Chiral Calixarenes as Modifiers of Stationary and Mobile Phases in HPLC

By

Michael Sullenberger

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Abstract

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and Mobile Phases in HPLC

Michael Sullenberger
Master of Science
Youngstown State University

The class of basket-shaped macrocyclic compounds known as calix[n]arenes are of interest in chromatography due to their ability to form host/guest complexes. An area of limited exploration has been the use of chiral calixarenes to perform analytical separations of enantiomers. The interest in this work arises from the search for alternatives to cyclodextrins, which are used predominantly in the separation of enantiomeric mixtures. Two approaches using calixarenes are presented here. The first is the use of a synthesized chiral calixarene as a modifier of a reverse phase stationary phase in HPLC; the second is the use of the same calixarene as a modifier of the mobile phase in reverse phase HPLC.

Interactions between a non-chiral homologous series of compounds and the synthesized calixarene were determined to take place during both approaches. This was evidenced by the difference in retention times, capacity factors, and asymmetry factors when the synthesized calixarene was used as a modifier.

Acknowledgements

I would like to thank Dr. James Mike for his support and encouragement over the past two years. Without his steady guidance I would not have accomplished the goals of my graduate career. I also appreciate his sincere belief in the work that his group does. I also extend my sincerest thanks to Dr. Peter Norris. I certainly would not be in graduate school, or have discovered my desire to do organic chemistry were it not for his support and excellent teaching ability. I thank Dr. Daryl Mincey for reviewing this manuscript and for his dedication to the graduate program. I thank Ronnie Hefley for his support and the many great times we've had in graduate school. Last, but certainly not least, I extend my thanks to Dr. Bruce Levison. His dedication to this project is commendable in itself, but more than that, I appreciate the education he gave me in the practice of chemistry. The extensive list of organic as well as analytical techniques and strategies that I learned from him I will certainly carry with me into industry, but the jokes will probably have to stay here. Thanks, Bruce.

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Symbol	Definition	Units
mL	milliliter	$1x10^{-3}$ liters
μL	microliter	$1x10^{-6}$ liters
mg	milligram	$1x10^{-3}$ grams
M	molar	moles/liter
L	liter	
g	gram	
min.	minute	
°C	degrees Celsius	
HPLC	High Pressure Liquid Chromatography	
GC	Gas Chromatography	
TLC	Thin Layer Chromatography	
NMR	Nuclear Magnetic Resonance	
UV/Vis	Ultraviolet/Visible	
MHz	megahertz	10^6 cycles/second
i.d.	internal diameter	
C_{18}	octadecyl	
CE	capillary electrophoresis	

CHAPTER 1

Introduction

Calixarenes

The class of compounds known as calix[n]arenes has been known for some time. They are oligomers composed of repeating phenolic units bonded through methylene bridges. Gutsche named the calixarenes according to their structure, which is traditionally thought of as being in a cup shape (1). *Calix* means cup in Greek, the arene part of the name arises from the arene functionality that is present, and *n* designates the number of repeating phenolic units.

The structure shown in Figure 1 is that of calix[4]arene, the red atoms designating the hydroxyl functionalities from the phenols. There are two commonly referred to parts of the calixarene structure, those being the "upper rim" or the *para* position of the benzene rings, and the "lower rim", or the hydroxyl portion of the calixarene. Calixarenes are often referred to as a third class of macrocycle, with crown ethers (Figure 3) and cyclodextrins being the first two. Like their related compounds, the calixarenes have the ability to form host/guest complexes with various species of atoms and molecules (1,2). The mode of interaction is believed to be due to the guest molecules contact with the calixarenes pi electron system (3). Figure 2 depicts a host/guest complex formation between a generic molecule and p-*tert*-butylcalix[4]arene.

Unlike the cyclodextrins, calixarenes are not inherently chiral. Chirality must be introduced through synthetic modifications, and this can be accomplished in several ways. The first is through modification of the calixarene at the lower rim, where a functional heteroatom is already present. Another method is through modification at the *para* position of the calixarene, and a third method is through modification of the starting materials which go into the calixarene synthesis.

Calixarenes are of interest in chromatography due to their ability to form host/guest complexes. As has been demonstrated by use of cyclodextrins in chromatography, if host/guest complexes form during a separation, this can have a pronounced affect on the characteristics of the separation, and lead to new technologies in separation science. Because calixarenes are in many ways similar to cyclodextrins, especially in their mode of interaction with guest species, it is hypothesized that they can be used in place of, or in conjunction with, cyclodextrins to accomplish various separations.

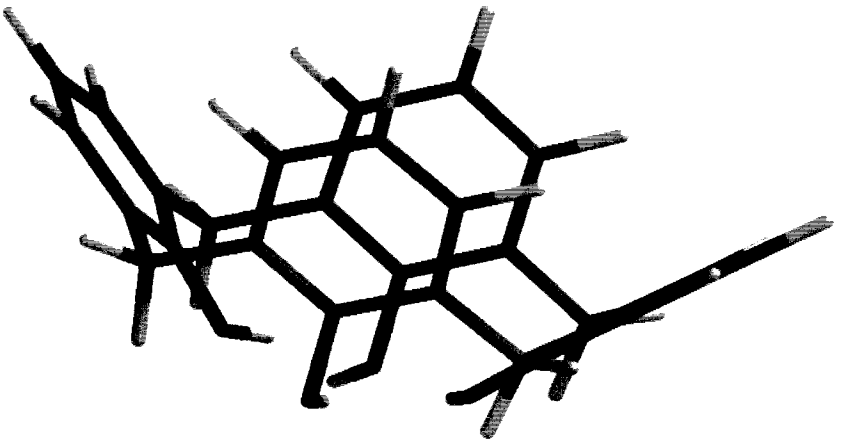


Figure 1: *calix[4]arene*

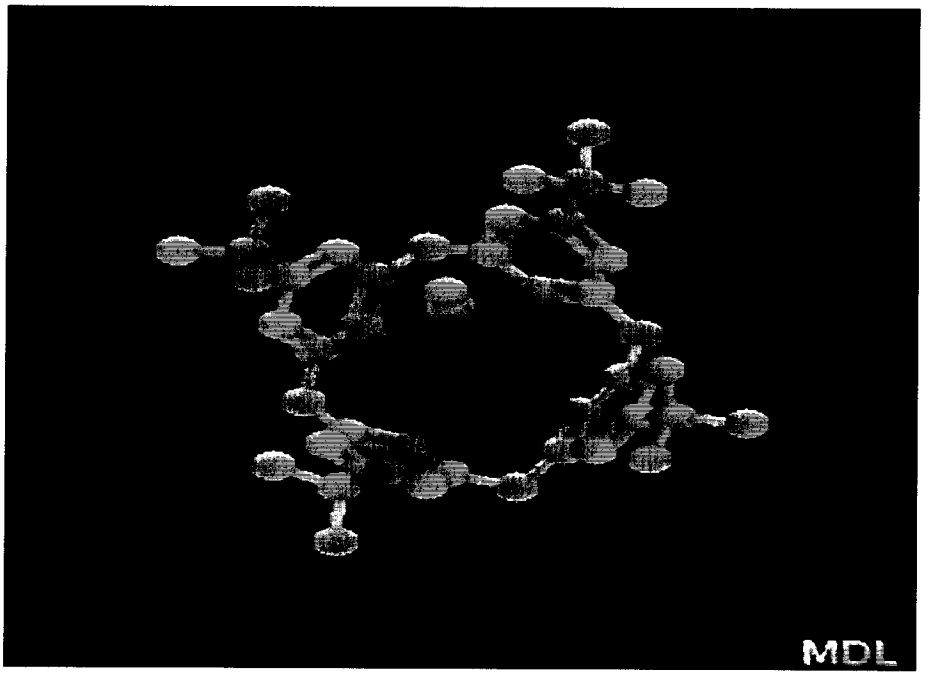
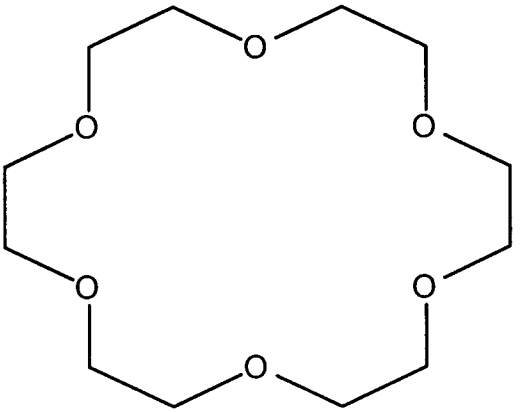


Figure 2: *p*-tert-*butylcalix[4]arene with guest*



18-Crown-6

Figure 3: *The crown ethers are structural relatives to the calixarenes*

HPLC

HPLC, or High Pressure Liquid Chromatography, is a widely used technique both in the analytical separation and quantification of compounds, and in the routine purification and collection of compounds. Although it is performed at high pressures (up to 5000psi), like other forms of chromatography, it is based on the partitioning of solutes between two immiscible phases, a stationary phase (packed in a stainless steel column) and a mobile phase (passed through the column). Separation is based on the individual solute's "preference" for either

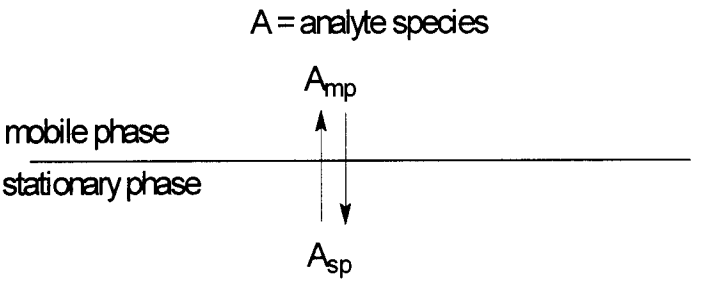
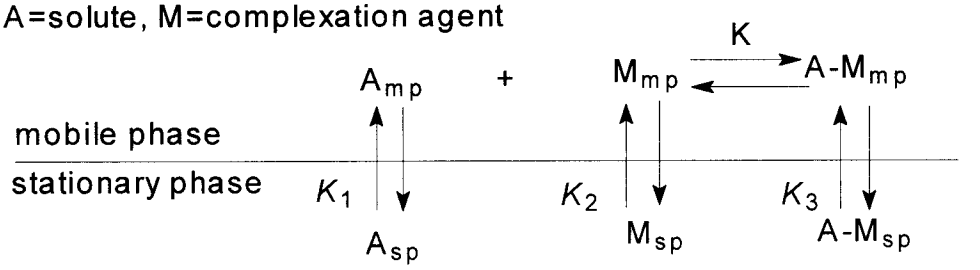


Figure 4: *Primary Equilibrium Process*

the mobile or the stationary phases (4). If a solute is more soluble in the mobile phase it elutes, or passes through the stationary phase, quickly; if it is more soluble in the stationary phase then it elutes more slowly. The time a solute spends in the column is known as the "retention time". This "preference" for one or the other phase is governed by pseudo-equilibrium processes which go on inside the column during a separation. The schematic shown above (Figure 4) demonstrates this partitioning process, where the analyte species establishes a pseudo equilibrium between the mobile phase and the stationary phase. This is relatively uncomplicated, and is known as the primary equilibrium. Any other equilibrium processes that occur are known as secondary equilibrium processes. There are many examples of this, but of particular interest to this research is the formation of host/guest complexes in both the mobile and stationary phase. If a host/guest complexation agent is added to the mobile phase, or to the stationary phase, and a host/guest complex forms between a solute and the agent, this would be deemed a secondary equilibrium process (Figure 5). Now, the solute is not only partitioning between the mobile and stationary phases, but also between the complexation agent and the mobile (or stationary) phase. This can have the effect of increasing or decreasing the amount of time that a solute will spend either in the mobile phase or the stationary phase.

A=solute, M=complexation agent



K_1=solutes equlibrium between mobile and stationary phase
K_2=complexation agents equilibrium between mobile and stationary phase
K=equilibrium between the unbound solute and the solute/agent complex
K_3=solute/agent complex between mobile and stationary phase

Figure 5: *Secondary Equilibrium Processes*

Chiral Separations

Chirality is thought of as molecular handedness. Chiral molecules differ only in the way in which atoms are arranged in three dimensions, and cannot be interconverted through bond rotation. When a molecule is chiral it will have two isomeric forms called enantiomers, each of which is the mirror image of the other but which are non-superimposable (5). If a molecule lacks planes or axes of symmetry, it is chiral. The most common example of this is sp^3-hybridized carbon, to which four different substituents are bonded. Based on the priority of the substituents surrounding the chiral atom, enantiomers are designated *R* or *S*.

