SYNTHESIS AND CHARACTERIZATION OF AMINO-DERIVED T-BUTYL-CALIX[4]ARENE BONDED PHASES FOR HPLC

by

Erica E. Eliser

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Synthesis and Characterization of Amino-derived t-butyl-calix[4]arene Bonded Phases for HPLC

Erica E. Eliser

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Erica E. Eliser Date

Approvals:

5/1/01

James H. Mike, Ph.D., Thesis Advisor Date

5/1/01

Steven M. Schildcrout, Ph.D., Committee Member Date

5/1/01

Larry S. Curtin, Ph.D., Committee Member Date

5/2/01

Peter J. Kasvinsky, Ph.D., Dean of Graduate Studies Date

ABSTRACT

The cone conformation of a calixarene has the perfect shape to receive molecules for host-guest complexation, which makes these molecules desirable in chromatography. Host/guest complexation is the ability of a host molecule (calixarene) to interact with a guest molecule (PAH's) or a guest ion (Na^+). The objective of this research was the development of a reliable method for covalent attachment, with high surface coverage, of p-tert-butylcalix[4]arenes to the surface of silica particles to utilize their host/guest abilities. A new synthetic method has been developed based on imine formation between an amino-derived calixarene and amino-derived silica with a glutaraldehyde linker. Specific types of solute probes (homologous series of PAH's, alkyl benzenes, and aromatic amines) were used to test the effectiveness of the calixarene stationary phases to separate in the reversed-phased mode of chromatography. Chromatograms were compared before and after endcapping the stationary phase, and it was concluded that the tailing of the peaks and the non-linearity of the log k' vs. number of atoms in a homologue was indicative of host/guest complexation due to the tethered calixarenes.

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LIST OF SYMBOLS

Symbol	Definition	Units of Reference
mol	mole	6.022×10^{23} molecules
m o l	micromole	1×10^{-6} mole
m	meter	--
nm	nanometer	1×10^{-9} meter
m	micrometer	1×10^{-6} meter
mm	millimeter	1×10^{-3} meter
Å	Angstrom	1×10^{-10} meter
g	gram	--
mg	milligram	1×10^{-3} gram
L	liter	--
L	microliter	1×10^{-6} liter
mL	milliliter	1×10^{-3} liter
min	minute	60 seconds
s	second	--
s	microsecond	1×10^{-6} second
nA	nanoampere	1×10^{-9} ampere
°C	degrees Celsius	--
%	percent	--
Hz	hertz	s^{-1}
MHz	megahertz	1×10^{6} hertz
V	volts	--

LIST OF SYMBOLS

Symbol	Definition	Units of Reference
psi	pounds per square inch	--
ppm	parts per million	--
m/z	mass to charge ratio	--
h	height equivalent to a theoretical plate	--
u	linear flow rate	cm/min
K	thermodynamic equilibrium constant	--
Φ	volume ratio of the stationary phase and mobile phase	--
R	universal gas constant	8.314 J/ mole K
G°	standard Gibbs free energy change	--
$\overline{\Delta G}$	Gibbs free energy contribution of a homologue	--
S	entropy change	--
H	enthalpy change	--
k'	capacity factor	--

LIST OF ABBREVIATIONS

Symbol	Definition	Units of Reference
PAH	polyaromatic hydrocarbon	--
HPLC	high performance liquid chromatography	--
RPC	reversed phase chromatography	--
IR	infrared spectroscopy	--
FTIR	fourier transform infrared spectroscopy	--
NMR	nuclear magnetic resonance	--
CP/MAS NMR	cross polarization magic angle spinning NMR	--
TLC	thin layer chromatography	--
LC-MS	liquid chromatography-Mass Spectrometry	--
TMCS	trimethylchlorosilane	--
TMS	tetramethylsilane	--
APCI	atmospheric pressure chemical ionization	--
APT	attached proton test	--

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Chapter I

Introduction

Calixarenes

Johann Frederich Wilhelm Adolph von Baeyer was known as one of the greatest organic chemists of the nineteenth century. Baeyer had published a series of papers on the reactions of phenols with formaldehyde.[1] The resulting tar-like substances that he created would not dissolve in any solvents, which led to difficult purification problems. These compounds therefore went uncharacterized for many years. Phenol-formaldehyde chemistry would pique interest again years later, and determine what Baeyer was trying to discover in the late 1800's. Baeyer did not know it then, but he had opened the doors to the making of calixarenes.

