe used in a fluorescent sensor may be quenched or significantly increased when in the presence of a particular substance.[32] Some sensors allow for the measurement of free ions, such as Cl^-, Na^+ or Ca^{2+}, which by themselves are spectroscopically silent.[32] Probes used in this type of fluorescent sensor are called wavelength-ratiometric probes. When using this type of probe, the analyte concentration can be determined from the ratio of fluorescence intensities, either excitation or emission, at different wavelengths.[32] Another type of fluorescent sensor uses fluorogenic probes. These probes are not fluorescent or only very weakly fluorescent until a particular event occurs, for example enzymatic cleavage, at which point the probe become highly fluorescent.[32] This type of fluorescent sensor could be used to monitor the progress

of a reaction. Other probes may show a shift in fluorescence emission in the presence of particular substances or under particular environmental conditions.[32]

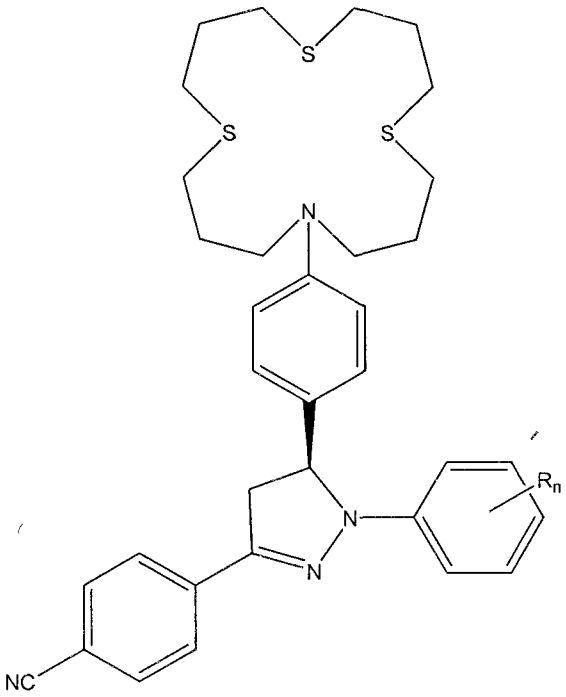
When designing a fluorescent sensor several factors should be considered. If the desired sensor is to successfully target a specific analyte, the proposed receptor must match in size and preferentially bind that specific analyte. It is also important when designing a fluorescent sensor to select a probe that will react under the conditions that will be studied. In other words, not all fluorophores are created equal. If the expectation is to monitor a change pH, only fluorescent probes sensitive to pH need be considered.

1.6 Tethered Probes

Sometimes probes are not merely part of an inclusion complex with another, larger, host molecule; they are actually directly linked to the host. Just as with un-tethered inclusion complexes, tethered inclusion complexes can also act as fluorescent sensors. There are some instances in which it may be desirable to have a fluorescent guest molecule that is covalently attached to a host molecule. In such cases, the close proximity of the host and guest moieties facilitates the formation of an inclusion complex, proving to be, at least in theory, a more efficient system than one in which the guest and host molecules exist as separate molecules.[17]

There are several types of fluorescent molecular sensors that can be constructed by tethering a fluorescent probe to a host molecule. Examples of tethered sensors include 1,3,5-triarylpyrazoline tethered to a 16-member thiazacrown molecule, shown in Figure 9.[35] This tethered molecule is used to detect copper(I). As

the sensor encounters copper(I) ions, the 1,3,5-triarylpyrazoline dissociates from the host cavities and the copper(I) ions become included and fluorescence emission is increased, therefore the more intense the fluorescence the more copper(I) ions are present.[35]



1a R_n=3-F
1b R_n=2,5-F_2
1c R_n=2,3,5-F_3
1d R_n=2,3,5,6-F_4
1e R_n=2,3,4,5,6-F_5

Figure 9. 16-Member thiazacrown molecule with 1,3,5-triarylpyrazoline tether.

Fluorescence based sensors may also be constructed using protein molecules as receptor. Fluorescence based sensors are particularly well suited for measurements within cells and in vivo as they permit non-invasive measurement of the analyte of interest, such as metal ions or metabolites.[36, 37] They may allow for real time tracking of the secretion of and dispersion of neurotransmitters, an action needed in modern neurobiology. One particular type of fluorescent biosensor may detect both zinc ions

and sulfonamides, depending on the binding protein used, either human carbonic anhydrase II or *apo*- human carbonic anhydrase II.[37] In detecting either zinc or sulfonamide, in the absence of the analyte, the intermolecular ligand binds to the binding protein, closing the biosensor, resulting in a dramatic increase in fluorescence emission, essentially "turning the light on." In the presence of zinc or sulfonamide, the biosensor is opened as the analyte displaces the intermolecular ligand, "turning the light off" as the fluorescence emission is greatly reduced.[37] Another type of fluorescent biosensor that detects sulfonamide involves tethering a fluorescent probe directly onto an enzyme, in close proximity to the active site on the enzyme. In the presence of sulfonamide, the probe dissociates and the sulfonamide binds to the enzyme, resulting in a very large decrease in the intensity of fluorescence emission, thus the more intense the fluorescence observed, the lower the sulfonamide concentration.[38] This is another fluorescence sensor that can be used on the surface of or within a living cell.

In another instance, (dimethylamino)nitrostilbene, a fluorescent dye, was tethered to a glass surface in order to monitor the interfacial region between the glass and a resin applied to the glass.[39] As the resin cured, the fluorescence emission of (dimethylamino)nitrostilbene not only increased in intensity, it also under went a blue shift.[39]

A tethered fluorescent sensor may also serve more than one purpose. A modified β-CD molecule with a tethered azobenzene fluorophore has been used as a gated ion channel in a phospholipid vesicle membrane.[40] The CD cavity is elongated by substitution of pentabutlene glycol anime at all seven primary hydroxyl

positions.[40] In the case of this tethered fluorescent sensor, 4-phenylazo-phenol is used as the gate and the tether is composed of four alkyl carbons from 1,4-dibromobutane.[40] A tether of this length allows for free movement of the gate, 4-phenylazo-phenol, in and out of the CD cavity. As shown in Figure 10, when in the trans conformation, the azobenzene moiety is included in the CD cavity and it acts as closed gate, only allowing for the transfer of cations across the membrane. After the tethered fluorescent sensor has been irradiated with UV light (~350 nm) for one minute, the azobenzene gate changes to the cis conformation. In the cis conformation, the azobenzene moiety dissociates from the CD cavity, allowing the transfer of larger ions across the membrane.[40] A change in the inclusion complex, and thus changes in the rate of ion transport, can be observed by a decrease in absorption and fluorescence emission.[40]

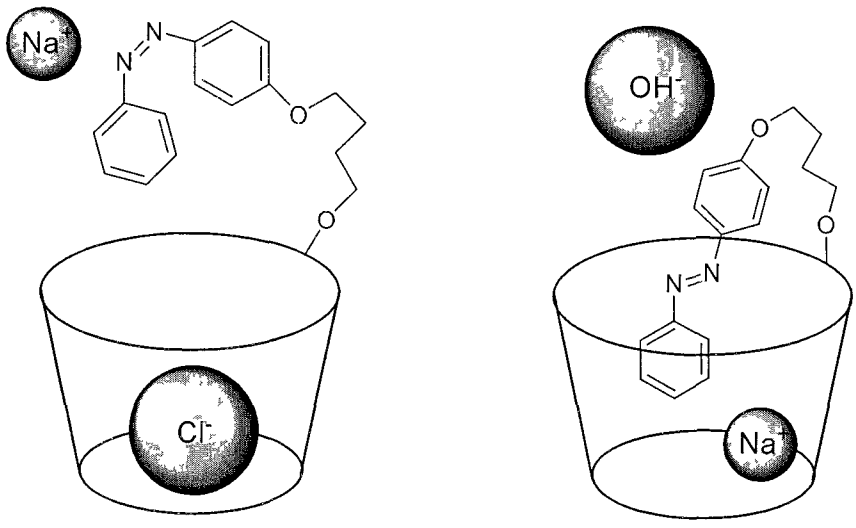


Figure 10. Schematic diagram of anion and cation transport through ion channel based on reference 40.

Tethered CD inclusion complexes may also be used as fluorescent sensors outside of a membrane. β-CD with a tethered naphthyl group has been used to detect not only the presence, but also the concentration of viologen molecules.[41, 42] An inverse correlation was observed between the intensity of fluorescence emission of the tethered CD and the concentration of methylalkyl viologen, the higher the viologen concentration, the greater the reduction in fluorescence emission that was observed.[41] This is how the tethered CD proposed in this Masters project will function, detecting an analyte and indicating at least relative concentration.

1.7 TNS as an Appropriate Fluorophore for a Supramolecular Sensor

ANS and TNS are in a class of substrate molecules that only weakly fluoresce in water, but fluoresce very strongly in organic solvents or when bound to molecules which offer a hydrophobic environment, making it very obvious to determine if they are part of an inclusion complex or not. In 1954, Weber and Laurence determined that 2,6-TNS and 1,8-ANS were not fluorescent in water, but underwent significant fluorescence enhancement when they were adsorbed onto bovine serum albumin.[31, 43] That same year, Fildes et al. proposed a fluorometric methodology which detected albumin using ANS.[31] 2,6-TNS may be used to assess the relative polarity of a solvent.[44] TNS is currently a widely used fluorescent probe as it has been shown to have a very large, polarity induced fluorescence enhancement.[32, 45, 46] In 1977, 2,6-TNS was observed to undergo a 25 fold increase in fluorescence enhancement upon inclusion within the cavity of β-CD and a 571 fold increase in fluorescence enhancement upon inclusion within the cavity of the modified CD, poly-acryloyl-β-

CD.[45] In 1976, inclusion complexes were formed by the inclusion of 2,6-TNS within the cavities of each of α-CD, β-CD and γ-CD.[46] It was determined that 2,6-TNS formed a 1:1 host:guest inclusion complex with α-CD, while it formed 2:1 complexes with both β-CD and γ-CD.[46] 2,6-TNS is unable to form a 2:1 inclusion complex with α-CD because of size constraints. The inner diameter of the α-CD cavity is simply too small to include the naphthalene portion of 2,6-TNS. With β-CD and γ-CD, the cavities are of sufficient size to accommodate both portions of 2,6-TNS, the naphthalenesulfonyl group and the toluidinyl group. A sensor composed of 2,6-TNS tethered to a CD may prove to be useful in multiple applications.

It should be noted that the structures of 2,6-ANS and 2,6-TNS vary by a single methyl group in the C-4 position of the single benzene ring, see Figure 6 b and c. If the probe is tethered to the CD at this location it would not be possible to determine if the starting material was in fact 2,6-ANS or 2,6-TNS by simply looking at the structure of the final tethered product. This may offer additional approaches in the synthesis of the desired product.

1.8 Specific Goals of Project

The goals of this Masters project are to investigate synthetic methods for tethering a fluorescent probe, either ANS or TNS, to a CD and to evaluate the properties of the tethered probe and its potential effectiveness as a fluorescent molecular sensor. The fluorescent probe, be it ANS or TNS, should be tethered to a secondary hydroxyl group on a CD. This should allow for the probe to move in and out of the CD cavity with more freedom as the secondary hydroxyl groups are on the

larger rim of the CD. The probe should be tethered at the C-4 position of the single benzene ring. This would make it more likely that the naphthalene portion of the probe would be able to enter the CD cavity and in doing so would promote the desired substantial increase in fluorescence emission. There should be only one probe tethered to each CD molecule. If multiple probe molecules are tethered to a single CD molecule, there may be issues with the probes entering the cavity; the probe molecules may hinder each other's movement. By designing the probe in such a fashion as to promote ease of movement of the probe in and out of the CD cavity, the effectiveness of the sensor is promoted. If the probe is able to freely move in and out of the CD cavity without any hindrance, the change in fluorescence emission should be very distinctive and extremely large, making for very obvious on and off position of the "switch". The length of the tether will be adjustable. The ability to change the length of the tether should allow for the determination of the most efficient sensor. Each molecular sensor should be self contained, that is each fluorescent probe should form an inclusion complex with the CD it is tethered to, as opposed to inclusion complexes forming between sensor molecules. The ultimate goal of this project is to synthesize a reusable, fluorescent, molecular sensor, with an extremely large dynamic range, *i.e.* the difference in intensity of the "on" *versus* the "off" signals, larger than any such sensor previously reported. Developing a tethered system is an important first step in achieving these ultimate goals.

2 Experimental

For this work all samples for fluorescence spectroscopy were prepared in deionized water or phosphate buffer. The buffer was prepared by dissolving 8.44 g (0.0620 mole) of potassium dihydrogen phosphate and 6.60 g (0.0379 mole) of potassium hydrogen phosphate in 1.00 L of deionized water. Table 2 contains a list of all chemicals used. Unless otherwise stated, all chemicals were used as received. Absorbance was measured on a Varian Cary 50 Bio UV-Visible Spectrometer. Fluorescence emission was measured on a Perkin Elmer LS55 Luminescence Spectrometer or a Photon Technology International Fluorimeter. Nuclear magnetic resonance (NMR) spectroscopy was conducted on a Bruker Avance 300 MHz Nuclear Magnetic Resonance Spectrometer. Infrared (IR) spectroscopy was performed using a Bruker Alpha FTIR Spectrometer. Mass spectroscopy analysis was done on a Thermo HUPLC Accela Mass Spectrometer. Samples were freeze dried on a Virtis Benchtop Freeze Drier.

Table 2. Chemicals used.

Chemical	Purity	Supplier
1,4-Diiodobutane	99+%	Aldrich
1.8-ANS	97%	Aldrich
2,6-ANS	97%	Aldrich
Dimethyl-β-CD	1.6-2.0 moles CH_3/ anhydoglucose unit	Aldrich
2,6-TNS	≥97%	Fluka
4-Dimethylaminopyridine	99%	Aldrich
Acetic acid	99.7%	Fisher Scientific

Acetone	99.7%	Caledone
Acetonitrile	99.8%	Sigma-Aldrich
Acetyl chloride	98%	Aldrich
Ethanol	100%	Fisher Scientific
Benzene	99%	Aldrich
Bromoacetyl bromide	98%	Aldrich
Chloroform	99.8%	Sigma-Aldrich
Cyclohexane	≥98%	Eastman
Deuterium oxide	99.9%	Aldrich
Dimethyl sulfoxide-d_6	99.9%	Aldrich
Di-*tert*-butyl dicarbonate	97%	Aldrich
Ethyl acetate	99.5%	Caledon
Hydrochloric acid	37%	Fisher Scientific
Methanesulfonyl chloride	99.7%	Aldrich
Methanol	99.8%	Aldrich
N,N-Dimethylformamide	99.8%	Aldrich
N-bromosuccinimide	99%	Aldrich
Potassium dihydrogen phosphate	≥99.0%	Sigma
Potassium hydrogen phosphate	≥99.5%	Fluka
Pyridine	99+%	Aldrich
Sodium chloride	98+%	Aldrich
Sodium hydride	60%	Aldrich
Sodium hydrogen phosphate	100.2%	Fisher Scientific
Sodium hydroxide	97+%	Sigma-Aldrich
Sodium sulfate	≥99%	Sigma
t-Butanol	99.8%	Fisher Scientific
Toluene	99.7%	OmniSolv
p-Toluenesulfonyl chloride	98%	Aldrich
Triethylamine	99.5%	Aldrich
α,α'-Azobis-(isobutyronitrile)	98%	Sigma-Aldrich

Hydroxypropyl-β-CD	98%	Aldrich
β-CD	98%	Aldrich

2.1 Intermolecular Host-Guest Binding Studies

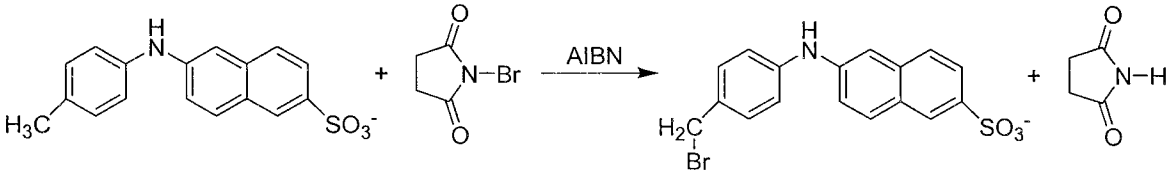
Probe solutions of 0.03 mM were prepared in phosphate buffer; the probes used in this study were 1,8-ANS, 2,6-ANS and 2,6-TNS. The absorbance of each solution was measured to ensure that it was in the proper range for fluorescence studies, between 0.20 and 0.40 at 330 nm, and the solutions were diluted as required with additional phosphate buffer solution. The probe-buffer solution was used to prepare CD solutions, with dimethyl-β-CD and β-CD, ranging from 0 to 10 mM; see Table 3 for probe-CD combinations studied. Dimethyl-β-CD was chosen as the modified β-CD for these binding studies due to its potential use in the synthesis of the desired tethered sensor. For each solution, the appropriate mass of the CD of interest was weighed in a small vial then 3.00 mL of probe solution was added and the solution was shaken until the CD was completely dissolved. The fluorescence of this series of solutions was measured from 350-600 nm, with an excitation wavelength of 330 nm. The integrated area of each spectrum was determined and used to determine the fluorescence enhancement from the CD. Fluorescence enhancement (F/Fo) is defined as the integrated area of the fluorescence spectrum of the probe in the presence of CD (F) divided by that of the probe in the absence of CD (F_O). This data was used to determine the host:guest ratios and association constants for the various inclusion complexes.

Table 3. CD-probe combinations studied.