S enantiomer R enantiomer

Figure 6: *R versus S enantiomers*

As shown above (Figure 6), no possible bond rotation could convert the form on the left to the form on the right, other than this, the molecules are identical. Further, enantiomers have identical physical properties, which makes resolution of enantiomers difficult, and possible only in an environment that is itself chiral. One such environment is plane-polarized light, which is why a polarimeter may be used to distinguish enantiomers optically. Another method is to interact a mixture of enantiomers with other chiral molecules to form diastereomeric pairs, which will have different physical properties and can thus be separated, or resolved.

Fractional crystallization was a popular choice for such resolution, but more recently chromatography has become a useful method for enantiomeric separations. A racemic mixture (equal mixture of both enantiomers) can be coupled to an optically active compound to form a diastereomeric pair, as described above, and then separated using HPLC, or some other chromatographic method. This is usually faster and more efficient than fractional crystallization (5). Even more useful is the more common method of binding a chiral substrate to the surface of, or packing such a substrate inside of, a

chromatography column, as in chiral GC and HPLC. This way, a mixture of enantiomers may be passed through the column, and based on the individual enantiomer's affinity for the column packing, they may be separated and even collected in HPLC. This can be visualized as follows (Figure 7) (5):

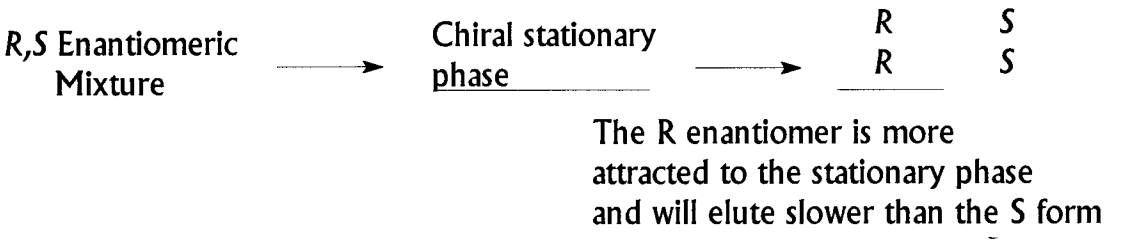


Figure 7: *Chiral Chromatography*

CHAPTER 2

Statement of the Problem

As discussed above, it is known that calixarenes form host/guest complexes in a manner similar to the cyclodextrins. However, as was also discussed above, calixarenes are not inherently chiral. By introducing chirality into the calixarenes, perhaps, like the cyclodextrins, a chiral host/guest complex will form preferentially with one enantiomer (*R* or *S*) and allow resolution of the enantiomers.

Because there have been few investigations of chiral calixarenes for use as possible chiral resolving agents, the purpose of this research was to devise synthetic routes to chiral calixarenes and to subsequently use them as either mobile phase additives or as bound stationary phases, for the purpose of enantiomeric separations using HPLC. There were several subproblems, which included devising and carrying out the synthetic routes to novel chiral calixarenes, determining the solubility properties of the chiral calixarene, and characterizing the calixarene, both spectroscopically and chromatographically. Finally it was a goal to determine the calixarenes efficacy as a chiral resolving agent using HPLC. Differences in retention times, capacity factors, and asymmetry factors when the calixarene was used in the separation of nonchiral solutes versus when it was not, indicated that host/guest complexes had formed. The appearance of two peaks during the separation of enantiomers when the calixarene is used would indicate a chiral separation.

CHAPTER 3

Historical

Calixarenes, a class of cup-shaped molecules (1), are of interest in chromatography because of their demonstrated ability to form inclusion complexes. Chiral calixarenes are able to perform similarly, by forming inclusion complexes with one enantiomer preferentially allowing their use as chiral resolving agents. Chiral calixarenes have been developed to achieve enantiomeric separations in CE, or capillary electrophoresis (6,7), a hybrid method of electrophoresis and chromatography. In the first example (6), a water-soluble chiral *N*-acylcalix(4)arene amino acid derivative was synthesized and used as a mobile phase additive in capillary electrophoresis. The calix allowed successful resolution of a racemic mixture of (+/-)-1,1'-binaphthyl-2,2'-diyl hydrogen phosphate. In a similar report (7), *p*-sulfonic calix[4]arene was added to the CE buffer solution and successful separation of various positional isomers was facilitated. This work also reported that resolution of the isomers increased as the concentration of the calixarene was increased.

Calixarenes have also been used as mobile phase additives in HPLC (8). Here, the sodium salts of *p*-sulfonic calix[4]arene and *p*-sulfonic calix[6]arene were shown to influence the retention characteristics of 2-,3-, and 4-nitrophenols during a reverse-phase HPLC separation.

While use of calixarenes as mobile phase additives seems predominant, they have also been used as bound stationary phases in HPLC (9). In this case

two stationary phases were synthesized and characterized using ^{29}Si and ^{13}C solid-state NMR experiments. The main focus of this research was to demonstrate this characterization method, but it also provided two distinct synthetic strategies to prepare silica-bonded calixarenes.

Numerous synthetic methods for making chiral calixarenes have been reported, the earliest being in 1990, by Bohmer, et al. (2). It was reported that asymmetrical superpositioning of substituents at the upper and lower rims of the calix made the overall calix chiral. In January 1994, Ferguson, et al., reported the synthesis of inherently chiral calix[4]arenes (10). Nineteen atropisomers (conformational isomers) of calix[4]arene were synthesized and characterized by ^{1}H and ^{13}C NMR, and some by x-ray crystallography. In 1995, Bayard, et al., reported the synthesis of calix[4]arenes bearing chiral methoxymethyl-pyrolidine groups on the upper rim (11). The focus of this research was to determine which conformation the calix would adopt with these substituents attached, which was determined by 2D ^{1}H NMR, as well as to provide a synthetic method to yield chiral calixarenes. Also in 1995, Iwanek, et al., described a new method for making chiral calixarenes via the Mannich reaction (12). In this instance, chiral α-aminoalcohols were reacted with a calix[4]arene substrate to yield a chiral calix product. In 1998, Vysotsky, et al. (13), reported three new inherently chiral calixarenes. The method was similar to Bohmer's. Ethyl and diethoxyphosphoryl groups were attached at the lower rim and bromine atoms at the upper rim of a calix[4]arene, which provided the necessary asymmetrical superposition of substituents needed to make the calixarene chiral. Recently, in 1999, Soi, et al.,

reported the synthesis of new chiral calixarenes based on (*p*-hydroxy-phenyl)-menthone (14). Starting with enantiomerically pure *R*-(+)-pulgeone, three chiral calixarenes were prepared, calix[5], [6], and [8].

Since a large number of chiral calixarenes have been synthesized, this has given researchers the opportunity to study the inclusion characteristics of these calixarenes, and to determine their possible effectiveness as chiral resolving agents in either the stationary or mobile phase of chromatography.

The purpose of this research was to develop synthetic methods to novel chiral calixarenes, and to evaluate their use as chiral resolving agents in High Pressure Liquid Chromatography, either as mobile phase additives of as bound stationary phases.

CHAPTER 4

Materials and Methods

Reagents for the Synthesis

THF was purchased from Fisher Scientific (Pittsburgh, PA) and was distilled over CaH_2. Purum-grade trifluoroacetic acid and 4-*tert*-butylcalix[4]arene were purchased from Fluka (Milwaukee, WI), *O*-*t*-butyl-L-serine-*t*-butyl ester hydrochloride (99+%) was purchased from Chem-Impex International (Wood Dale, IL), and sodium hydride (60% in mineral oil), ethylbromoacetate, tetramethylammonium hydroxide (10 wt.% in water), the coupling reagent HATU, di-isopropylethylamine, and DMF were purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI) and were used as received.

Reagents for HPLC

Solvents for HPLC were of HPLC quality from Fisher Scientific (Pittsburgh, PA). The amino acid calixarene derivative was synthesized at Youngstown State University. Benzene, naphthalene, anthracene, tetracene, D,L-methylmandelate, D,L-aspartic acid, and D,L-tryptophan were purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI).

Apparatus for HPLC

A reverse phase HPLC unit consisting of two Beckman 110B pumps, a Beckman 406 analog interface module, a Beckman Model 166 UV detector, a

Shimadzu RF-535 fluorimeter, and Beckman System Gold Software (V. 5.10) was used to collect HPLC data. The column used was a Phase Separations 5μ ODS2 C-18 reversed phase analytical column (4.6mm id x 250mm) and the injection loop was 20μL.

Apparatus for NMR and MS

NMR spectra (^{1}H and ^{13}C) were recorded using a Varian Gemini 2000 400MHz spectrometer. $CDCl_3$ or d_6-DMSO were used as the solvents; the chemical shifts were reported in parts per million downfield from TMS. Mass spectra were collected using a Bruker Esquire LC/MS electrospray MS unit utilizing acetonitrile as the protonation solvent, e.g. to obtain the $M\text{-}H^+$ ion.

Apparatus for Fluorescence

A Shimadzu RF-5000U fluorimeter was used to obtain the fluorescence spectrum of the final calixarene product, as well as the fluorescence spectra of tetracene. Fisher Brand 14-385-918A quartz glass fluorescence cuvets were used.

Apparatus for UV

A Hewlett Packard 8453 UV/Vis spectrophotometer was used to collect the UV spectrum of the final calixarene product. The same sample for fluorescence was used in the UV experiment.

CHAPTER 5

Experimental

Synthesis of 4-*tert*-butylcalix[4]arene *O,O',O'',O'''*tetraacetate

To a 500mL round bottom flask at 0°C under argon was added 4-*tert*-butylcalix[4]arene (5 g, 0.0015 mol) and THF (dry, 150 mL) with stirring. Sodium hydride (60%, 10 eq., 3.0 g) was added. The solution was stirred 15 min at 0°C, then brought to reflux (0.5 hr) to ensure complete deprotonation. To this solution was added ethylbromoacetate (8.6 eq., 7.0 mL) by syringe. The resulting solution was stirred and refluxed under argon for 20 hrs. The solution was then brought to room temperature and neutralized using aqueous ammonium chloride. The layers were separated, and the aqueous layers were washed with ethyl acetate. The organic layers were combined and evaporated to about 100 mL. This solution was then stirred while ethanol was added to cause precipitation of the product. The product was suction filtered, washed with ethanol, and oven dried at 100°C (1 hour) to give 5.15g of pure product.

^{1}H NMR in $CDCl_3$: d 6.8 (s, 8H), 4.8 (s, 8H), 4.2 (q, 8H), 3.2 and 4.9 (dd, 8H), 1.3 (t, 12H), and 1.1 (s, 36H).

Synthesis of 4-*tert*-butylcalix[4]arene *O,O',O'',O'''*tetracarboxylate

To a round bottom flask was added 4-*tert*-butylcalix[4]arene *O,O',O'',O'''*tetraacetate (2.014 g, 0.002 mol), THF (100 mL), and tetramethylammonium hydroxide (10 wt. % in water, 100 mL). The solution was

refluxed 24 hours, brought to room temperature, then acidified by addition of conc. HCl to pH ~2. The resulting solution was evaporated to cause precipitation of the product, which was suction filtered, washed with water, and oven dried to give 1.8 g of product.

^{1}H NMR in d_6-DMSO: d 6.8 (s, 8H), 4.6 (s, 4H), 3.2 (d, 8H), and 1.0 (s, 36H).

Synthesis of 4-*tert*-butylcalix[4]arene *O,O',O'',O'''*tetra-L-serine derivative

To a round bottom flask at 0°C with stirring was added 4-*tert*-butylcalix[4]arene *O,O',O'',O'''*tetracarboxylate (1.8 g, 2.125 mmol), DMF (80 mL), diisopropylethylamine (8 eq., 17 mmol, 3.0 mL), and HATU (4.1eq., 8.5 mmol, 3.57 g). *O-t*-butyl-L-serine-*t*-butyl ester hydrochloride (4.1 eq., 8.5 mmol, 2.125 g) was then added. The solution was stirred at 0°C for 1hr. then brought to room temperature and stirred for 3 hrs. Water was added to precipitate the product, which was suction filtered and air-dried. The crude product was eluted over silica (20g) using 20/80 THF/hexane. The solvent was evaporated to give 3.3 g of pure product.

^{1}H NMR in $CDCl_3$: d 7.6 (d, 4H), 6.8 (s, 8H), 4.7 (m, 4H), 3.3 (d, 8H), 1.5 (s, 36H) 1.15 (s, 36H) 1.05 (s, 36H).