Calixarenes are synthesized through a condensation reaction between p-alkyl phenols and formaldehyde under basic conditions. The exact mechanism is still not understood, but possibilities have been proposed by Gutsche.[1] The reaction can create ring sizes that range from four phenyl groups to fourteen. The number of phenyl rings is indicated by a bracketed number in its name. Also included are additional functional groups and their position on the ring. Figure 1.1 is a calixarene that contains four phenyl groups with tertiary butyl groups in the *para* position.

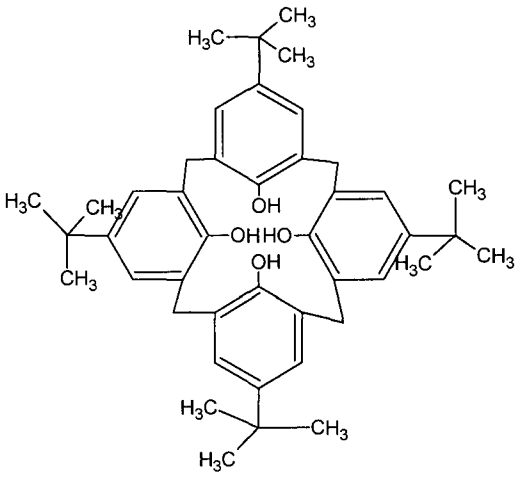


Figure 1.1: p-*tert*-butylcalix[4]arene

Synthetically, calixarenes with an even number of phenyl groups are more favored than those with an odd number of groups. The even numbered calixarenes form a more upright, stable cup formation while odd numbered calixarenes form a more planar structure. Under most reaction conditions, the calix[4]arene has been determined to be the thermodynamically favored product.[2] Calixarenes can change orientations of the phenyl rings through the free rotation of the bonds of the methylene bridges. These orientations are known as conformers. There are four possible conformers of a calix[4]arene in the solid state; the cone, partial cone, 1,3 alternate and 1,2 alternate (Figure 1.2).

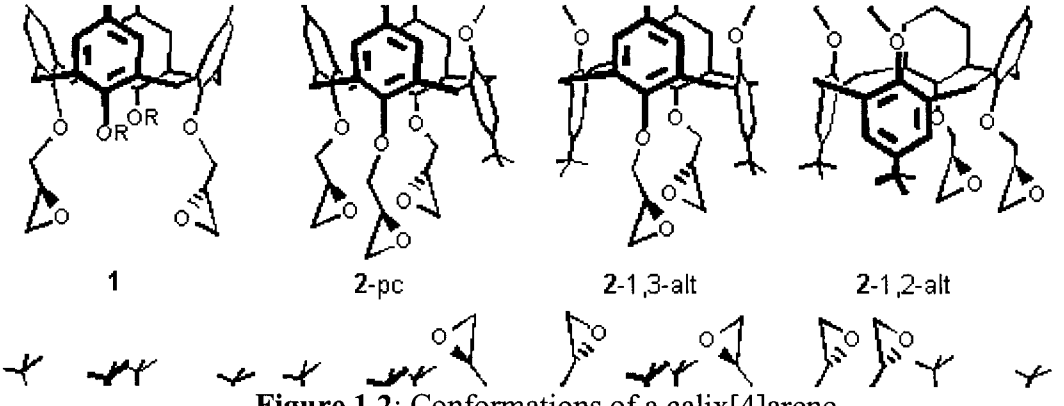


Figure 1.2: Conformations of a calix[4]arene

In solution, the calix[4]arene exists in the cone conformation due to the stability of the intramolecular hydrogen bonding between the hydroxyl groups. When the hydroxyls are derivitized, the cone conformation is still present and is the most thermodynamically favored conformation.