CD	Probe
2,6-dimethyl-β-CD	1,8-ANS
2,6-dimethyl-β-CD	2,6-ANS
2,6-dimethyl-β-CD	2,6-TNS
β-CD	2,6-TNS

2.2 Modification of Potential Guest Molecules

2.2.1 Attempted Bromination of TNS



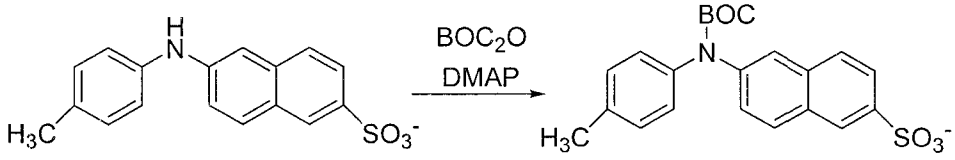
N-bromosuccinimide (NBS) was recrystallized following the published procedure.[47] 5.87 g (0.0330 mole) of NBS was dissolved in 60 mL of boiling deionized water. This solution was filtered by gravity then placed in an ice bath for 2 hours. The resulting crystals were filtered under vacuum and washed with ice cold deionized water. The NBS crystals were then placed under vacuum over anhydrous calcium chloride for 2 days to thoroughly dry.

0.5458 g (1.739×10^{-3} mole) of 2,6-TNS was dissolved in 12.3 mL chloroform along with 0.5261 g (2.956×10^{-3} moles) of recrystallized NBS and 0.0034 g (2.071×10^{-5} moles) of α,α'-azobis-(isobutyronitrile) (AIBN). This reaction mixture was refluxed at 60°C under argon for 1 hour then cooled to 10°C. The resultant precipitate was filtered under vacuum and washed with ice-cold deionized water The filtrate was reduced in volume to approximately 10 mL by rotary evaporation then

freeze-dried. A yield of 0.1270 g was obtained. Small portions of the two solids were prepared for NMR analysis in deuterium oxide.

The fact that 2,6-TNS was not particularly soluble in chloroform may have resulted in the failure of this reaction. It was thought that attempting this reaction in a different solvent might improve the outcome. The solubility of 1,8-ANS was also investigated as a possible alternative fluorescent probe. 3.0 mL of each solvent was combined with approximately 0.0010 g of 2,6-TNS (3.2×10^{-6} mole) and 1,8-ANS (3.3×10^{-7} mole). The solvents used were: chloroform, methanol, acetonitrile, acetic acid, benzene, cyclohexane and toluene. Although 2,6-TNS and 1,8-ANS were not overly soluble in chloroform, it was observed that they were both appeared more soluble in it than in any other solvent listed, and 1,8-ANS did not appear any more soluble in chloroform than 2,6-TNS.

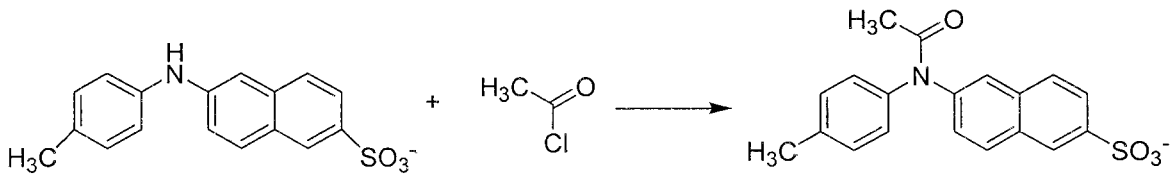
2.2.2 Attempted Protection of TNS with BOC



0.8069 g (2.571×10^{-3} mole) of 2,6-TNS was combined with 2.1399 g (9.8048×10^{-3} mole)of di-*tert*-butyl dicarbonate (BOC_2O) and 0.2327 g (1.905×10^{3} mole) of 4-dimethylamino pyridine (DMAP) in 6.0 mL of acetonitrile. This mixture was stirred overnight under argon. 10.0 mL of deionized water was added to the reaction mixture and it was again left overnight. This mixture, in which some crystals had formed, was then washed with 8.8 mL of ethyl acetate. The

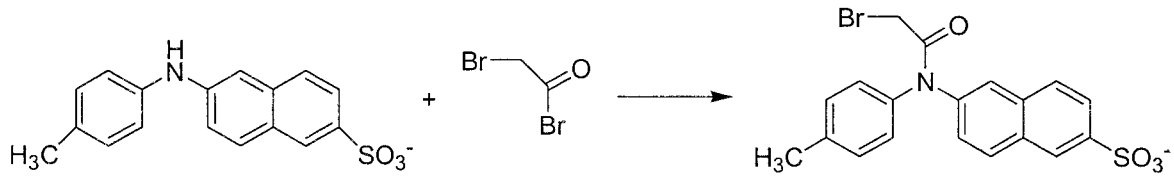
crystals did not dissolve; they were filtered by gravity from the aqueous layer after separation. The fluorescent product was eluted through Isolute© HM-N pre-packed solid phase extraction columns to remove the water from the product and then extract the product into ethyl acetate. The aqueous layer was poured onto two columns, each with a solvent capacity of 20 mL, and a total of 250 mL of ethyl acetate was used to elute the product. Elution was continued until the columns no longer fluoresced under ultraviolet (UV) light. This ethyl acetate solution was placed in a separatory funnel and 85 mL of 1 molar hydrochloric acid was added. This mixture was vigorously shaken for two full minutes, at which point the aqueous layer was removed and placed under a UV light to determine if any of the fluorescent product was dissolved in it. As the only visible fluorescence was in the organic layer, it was washed with 84 mL of saturated sodium chloride solution. Again the aqueous layer was removed and placed under a UV light to check for fluorescence. The organic layer was washed three additional times with approximately 85 mL of sodium chloride solution. Each time only the organic layer displayed any fluorescence. The organic layer was then dried over anhydrous sodium sulfate. The dried organic solution was filtered by gravity and concentrated by rotary evaporation until only a few drops of liquid remained. This mixture was then freeze dried. A yield of 0.0940 g was obtained. A small sample of the product was dissolved in deuterium oxide for NMR spectroscopy. A small sample was also analyzed by infrared spectroscopy.

2.2.3 Attempted Protection of TNS as Acetamide



0.2467 g (7.872×10^{-4} mole) of 2,6-TNS was combined with 0.2831 g (0.1968 mole) sodium hydroxide in 5.7 mL deionized water. This mixture was stirred for several minutes under nitrogen gas then 0.430 mL (6.05×10^{-3} mole) acetyl chloride was added. This reaction mixture was allowed to stir for approximately one hour and was then freeze dried. 2.0 mL of 10% hydrochloric acid was added to 0.2915 g of the resulting product. This product was stirred for several minutes then freeze dried again to give an intractable black tar-like material.

2.2.4 Attempted Reaction of TNS with Bromoacetyl Bromide

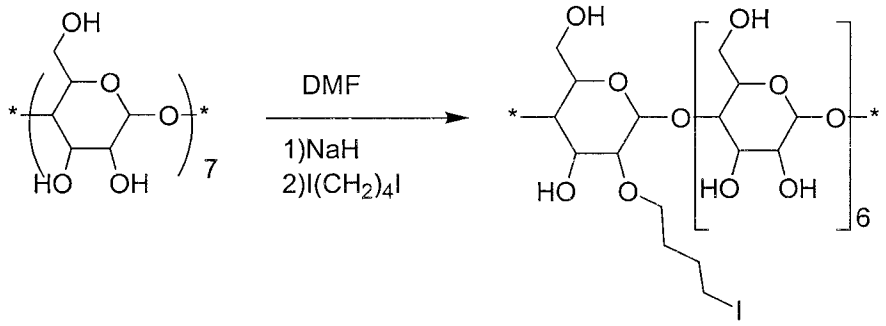


0.2826 g (9.003×10^{-4} mole) 2,6-TNS was stirred in 8.0 mL of pyridine under nitrogen gas until the solid was dissolved. 0.08 mL (9.21×10^{-4} mole) of bromoacetyl bromide was added and the solution was stirred for several minutes. A few drops of deionized water were added to the reaction mixture. It was then concentrated to approximately half of the original volume in a rotary evaporator. 3.0 mL deionized water was added to the reaction mixture. The reaction mixture was concentrated again to approximately 3 mL on the rotary evaporator and then freeze dried.

A small sample of the resulting product was dissolved in deuterated dimethyl sulfoxide (DMSO-d_6) for NMR analysis. The product was also analyzed by Attenuated Total Reflectance (ATR) IR spectroscopy. 0.3045 g of product was dissolved in 10.0 mL of deionized water at 85°C. This solution was cooled to room temperature then placed in the refrigerator to recrystallize. As no precipitate formed, 3.0 mL of methanol was added and the solution was returned to the refrigerator. After several days in the refrigerator, no precipitate had formed so the solution was concentrated by rotary evaporation in the hopes of saturating the solution, thereby forcing some of the product to crystallize out of solution. As the solution concentrated, the mixture began to bear a strong resemblance to the tar-like product obtained previously with acetyl chloride. As acetylating 2,6-TNS was not proving to be a successful endeavor, investigations were made into modifying the CD.

2.3 Modification of β-CD

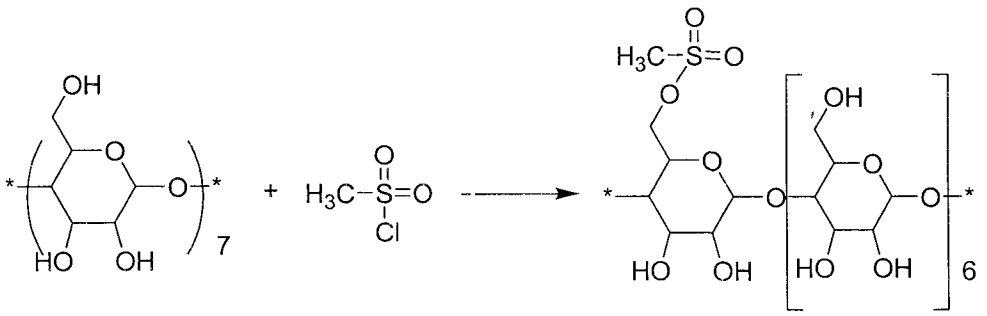
2.3.1 Reaction of β-CD with Diiodobutane



According to the published procedure,[48, 49] 5.1153 g (4.5108×10^{-3} mole) of oven dried β-CD was added to 30 mL of freshly distilled N,N-dimethylformamide (DMF). To this solution, 0.1097 g (2.742×10^{-3} mole) of sodium hydride was added.

This reaction mixture was stirred overnight under nitrogen gas. 0.583 mL (2.58×10^{3} mole) of 1,4-diiodobutane was added to this reaction mixture, which was then stirred for 5 hours. 480 mL of ice cold acetone was added to the reaction mixture to form a precipitate. The solid was filtered under vacuum and washed with an additional 145 mL of ice cold acetone. The solid was dissolved in as little deionized water as possible, 11 mL, and this solution was poured onto a size exclusion Sephadex chromatography column. The Sephadex column had previously been prepared as follows: 17.8243 g of dry Sephadex G-15 powder was combined with 82 mL deionized water and left to stir for approximately 2 hours in a boiling water bath. The column was constructed using a clean, dry burette. Approximately 1 cm of cotton ball was gently packed at the bottom of the column. Clean, dry sand was poured into the column to a height of approximately 1 cm. The swelled Sephadex was cooled to room temperature then poured into the column. While the Sephadex gel was packing within the column, the excess water was drained off of the column. The prepared column had a diameter of 3.0 cm and a height of 12.5 cm. Deionized water was used to elute the product. Gentle pressure was applied to the column to facilitate the elution of the product through the column. The yellow band was collected and freeze-dried in order to obtain a solid; 2.6979 g of product were obtained. This solid product was analyzed by NMR spectroscopy.

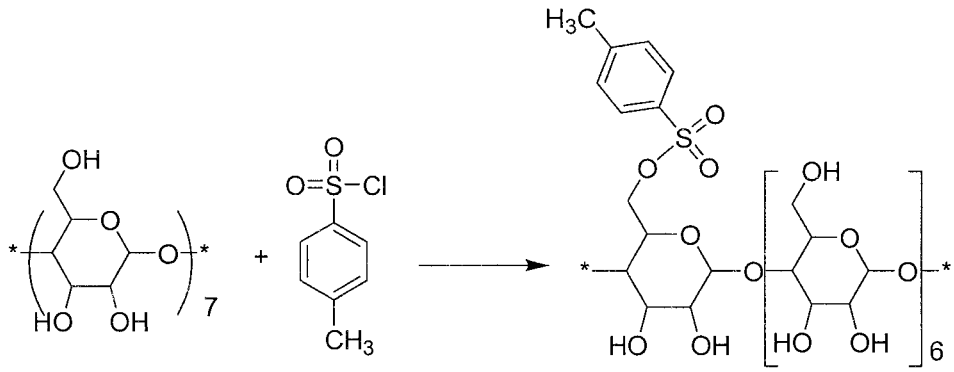
2.3.2 Attempted Reaction of β-CD with Methanesulfonyl Chloride



9.724 g (8.5750×10^{-3} mole) of oven dried β-CD was dissolved in 19.0 mL pyridine while under nitrogen gas. The reaction mixture was placed in an ice bath and allowed to stir for approximately 10 minutes. At this point, the reaction mixture had turned into a gel. To liquefy this mixture, the reaction flask was removed from the ice bath and placed in a hot water bath (45°C). After 15 minutes, some liquid was again visible, at this point, a further 6.0 mL of pyridine were added to the reaction flask and the remaining solid was broken apart with a spatula. This mixture was left to stir in the hot water bath for 20 more minutes. Once the mixture was completely liquid again, it was removed from the hot water bath and left to cool to room temperature. It was then placed in a cold water bath. 1.0 mL (0.013 mole) methanesulfonyl chloride (Mesyl-Cl) was added at a rate of one drop every 7-10 seconds. As the drops of Mesyl-Cl were added to the reaction flask, ice was added to the cold water bath. By the time approximately 0.35 mL of Mesyl-Cl was added, the cold water bath had reached 0°C. The reaction mixture was left to stir in the ice bath for 30 minutes. The reaction mixture was then removed from the ice bath and allowed to return to room temperature. It was left to stir overnight. The reaction mixture was concentrated to approximately 5 mL by rotary evaporation. 220 mL of

cold methanol was added. The resulting mixture was filtered by gravity. The collected solid was dissolved in 13.0 mL 85°C deionized water. This solution was cooled to room temperature. 65.0 mL methanol was added to facilitate the formation of precipitate. The precipitate was collected via gravity filtration. It was dissolved in 5.0 mL 87°C deionized water. 75 mL cold methanol was added and the mixture was placed in the refrigerator. The resultant precipitate was filtered by gravity and washed with approximately 50 mL of cold methanol. 0.261 g of product was obtained. A small sample was dissolved in deuterium oxide for NMR analysis.

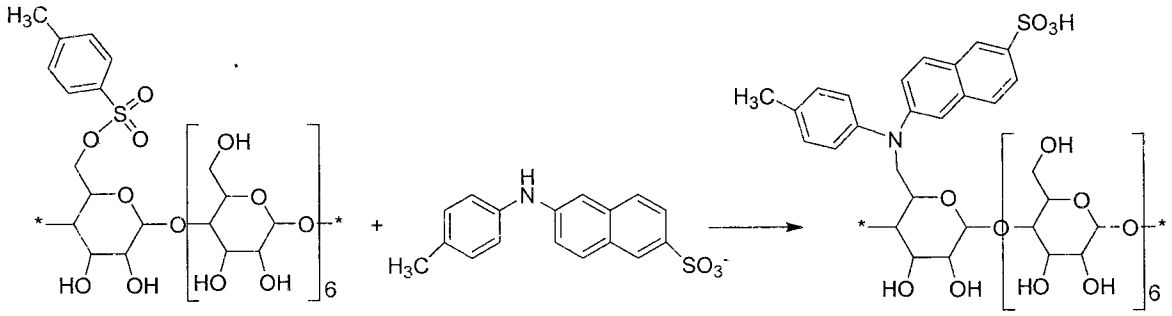
2.3.3 Tosylation of β-CD



9.9447 g (8.7696×10^{-3} mole) of oven dried β-CD was dissolved in 35.0 mL pyridine; the solution was stirred under nitrogen gas for approximately 10 minutes. The reaction flask was placed in an ice bath and allowed to stir for an additional 10 minutes. As when the Mesyl-Cl reaction was attempted, the reaction mixture changed from a liquid to a solid gel when in the ice bath. The gel was dealt with in a similar manner. It was heated, broken apart, 6.0 mL of additional pyridine was added then the solution was cooled to room temperature then placed in a cold water bath.

3.5081 g (0.018401 mole) *p*-toluenesulfonyl chloride (Tosyl-Cl) was slowly added to the reaction mixture. As with the Mesyl-Cl reaction, ice was added to the cold water bath during the addition of Tosyl-Cl, until the ice bath was at 0°C. This mixture was stirred for 30 minutes in the ice bath before being removed and allowed to warm to room temperature. The reaction mixture was left to stir for two days. 140 mL cold acetone was added to the reaction mixture. The precipitate that formed was collected by gravity filtration and washed with an additional 40 mL of cold acetone. The collected solid was dissolved in 12.5 mL of deionized water at 55°C. 150 mL cold acetone was added to this solution. The precipitate that formed was collected by gravity filtration and washed with 60 mL cold acetone. The collected solid was dissolved in 20 mL of deionized water at 68°C, cooled to room temperature, then placed in the refrigerator for 45 minutes. The precipitate that formed was filtered by gravity filtration and dissolved in 20 mL of deionized water at 70°C and again placed in the refrigerator, this time for 1.5 hours. The precipitate that formed was collected via gravity filtration and then freeze dried. A yield of 2.1927 g was obtained. A small sample of the product was dissolved in DMSO-d_6 for NMR analysis.

2.4 Reaction of Tosylated β-CD with 2,6-TNS



1.1205 g (8.6928×10^{-4} mole) of tosylated β-CD (Tosyl-CD) was dissolved in DMF; the solution was stirred under nitrogen gas. The reaction flask was placed in a 95°C bath. 0.28 mL (2.0×10^{-3} mole) triethylamine and 0.3161 g (1.007×10^{-3} mole) 2,6-TNS were added to the solution. The reaction mixture was left to stir; the oil bath temperature was maintained between 94 and 98°C.