Synthesis of hydrolyzed 4-*tert*-butylcalix[4]arene *O,O',O'',O'''*tetra-L-serine derivative

To a round bottom flask was added the 4-*tert*-butylcalix[4]arene *O,O',O'',O'''*tetra-L-serine derivative (1.0 g, 0.6 mmol) and a trifluoroacetic acid / water mixture (15 g TFA / 0.5 g water). The solution was stirred one hr. Water was added to precipitate the product, which was suction filtered, washed with water and air-dried to give 0.65 g product.

^{1}H NMR in d_6-DMSO: d 7.6 (d, 4H), 6.7 (s, 8H), 4.6 (broad m, 4H), 4.4 (s, 8H), 3.4 (broad s, 4H), 3.1 and 3.7 (dd, 8H), 1.5 (s, 36H), and 1.0 (s, 9H).

Sample Preparation for HPLC

The homologous series sample was prepared by dissolving benzene (10 mg), naphthalene (1 mg), anthracene (1 mg), and tetracene (1 mg) in methanol (100 mL). Tetracene was dissolved in a minimal amount of THF and added to the methanol solution.

Sample Preparation for UV and Fluorescence Studies

The UV and Fluorescence spectra of the hydrolyzed final product were obtained by dissolving the product in methanol (HPLC grade) at a concentration of 5 mg / 3 mL. Appropriate wavelengths for detection were decided upon after the following information was reviewed. It was determined that an excitation wavelength of 255nm could be used to detect all the solutes. The emission wavelength however would have to be changed during each separation (after

naphthalene eluted) to detect anthracene and tetracene. 400nm was used to detect benzene and naphthalene, and 470nm was used to detect anthracene and tetracene.

Compound Name:	Excitation (nm)	Emission Possibilities (nm)
Benzene	225-260	275-450
Naphthalene	250-285	325-450
Anthracene	250, 325-375	375-500
Tetracene	251, 288, 453, 472	470-510 @ any of the Exc's
Synthesized Calixarene	265	minimal response at 305
	292	305

Table 1: *Fluorescence Information for Mobile Phase Studies*

Stationary Phase Studies

The homologous series of compounds was first separated without the calixarene adsorbed to the stationary phase, using 50/50 THF/water as the mobile phase. To immobilize the calixarene onto the column, a 0.001M solution of the calixarene in methanol was prepared and pumped over the column for 1 hour at 0.5 mL/min. The mobile phase was then changed to water, which was pumped over the column for 15 minutes at 1 mL/min. The mobile phase was changed back to 50/50 THF/water, and the system was allowed to equilibrate. Since the calixarene was insoluble in water and in THF/water, once the mobile

phase was changed to water, any calixarene remaining in the stationary phase became "immobilized." The homologous series was then separated with the calixarene immobilized on the stationary phase. A flow rate of 1.0 mL/min was used, and injection volumes were 20μL of sample. After each sequence of separations employing THF as a mobile phase constituent, the column was washed with methanol at a flow rate of 1mL/min for not less than 30 minutes. This was necessary to avoid column degradation that results from the presence of THF.

Mobile Phase Studies

For the mobile phase studies two solutions were prepared. The first was 80/20 methanol/water with no calix additive; the second was 80/20 methanol/water, which was 0.001M in calix additive. Both solutions were filtered through 0.45μ membrane filters from Alltech Associates (Deerfield, IL) prior to use. First, using 80/20 methanol/water, the homologous series was separated, then the mobile phase was changed to the spiked 80/20 methanol/water, the system was allowed to equilibrate, and the homologous series was separated again. Because the calixarene had a high absorbance in the UV range, fluorescence was used to detect the homologous series. An excitation wavelength of 255nm was used for all solutes, an emission wavelength of 400nm was used to detect benzene and naphthalene. Emission at 470nm was used to detect anthracene and tetracene. A flow rate of 1.0 mL/min was used, and injection volumes were 20μL of sample.

HPLC Analysis for Purity of the Calixarene

The purity of the calixarene product was analyzed using HPLC at two different wavelengths, 230nm and 254nm. Methanol was used as the mobile phase, and a flow rate of 1 mL/min and injection volume of 20μL was used. The calixarene was dissolved in methanol to a concentration of 1 mg/mL.

Analysis of Column Polarity

Column polarity was analyzed both without calixarene on the column and with calixarene on the column. A mobile phase of 50/50 THF/water was used to preserve the calixarene's presence on the column. The reverse phase test mixture used consisted of uracil, phenol, *N,N*-diethyltoluamide, and toluene. Uracil was used as a dead time marker, phenol was a weak acid, *N,N*-diethyltoluamide was a weak base, and toluene was used to evaluate the reverse phase characteristics of the column.

Sample Preparation for Chiral Separations

Samples to examine chiral separations included D,L-methylmandelate, D,L-tryptophan, and D,L-asparatic acid. The samples were prepared by dissolving 5 mg of each compound in 10-20 mL of the 50/50 THF/water mobile phase.

Chiral Separation Studies

Once the appropriate method of immobilizing the calixarene on the column was discovered, racemic mixtures of methylmandelate, aspartic acid, and tryptophan were separated on the "functionalized" stationary phase. Flow rates of 1 mL/min were used in each case, and the injection volume in each case was 20μL. Again, after each sequence of separations using THF in the mobile phase, the column was washed in methanol for not less than 30 minutes.

The mobile phase approach, e.g. using the calixarene as a chiral mobile phase additive was not investigated due to the loss of material (calixarene) that is inherent in this method.

CHAPTER 6

Results and Discussion

Synthesis of 4-*tert*-butylcalix[4]arene *O,O',O'',O'''*tetraacetate

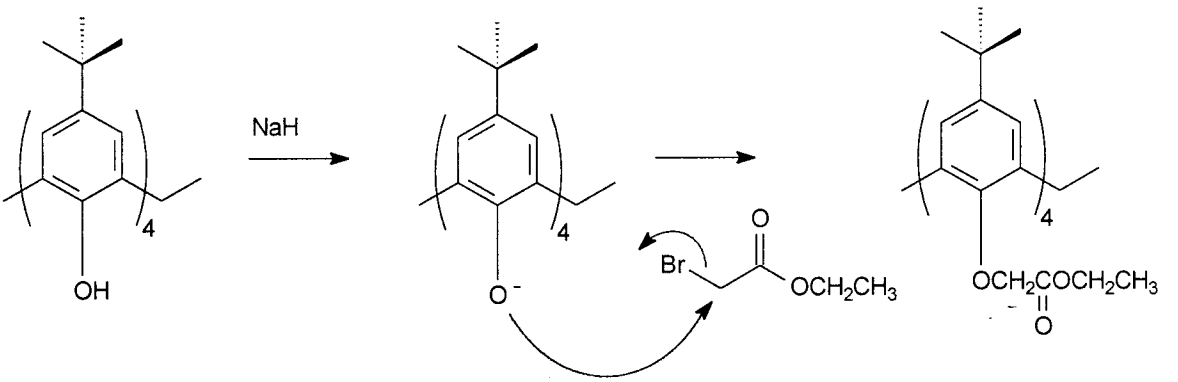


Figure 8: *Mechanism of formation of 4-tert-butylcalix[4]arene O,O',O'',O'''tetraacetate*

The literature procedure (6) described by Pena worked well, but needed some modification to determine appropriate equivalents of reactants and recrystallization conditions for the product. It was found that adding ethanol to the evaporated reaction mixture effectively precipitated the product, which could then be gravity filtered and washed with ethanol. This successfully purified the product, as was evidenced by one spot on a TLC plate using 80/20 hexane/THF as the eluant. The ^{1}H NMR (shown below) also correlated with the known compound. Yield was 71%. Figure 8 shows the mechanism of formation of this product, and Figure 9 shows the ^{1}H NMR of the product.

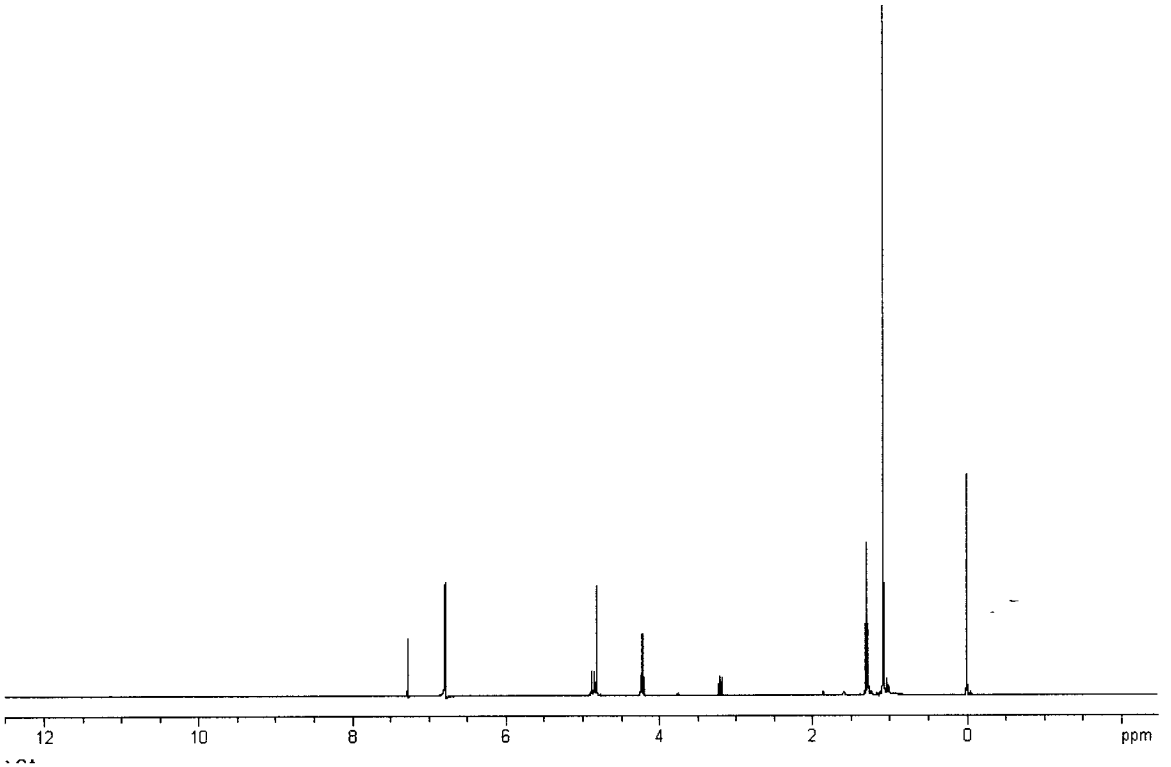


Figure 9: *^{1}H NMR of 4-tert-butylcalix[4]arene O,O',O'',O'''tetraacetate*

Synthesis of 4-*tert*-butylcalix[4]arene *O,O',O'',O'''*tetracarboxylate

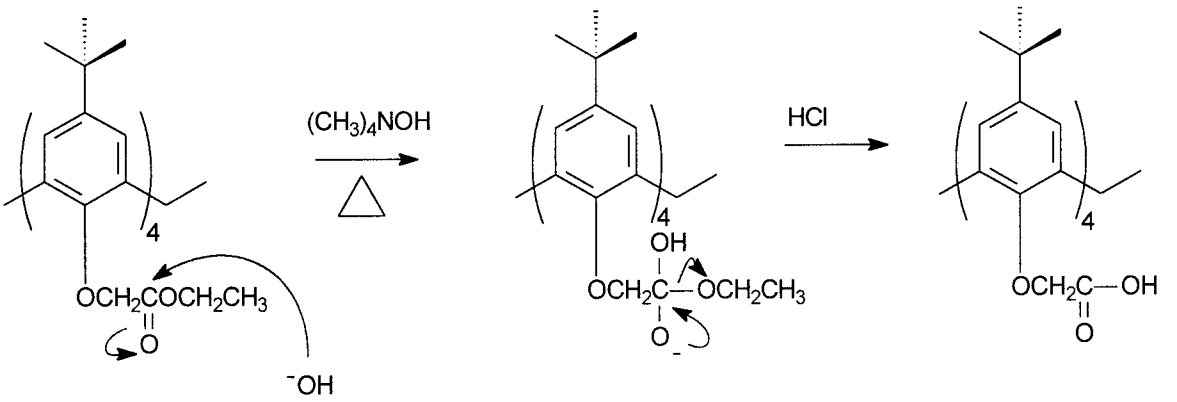


Figure 10: *Mechanism of formation of 4-tert-butylcalix[4]arene O,O',O'',O'''tetracarboxylate*

Figure 10 shows the mechanism of formation of this product, and again, the procedure described in the literature worked well, however the product failed to precipitate upon addition of HCl. To cause precipitation the entire contents were rotary evaporated until the organic solvent was sufficiently removed to allow precipitation of the product. Analysis by TLC was impossible because a suitable solvent could not be found to elute the product up the plate. This was expected due to the presence of four carboxylate groups. The ^{1}H NMR (Figure 11), taken in d_6-DMSO, matched well with the expected spectrum of this compound. Yield was 100%.