The cone conformation has the ideal shape to receive molecules in its cavity for host-guest complexation, which makes these molecules desirable for chromatographic separations.

Synthesis of a Stationary Phase

Chromatographic stationary phases for HPLC are generally synthesized on a base material composed of spherical, ultrapure particles of silica. The particles have very high surface areas and range in size from about 3 μm to about 10 μm and vary in their porosity from about 80Å to more than 1000Å. The phases are generally made by reaction of the silanol groups on the silica surface with various functional groups. Functional groups that can be attached include hydrocarbons, amines such as propylamine, nitriles such as propyl or benzyl nitrile, alcohols, and phenyl compounds. Each of these will give the resulting stationary phase a unique character for separation of various mixtures. One may also attach "tethers", as in this work, to the silica surface.

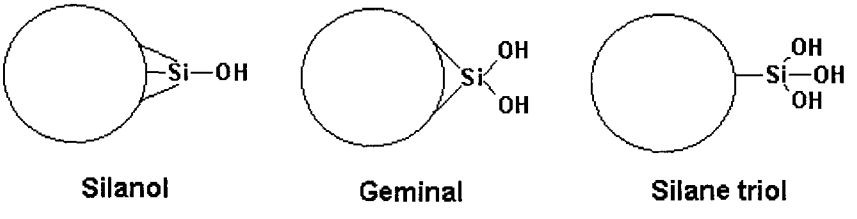


Figure 1.3: Orientation of hydroxyls on a silica surface

Silica is composed of a network of silicon (Si) and oxygen (O) atoms, where the oxygens at the surface can form hydroxyl (-OH) groups. There are several ways that a

hydroxyl can be oriented on the silica surface.[3] (Figure 1.3) Silanols can have one, two or three hydroxyl group attached to the Si atom on the surface. Mono-silanols are desired for ideal attachments because the position of the -OH group is ideal for maximizing the surface concentration of a tether.

Geminal silanol groups have two -OH groups free on one Si atom, while a silane triol has three -OH groups stemming from one Si atom. These extra groups can pose a problem in attachment and surface coverage of a derivatizing agent. Heating the silica at high temperatures can maximize the amount of mono-silanols available for attachment by eliminating water off the surface and slowly letting the water condense back on the surface to form the –OH groups. Using a tether that has only one reactive end is another way to achieve a high surface coverage. This limits the tether to reacting with only the hydroxyl groups on the surface and not to other tethers in solution.

Tethers can be attached in either of two ways, a direct bond to a Si atom or bonded to the Si atom using a bridge atom. The three most common bridging groups are an O (ester/ether), an N (amine), or an organosilane.[3] An ester/ether can be made by reacting an alcohol with the hydroxyl group. An amine can be introduced by first chlorinating the silica surface, and then introducing an amine to attach through a replacement reaction with the chlorine. An organosilane can be directly introduced to the silica to react with the hydroxyls on the surface. (Figure 1.4)

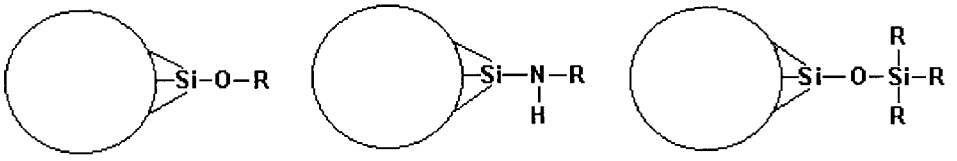


Figure 1.4: Types of attachment

Figure 1.5 shows an example of the way tethers can attach to the silica surface and cause poor surface coverage due to a lack of available active sites. Ethoxy groups are reactive to –OH groups. If several ethoxy groups are present on the tether, they