After one week, the reaction mixture was removed from the oil bath and brought to room temperature. In order to determine which solvent(s) were optimal for chromatography of the product, thin layer chromatography (TLC) was performed on two portions of the reaction mixture as well as 2,6-TNS. For the first portion of the reaction mixture, four drops of the reaction mixture were diluted with 1.0 mL deionized water. For the second portion, 7 mL of cold acetone was added to 1.0 mL of the reaction, this mixture was placed in the refrigerator to facilitate the formation of precipitate. The precipitate that formed was filtered by gravity and rinsed with cold acetone. This filtered solid and 2,6-TNS were each dissolved in deionized water. The three solutions were each placed on four separate silica gel TLC plates and thoroughly dried with a heat gun. The TLC plates were placed in solvent tanks containing ethyl acetate, a 1:9 mixture of ethanol and ethyl acetate, a 1:1 mixture of

ethanol and ethyl acetate and a 5:4:3 mixture of *t*-butanol, ethanol and water. The diluted reaction mixture, recrystallized product and 2,6-TNS solutions were applied to a fifth TLC plate; the plate was thoroughly dried before being placed in a solvent tank containing 3:2 mixture of ethyl acetate and ethanol. After this solvent mixture had migrated to the top if this TLC plate, it was removed and dried. This plate was then rotated 90° and placed in a second solvent tank containing a 1:9 mixture of deionized water and ethanol

90 mL of cold acetone were added to the remaining reaction mixture, it was then placed in the refrigerator for several hours during which time a precipitate formed. This precipitate was filtered by gravity and washed with approximately 40 mL of cold acetone. 1.308 g of product was obtained. Chromatography was used to purify the product. The filtered solid was dissolved in 18.2 mL of deionized water and a heaping scoop of Isolute© diatomous earth granules were added. The water was removed by evaporation on a rotary evaporator. Once the solid was dry, it was compacted into a plastic column and connected to a pre-packed 12 g Isolute© silica gel column. The column was primed with ethyl acetate and placed on the chromatography apparatus. The solvents used for elution were as follows: ethyl acetate, 1:9 ethanol:ethyl acetate, 3:7 ethanol:ethyl acetate, 1:1 ethanol:ethyl acetate, 3:1 ethanol:ethyl acetate, ethanol, 1:9 deionized water:ethanol, 1:4 deionized water:ethanol and finally 3:7 deionized water:ethanol. The eluant was collected in numbered test tubes; TLC was performed on the contents of these test tubes using a solvent tank containing 1:9 deionized water:ethanol to determine which fractions contained fluorescent product. Those fractions which contained fluorescent product

were combined, they had been collected when the solvent mixture was 1:9 deionized water:ethanol and then 1:4 deionized water:ethanol. This mixture was concentrated to approximately 25 mL by rotary evaporation then placed on the freeze drier. 0.0414 g of material was obtained. A small sample was dissolved in deuterium oxide for NMR analysis. A sample was dissolved in deionized water for mass spectroscopy.

This experiment was repeated on a larger scale to determine if the reaction was reproducible and to obtain more product.

2.5 Fluorescence of product

The fluorescent properties of the reaction product were investigated. Solutions containing 2,6-TNS and reaction product were prepared in nanopure water. Absorbance was measured and the solutions were diluted as needed until the absorbance at 330 nm was between 0.20 and 0.40. The 2,6-TNS stock solution was then used to prepare solutions containing β-CD and hydroxypropyl-β-CD. Hydroxypropyl-β-CD was chosen as the modified CD for this study because previous research in our group has shown that it consistently provides the largest enhancement of polarity-sensitive fluorescent probes. The fluorescence of these four solutions was measured from 350 – 600 nm with an excitation wavelength of 330 nm on the two fluorimeters. Solutions containing 2,6-TNS and the reaction product were also prepared with phosphate buffer. The absorbance was measured and solutions were diluted as before. This 2,6-TNS was used to prepare CD solutions containing β-CD, hydroxypropyl-β-CD and dimethyl- β-CD. The reaction product solution was used to

prepare a β-CD solution. The fluorescence emission of these solutions was measured on the two fluorimeters from 350 – 600 nm with an excitation wavelength of 330 nm.

An experiment was also conducted investigating the effect of pH on the fluorescence emission of 2,6-TNS and the reaction product. Phosphate buffer was used to prepare solutions containing 2,6-TNS and the reaction products from both attempts of the TNS-CD experiment. Monobasic potassium phosphate was added to the solutions to reduce the pH of the solutions and dibasic potassium phosphate (or dibasic sodium phosphate) was added to the solutions to increase the pH. As the potassium phosphate was added to the solutions, the pH was monitored using an Accumet pH Meter 910. Portions of the solutions were collected at a range pHs, from approximately 5 – 9, and the fluorescence was measured on the Perkin-Elmer spectrometer.

3 Results & Discussion

3.1 Intermolecular Host-Guest Binding Studies

Solutions of 1,8-ANS, 2,6-ANS and 2,6-TNS were all prepared in phosphate buffer. After confirming the concentrations of the probe solutions by measuring the absorbance, they were used to prepare series of solutions containing a range of CD concentrations. The fluorescence of the CD-probe solutions was measured and the integrated areas of the spectra determined. This work was conducted to determine the host:guest stoichiometry and association constants of the resulting inclusion complexes.

As shown in Figures 11, 12, 13 and 14, the fluorescence emission of 1,8-ANS, 2,6-ANS and 2,6-TNS is greatly increased upon inclusion within the cavity of a CD. This is due to the difference in polarity inside and outside of the CD cavity. The sensitivity to this difference in polarity can vary from probe to probe and is described as the polarity sensitivity factor (PSF).[16, 50, 51] The PSF is the total fluorescence of a probe in ethanol compared to the total fluorescence of the probe in water, corrected for the difference in the absorbance (A) of the two probe solutions. It can be calculated by Equation 3. It has been previously determined that the PSF values for 1,8-ANS and 2,6-ANS were 197 and 120.[50, 51] For 2,6-TNS the PSF value was determined to be over 200 (B. D. Wagner, unpublished results).

$$PSF = (F_{EtOH} / F_{H2O}) \times (A_{EtOH} / A_{H2O}) \qquad \textbf{Equation 3}$$

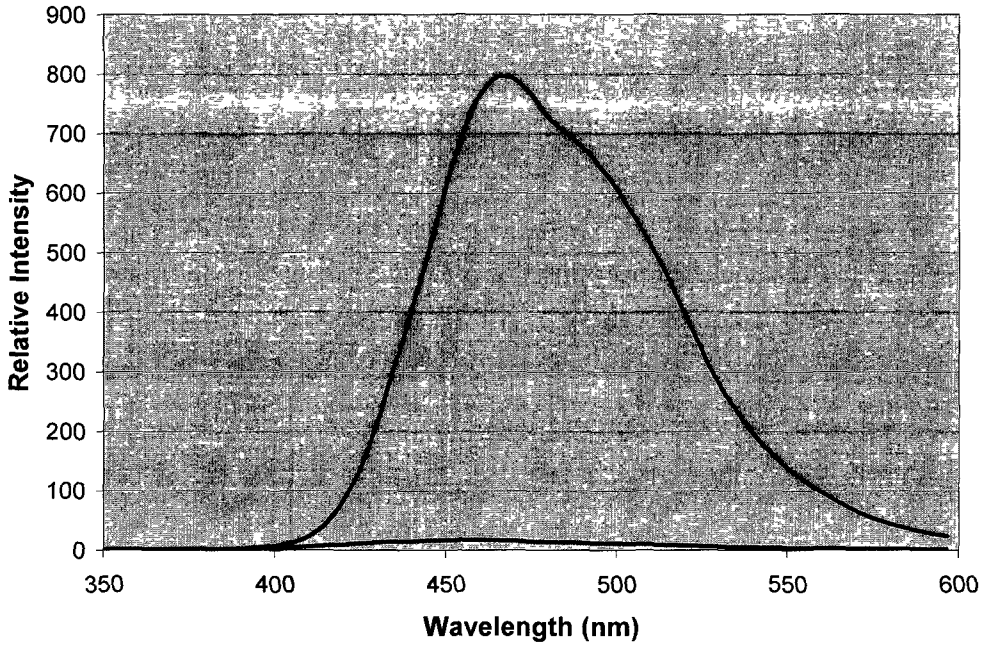


Figure 11. Fluorescence emission of 1,8-ANS (blue line) and 1,8-ANS + 10 mM 2,6-dimethyl-β-CD (red line); excitation wavelength: 330 nm.

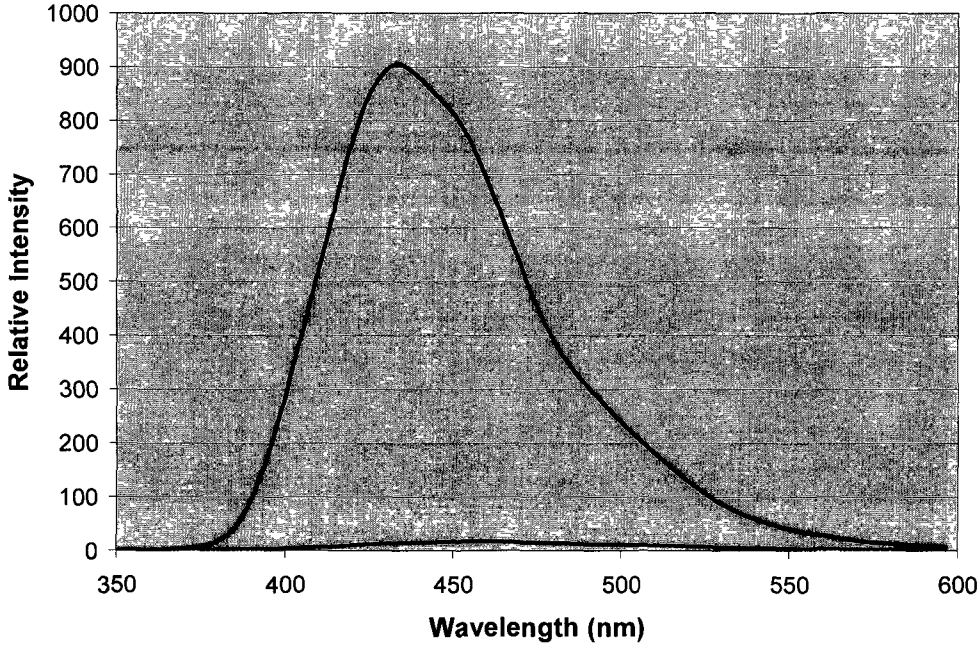


Figure 12. Fluorescence emission of 2,6-ANS (blue line) and 2,6-ANS + 10 mM 2,6-dimethyl-β-CD (red line); excitation wavelength: 330 nm.

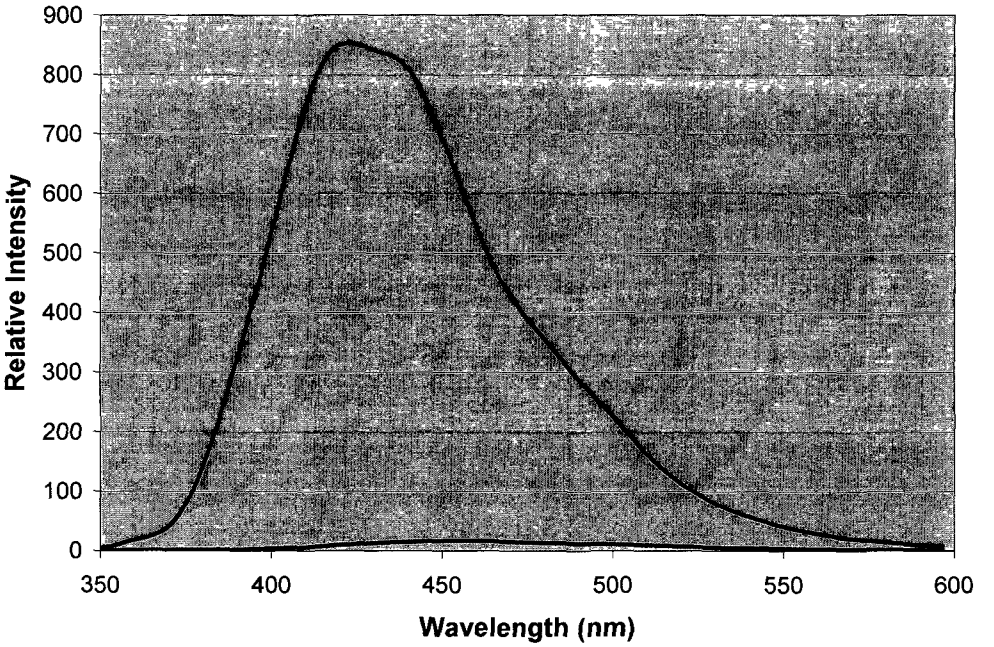


Figure 13. Fluorescence emission of 2,6-TNS (blue line) and 2,6-TNS + 10 mM dimethyl-β-CD (red line); excitation wavelength: 330 nm.

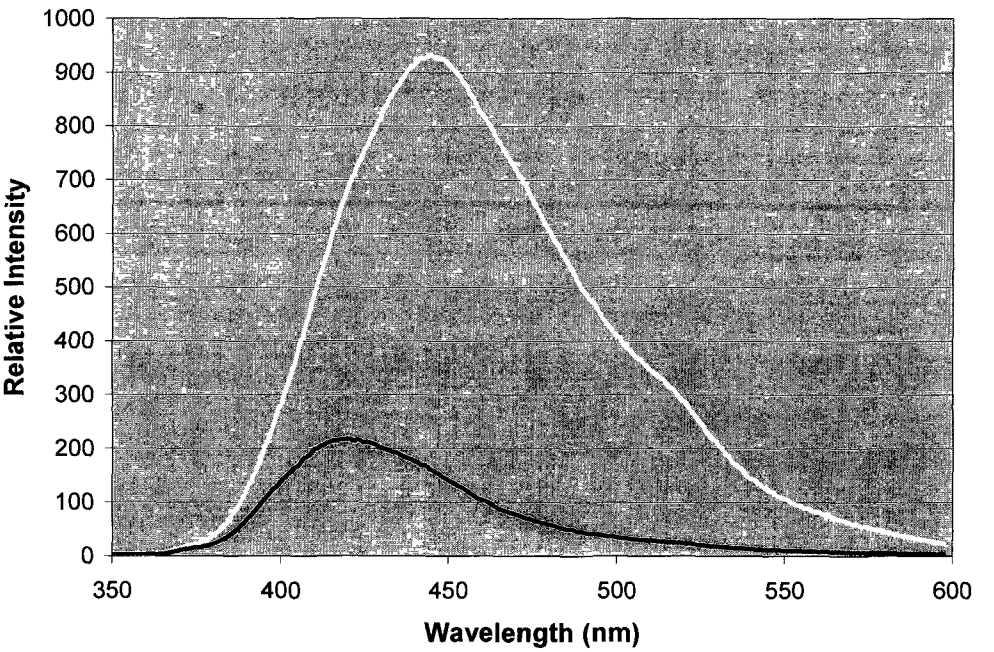


Figure 14. Fluorescence emission of 2,6-TNS (blue line) and 2,6-TNS + 10 mM β-CD (yellow line); excitation wavelength: 330 nm.

To determine the host:guest stoichiometry and association constant(s), the integrated areas of a series of CD-probe solutions were used to determine the fluorescence enhancement, F/F_O, of each CD-probe solution in the series. Two plots were constructed, the first comprised of F/F_O values for the CD-probe solution series versus CD concentration (known as a fluorescence titration), shown in Figures 15, 17, 19 and 21; the second a double reciprocal plot, $1/(F/F_O-1)$ versus 1/[CD], shown in Figures 16, 18, 20 and 22. If the double reciprocal plot was linear, the observed fluorescence enhancement was the result of a 1:1 host-guest inclusion complex.[51, 52] If the double reciprocal plot was not linear, the observed fluorescence enhancement was the result of a higher order inclusion complex.[51]

Once it was determined if the inclusion complex was of 1:1 or higher complexation, it was possible to determine the association constant(s) for the complex. For 1:1 inclusion complexes, Equation 4 was used.[16, 17, 52, 53] If the inclusion complex was of higher complexation, Equation 5 was used; Equation 5 is the fit equation for a 2:1 host:guest inclusion complex.[16, 17, 54] Although a non-linear double reciprocal plot only indicates a higher order-complex than 1:1, and not necessarily 2:1, in the current case, a 2:1 complex is a likely complexation model. This is a result of the long, linear geometry of the guest (allowing for complexation of a single guest by two hosts), the fact that there are two aromatic moieties (the aniline and the naphthyl groups), each of which could be included in a CD host, and the fact that the guests are anionic, making inclusion of two guest in one host (*i.e.* a 1:2 host:guest complex) very unlikely due to electrostatic repulsion. Two computer programs written in the Wagner laboratory were used to determine the association

constants contained within Equations 4 and 5 for 1:1 and 2:1 models based on F/F_O values and CD concentrations.

$$\frac{F}{F_\circ} = 1 + \left(\frac{F_\infty}{F_\circ} - 1\right)\frac{[CD]K}{1 + [CD]K}$$

Equation 4

$$\frac{F}{F_\circ} = \frac{1 + \left(\frac{F_1}{F_\circ}\right)K_1[host] + \left(\frac{F_2}{F_\circ}\right)K_1K_2[host]^2}{1 + K_1[host] + K_1K_2[host]^2}$$

Equation 5

Based on the double reciprocal plots, it was determined that the 2,6-dimethyl-β-CD:1,8-ANS inclusion complex and the 2,6-dimethyl-β-CD:2,6-ANS inclusion complex were both 1:1 complexes, see Figure 15 and 17. The 2,6-dimethyl-β-CD:2,6-TNS inclusion complex and the β-CD:2,6-TNS inclusion complex were determined to be higher-order complexes, and therefore fit as 2:1 complexes, see Figure 20 and 22. Based on equations 1 and 2, the following association constants were determined for the inclusion complexes studied.