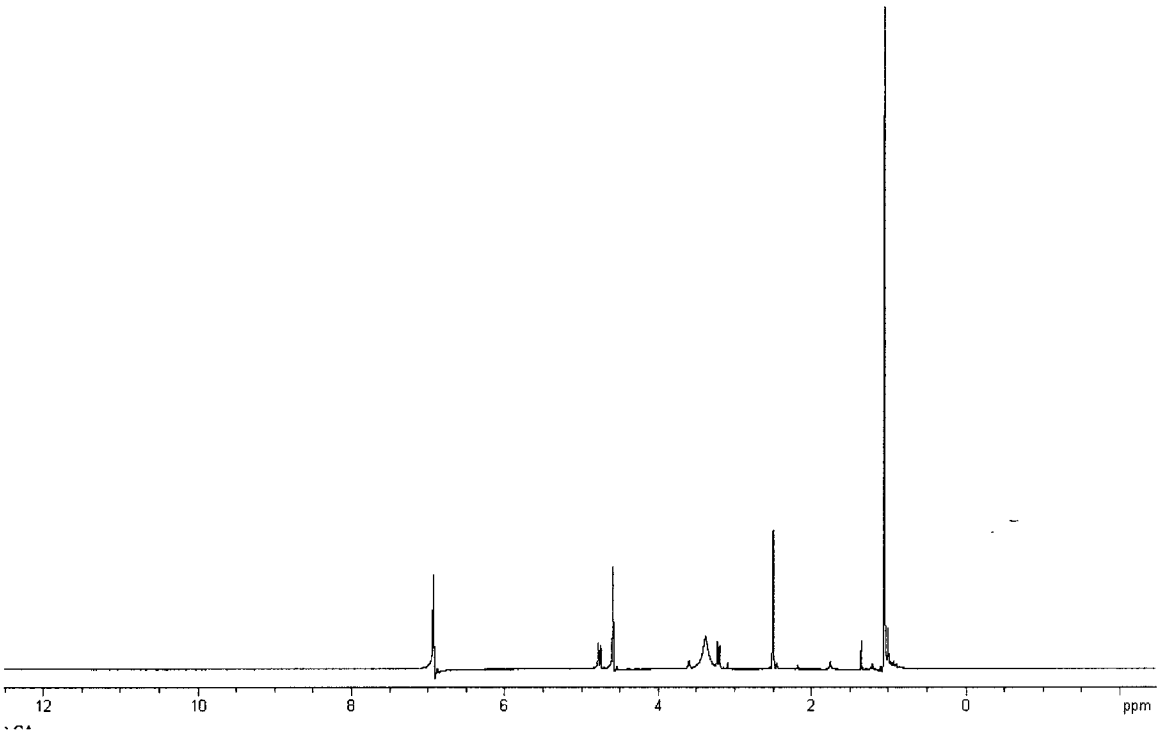


Figure 11: *^{1}H NMR of 4-tert-butylcalix[4]arene O,O',O'',O'''tetracarboxylate*

Synthesis of 4-*tert*-butylcalix[4]arene *O,O',O'',O'''*tetra-L-serine derivative

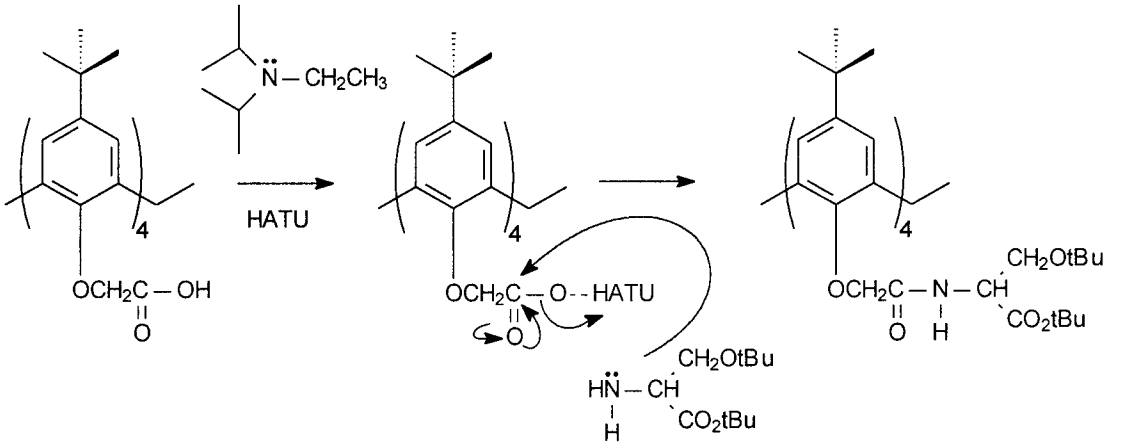


Figure 12: *Mechanism of formation of 4-tert-butylcalix[4]arene O,O',O'',O'''tetra-L-serine derivative*

Figure 12 shows the mechanism of formation of the addition product. This synthesis went smoothly, and needed no modification from the literature. The product was readily purified by flash chromatography to reveal one spot on a TLC plate using 80/20 hexane/THF as the eluant. The ^{1}H NMR (Figure 13) also revealed that the reaction worked well and that the product was pure. Electrospray MS (Figure 14) reported a m/z of 1679, which was indicative of the $M\text{-}H^{+}$ ion. Yield was 92%.

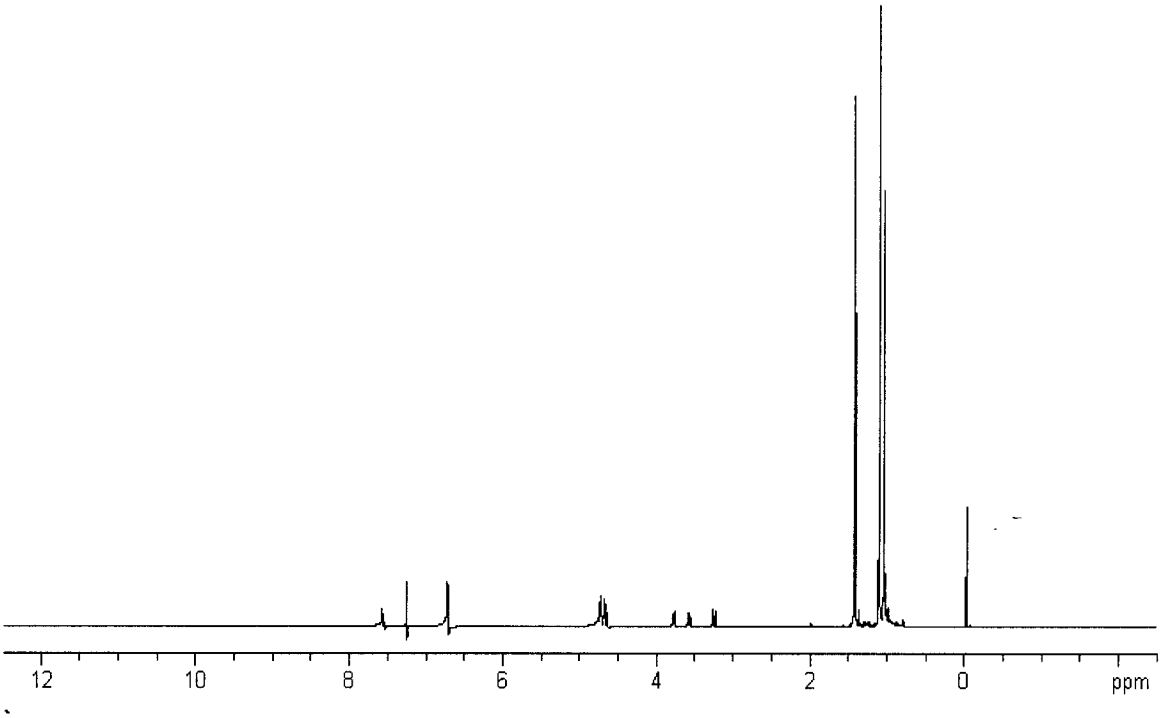


Figure 13: *^{1}H NMR of 4-tert-butylcalix[4]arene O,O',O'',O'''tetra-L-serine derivative*

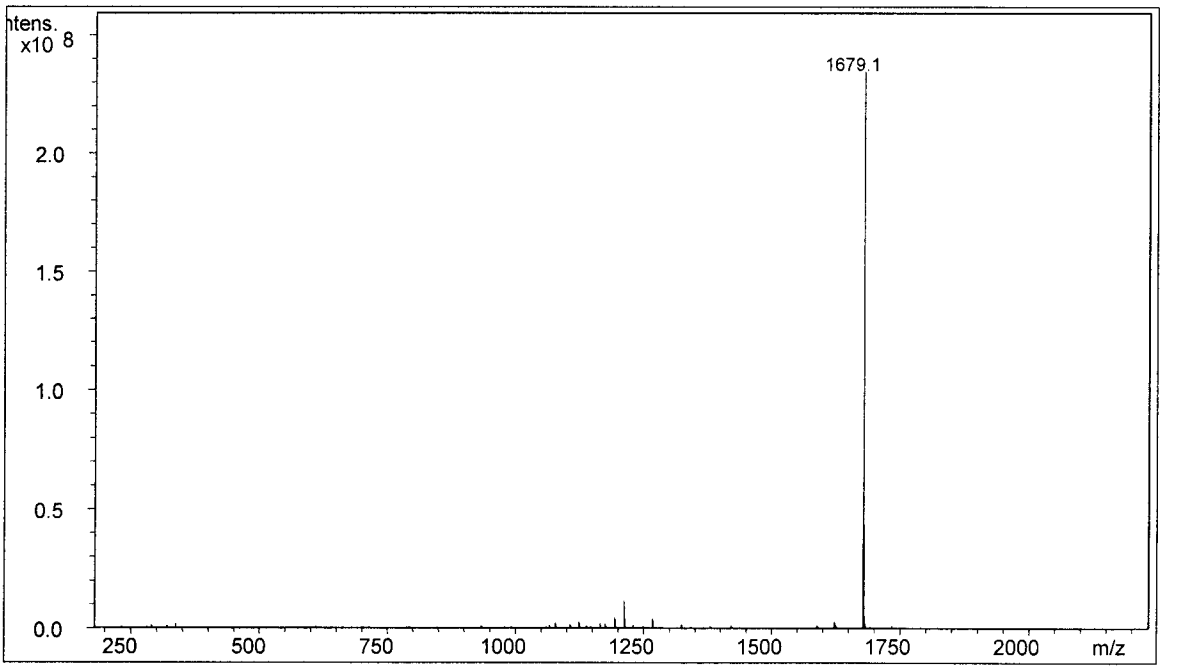


Figure 14: *MS of 4-tert-butylcalix[4]arene O,O',O'',O'''tetra-L-serine derivative*

Synthesis of hydrolyzed 4-*tert*-butylcalix[4]arene *O,O',O'',O'''*tetra-L-serine derivative

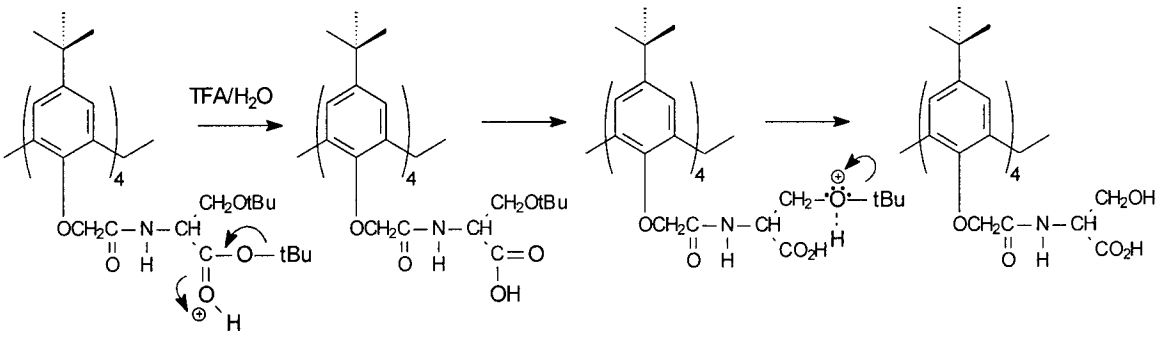


Figure 15: *Mechanism of formation of hydrolyzed 4-tert-butylcalix[4]arene O,O',O'',O'''tetra-L-serine derivative*

Figure 15 depicts the mechanism of formation of the hydrolysis product. This deprotection sequence took some experimentation to determine the correct conditions, but it was eventually determined that per one gram of the protected product, a mixture of 15g TFA and 0.5g water worked well to hydrolyze both the *t*-butyl ester and also the *t*-butyl ether. The role of water in this reaction was not clear. Under usual conditions, a neat TFA hydrolysis should hydrolyze both the ether and ester, isobutylene gas would be given off, and the TFA would be regenerated. However, upon trying this hydrolysis using neat TFA, i.e. no water, the NMR of the product generated was unable to be interpreted. The hydrolyzed product could not be purified by flash chromatography, and analysis for purity by TLC was impossible. This was expected due to the presence of eight hydroxyl groups per molecule. The ^{1}H NMR (Figure 16) correlates to the expected structure, but the electrospray MS (Figure 17) revealed the presence of impurities. A m/z of 1212 was the most intense peak in the mass spectrum,

which corresponded to the loss of a hydroxyl (17amu) functionality from the product, which is calculated to have a mass of 1229amu. Yield was 89%.