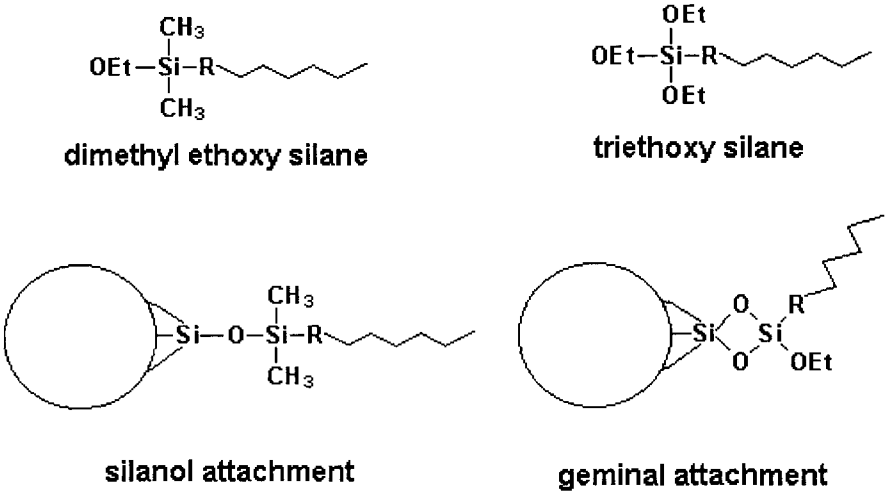


Figure 1.5: Examples of silane attachment

have the ability to react with surrounding Si groups as well. This can limit the number of tethers that can attach to the surface. The ethoxy groups can also react with each other, causing a layering effect of the tether. (Figure 1.6) This can increase the carbon content of a stationary phase and give an inaccurate indication of how many active sites are available on the surface. A dimethyl linker prevents this layering from happening because methyl groups are not as reactive to silanol groups as ethoxy groups. As a result of using a dimethyl linker, there should be one tether for each Si atom, which represents

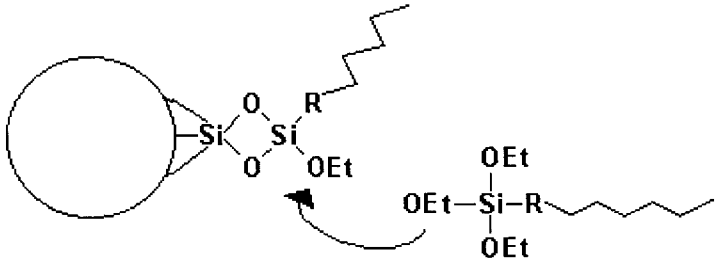


Figure 1.6: Ethoxy layering effect

the ideal maximum coverage.

In reality, the presence of methyl groups on a dimethyl linker prevents some silanol groups from being tethered. Methyl groups are sterically large, creating a "methyl umbrella". (Figure 1.7) This umbrella can cover free –OH groups and prevent other tethers from attaching. These free –OH groups are very polar and can interact with solutes introduced to the stationary phase during separations, causing mixed-mode interactions and tailing peaks on the chromatograms. To prevent mixed-mode interactions, a smaller, highly reactive silane, like trimethylchlorosilane, can be added to react with residual hydroxyl groups to form non-reactive sites. This addition of an extra silane is called "endcapping" and should be done only after all modifications to the silica surface have been achieved.

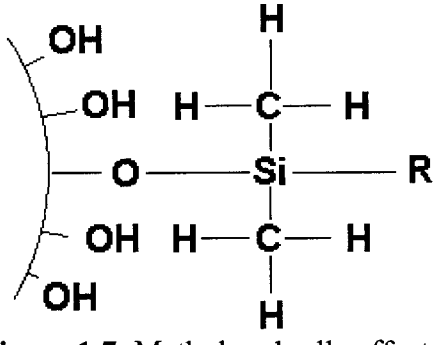


Figure 1.7: Methyl umbrella effect

The advantage of chemically modified silica over unmodified silica is that changing functional groups on the adsorbent can change the polarity of the stationary phase and the mode of interaction is changed from surface adsorption to bulk absorption, which is more reproducible in its interactions. The hydroxyl groups on the silica surface make a very polar stationary phase. Depending of the type of modifier, the polarity of the stationary phase can be altered. The addition of a tether can make the silica surface less polar, which can make separations of solutes easier by altering the polarity of the mobile

phase. The more non-polar stationary phase will prevent peak tailing associated with polar solutes in normal phase chromatography, as well as retain non-polar solutes for separation based on the polarity of the mobile phase.