Table 4. Average fluorescence enhancement values and association constants for the inclusion complexes.

Inclusion complex	**F_{max}/F_O**		**K**	
2,6-dimethyl-β-CD:1,8-ANS[a]	115±1		320±8 M^{-1}	
2,6-dimethyl-β-CD:2,6-ANS[b]	30±10		5200±300 M^{-1}	
	F_1/F_O	**F_2/F_O**	**K_1**	**K_2**
2,6-dimethyl-β-CD:2,6-TNS[b]	20±20	50±50	11000±6000 M^{-1}	50±30 M^{-1}
β-CD:2,6-TNS[a]	2.98±0.03	11.24±0.05	5600±200 M^{-1}	46±3 M^{-1}

a = average of 2 experiments; b = average of 3 experiments

The inclusion complexes of 1,8-ANS and 2,6-ANS with dimethyl-β-CD were both determined to be 1:1 host:guest complexes. This is based on the linear nature of the double reciprocal plots and the degree of fit to Equation 4, shown in Figures 14 and 16. For 1,8-ANS:diemthyl-β-CD the average χ^2 value was 0.0055; for 2,6-ANS:dimethyl-β-CD the average χ^2 value was 0.0062. These values demonstrate the high quality of fit between the fit line and the actual data points, the lower the χ^2 value, the better the fit. For the double reciprocal plots, the average regression constants were 0.9986 for the 1,8-ANS plot and 0.9982 for the 2,6-ANS plot, demonstrating the high degree of linearity of both plots. The K values of these ANS:dimethyl-β-CD inclusion complexes were larger than previously published values for ANS:β-CD inclusion complexes[50] listed in Table 5. This indicates stronger intermolecular bonding within the modified β-CD inclusion complexes.

Table 5. Average K values of ANS:CD inclusion complexes from this work and the literature.

	Dimethyl-β-CD	**β-CD***
1,8-ANS		
K	320±8 M^{-1}	80 M^{-1}
2,6-ANS		
K	5200±300 M^{-1}	1350 M^{-1}

*β-CD values[50]

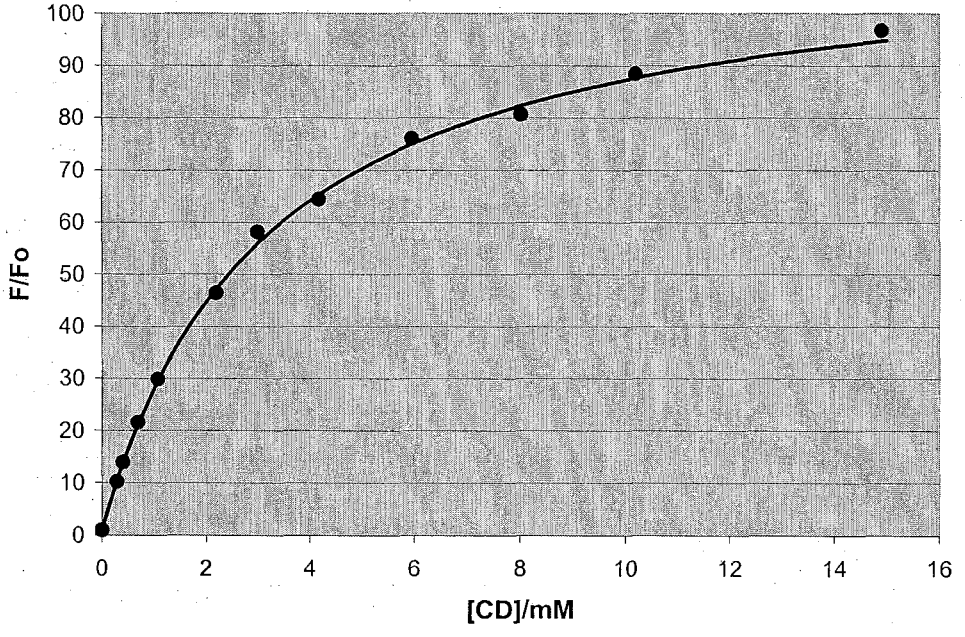


Figure 15. Fluorescence titration plot of the enhancement of 1,8-ANS as a function of 2,6-dimethyl-β-CD concentration. The solid line shows the fit to Eq. 4, with $F_{max}/F_O = 115$, $K = 310\ M^{-1}$.

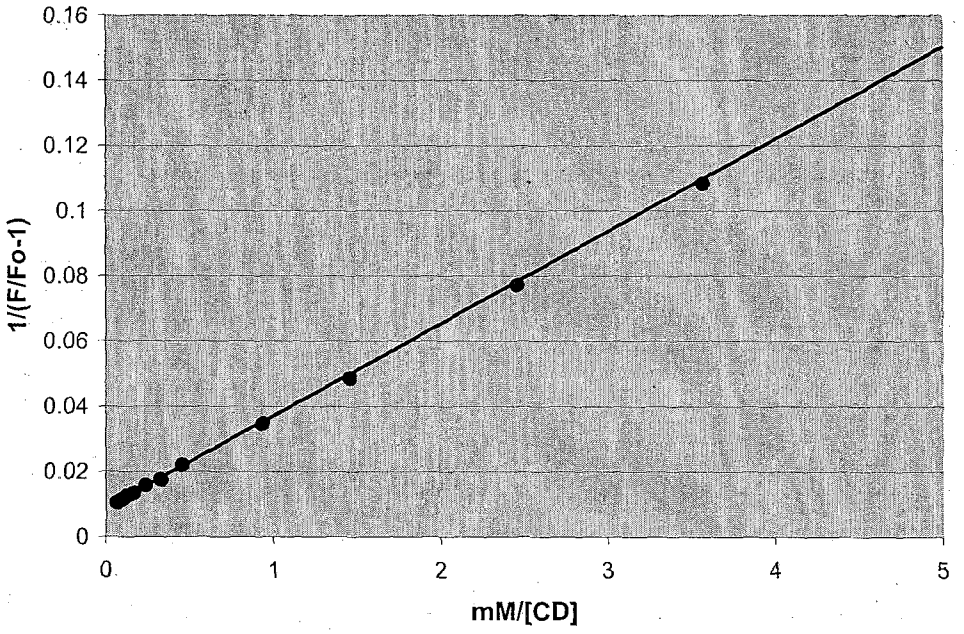


Figure 16. Double reciprocal plot of 1,8-ANS enhancement in 2,6-dimethyl-β-CD.

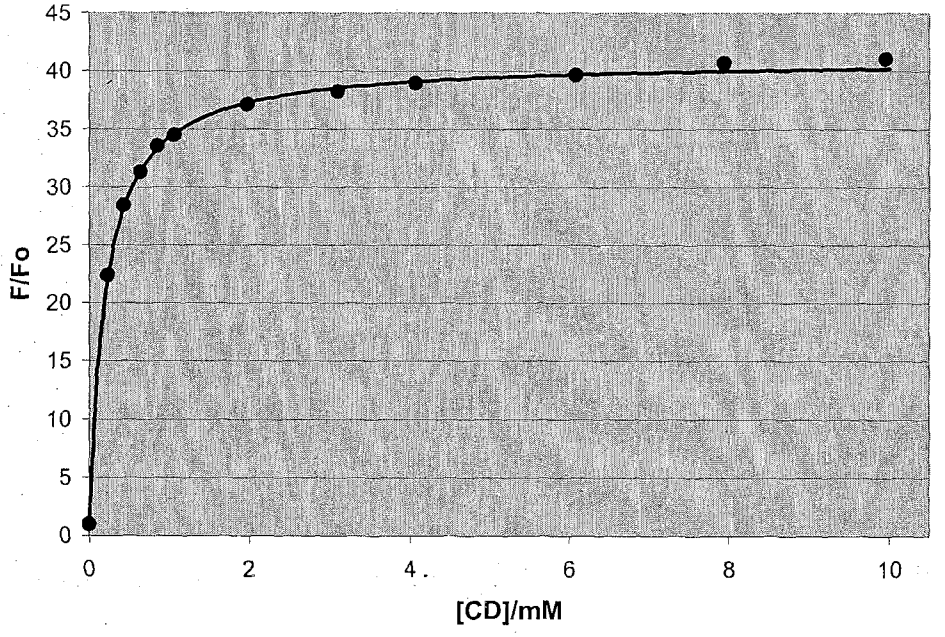


Figure 17. Fluorescence titration plot of the enhancement of 2,6-ANS as a function of 2,6-dimethyl-β-CD concentration. The solid line shows the fit to Eq. 4, with $F_{max}/F_O = 41$, $K = 4900\ M^{-1}$.

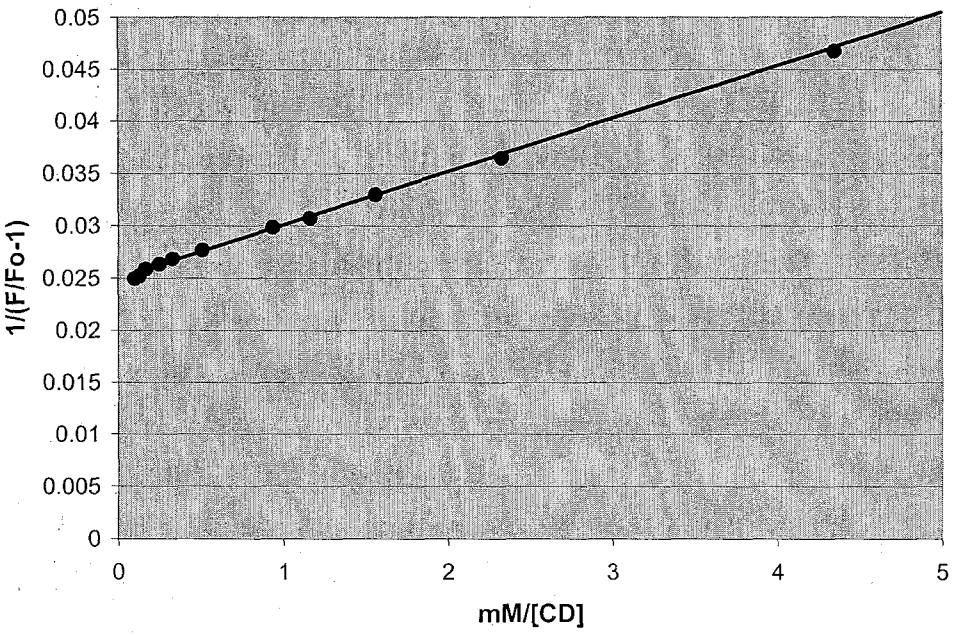


Figure 18. Double reciprocal plot of 2,6-ANS enhancement in 2,6-dimethyl-β-CD.

Based on the non-linear double reciprocal plot, shown in Figure 20, the 2,6-TNS:dimethyl-β-CD inclusion complex was determined not to be a 1:1 complex; they involve higher order complexation. When fit to Equation 5, there was a high degree of agreement between the data and the fit line, shown in Figure 19. The average χ^2 value for this complex fit to Equation 5 was 0.0061.

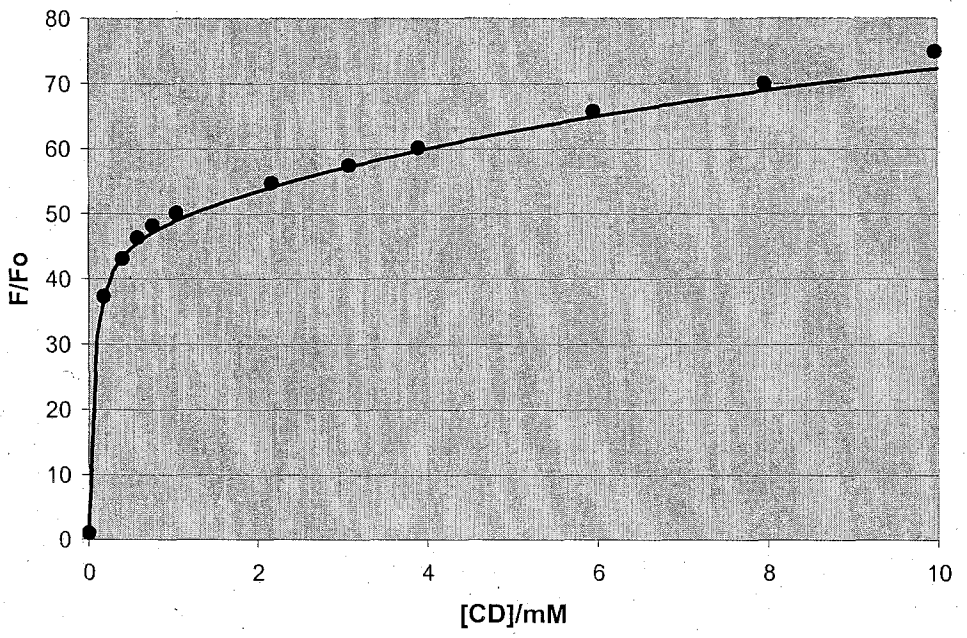


Figure 19. Fluorescence titration plot of enhancement of 2,6-TNS as a function of 2,6-dimethyl-β-CD concentration. The solid line shows the fit to Eq. 5 with $F_1/F_O = 47$, $F_2/F_O = 110$, $K_1 = 18000\ M^{-1}$, $K_2 = 72\ M^{-1}$.

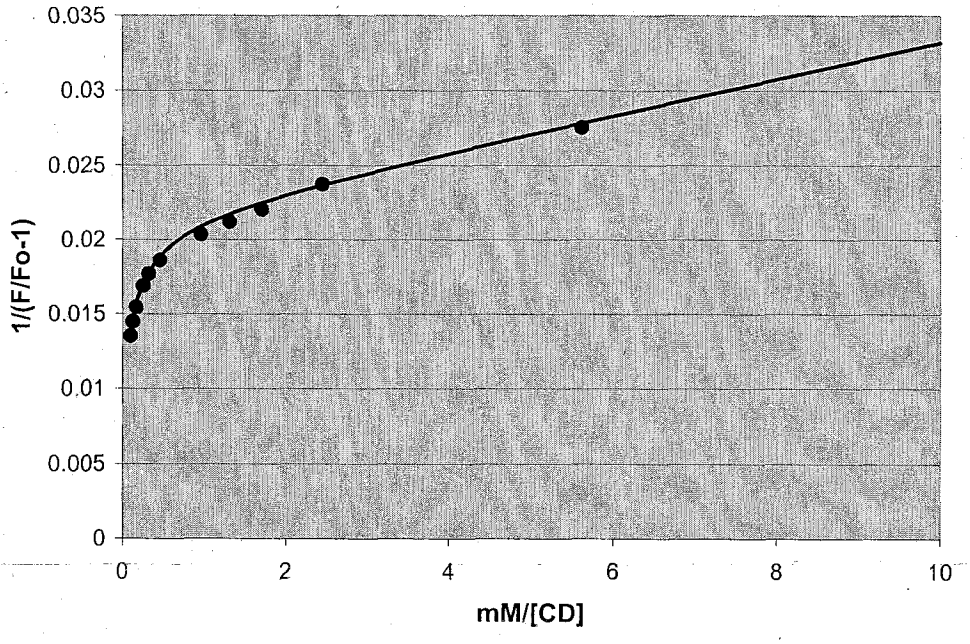


Figure 20. Double reciprocal plot of 2,6-TNS enhancement in 2,6-dimethyl-β-CD.

The inclusion complex of 2,6-TNS and β-CD had previously been determined to be a 2:1 inclusion complex.[45, 46, 55] In this investigation, the non-linear double reciprocal plot, shown in Figure 22, indicated a higher order complexation than 1:1. When fit to Equation 5, there was very good agreement between the fit line and the data, the average χ^2 value was 0.0020. Published K values for 2,6-TNS:β-CD, K_1 = 3737 M^{1} and K_2 = 149 M^{-1},[55] were different from those obtained in this work, K_1 = 5600±200 M^{-1} and K_2 = 46±3 M^{1}, but showed a similar pattern, with K_1 being much larger than K_2. The K values for the 2,6-TNS:dimethyl-β-CD inclusion complex were compared to those of the 2,6-TNS:β-CD inclusion complex. As with the ANS inclusion complexes, intermolecular bonding was much stronger within the modified β-CD than with the unmodified β-CD, indicating that the modified β-CD is the better potential host to be used in the tethered fluorescence sensor, as this will give the largest enhancement for the on versus the off signal.

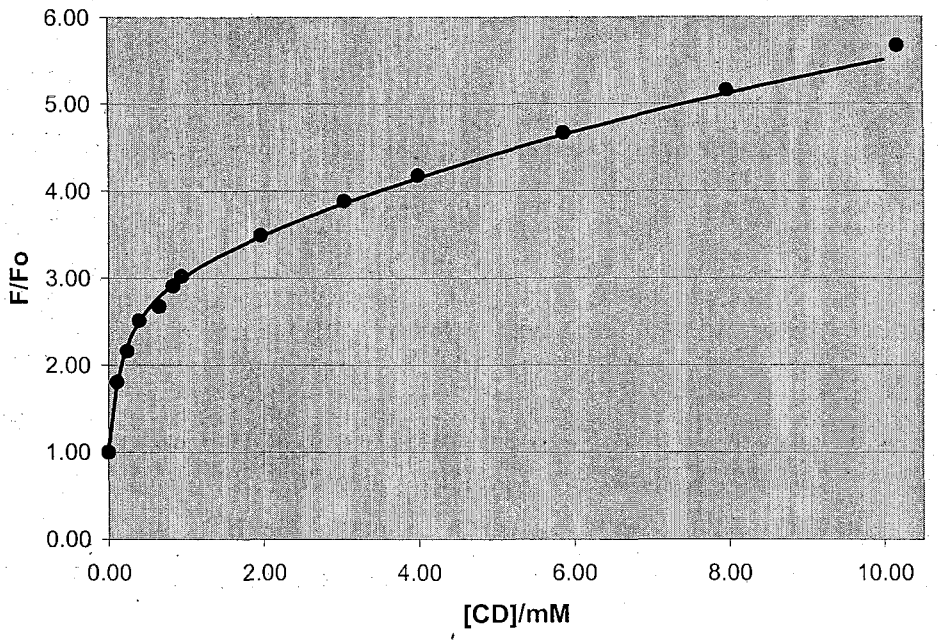


Figure 21. Fluorescence titration plot of the enhancement of 2,6-TNS as a function of β-CD concentration. The solid line shows the fit to Eq. 5, with $F_1/F_O = 2.95$, $F_2/F_O = 11.27$, $K_1 = 5800\ M^{-1}$, $K_2 = 47\ M^{-1}$.