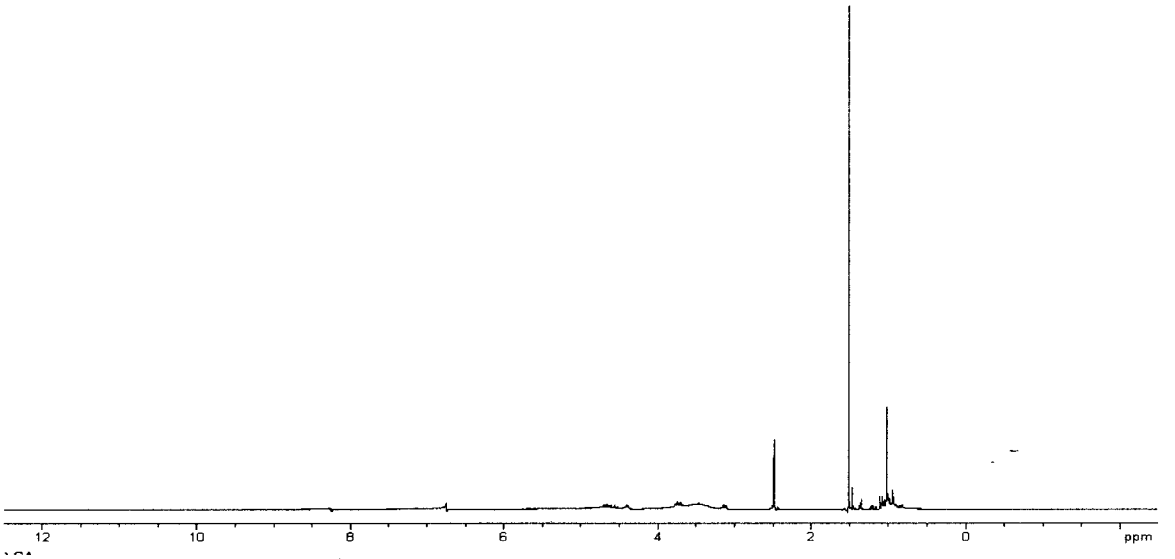


Figure 16: *^{1}H NMR of hydrolyzed 4-tert-butylcalix[4]arene O,O',O'',O'''tetra-L-serine derivative*

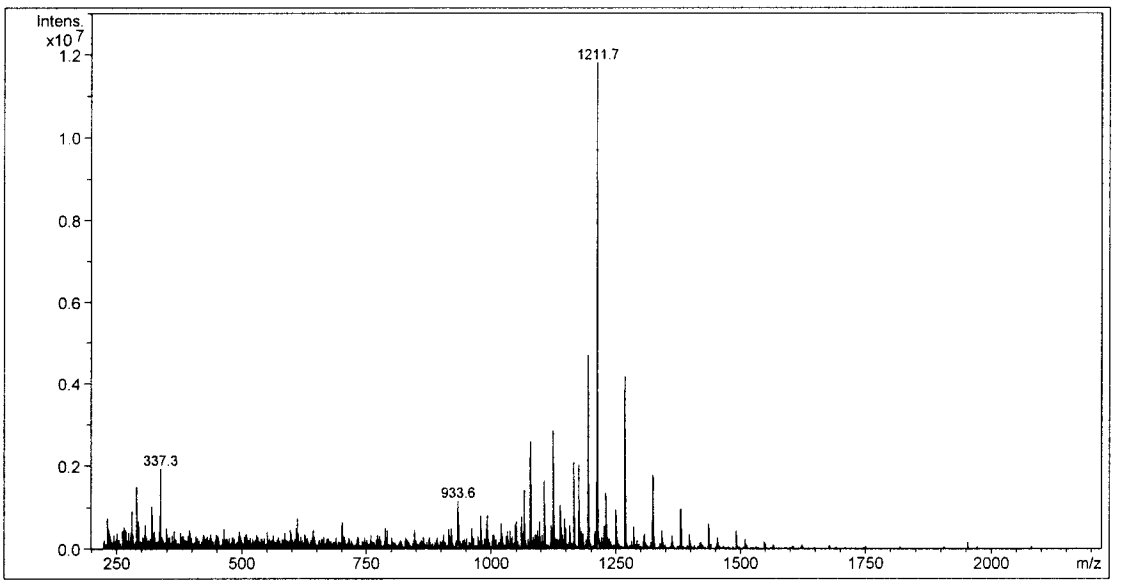


Figure 17: *MS of hydrolyzed 4-tert-butylcalix[4]arene O,O',O'',O'''tetra-L-serine derivative*

The UV and Fluorescence Spectra of the hydrolyzed 4-*tert*-butylcalix[4]arene *O,O',O'',O'''*tetra-L-serine derivative

The UV spectrum for the product calixarene is shown below. The absorption maximum was at 235nm, and a second strong band was observed at 275nm. A scan from 180nm to 500nm was performed. The solvent was methanol. A methanol blank was scanned and subtracted from the sample spectrum to give the resulting spectrum shown below.

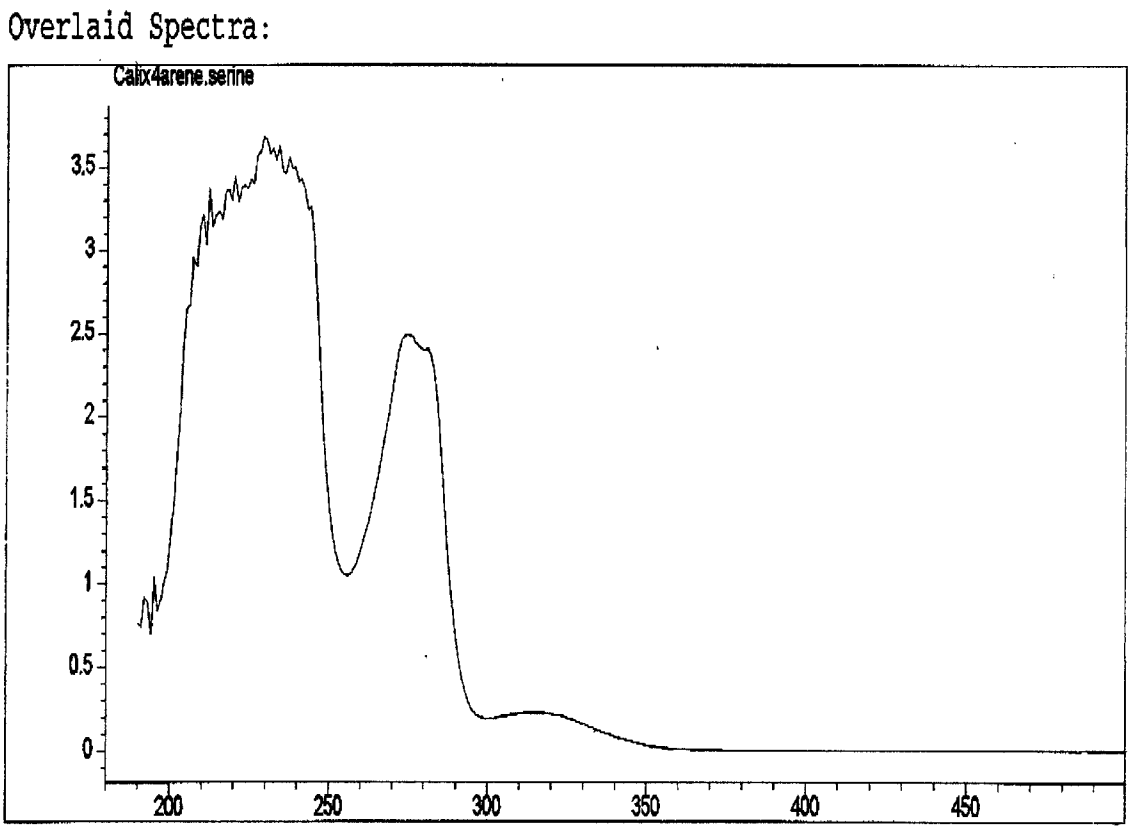


Figure 18: *The UV absorbance spectra of the hydrolyzed 4-tert-butylcalix[4]arene O,O',O'',O'''tetra-L-serine derivative*

The fluorescence spectrum, excitation at 292nm, shows emission at 305.6nm and also a second band at approximately 610nm. The second band was thought to be an overtone of the first. This is a typical spectrum for the calixarenes, and the fact that fluorescence was observed was a good indication that the calixarene product was in the desired cup conformation. That the calixarenes fluoresce is thought to be due to the delocalization of the π electrons throughout the structure. If the cup conformation was not adopted by the calixarene, then this delocalization would not be possible, and no fluorescence would have been observed.

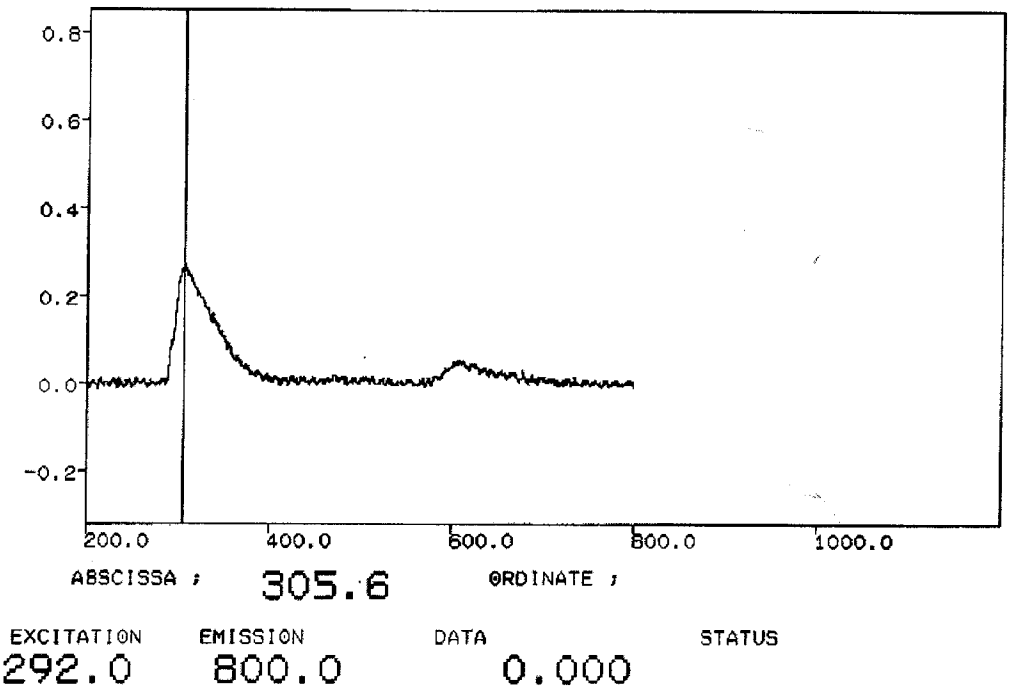


Figure 19: *The fluorescence spectrum of* the *hydrolyzed 4-tert-butylcalix[4]arene O,O',O'',O'''tetra-L-serine derivative at 292nm*

Introduction to the Chromatography Studies

To study the effects of the calixarene derivative on the retention of solutes, a homologous series of solute probes was used. As explained in the sample preparation section, the series chosen consisted of benzene, naphthalene, anthracene, and tetracene. This series was chosen because of previous research (15), which indicated that nonchiral calixarenes and these compounds form host/guest complexes. Before a chiral separation was attempted using the synthesized calixarene, it was necessary to prove that the calixarene was capable of forming host/guest complexes with nonchiral solutes. Only after this was proven could a reasonable conjecture be made as to what type of chiral molecules might be able to be separated using this methodology. In each case presented, the elution order was benzene, naphthalene, anthracene, and lastly tetracene.