Not only can polarity be changed by the addition of a modifier to the silica surface to improve separations, but molecules that separate through other methods as well can be attached. Adding a basket-shaped molecule, like a cyclodextrin or a calixarene, to a stationary phase allows separations to occur on a basis other than polarity. The bowl shape of a calixarene has the ability to accept molecules and retain them based on a solute's physical properties, as well as charge. Solutes that have the same size and depth as the cavity of the calixarene will retain well, while other solutes that do not meet these specifications will not. This phenomenon is known as host-guest complexation.

Host/Guest Complexation

Host/guest complexation refers to the ability of a host molecule (calixarene) to interact with a guest molecule (e.g., polycyclic aromatic hydrocarbons, PAHs) or a guest ion (e.g., Na^+). In the case of calixarene bonded stationary phases, the length of time a molecule stays within the "cup" and the reverse phase character of the stationary phase both contribute significantly to solute retention. Molecules that are too large, or do not have appropriate interactions to form a host-guest complex would elute off the column faster than a smaller molecule, or one that interacts strongly through host-guest complexation. PAH's can be effectively separated using calixarene bonded stationary phases because of their range of size, their aromatic character, and overall neutrality, all of which favor host-guest complexation with calixarenes. The persistence at which the guests are held within the calixarene depends on the number of monomer units

composing the calixarene series. For example, calixarene tetramers and hexamers hold guests very tightly compared to other sizes of calixarenes.[4]

The type of calixarene used for separations can also control the type of host/guest formations that will occur. Chiral calixarenes can be used to separate enantiomers through the specific shape and the position of each enantiomer.[13-16] Chiral calixarenes have the ability to retain these similar molecules differently based on their orientation and the secondary interactions that occur once inside the cup. This type of separation is highly favorable in uses for biological systems where enantiomers are commonly encountered and enantiomeric separations are essential to gauging biological activity.

Reversed-phase High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) is a separation technique that is based on the interaction of two phases: a stationary phase and a mobile phase. The mobile phase is pumped at a high pressure through a uniformly packed column of stationary phase. Samples injected onto a column separate based on differences in the magnitude of their components' interactions with the stationary phase and the mobile phase. The more a solute interacts with the stationary phase, the longer a sample will take to elute from the column.

There are three fundamental types of interactions that a solute can have with a phase boundary to induce separations; ionic, van der Waals, and hydrogen bonding. Ionic interactions are forces that are controlled by the attractive forces of oppositely charged molecules or repulsive forces of similarly charged molecules. Van der Waals interactions are weak forces that involve induced temporary (London forces) or

permanent dipole interactions (dipole forces) between a solute and a phase. Hydrogen bonding involves molecules that have hydrogen bonded to an O, N or F atom. These interactions only occur with other molecules containing O, N, or F. These three interactions dictate the retention of solutes in an HPLC column, and depending on the type of a particular phase, they can result in longer or shorter retention times of a solute on a chromatographic column.