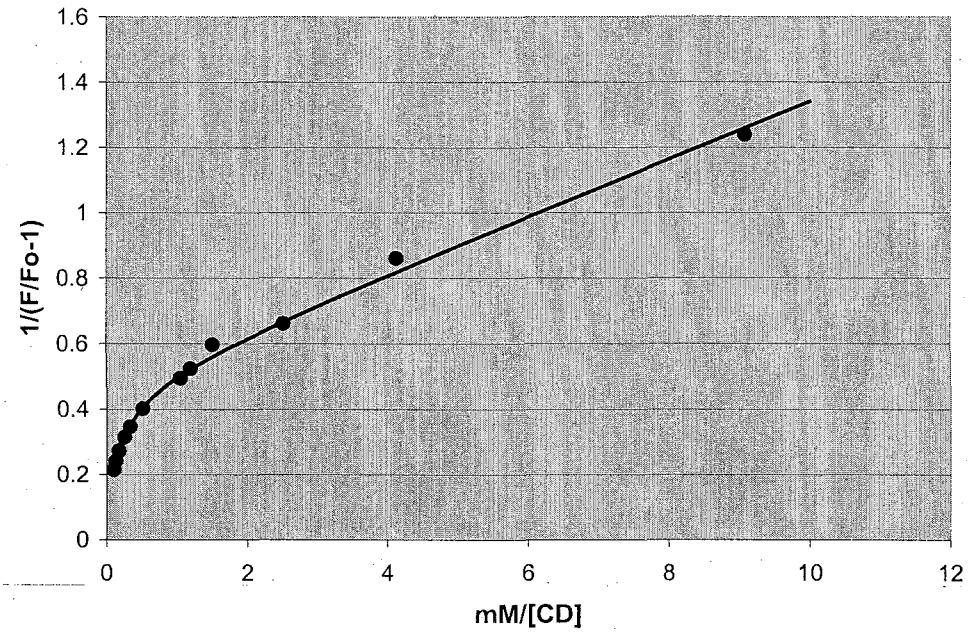


Figure 22. Double reciprocal plot of 2,6-TNS enhancement in β-CD.

Based on this information it was determined that 2,6-ANS and 2,6-TNS bind to CDs better that 1,8-ANS. This is most likely due to the shape of the probes. The more linear and streamlined shape of 2,6-ANS and 2,6-TNS is more conducive to entering the CD cavity than the bulky shape if 1,8-ANS. When comparing the fluorescence enhancement of the inclusion complexes, 1,8-ANS and 2,6-TNS undergo the largest increase in fluorescence. As a result of its large binding constant and its large increase in fluorescence enhancement upon inclusion in a CD cavity, it was determined that 2,6-TNS would be the best probe to tether to a CD. The large binding constant should allow for increased selectivity in a fluorescent sensor as only molecules with a higher binding constant should be able to displace the probe from the CD cavity. Also, the dramatic increase in fluorescence emission upon inclusion will result in the achievement of the goal of having a large, very obvious difference between the on and off positions of the sensor.

3.2 Modification of the Guest for Attachment to the CD

Initial attempts to tether 2,6-TNS to β-CD dealt with modifying the 2,6-TNS molecule so that it could be attached to a β-CD molecule or other derivative.

3.2.1 Bromination of TNS

The first approach attempted involved the bromination of the methyl group on the 2,6-TNS molecule. If a tether could be established at this position, it was thought that the methyl group could act as a one carbon "link" in the tether. Also, at this position the tether would not interfere with movement of the probe in and out of the

CD cavity, thus not hindering the performance of the fluorescent sensor. It was also expected that modification of this position of the TNS would have little effect on its fluorescence behavior.

The technique for bromination of the 2,6-TNS methyl group was based on that of Dubey and Kumar.[56] This group performed a radical bromination of 7,8,9,10-tetrahydrobenzo[f]quinoline with NBS in the presence of AIBN. In their work, this resulted in a mixture of two regioisomers, with bromination occurring at either the C-7 or C-10 benzylic position, as shown in Figure 23. The desire of this work was to perform a single bromination of the methyl group of 2,6-TNS and since the methyl group on 2,6-TNS is the only benzylic position, that is where the bromination should take place, so this approach was tried.

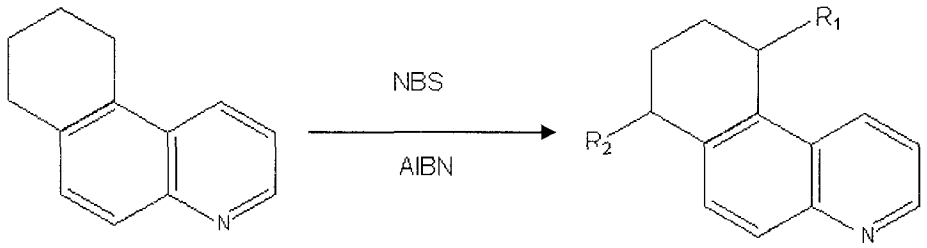


Figure 23. Radical Bromination of 7,8,9,10-tetrahydrobenzo[f]quinoline with NBS in the presence of AIBN.[56]

The first step was to purify the NBS. This was successfully done according to the protocol outlined in *Purification of Laboratory Chemicals, 2nd Edition.*[47] The purified NBS was then refluxed with 2,6-TNS and AIBN in chloroform then cooled. The precipitate that formed was collected and dried. ^{1}H NMR analysis was conducted in deuterium oxide. It was determined that the solid collected was in fact unreacted 2,6-TNS. The reaction was attempted several times, ensuring that all glassware was thoroughly dried, extending the time for refluxing and increasing the amount of chloroform used, but the same result was obtained each time, 2,6-TNS remained unreacted.

One question that arose from this work was whether the low solubility of 2,6-TNS in chloroform was affecting the success of this reaction. In order to answer this question, an investigation into the solubility of 2,6-TNS in various organic solvents was conducted. The solubility of 1,8-ANS was also investigated, in case a change of probe would be warranted. The two probes were combined with the solvents listed in section 2.2.1. The two probes were insoluble or only very slightly soluble in most of the solvents. 2,6-TNS was most soluble in chloroform and acetonitrile. 1,8-ANS was not more soluble than 2,6-TNS in any solvent, so 2,6-TNS continued to be the probe used in these experiments.

3.2.2 Protection of Anilino Group of TNS with BOC_2O

It was theorized that one of the possible reasons for the failure of the bromination reaction could be quenching of the radical reaction by the secondary

amino group of 2,6-TNS. Accordingly, an attempt was made to protect the amino group before attempting to brominate the methyl group.

The reaction to protect the amino group was based on the method published by Theon *et al.*[57, 58] The intention was to protect the amino group of 2,6-TNS with a *tert*-butoxycarbonyl (BOC) group. 2,6-TNS was combined with BOC_2O and DMAP, this mixture was left to stir overnight. Deionized water was added to the reaction mixture and it was again left to stir overnight. After washing with ethyl acetate, the aqueous layer was separated and the product was isolated using solid phase extraction columns. Ethyl acetate was used to elute the columns. The columns were constructed from a translucent plastic, so it was possible to monitor the movement of the fluorescent product through the column with a hand held UV-light. Once all of the fluorescent product had been collected from the column, the elute was washed with hydrochloric acid in the hope of extracting any unreacted TNS. Once the organic and aqueous layers had separated, they were each placed under a UV-light in order to determine where the fluorescent product was. As only the organic layer fluoresced, the aqueous layer was discarded and work continued with only the organic layer. The fluorescent organic layer was washed four times with sodium chloride solution. After each washing with sodium chloride, the organic and aqueous layers were separated and placed viewed under a UV-light to double check the location of the fluorescent product; it remained in the organic layer after each washing. The organic layer was then dried over anhydrous sodium sulfate to remove any remaining water. After filtering off the sodium sulfate, the filtrate was concentrated by rotary evaporation, to remove the bulk of the solvent; it was then

dried under vacuum. The product was analyzed by infrared and NMR spectroscopy. In comparing the spectra from the product to those of unreacted 2,6-TNS, it was determined that the fluorescent product was, in fact, simply a small amount of unreacted 2,6-TNS. This methodology was repeated several times, extending reaction times to a full 24 hours, but the same result was obtained each time.

3.2.3 Protection of Anilino Group of TNS With Acetyl Chloride

Following the methodology of Vogel,[59] 2,6-TNS was combined with sodium hydroxide and acetyl chloride and the resulting product acidified. As the product of this reaction was a black, tar-like substance, there was not much hope that the amine group of 2,6-TNS had become acetylated. ATR-IR spectroscopy and NMR spectroscopy of the product were conducted. Based on this information it was determined that the product was not acetylated 2,6-TNS. The reaction was attempted again, but the same result was obtained.

3.2.4 Reaction of TNS With Bromoacetyl Bromide

Brominating the methyl group of 2,6-TNS in order to provide a site for a tether was proving to be unsuccessful, as was protecting the amine group in order to avoid interference with the reaction of the methyl group. With the knowledge that the amine group of 2,6-TNS is potentially reactive, the possibility of positioning a tether there was investigated.

The original hypothesis for this project was that 2,6-TNS would be best tethered through the methyl group as this would limit any hindrance on the movement

of the probe in and out of the CD cavity If 2,6-TNS were to be tethered through the amine group, a longer tether may be required in order to facilitate the movement of the probe. It was also thought that by tethering through the methyl group, the tether would not interfere with the linear shape of the probe and thus, would not interfere with the binding between probe and CD. If the 2,6-TNS is tethered through the amine group, the strength of the binding between probe and CD may be adversely affected.

However, given the difficulties encountered in the activation of the methyl group, the reaction of 2,6-TNS with bromoacetyl bromide was attempted. If this reaction was successful, it would produce a molecule with a good leaving group, bromine, which could be used to attach the probe to a CD.

2,6-TNS and bromoacetyl bromide were stirred together in pyridine for several minutes. A few drops of deionized water were added to the reaction mixture to halt any further reaction. The reaction mixture was concentrated to approximately half of its original volume. Deionized water was added to the concentrated reaction mixture and it was again concentrated; this was done as a means of removing the pyridine. The mixture was then freeze dried. Attempts to recrystallize the product were made; the dried product was dissolved in warm deionized water and placed in the refrigerator. As no precipitate formed, a small amount of methanol was added to the solution to facilitate precipitation. Still no precipitate formed so the solution was concentrated by rotary evaporation. The hope was that by concentrating the solution, it would reach the saturation point and the product would be forced out of solution. As the solvent evaporated, solid did begin to form. Unfortunately the solid that

formed looked very similar to the tar like substance that resulted from the reaction of 2,6-TNS and acetyl chloride. The solid was freeze dried and analyzed by ATR-IR and NMR spectroscopy (in DMSO-d_6). The analysis indicated that the bromoacetyl group had not reacted with the 2,6-TNS molecule. Repeated attempts to complete this reaction were made with extreme caution being made to ensure all components involved were dry. The reaction was also attempted on an increased scale and the bromoacetyl bromide was added very slowly to the dissolved 2,6-TNS. All further attempts produced the same result, a black intractable material.

It was hypothesized that the acetyl molecules were too reactive to successfully bond to the 2,6-TNS in the desired fashion. It is possible that the acetyl chloride and bromoacetyl bromide underwent side-reactions to give the black tar-like product.

Modification of the 2,6-TNS molecule was proving to be unsuccessful. It was therefore thought that it may be advantageous to first modify the CD then attach the probe through a substitution reaction.

3.3 Modification of the CD Host for Attachment of the Modified Guest

Original investigations into the synthesis of a fluorescent sensor of the desired design focused on modification of 2,6-TNS. It was thought that bromination of 2,6-TNS would allow for a substitution reaction with dimethyl-β-CD at the un-methylated hydroxyl group on the C-3 position. As bromination of 2,6-TNS was proving unsuccessful, alternative methods of initiating a tether on β-CD, natural or modified, were investigated. Several techniques were identified as possible means of

modifying β-CD in such a way as to be advantageous in achieving the desired design of fluorescent sensor.

3.3.1 Reaction of CD with Diiodobutane

The first CD modification method to be attempted was outlined by Park *et al.*[48] and based on work conducted by Rong and D'Souza.[49] With this technique, a viologen was tethered to the secondary face of β-CD via an alkyl chain, as shown in Figure 24. This technique seemed to fit very well with the goals of this project. First, the probe was tethered by a butyl and an octyl chain. This would be beneficial as it is not known what length of tether would be best if the tether is to be attached to the amine group of 2,6-TNS. Also, in this case, the tethers are simple alkyl chain, so there should be little chance of the tether interfering with the inclusion of the probe. In this technique a single viologen is tethered to each β-CD molecule on its secondary face which is of particular importance if 2,6-TNS is to be tethered by the amine group. In order to facilitate the movement of 2,6-TNS in and out of the CD, it may be necessary for the probe to be tethered to the wider cavity rim of the CD. While the desired structure of the fluorescent sensor being synthesized does not dictate a preference of substitution at the C-2 position over the C-3, position, the goal is to substitute a secondary C. This methodology was pursued because the alkyl chain was selectively tethered at the 2-C position, substituting a secondary hydroxyl group.

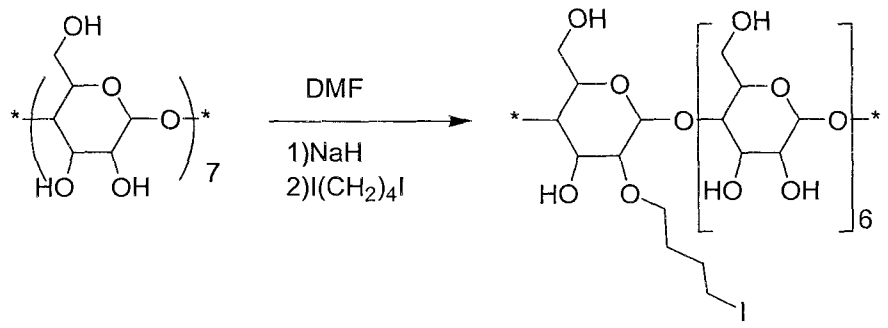


Figure 24. Reaction published by Park *ét al.*[48]

One potential drawback to this methodology is the low yield reported. Park *et. al.*'s synthesis resulted in an overall yield of 3-4%, though they did not publish the yields of the intermediates [48]

Following Park's method, dry β-CD was reacted with sodium hydride and 1,4-diiodobutane in order to introduce the four-carbon tether onto which the 2,6-TNS could be substituted. The reaction product was precipitated out of solution by the addition of acetone. Purification of the reaction product was performed using a Sephadex size exclusion chromatography column. As the product eluted through the column, a yellow band was observed as expected.[49] This band was collected and freeze dried to obtain a solid product. This solid was analyzed by NMR (in both deuterium oxide and DMSO-d_6) and mass spectroscopy. The isolated product was determined to be unmodified β-CD. The reaction was attempted several more times. In the further attempts of this reaction, purified reagents were used, the scale of the reaction was increased and the reaction time was increased, but the end result remained the same, the CD was not modified as required.

Future work dealing with this methodology may include investigations using dimethyl-β-CD. Dimethyl-β-CD has methyl groups protecting the primary hydroxyl

groups and the 2-position hydroxyl groups. Having the hydroxyl groups protected may help to facilitate the substitution of a 3-position hydroxyl group with an alkyl chain, thus creating the desired tether.

The original goal of this project was to tether a fluorescent probe onto the secondary hydroxyl (larger) cavity rim of β-CD as a means of trying to facilitate the entry of the probe into the CD cavity. The lack of success in achieving this goal spurred an investigation into the possibility of tethering the probe onto the less sterically hindered primary cavity rim of β-CD.

3.3.2 Reaction of CD With Mesyl Chloride

As the primary hydroxyl groups of β-CD are more reactive towards substitution than the secondary hydroxyl groups, an attempt to attach a tether to the primary face of CD was made. The strategy used was to convert the less hindered primary hydroxyl group into a mesylate leaving group. The methodology used was similar to that used to attach a tosyl group to the primary hydroxyl of β-CD,[60] however, given the steric bulk of the incoming nucleophile (the 2,6-TNS), the less hindered mesylate was selected as the leaving group.

Dried β-CD was dissolved in a small amount of pyridine by warming in a water bath then cooled in a cold water bath. An ice-bath was not used as this resulted in solidification of the solution. Mesyl chloride was added to the cold reaction flask in a dropwise manner in order to help facilitate the formation of mono-mesylated β-CD. As drops of mesyl chloride was added to the reaction flask, ice cubes were added to the cold water bath. By the time approximately one third of the mesyl

chloride was added to the reaction flask, the bath had reached 0°C. Once the mesyl chloride was added, the mixture was stirred in the ice bath for approximately 30 minutes; it was then allowed to return to room temperature and stirred overnight. The reaction solution was concentrated by rotary evaporation then cold methanol was added to precipitate the product out of solution. The precipitate was collected and dissolved in hot deionized water. Once this solution had been brought to room temperature, methanol was added to precipitate the product out of solution. The resulting precipitate was collected and recrystallized a second time in the same manner. The product was analyzed by NMR spectroscopy. It was not mesylated β-CD. The reaction was attempted again. In the repeated attempts, steps were taken to try to slow the reaction of mesyl chloride as much as possible. The mesyl chloride was added more slowly and the solution was not removed from the ice bath, instead the reaction flask remained in the ice bath when it was left to stir over night, only returning to room temperature when the ice bath did. These technique modifications did not alter the outcome; the β-CD did not become mesylated.