Results from the Stationary Phase Studies

The chromatographic conditions were optimized in such a way as to get a reasonable separation between each member of the homologous series that would allow any recognition of a difference between the cases where the calixarene was involved and those cases where the calixarene was not involved. This was achieved primarily by experimentation with various mobile phase solvents and various mobile phase concentrations. Since the product is insoluble in both acetonitrile and THF, both were used in the stationary phase experiments. However, it was known that acetonitrile readily occupied the cavity

of the calix[4]arene system. For this reason, when acetonitrile was used in the mobile phase, there was no observed difference in the retention of the members of the homologous series when the calixarene was immobilized on the stationary phase and when it was not immobilized on the stationary phase (Figure 20). Since THF did not occupy the cavity of the calix[4]arene system, it was chosen as an appropriate mobile phase solvent for the stationary phase experiments. Various concentrations of THF and water were experimented with, however a 50/50 mix of both solvents gave good results, as shown below (Figure 21).

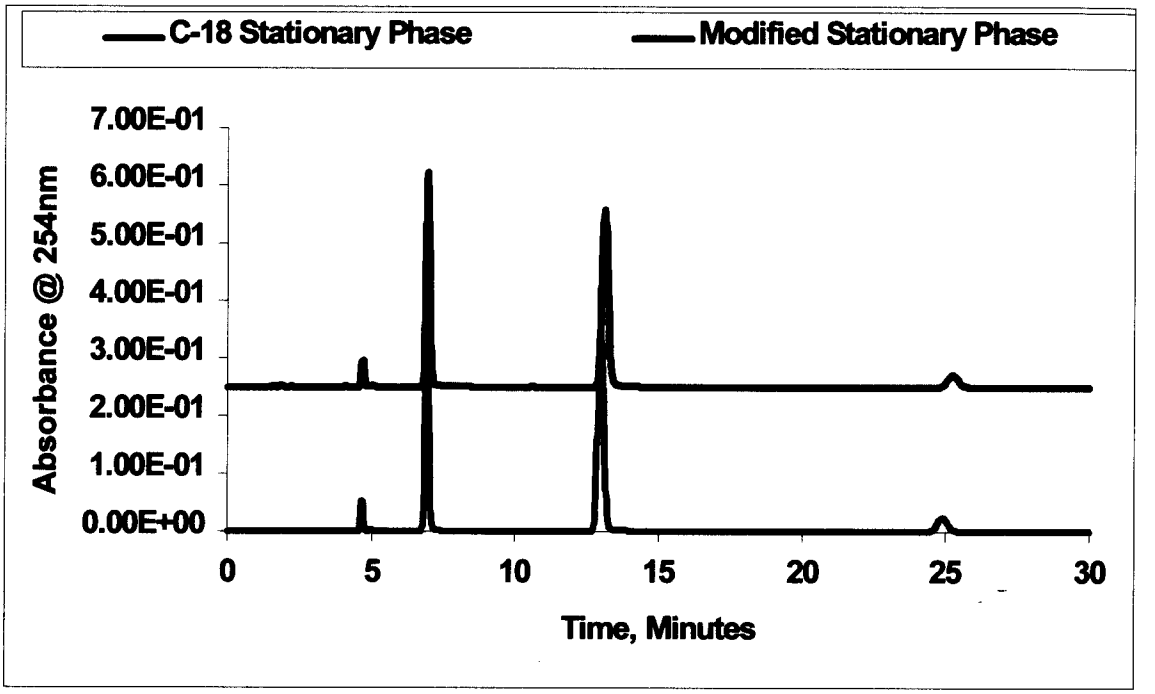


Figure 20: *ACN based mobile phase*

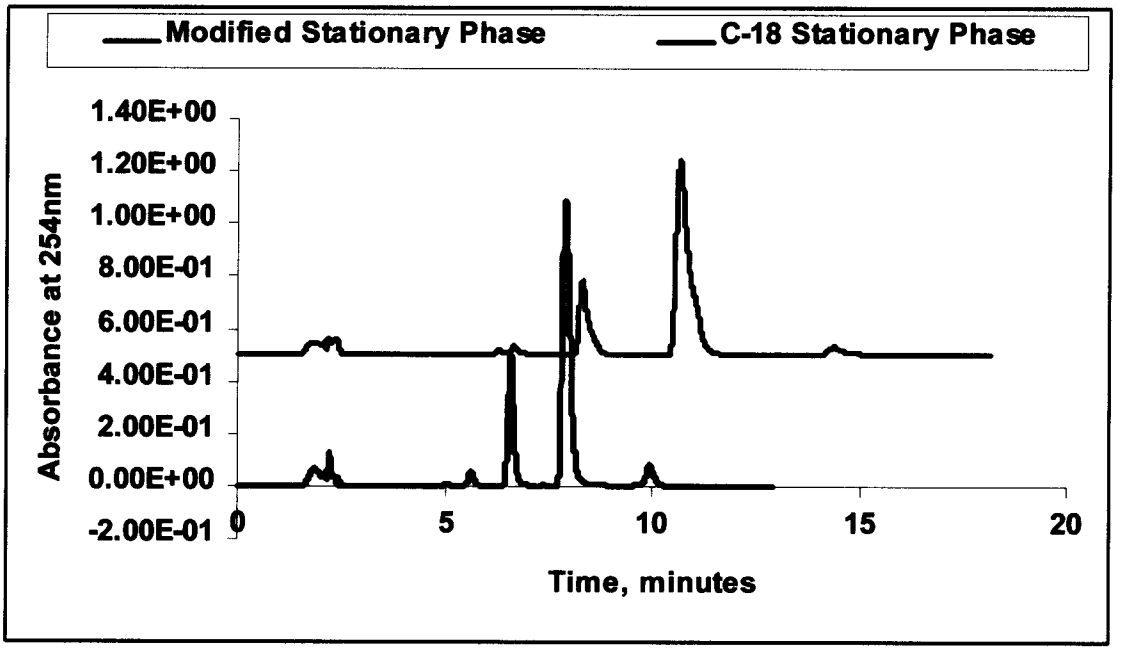


Figure 21: *THF based mobile phase*

Results from the Mobile Phase Studies

Again, during the mobile phase experiments, a reasonable separation between the members of the homologous series was sought, so as to observe any differences in retention characteristics. For the mobile phase studies, the limiting factor was the amount of water that could be used in the mobile phase before the calixarene precipitated. At water concentrations above 80/20 methanol/water, the calixarene precipitated. For this reason, a mobile phase concentration of not less than 80% methanol was chosen, to ensure that the calixarene would not precipitate during the trials. Another aspect of the mobile phase trials was the use of fluorescence detection of the homologous series. Shown below (Figure 22) are the results from the mobile phase experiments.

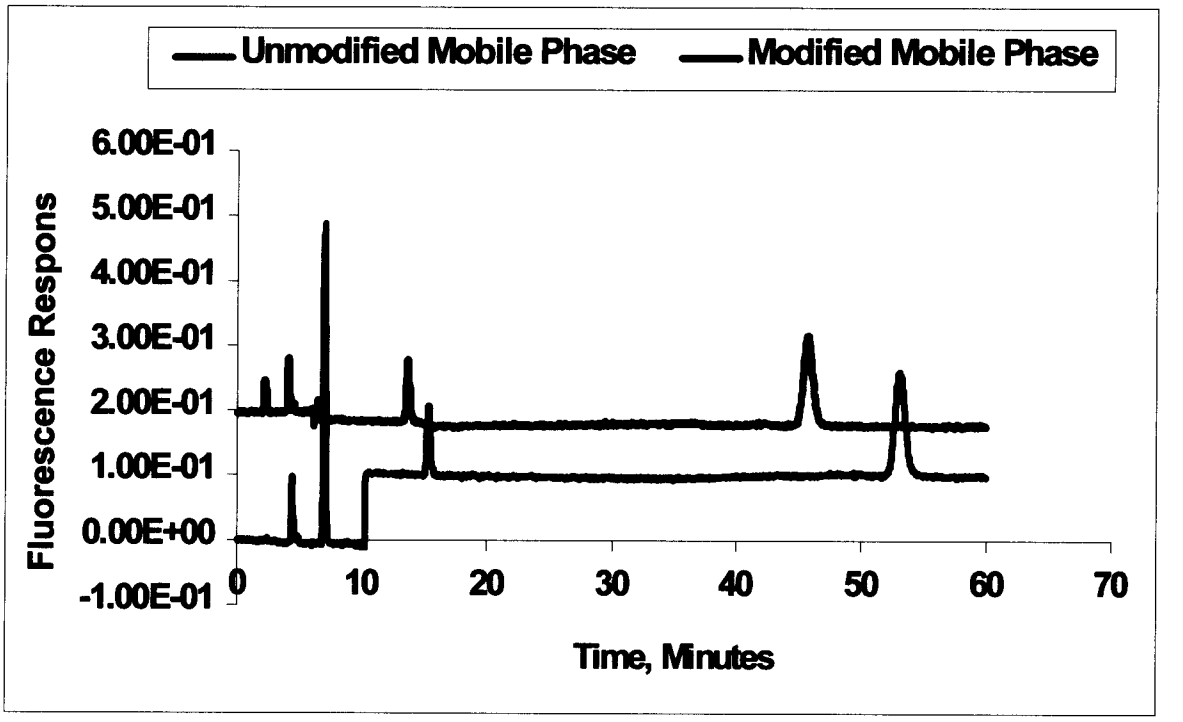


Figure 22: *The mobile phase results*

Shown on the next page (Table 2) is a chart that summarizes the results from the chromatography experiments. Each experiment was performed in triplicate to ensure repeatability. Corrected retention times were calculated by the formula:

$$t_r' = t_r - t_m$$

where t_r is the observed retention time of each solute and t_m is the dead time. Capacity factors, or k' values, were calculated according to the formula:

$$k' = t_r'/t_m$$

Asymmetry factors were calculated according to the formula:

$$A_s = b/a$$

where *b* is the width from the center of the peak to the end of the peak at 10% the peak height and *a* is the width from the center of the peak to the beginning of the peak at 10% the peak height.

This analysis was done to compare retention times for each solute when the calixarene was involved in the separation and when it was not. Changes in retention time, and therefore capacity factor, can indicate the presence of host/guest interactions. Peak shape can also indicate host/guest interactions and therefore asymmetry factors were calculated as well. An asymmetry factor of 1 indicates a perfectly symmetrical peak. Non-symmetrical peaks arise from a variety of factors, one of which is host/guest interactions. When host/guest complexes form, the kinetics of the guest molecule entering the host can cause non-symmetrical peaks, therefore observation of severely non-symmetrical peaks is a good indication of host/guest complex formation.

Table 2: *Summary of Chromatographic Results*

Stationary Phase:

Without Calix Adsorbed

Analyte	t_r ave. (n=3)	k'	A_s
Benzene	5.673	1.837	1.5
Naphthalene	6.634	2.317	1.25
Anthracene	7.990	2.995	1
Tetracene	10.07	4.036	1.25

With Calix Adsorbed

Analyte	t_r ave. (n=3)	k'	A_s
Benzene	6.639	2.320	1.5
Naphthalene	8.289	3.144	2
Anthracene	10.61	4.306	1.7
Tetracene	14.30	6.153	2.2

Mobile Phase:

No Calix Additive

Analyte	t_r ave. (n=3)	k'	A_s
Benzene	4.262	1.131	1
Naphthalene	6.923	2.462	1
Anthracene	15.28	6.644	1
Tetracene	53.05	25.53	1

0.001M Calix Additive

Analyte	t_r ave. (n=3)	k'	A_s
Benzene	2.203	0.102	1
Naphthalene	4.106	1.053	1
Anthracene	13.72	5.86	1
Tetracene	45.67	21.83	1.5

HPLC Analysis for Purity of the Calixarene

Shown below are two chromatograms of the hydrolyzed 4-*tert*-butylcalix[4]arene-*O,O',O'',O'''*tetra-L-serine derivative. Two wavelengths were used in the determination of the product's purity. Response at 230nm (Figure 23) was significantly better than at 254nm (Figure 24). Both chromatograms clearly show the presence of two compounds. This has not been significantly investigated, but it is believed to be due to the presence of two conformations of the product. If this were the case, the very polar region of the calixarene (the amino acid portion) would not be *as* polar if a second conformation existed, where one or more of the amino acid functionality's was away from the others. This would change the overall polarity *and* the location of the polar sites on the molecule, which is why two (or more) peaks could be expected to appear.