Once a solute is introduced on a column, it passes through the column with the eluent, where diffusion of the band occurs. The elution band statistically approximates a Gaussian curve (Figure 1.8). This band broadening due to diffusion determines the chromatographic efficiency of a column: how well and fast a separation can be made. This process is expressed as " the height equivalent to a theoretical plate," and is termed h. The factors that influence h are expressed as the van Deemter equation [30]:

$$h = A + B/u + Cu$$

where A is eddy diffusion, B is longitudinal diffusion, C is mass transfer and u is the linear flow rate usually reported in cm/min. Eddy diffusion is a result of the differences in mobile phase velocity due to the different flow paths a solute molecule follows between silica particles. This is independent of the flow rate. Longitudinal diffusion

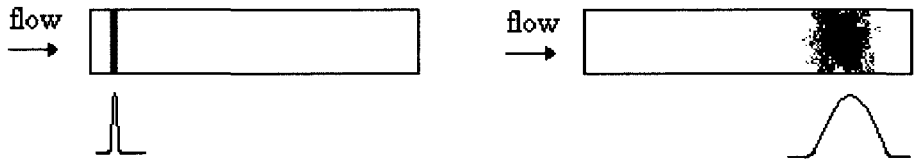


Figure 1.8: Diffusion bands over time on a column

is a process that results from diffusion in the direction of the mobile phase in a random, Gaussian manner. As the linear flow rate increases, the solutes have less time to diffuse on a column, so there is an inverse relationship of the flow rate and h due to longitudinal

diffusion. Diffusion due to mass transfer effects is a non-equilibrium diffusion that is dependent on flow rate. Solutes have the ability to partition between the mobile and stationary phase, but not necessarily at the same rate. Molecules that partition at a slower rate will retain longer on the column. Extracolumn broadening can occur in addition to these combined effects. Fittings, connective tubing, and detector cells that are not fitted properly can create extra volumes where mixing can occur. These effects can affect resolution and accuracy of separations.

There are two modes of HPLC that are commonly performed: normal phase and reversed phase. In normal phase chromatography, the stationary phase is more polar than the mobile phase. With normal phase HPLC, the least polar analyte is eluted first. This mode of separation is suitable for polar molecules that are either insoluble in organic solvents or that bind strongly to inorganic oxide absorbents.[4] In reversed phase HPLC, (RPC) the stationary phase is more non-polar than the mobile phase. Here, the least polar analyte is eluted last.

Types of Reversed Phase Chromatography

In RPC, neutral and ionic solutes can be separated at the same time, due to secondary equilibria such as ion suppression, ion pairing, acid/base complexation as well as host/guest complexation. Retention of solutes by simple interactions between the stationary phase and the mobile phase should result in Gaussian peaks. If any other interactions are involved, the peaks will skew to one side. This is known to be caused by mixed mode retention, which simply means that more than one type of interaction is occurring. If tailing peaks are present in a chromatogram, then more than one mode of retention is responsible for its shape. To determine which modes are involved in the

retention, it is necessary to eliminate all other possible forms of equilibria except for the desired retention mechanism.

Ion suppression deals with the extent of ionization of a weak acid or base in aqueous solution, which can be controlled by buffering the pH of the mobile phase. The ions in solution would interact with the stationary phase causing band-broadening effects. Lowering the pH of the mobile phase would force the weak acid to stay unionized, while raising the pH would have the same effect on weak bases.

Free ions introduced into the mobile phase can eliminate band-broadening effects as well. By adding an oppositely charged ion into the mobile phase, a "neutral complex" can form through electrostatic interactions. This will prevent further stationary phase interactions. This type of secondary equilibria is known as ion pairing. The "complex" is the attraction of the oppositely charged ions to each other, more so then to the stationary phase, but the ions do not form a chemical bond.