As the multiple attempts to bind a mesyl group onto the primary hydroxyl of β-CD were ineffective, a literature search was conducted into mesyl groups bound to CD in any manner. Very few references to this type of reaction were found. In all of these cases, mesyl groups were bound to all seven primary hydroxyl groups of β-CD.[60]

In contrast, there are several reports of the selective attachment of one tosyl group to a primary hydroxyl group of β-CD. As a result, it was thought that using a tosyl group as a leaving group, may in fact be the best option at this time, even

though its steric bulk might make the substitution reaction with 2,6-TNS more difficult.

3.3.3 Reaction of CD With Tosyl Chloride

The methodology outlined by Ihara *et al.*[60] was followed to monotosylate β-CD.

β-CD was reacted with tosyl-chloride. A precipitate formed when acetone was added, but when isolation of the precipitate was attempted, it changed from a loose solid to a substance resembling sticky caramel in both texture and color. This caramel-like substance was recrystallized from water to give an off-white colored solid. A small sample of this solid was analyzed by NMR spectroscopy in DMSO-d_6. The resulting ^{1}H and ^{13}C NMR spectra were in agreement with the published work,[60] and as such indicated that monotosylated β-CD was indeed made, see Figure 25. ^{1}H NMR (DMSO-d_6), 300 MHz: δ 2.42 (s, 3H, H-9), 2.50 (s, 2H, DMSO solvent peak), 3.31-3.63 (m, 60H, 42H: 2, 2', 3, 3', 4, 4', 5, 5', 6, 6'-positions with the remainder of H's from water), 4.19-4.53 (m, 6H, OH-6), 4.76-4.83 (overlapping s, 7H, H-1, 1'), 5.73 (s, br, 14H, H-2, 2', 3, 3'), 7.42 (d, *J*=7.8, 2H, H-7), 7.74 (d, *J*=7.8 2H, H-8); Figure 26 shows position assignment.

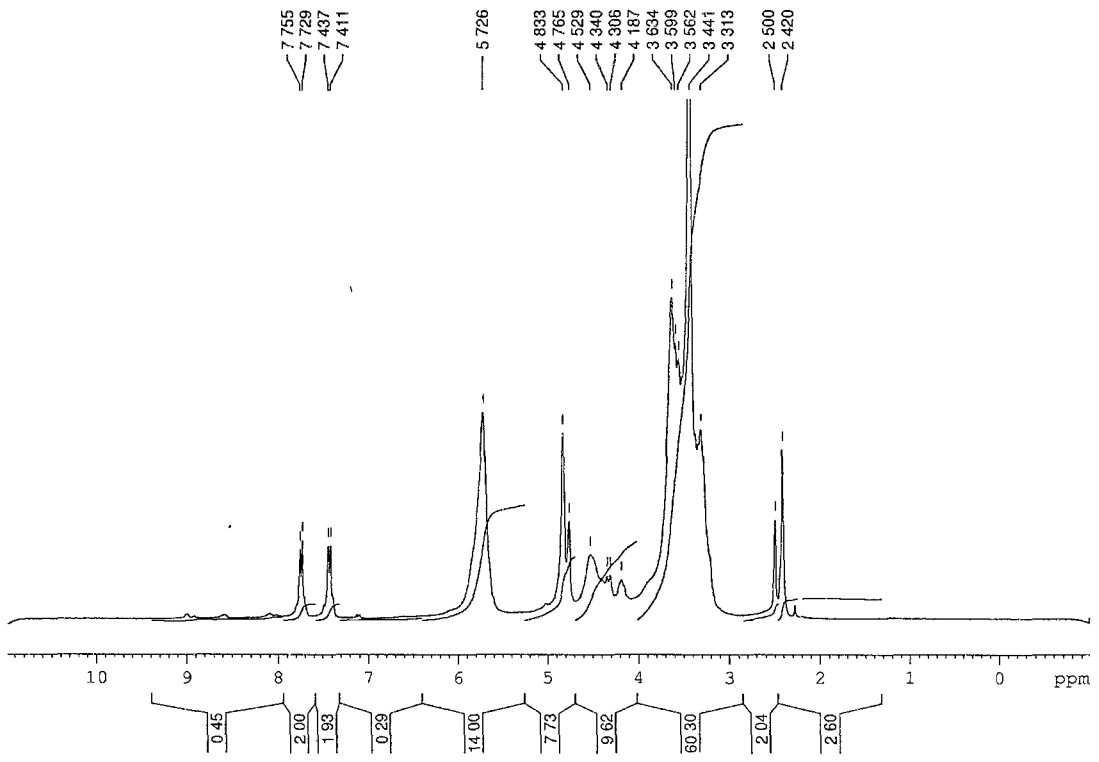


Figure 25. ^{1}H NMR spectrum of monotosylated β-CD.

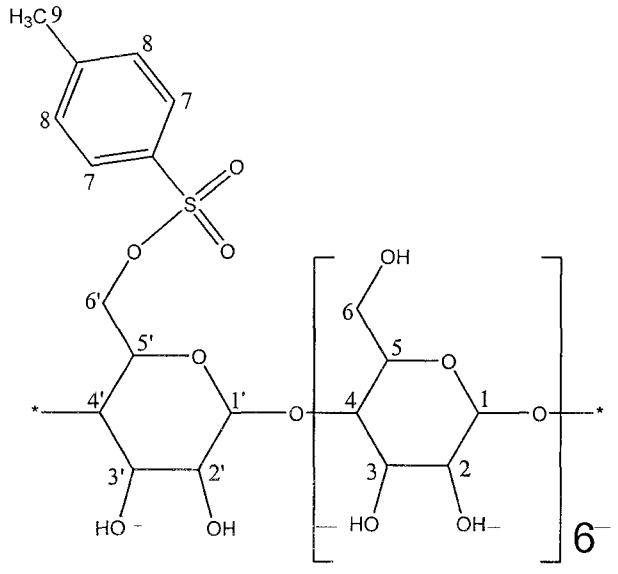


Figure 26. Illustration depicting position assignments of mono-tosylated β-CD.

Mono-tosylated β-CD was successfully synthesized, but tosylation occurred as expected at the primary position. Further investigations should be made into tosylating β-CD at the 3-position, perhaps by using dimethyl-β-CD as the 2- and 6-position hydroxyl groups are protected, leaving the 3-position hydroxyl group open for reaction.

As β-CD with a leaving group in the primary position had been obtained, it was now time to attach the fluorescent probe.

3.4 Attachment of the Tosylated β-CD to 2,6-TNS

As work was conducted to attach 2,6-TNS to β-CD by the tosyl group, it was acknowledged that this would result in the 2,6-TNS being attached in an undesirable manner. If the tosyl group acts as a leaving group, the 2,6-TNS will be tethered by a single carbon chain to β-CD. It is possible that even if 2,6-TNS does become tethered, no fluorescence enhancement will be seen as 2,6-TNS may not be able to maneuver into the β-CD cavity as the tether may be too short. If that is the case, methods for increasing the length of the tether will need to be investigated.

In order to attach 2,6-TNS to the modified β-CD, the two compounds were reacted together with a small amount of base, triethylamine. The reaction mixture was maintained between 92 and 98°C for one week in order to facilitate the reaction. After three days, thin layer chromatography was performed on the reaction mixture and unreacted 2,6-TNS. Three solutions were made, the first was 2,6-TNS dissolved in deionized water. The second contained 4 drops of the reaction mixture diluted to 1.0 mL with deionized water. For the third solution, acetone was added to a portion

of the reaction mixture in order to crash the product out of solution. This solidified product was dissolved in deionized water. The three solutions were spotted onto silica gel TLC plates and thoroughly dried. The TLC plates were placed into four different solvent tanks containing ethyl acetate, 1:9 ethanol:ethyl acetate, 1:1 ethanol:ethyl acetate nd 5:4:3 *t*-butanol:ethanol:water. The purpose of this small TLC study was to determine if any of the 2,6-TNS had reacted and what solvent(s) should be used to elute the product from a chromatography column.

Based on the TLC work, it was suggested that there was some modified 2,6-TNS present. This was indicated by a fluorescent compound with a smaller retention factor (R_f) than the unreacted 2,6-TNS, indicating that the fluorescent compound was more polar than 2,6-TNS. It also suggested that before the reaction mixture was separated by chromatography, one recrystallization of the product should be performed. This removed a large portion of the unreacted 2,6-TNS from the reaction mixture. In considering which solvent(s) should be used for chromatography, the R_f values of each compound in the different solvents were compared. It was hypothesized that an ethyl acetate/ethanol mixture would remove any remaining 2,6-TNS and a water/ethanol mixture would remove the reaction product from the column. In order to confirm this, a fifth TLC plate was prepared with the same compounds, the diluted reaction mixture, the recrystallized product and unreacted 2,6-TNS. The plate was first placed in a solvent tank containing a 3:2 mixture of ethyl acetate and ethanol. In this case, the 2,6-TNS had an R_f value of 0.92. Some of the product migrated up the TLC plate and had an R_f value equal to that of 2,6-TNS. It was assumed that this was unreacted 2,6-TNS contaminating the

reaction product. A second spot with an R_f value of 0.29 was also observed for the reaction product. The TLC plate was then thoroughly dried and placed in a solvent tank containing a 1:9 mixture of deionized water and ethanol. Under these conditions, the reaction product migrated up the TLC plate; the R_f value was 0.95. It was determined that the first solvent used in a gradient elution of the reaction product would be ethyl acetate. Ethanol would be added to the ethyl acetate until 100% ethanol is achieved then deionized water would be added to the ethanol. This should allow for the unreacted 2,6-TNS to be collected separately from the reaction product.

The solid was collected by the addition of acetone and cooling of the reaction mixture. This solid was then purified by chromatography. In viewing the chromatography column and collected eluant fractions under UV-light, it was possible to see fluorescence from 2,6-TNS as well as the fluorescent product. It was determined that fractions collected when the solvent mixture was 1:9 deionized water:ethanol and 1:4 deionized water:ethanol contained the fluorescent product. These fractions were combined and concentrated by rotary evaporation then freeze dried. The dried product was analyzed by NMR and mass spectroscopy. ^{1}H NMR (D_2O, 300MHz): 1.24 (m, 3H, H-9), 2.32, 2.40, 2.52 (3 s, 2H), 3.55-3.95 (m, 77H, 49 H: 1, 1', 2, 2', 3, 3', 4, 4', 5, 5', 6,6'-positions with the remainder of the H's from water), 5.78 (s, br, 14H, OH-2, 2', 3, 3'), 6.79-8.45 (m, 10H, H-7, 8, 9, 10, 11, 12, 13, 14, 15); ^{13}C NMR ((D_2O, 75MHz): δ 60.41, 72.42, 72.54, 73.58, 81.48, 102.48, 125.72, 129.77. Figure 29 shows position assignments. For the ^{13}C NMR spectrum, peaks can be assigned as: C-6, C-5, C-2, C-3,C-4, C-1, which are consistent with β-CD[62] and the two small peaks in the aromatic region are indicative of the presence of

TNS. The NMR spectra suggest the presence of β-CD as well as 2,6-TNS, but it is not clear whether the two molecules are actually tethered together or if they exist as an inclusion complex. It is thought that an inclusion complex would have dissociated while isolating the product by chromatography and the presence of both host and guest molecule in the isolated product says that they are in fact attached, but this can not be confirmed by NMR.

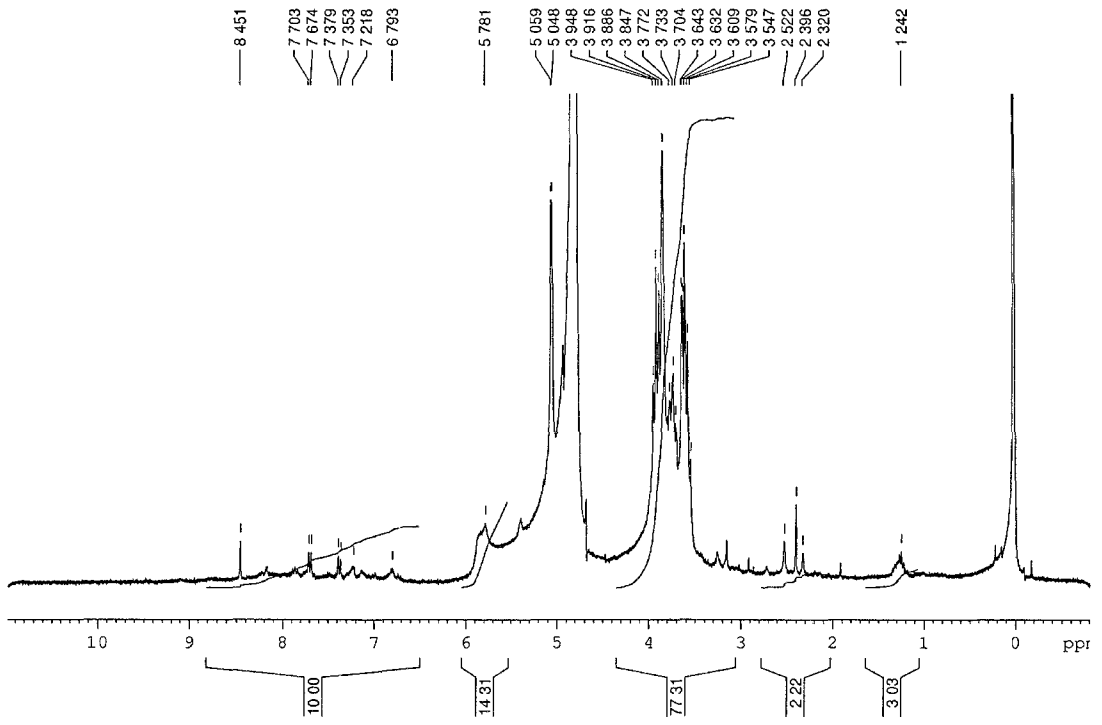


Figure 27. ^{1}H NMR spectrum of reaction product.

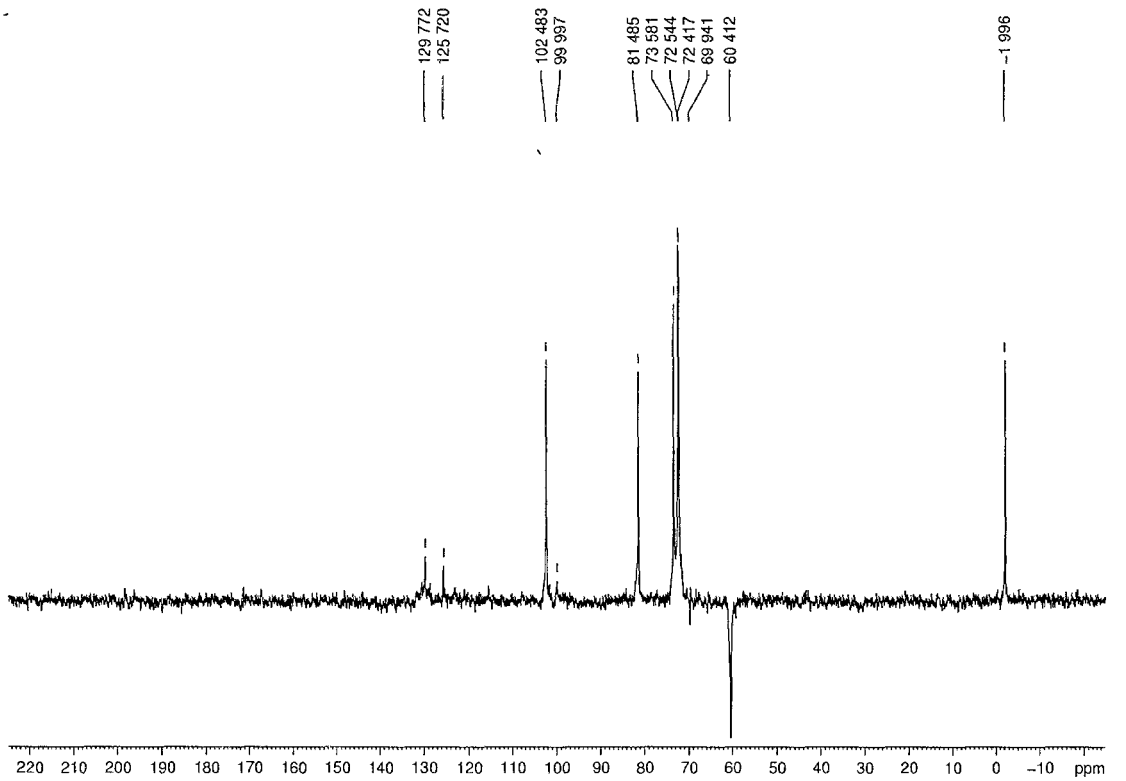


Figure 28. ^{13}C NMR spectrum of reaction product.