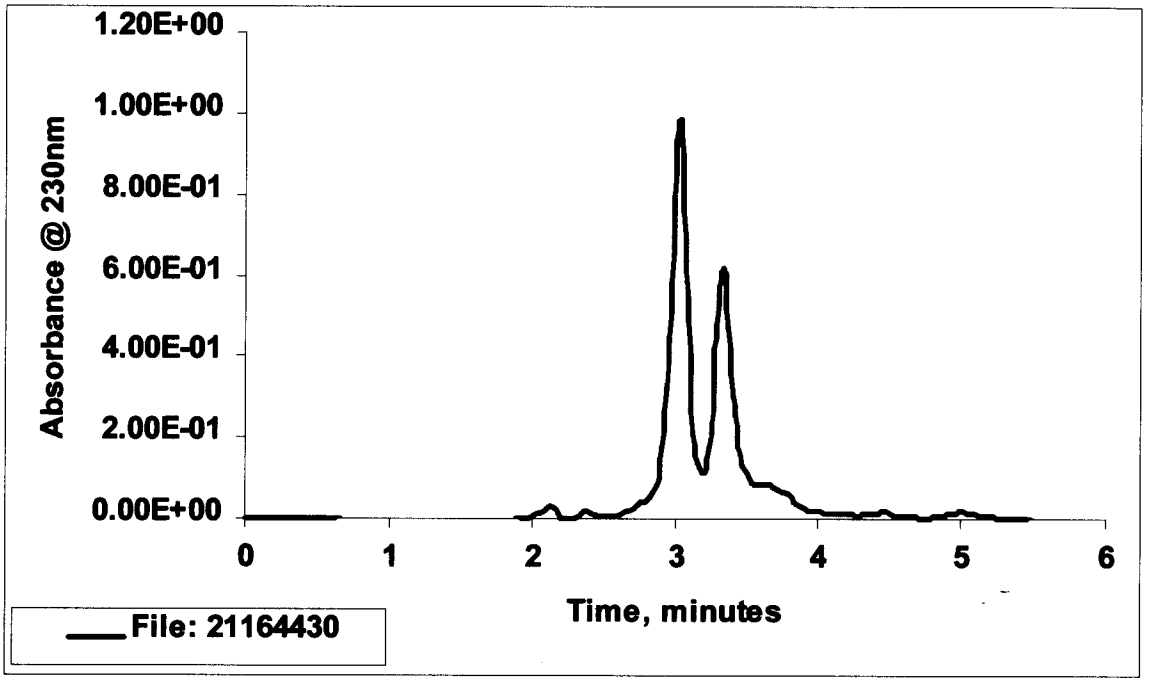


Figure 23: *HPLC analysis for purity of the hydrolyzed 4-tert-butylcalix[4]arene O,O',O",O"'tetra-L-serine derivative at 230nm*

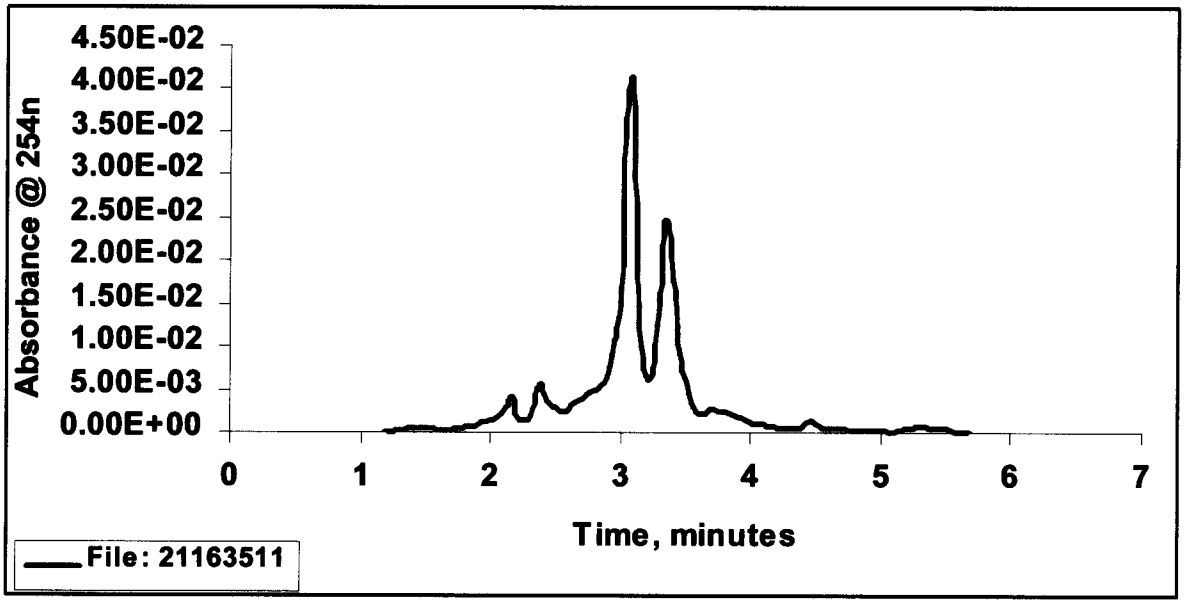


Figure 24: *HPLC analysis for purity of the hydrolyzed 4-tert-butylcalix[4]arene O,O',O",O"'tetra-L-serine derivative at 254nm*

Analysis of Column Polarity

The polarity of the stationary phase was analyzed both when the calixarene was immobilized on the column and when it was not. This was done to detect any polarity change. Figure 25 shows the results from no calixarene presence on the column, and Figure 26 shows the results when the calixarene was immobilized on the column. The elution order in each case is uracil, phenol, *N,N*-diethyltoluamide, and toluene. No difference in retention was observed when the calixarene was immobilized on the column (Figure 25) and when it was not (Figure 26). This is expected since the calixarene has both polar and non-polar portions to the structure, therefore no overall change in the stationary phase's polarity was expected or observed.

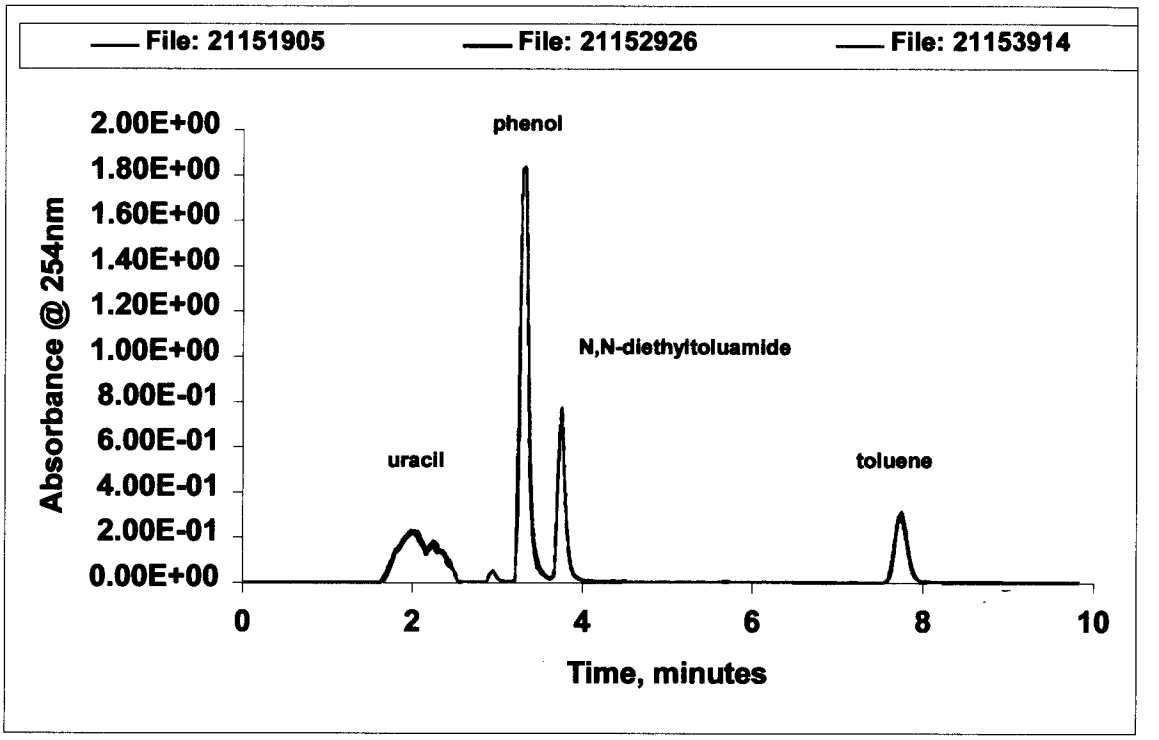


Figure 25: *HPLC analysis of column polarity-unmodified stationary phase*

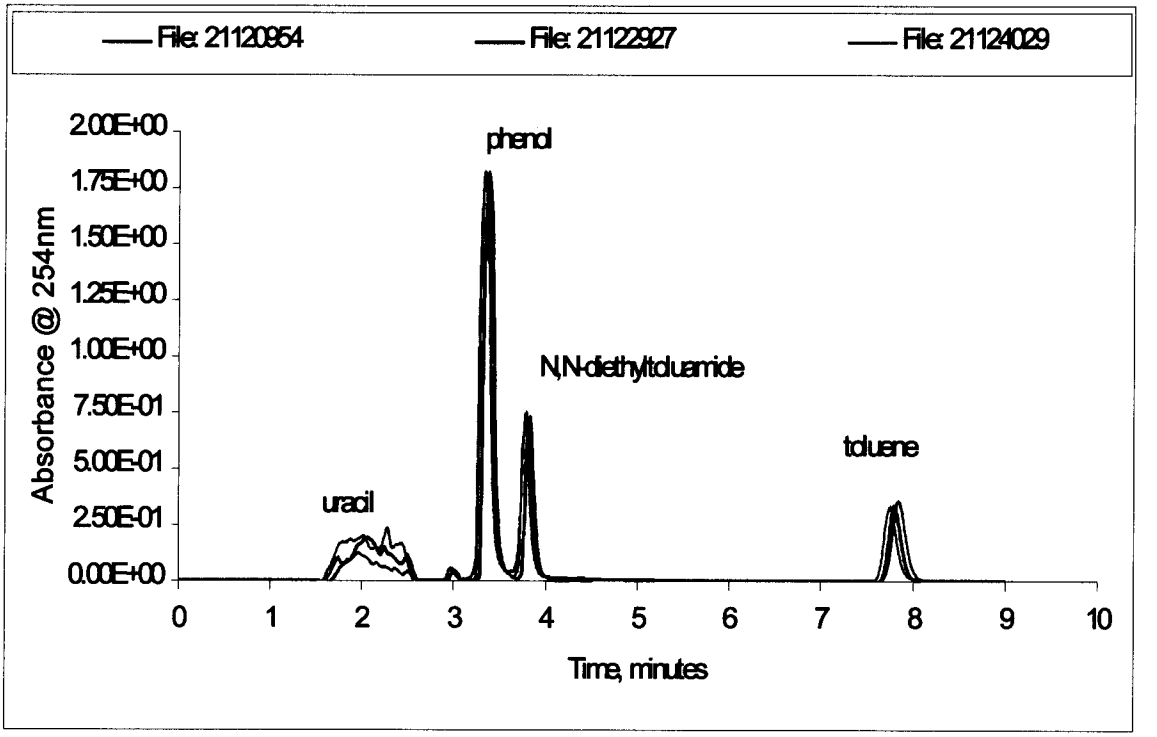


Figure 26: *HPLC analysis of column polarity-modified stationary phase*

Results from the Chiral Separation Experiments

Figures 27, 28, and 29 show the results of the attempted chiral separations. Methylmandelate, aspartic acid, and tryptophan were chosen because they contain aromatic functionality, which was believed to possibly allow them to form a host/guest complex with the calixarene, and the chiral portion of each molecule could then possibly interact with the amino-acid (chiral) portion of the calixarene. D-methylmandelate was unresolved from the L-methylmandelate (Figure 27), D,L-tryptophan (Figure 28) shows the beginning of a possible resolution into two peaks, and the chromatogram of D,L-aspartic acid (Figure 29) shows that the aspartic acid was unretained.