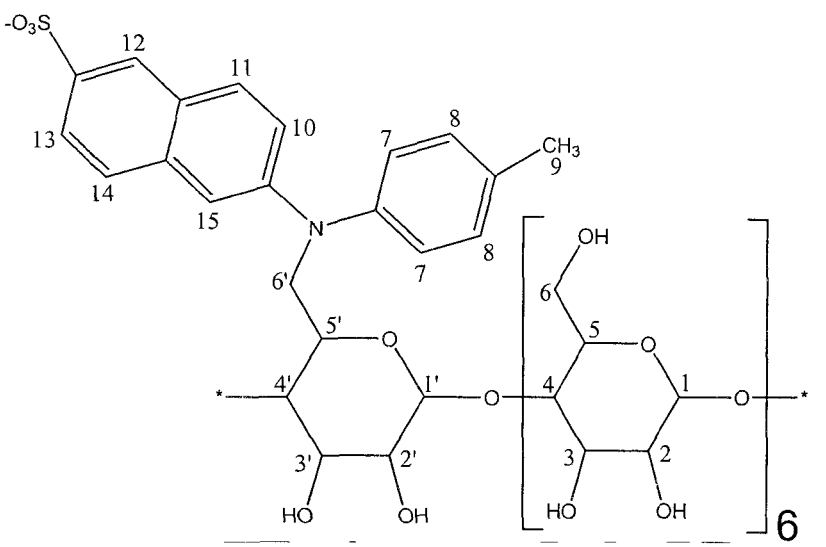


Figure 29. Illustration depicting position assignments of reaction product.

Mass spectra of 2,6-TNS, β-CD and the reaction product are shown for the positive and negative modes, in Figures 30, 31 and 32, respectively. The positive mode, positive ions are detected; in negative mode, negative ions are detected.

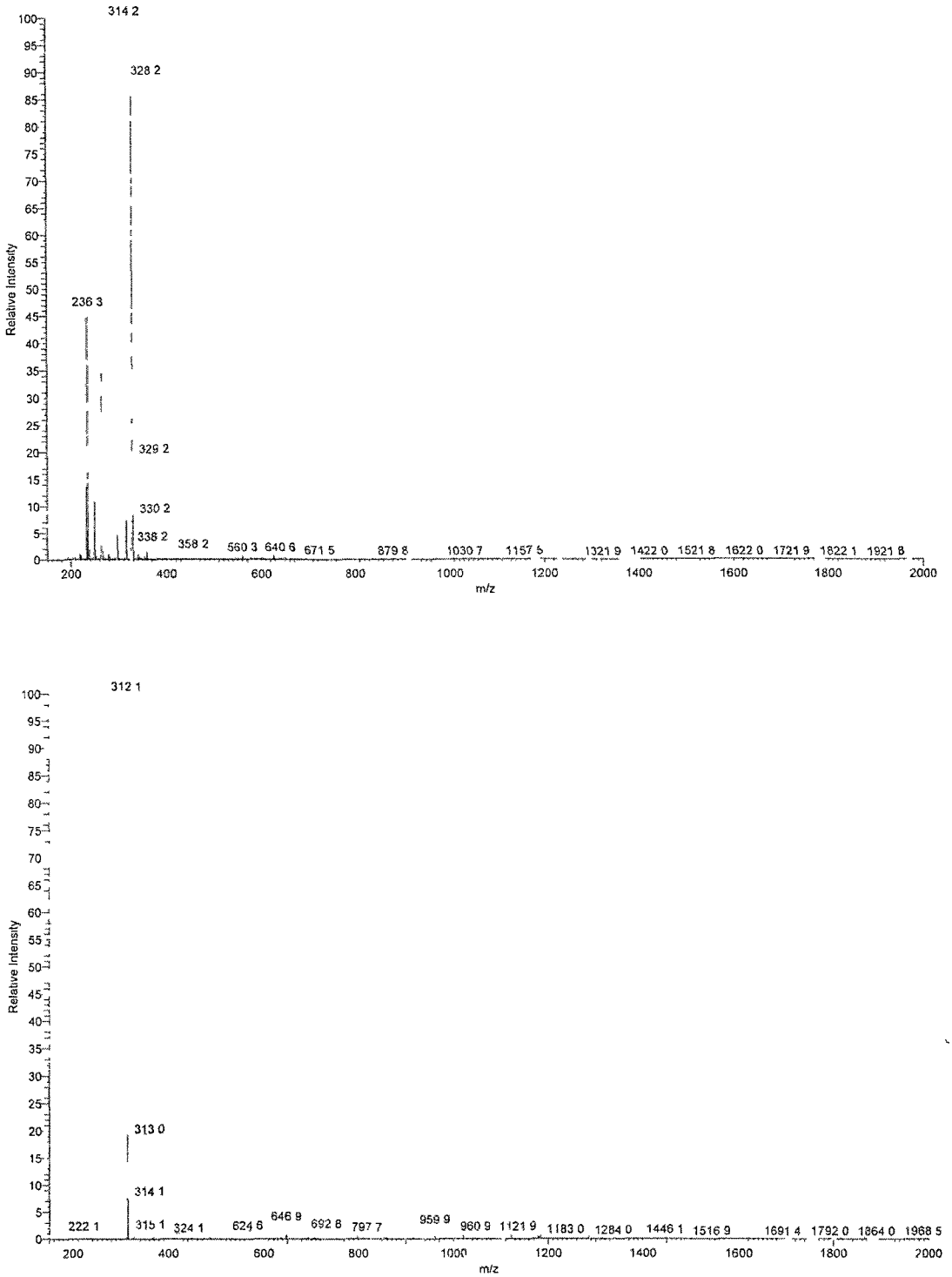


Figure 30. Mass spectra of 2,6-TNS, positive mode (top) and negative mode (bottom).

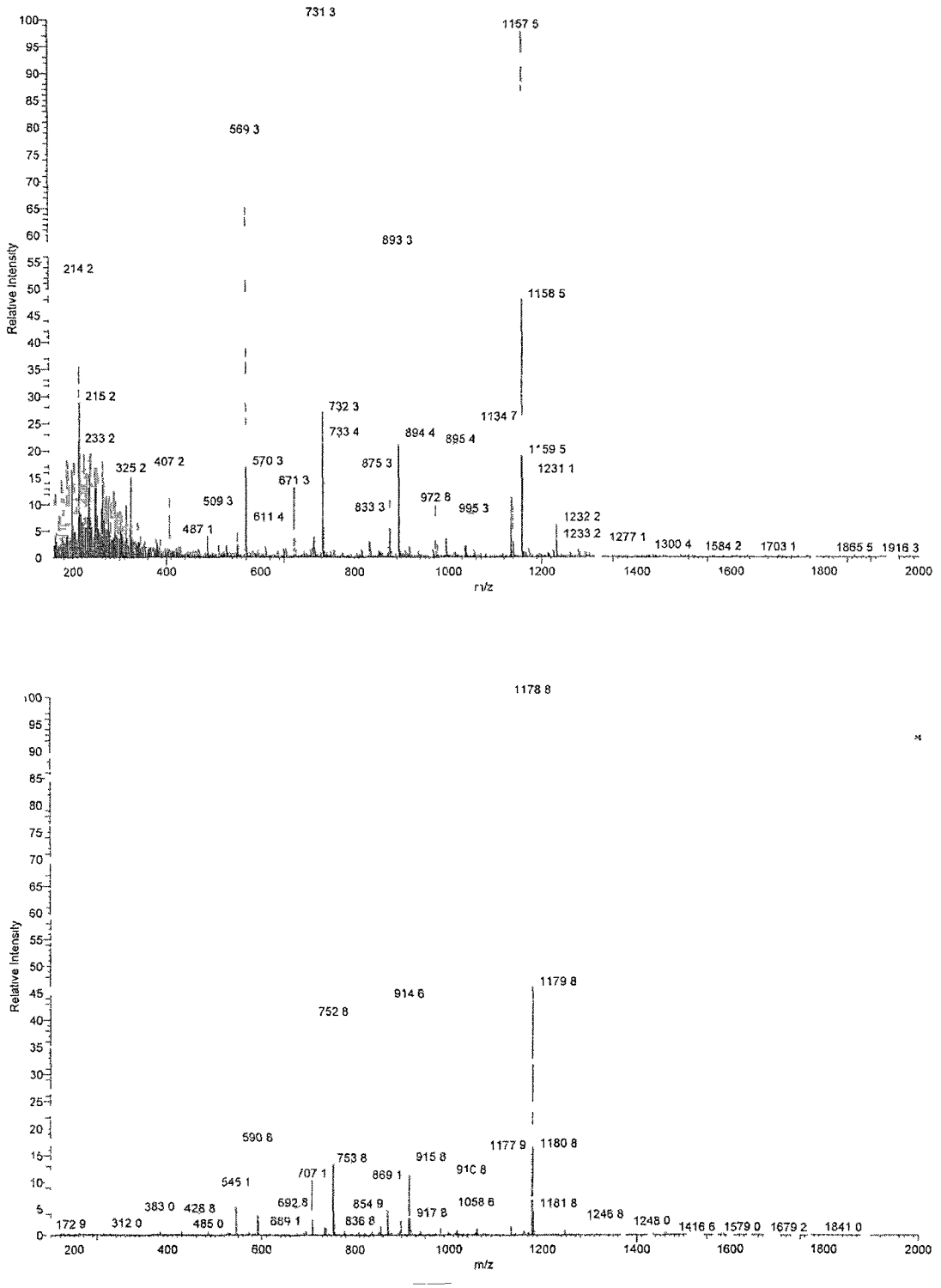


Figure 30. Mass spectra of β-CD, positive mode (top) and negative mode (bottom).

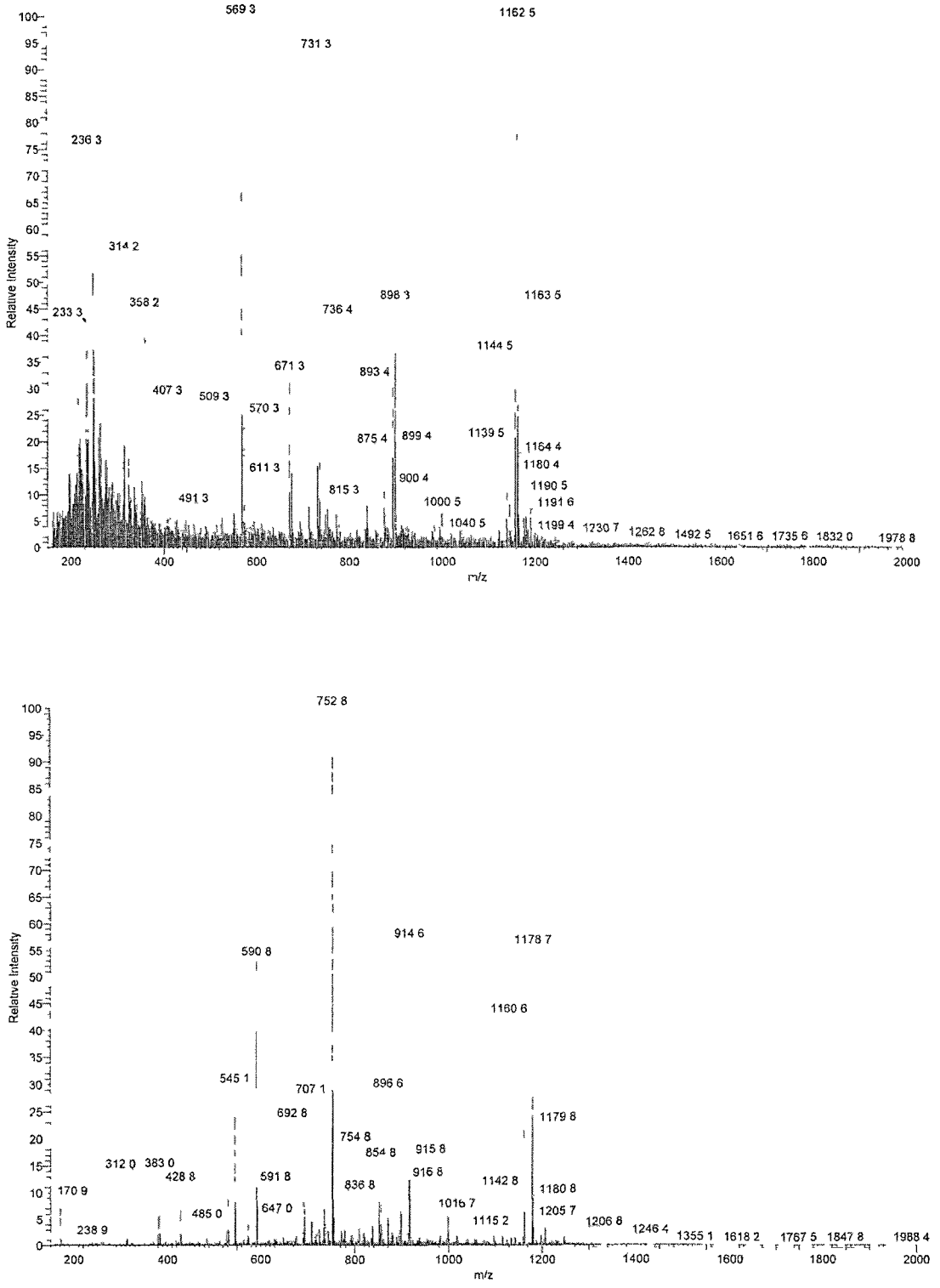


Figure 32. Mass spectra of the reaction product, positive mode (top) and negative mode (bottom).

Peak assignments are given in the Table 6.

Table 6. Mass spectrometry peak assignments.

2,6-TNS		
Positive mode	314.2	2,6-TNS + H
	236.3	Associated with 2,6-TNS
Negative mode	312.1	2,6-TNS – H
β-CD		
Positive mode	1157.5	β-CD + Na adduct; M/1
	893.3	(β-CD + Na adduct) – 264
	713.3	((β-CD + Na adduct) – 264) – glucose monomer
	569.3	β-CD + Na adduct; M/2
Negative mode	1178.8	(β-CD – H) + (Formic acid –H) adduct; M/1
	914.6	((β-CD – H) + (Formic acid –H) adduct) – 264
	752.8	(((β-CD – H) + (Formic acid –H) adduct) – 264) – glucose monomer
	590.8	(β-CD – H) + (Formic acid –H) adduct; M/2
Reaction Product		
Positive mode	1162.5	(β-CD – OH *or* TNS) + (Formic acid –H) adduct; M/1
	898 3	((β-CD – OH *or* TNS) + (Formic acid –H) adduct) – 264

	736.4	(((β-CD – OH *or* TNS) + (Formic acid –H) adduct) – 264) – glucose monomer
	569.3	(β-CD - OH *or* TNS) + (Formic acid –H) adduct; M/2
	314.2	2,6-TNS + H
	236.3	Associated with 2,6-TNS
Negative mode	1178.7	(β-CD – H) + (Formic acid –H) adduct; M/1
	1160.6	(β-CD – OH *or* TNS) + (Formic acid –H) adduct; M/1
	914.6	((β-CD – H) + (Formic acid –H) adduct) – 264
	896.6	((β-CD – OH *or* TNS) + (Formic acid –H) adduct) – 264
	752.8	(((β-CD - H) + (Formic acid –H) adduct) – 264) – glucose monomer
	590.8	(β-CD – H) + (Formic acid –H) adduct; M/2
	312.0	2,6-TNS – H

The β-CD spectra demonstrates previously recognized fragmentation of β-CD.[63, 64] Figure 33 illustrates the fragmentation of β-CD which occurs, resulting in a mass loss of 264 units.

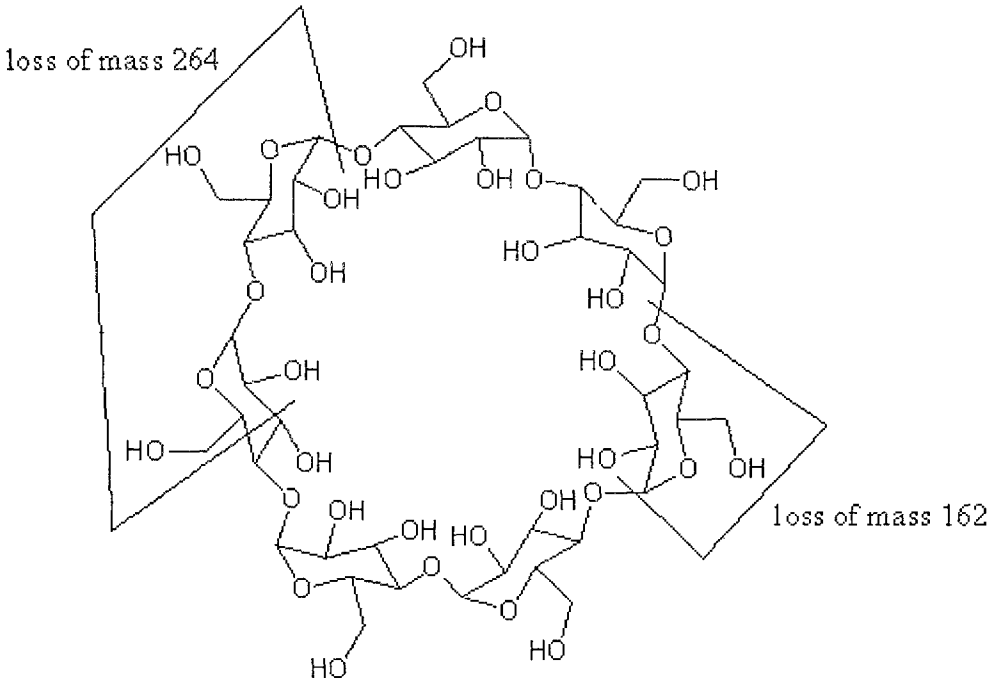


Figure 33. Illustration of β-CD fragmentation.

The mass spectrometry data suggests 2,6-TNS may have been tethered to β-CD, but it is not definitive. Peaks in the product spectra are consistent with those of β-CD and 2,6-TNS, indicating that both molecules are present. In the negative mode, there are two peaks of particular interest, 1160.6 and 896.6. These peaks could result from the fragmentation of β-CD or β-CD with a 2,6-TNS tether. It could be explained if 2,6-TNS was tethered to β-CD and fragmentation occurred at the 6'-position C and N and the remaining β-CD fragment formed an adduct with formic acid. At the same time, it could have formed if a primary hydroxyl group of an unmodified β-CD or the tosylate of tosyl-CD eliminated and the remaining β-CD fragment formed a formic acid adduct. As these peaks only appear in the sample spectrum, not in the β-CD spectrum, it could be argued that they are more likely to

have occurred as a result of fragmentation of tethered 2,6-TNS off of β-CD. It must also be acknowledged that mass spectrometry analysis does not always result in the formation of the same adducts every time, so it is possible that the peaks are the result of β-CD or tosyl-CD fragmentation.

3.5 Fluorescence of the Product

If the product is to be used as a fluorescent sensor, investigations must be made into its fluorescent properties. NMR and mass spectroscopy do not definitively conclude whether the product is simply an inclusion complex composed of 2,6-TNS and β-CD or if the two molecules are in fact tethered together. Comparison of the fluorescent properties of the reaction product to those of 2,6-TNS included in the cavity of β-CD may offer supporting evidence.

The absorption spectrum of the reaction product was measured and compared to that of 2,6-TNS in both nanopure water and phosphate buffer. The spectra are shown in Figure 34. The absorbance spectrum of the product was not the same as that of unreacted 2,6-TNS. This suggests that the probe was modified in some way, though it does not indicate how the probe was modified.

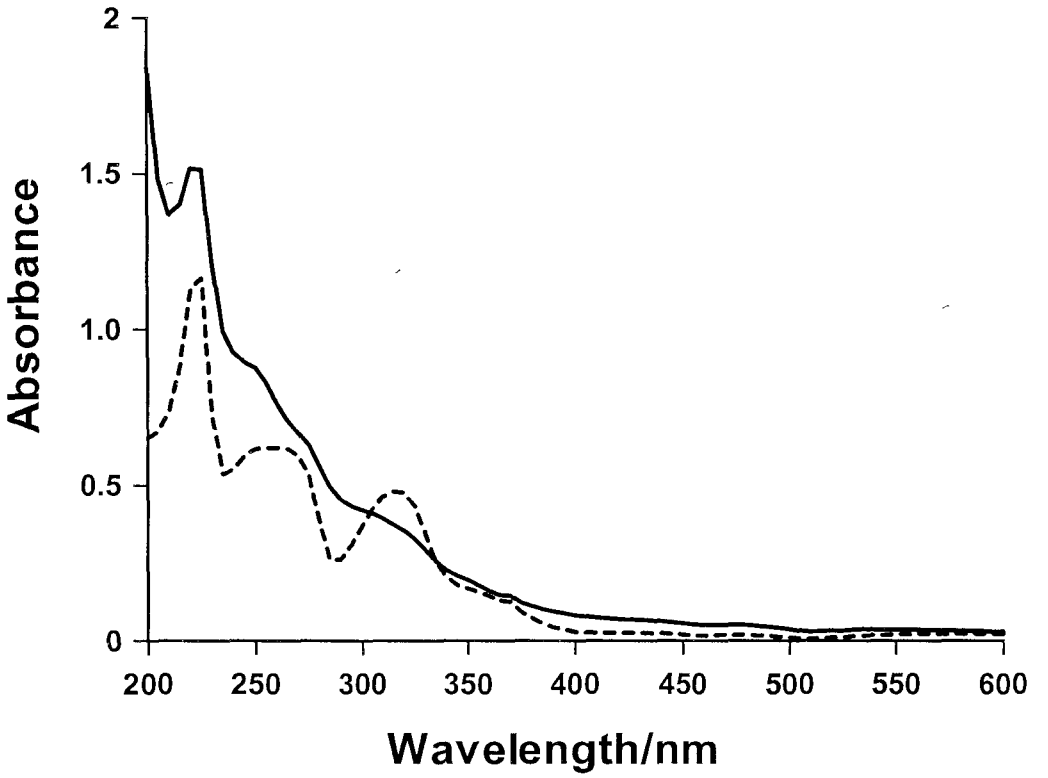


Figure 34. Absorbance spectra of 2,6-TNS (dashed) and the reaction product (solid) in nanopure water.

The fluorescence of the reaction product and 2,6-TNS was also measured, as well as that of the probe in the presence of CDs. Fluorescence analysis was conducted on both the Perkin-Elmer and PTI spectrometers; there was agreement in the spectra from both spectrometers. The spectra from 2,6-TNS and the reaction product are shown in Figure 35. It is clear that these fluorescence spectra are different, suggesting that the probe was modified. The spectrum of the product is broader, and less well resolved. This may be indicative of an impure product, or a mixture of products. When β-CD is added to these two solutions, an increase in the

intensity of fluorescence is observed in both cases. However, the increase observed for the unreacted 2,6-TNS is greater than that of the reaction product. This may give a clue as to how the probe in the reaction product is oriented. One reason that 2,6-TNS was chosen as the probe for these experiments is because its linear shape allows it to enter the CD cavity unhindered to form an inclusion complex. If 2,6-TNS is tethered to β-CD by its amine group there may be too much steric bulk to allow free movement of the probe in and out of the CD cavity. There also may be enough steric hindrance to reduce the likelihood that the tethered probe will become included in an additional, untethered β-CD molecule. Thus, these fluorescence results do not provide any specific information on the identity of the product, only that the 2,6-TNS has indeed been modified. It also does not provide any conclusive evidence that the 2,6-TNS is included within the cavity of the CD to which it is attached.

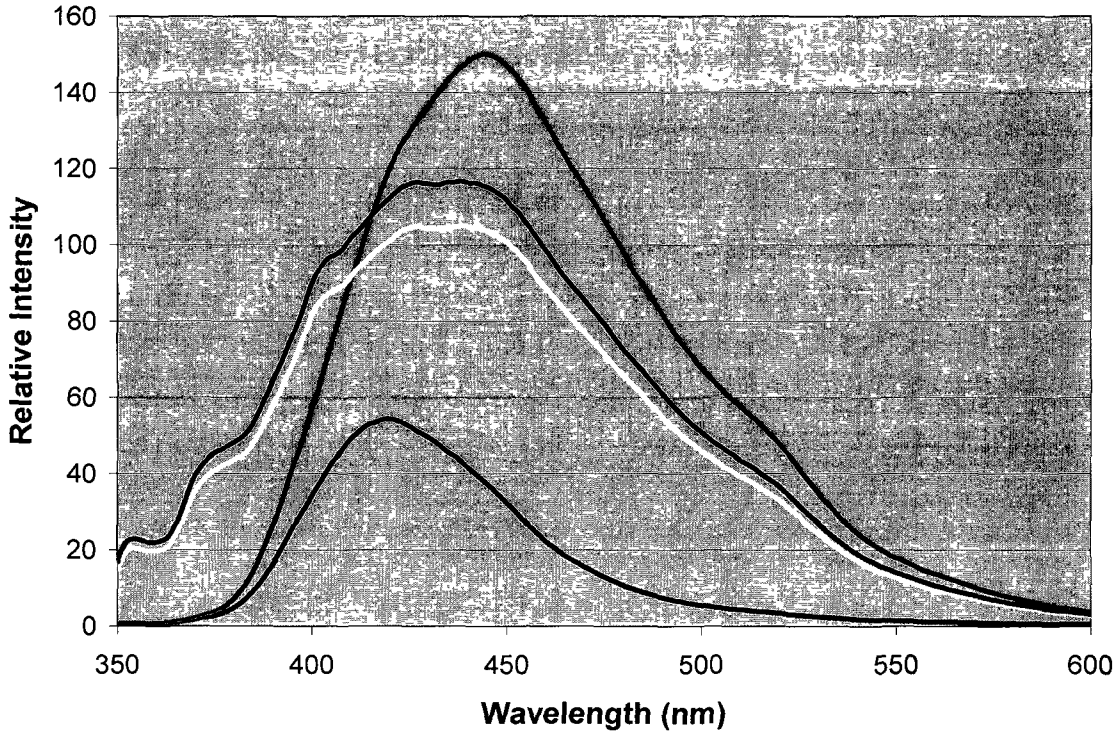


Figure 35. Fluorescence spectra of 2,6-TNS (blue line), 2,6-TNS + β-CD (red line), reaction product (yellow line) and reaction product + β-CD (green line), each dissolved in phosphate buffer.

The effect of pH on the fluorescence emission of 2,6-TNS and the reaction product was investigated. The fluorescence emission of the compounds was measured at a range of pHs, see Table 7 for pH values. As can be seen from this table and the fluorescence emission spectra in Figures 36 and 37, there was no consistent significant effect of pH on the product fluorescence. The observed behavior was similar to that observed for 2,6-TNS itself (note that 2,6-TNS was investigated at a higher pH range than was the product.) Thus, if the reaction product is in fact composed of 2,6-TNS tethered to β-CD, it will not function as an effective pH sensor

when tethered in this way, as the fluorescence emission was not greatly changed under the various pH conditions, as shown in Figures 36 and 37.

Table 7. pH, F, maximum intensity and wavelength at maximum intensity values of 2,6-TNS and product solutions.

pH	F	Maximum Intensity	$\lambda_{F,max}$ (nm)
2,6-TNS			
5.09	13121.21	187	421
6.05	23243.38	292	421
6.64	19422.31	297	417
7.87	17424.07	263	421
8.79	19760.85	297	422
9.90	51896.47	816	419
10.40	44626.74	696	417
Product (Replication 1)			
5.30	22757.56	231	422
5.77	24706.50	246	422
6.63	22180.92	204	427
7.65	24748.34	238	421
8.60	20207 28	199	425
Product (Replication 2)			
5.30	21971.44	224	423
5.74	21268.94	209	424
6.64	26475.01	257	420
8.03	21541.84	195	429
8.35	24055.72	220	438

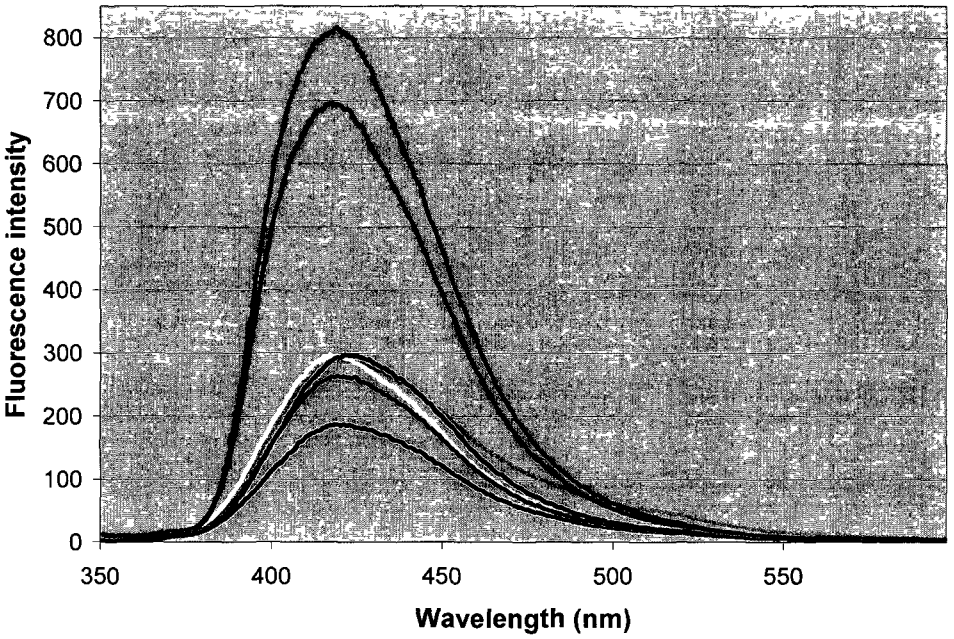


Figure 36. Fluorescence spectra of 2,6-TNS under various pH conditions; pH = 5.09 (blue), 6.05 (pink), 6.64 (yellow), 7.87 (green), 8.79 (purple), 9.90 (red) and 10.40 (orange).

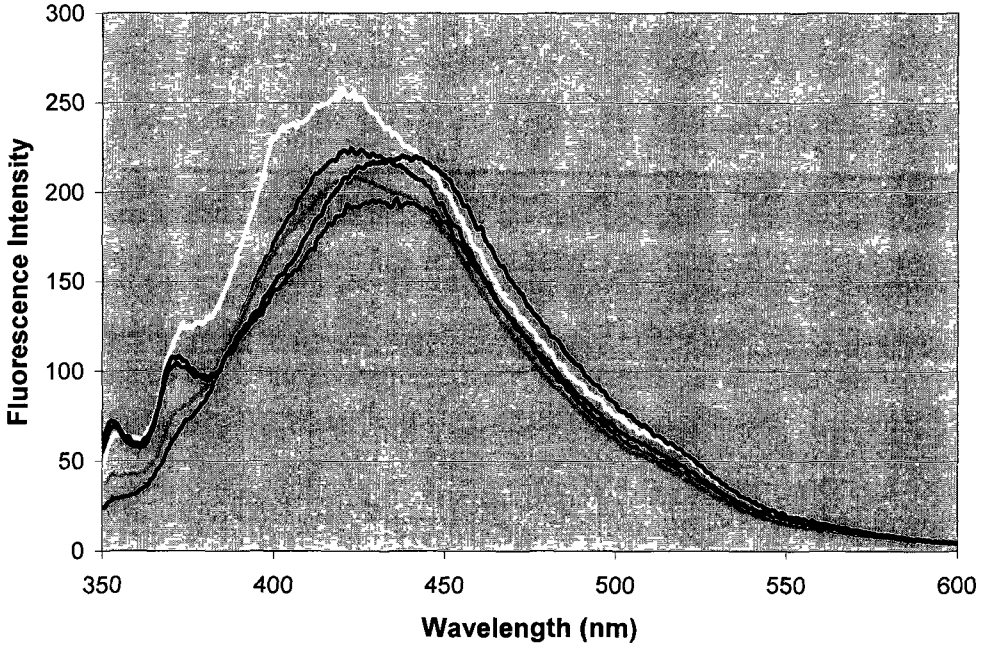


Figure 37. Fluorescence spectra of the reaction product under various pH conditions; pH = 5.30 (blue), 5.74 (pink), 6.64 (yellow), 8.03 (green), 8.35 (purple).

4. Conclusions

Intermolecular fluorescence-based binding studies were successfully performed for three candidate highly-polarity sensitive probes, namely 1,8-ANS, 2,6-ANS, and 2,6-TNS, in both parent and methylated modified β-CD. These results clearly demonstrated that 2,6-TNS gives the highest enhancement upon inclusion into CD cavities, and is therefore the best candidate for the polarity-sensitive guest to be tethered to a CD. Furthermore, comparisons of the results for the parent and methylated β-CD clearly indicated that not only was the fluorescence enhancement of 2,6-TNS much greater in the methylated β-CD, but the binding constants were as well. Both of these results indicate that the modified β-CD is the better choice to which to tether 2,6-TNS to create the desired fluorescence sensor, as this will give the largest difference in the on and off signals, and the stronger binding would in principle result in a greater selectivity – only target guests with very high affinity for the modified CD cavity could compete with the tethered 2,6-TNS, and thus be sensed by the proposed system. However, it is possible that the 2,6-TNS binds too strongly with the modified CDs, which might in fact result in a sensor which is too selective, i.e. it might not be possible to have a competing guest knock the 2,6-TNS out of the cavity. In this case, the parent β-CD itself would be more useful as the target host to which to tether β-CD, in spite of the lower difference between the resulting on and off position. For this reason, it would be ideal to make the tethered product with both parent and modified β-CD, in order to test their potential applications and selectivities as fluorescent sensors.

2,6-TNS is a very effective fluorescent probe, but it has not previously been tethered to a β-CD. There may be a reason this hasn't been done before. It proved very difficult to attach the probe to the CD and the reaction product synthesized within this work has not been definitively identified as a tethered complex, it is possible that it is just an inclusion complex. If 2,6-TNS is actually tethered to β-CD, it is not done so in a desirable manner. If it is tethered it is probably through the amine group, onto the primary face of the CD, by a very short tether composed of a single carbon. This would hinder the movement of the probe into and out of the CD cavity, and therefore reduce the efficacy of the molecule as a fluorescent sensor, for multiple reasons. First, tethering 2,6-TNS through its amine group disrupt the linear shape of the probe; this could reduce the likelihood that it would enter a CD cavity. Secondly, the primary face of a CD is smaller than the secondary face. This smaller opening may restrict the entry of a probe. The third reason the entry of a probe tethered in this fashion may be hindered is the length of the tether. The extremely short tether would greatly reduce the range of motion of the probe, analogous to waving a finger versus waving an arm.

Future work on the synthesis of a tethered fluorescent sensor composed of 2,6-TNS and β-CD should include investigations into the use of dimethyl-β-CD. As the 2- and 6-position hydroxyl groups are protected, it may be much more conducive to having the tether attached on the secondary rim of the CD cavity. In addition, as was clearly observed in the intermolecular binding fluorescence studies, a much larger enhancement of 2,6-TNS fluorescence can be obtained with the methylated modified β-CD than with the parent β-CD. It is possible that more success may be

achieved with the methodologies attempted within this project if dimethyl-β-CD is used in place of natural β-CD.

While all of the goals initially laid out for this Masters project were not achieved, success was achieved in moving towards the ultimate goal, synthesis of a reusable, fluorescent molecular sensor with an extremely large dynamic range. It was determined that the probe that should be tethered was 2,6-TNS due to its linear shape and its strong affinity for forming an intermolecular complex with β-CDs. Through the intermolecular studies, it was discovered that the probes, ANS and TNS, are more tightly bound within dimethyl- β-CD than natural β-CD. It was determined that synthesis techniques should begin with modification of the CD as opposed to modification of the probe. While not all of the techniques to modify β-CD were successful, they should not be abandoned, as more success may be achieved if the same techniques were attempted with a modified β-CD, *i.e.* dimethyl- β-CD. The knowledge gained through the work conducted during this Masters project will lay the ground work for future success in synthesizing the desired fluorescent molecular sensor.

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