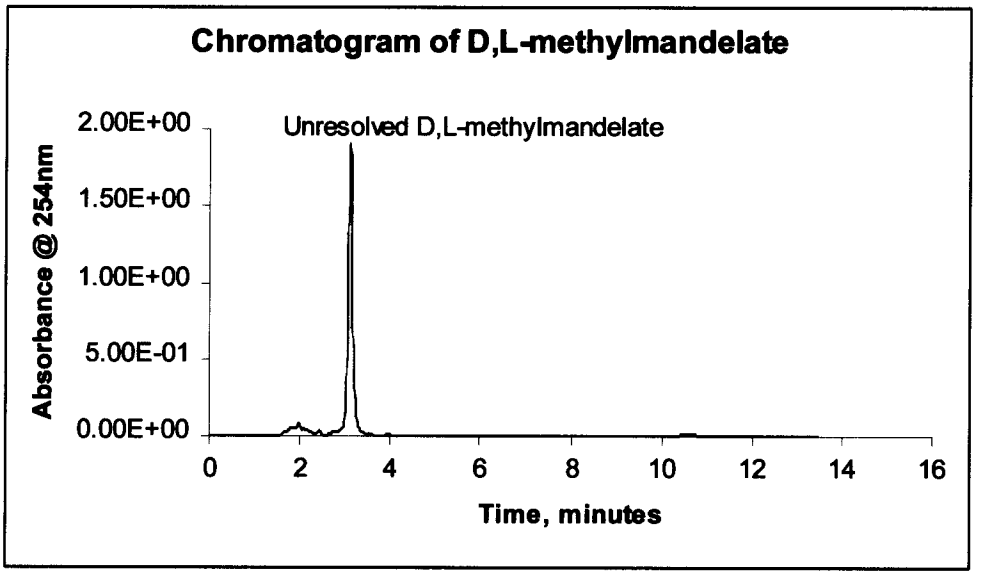


Figure 27: *Unsuccessful separation of D,L-methylmandelate*

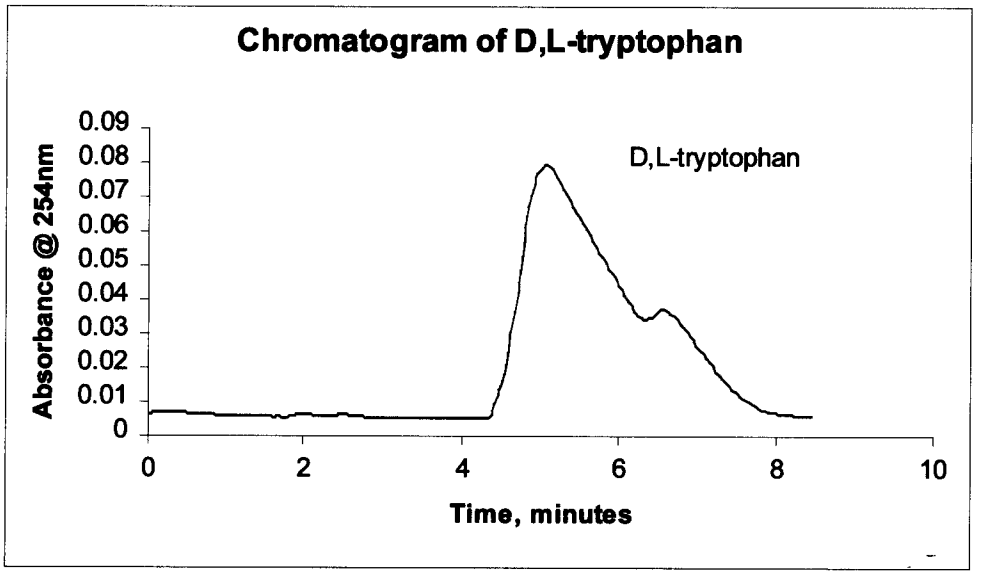


Figure 28: *Possible partial resolution of D- from L- tryptophan*

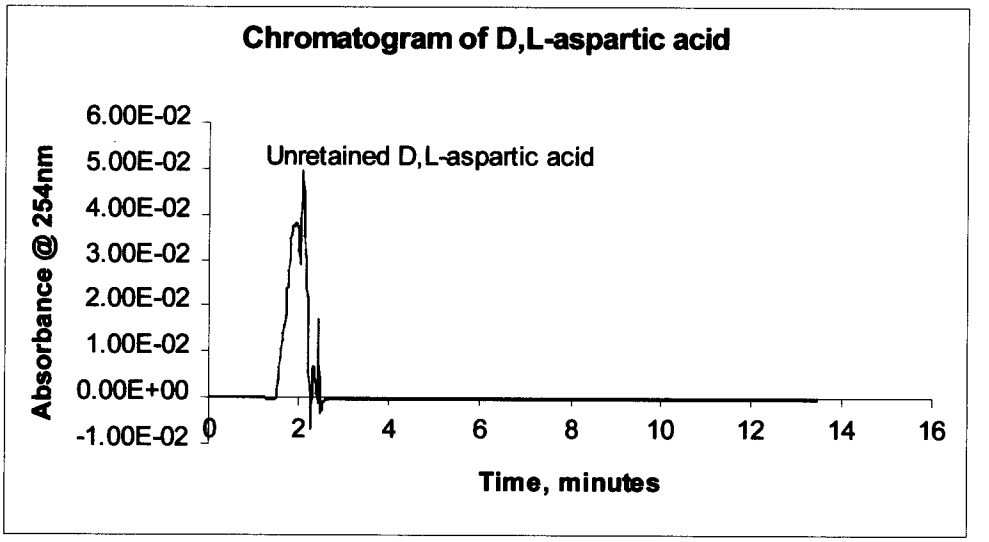


Figure 29: *Possible unsuccessful resolution of D- from L-aspartic acid*

CHAPTER 7

Conclusions

Although some modification was necessary, the synthetic methods described in the literature worked well, and allowed for successful preparation of a novel chiral calixarene.

Immobilization of the calixarene onto the stationary phase resulted in an increase in retention time and capacity factor for each of the solutes, as compared to the non-modified stationary phase. Each solute also tailed significantly when the calixarene was immobilized. These factors strongly suggested host/guest complex formation in the stationary phase. The secondary interaction of the solutes forming a host/guest complex with the calixarene was believed to be responsible for the tailing of each solute.

Addition of the calixarene to the mobile phase resulted in a reduction in retention time and capacity factor for each solute, as compared to the non-modified mobile phase. This strongly suggested host/guest formation in the mobile phase, and that the complex preferred the mobile phase.

Because it was demonstrated that the calixarene had the ability to interact with solute molecules both in the stationary and mobile phases, the calixarene is currently being investigated as a chiral mobile phase additive (CMPA) and as a stationary phase adsorbent to achieve chiral separations using HPLC. The results thus far have yielded some success (Figures 27, 28, and 29), and the groundwork has been laid for future work, for example, appropriate

immobilization conditions, mobile phase concentrations, and synthetic methods have been optimized so that this work may proceed further. D,L-methylmandelate and D,L-aspartic acid may be able to be resolved, but the conditions of these separations need to be optimized to allow longer retention times for each of these compounds.

Future Work

The obvious work that is necessary is to determine if chiral separations are possible using the methodology described above. Further, alternative mobile phases to THF will most likely have to be found, if the stationary phase method proves successful. This arises from the fact that THF is not a commonly used solvent in HPLC. It can degrade the column if left in the column, and is difficult to degas.

Some reverse phase preparative work needs to be done on the calixarene product to determine what structures the two peaks correspond to. A temperature gradient may be useful to better resolve the two peaks if they are different conformations of the same molecule. Reverse phase is necessary because the product calixarene will not move on silica gel. A better understanding of the purity and conformation of the product should be obtained before this work proceeds further. If this is not done, the hydrolysis method used here (TFA/water) should not be used as it may be damaging to the product, and may not fully hydrolyze the ether. A way to avoid this would be to use a mono-

protected amino acid, e.g. one *without* an alcohol protected as an ether, so that only an ester needs hydrolyzed.

Another area that needs work is to determine how much calixarene is present on the stationary phase. The simplest way to do this would be to immobilize the calixarene on the column, then elute it off using methanol, collect the methanol (after the detector) in a preweighed flask, and evaporate the methanol off, and reweigh the flask. Another mode of analysis, albeit destructive to the column, would be to take some of the packing material out of the column after the calixarene has been immobilized, and take fluorescence measurements on the material.

One further test that needs performed is to retest the stationary phase method of immobilization using THF. The chromatograms from these studies look promising, but unfortunately, they also may indicate that the column has a void or a blocked frit.

One last area of potential work is the synthesis of a carbohydrate/calixarene compound. Shown below is a scheme that was attempted earlier in this work using calix[6]arene. Due to complications in that synthesis arising from calix[6]arene, the following synthesis (16) is directed toward reaction of a protected and activated carbohydrate with p-*tert*-butylcalix[4]arene.

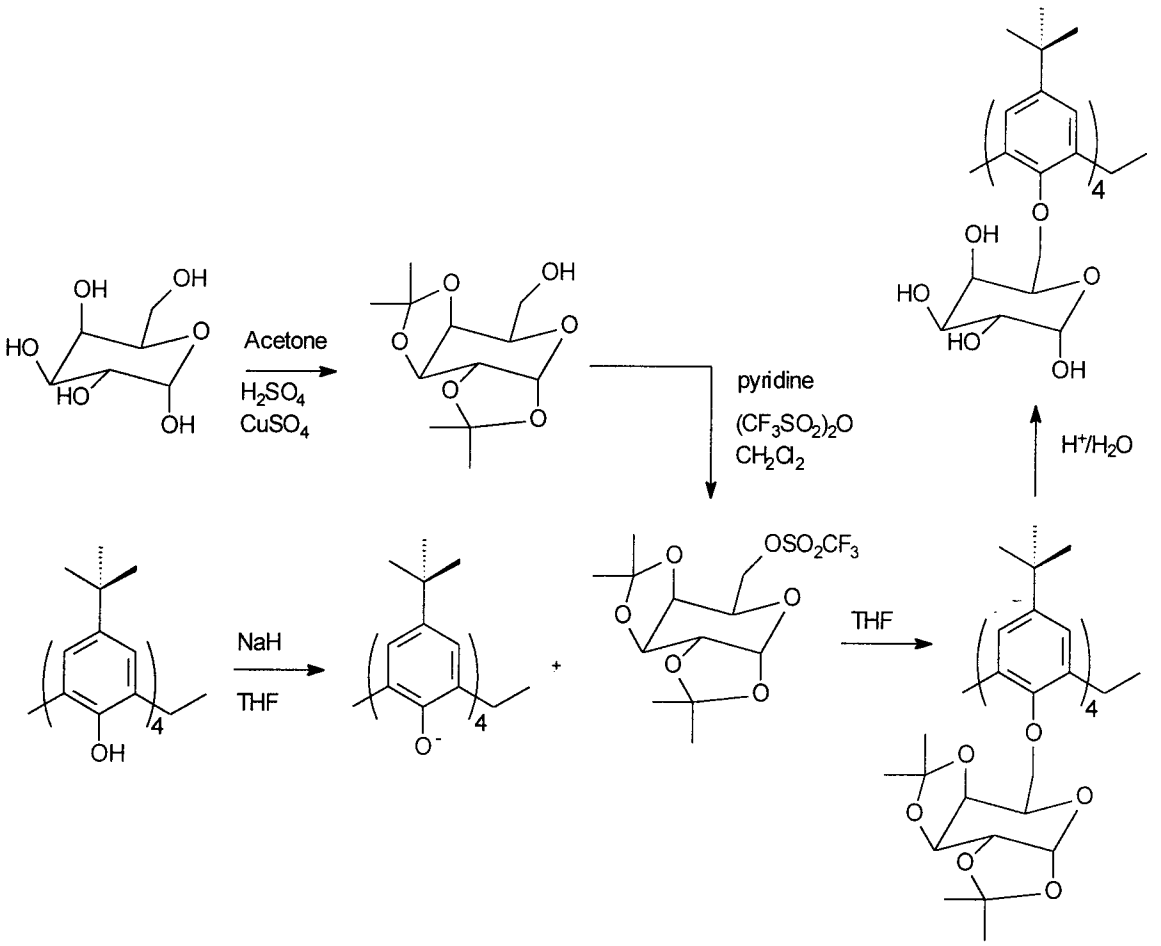


Figure 30: *Scheme to synthesize a carbohydrate/calixarene compound*

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