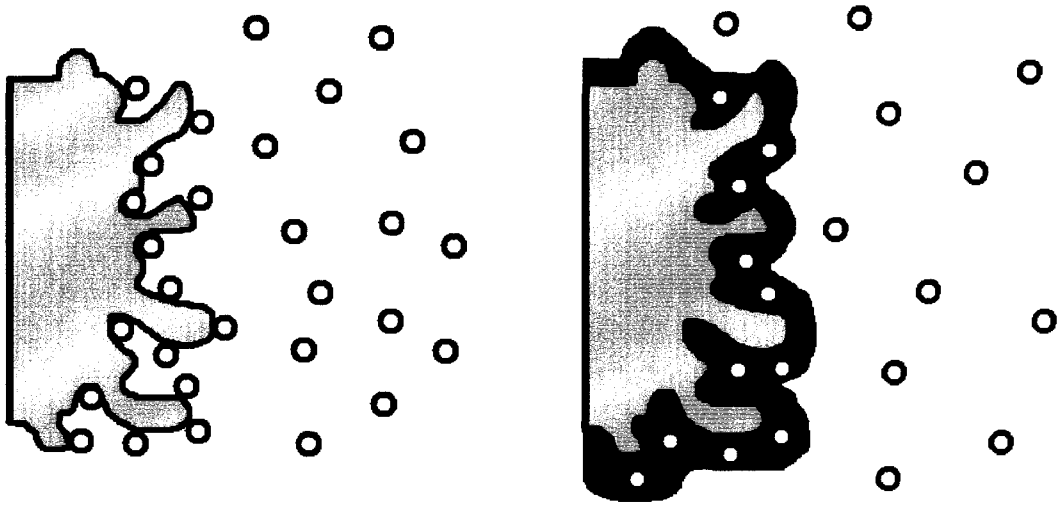
Complexation is the formation of Lewis acids/bases with the free ions. This is different from ion pairing because a complex is formed and can be collected off the column. After eliminating all other equilibria except host/guest complexation, if tailing peaks are still evident, it is an indication that complexes are forming with the solutes and the stationary phase.

There are other advantages for the uses of RPC as a separation mode in HPLC. By continually increasing the solvent strength, a more convenient elution time and sharper peaks can result. There are also improvements in solute resolution and solute detectability (detection limits are lowered and sensitivity to changes in analyte

concentration are improved). To explain how these phenomena can occur, it is necessary to examine the thermodynamics involved in chromatography.

Thermodynamics of Chromatography

There are two different fundamental approaches to describe the behavior of solute retention in RPC, absorption and adsorption (Figure 1.9). Absorption uses the idea that a



Adsorption Absorption

Figure 1.9

chemically bonded stationary phase forms a liquid layer coating around the silica particle. This concept theorizes that solute molecules can penetrate between the bonded tethers that make up the liquid layer and then return to the mobile phase. Adsorption is a process where the solute molecules can bond directly to the surface of the stationary phase. The solutes and solvent, therefore, compete for the active sites on the silica surface. What really happens on the surface is unclear and is probably a combination of both processes. Thermodynamics is used to describe what may actually happen on the surface to cause retention, by combining several factors.

A calixarene stationary phase is, for all intents and purposes, non-polar. A non-polar solute will interact with a non-polar stationary phase through weak dispersion

forces. These forces are expressed as the thermodynamic equilibrium constant, K, which can be represented in terms of the solute retention, k', and the volume ratio of the stationary phase and the mobile phase, ϕ [30].

$$k' = \phi K$$

When a non-polar solute is introduced by a polar mobile phase, a repulsion force of the solute and not the weak dispersion forces is responsible for the association of the solute and the stationary phase. This is known as the solvophobic theory. The retention of the solute can be expressed by the energy change for the binding of the solute to the stationary phase as:

$$ln\ k' = ln\ \phi - (\Delta G°/ RT) \text{ derived from}$$

$$\Delta G° = \Delta H° - T\Delta S° = - RT\ ln\ K$$

where R is the universal gas constant, T is absolute temperature, $\Delta G°$ is the standard free energy change, $\Delta H°$ and $\Delta S°$ are the standard enthalpy and entropy changes from the partitioning of the solute from the mobile phase to the stationary phase. The phase volume ratio factor, $ln\ \phi$ can be ignored due to the relative comparisons being made and is included as a constant, b in the equation [32]:

$$\Delta G° = n\ \overline{\Delta G} + b$$

where $\overline{\Delta G}$ is the free energy contribution of the addition of a homologue in a carbon atom series and *n* is the number of carbon atoms in a molecule or chain. This expression presumes a linear relationship between $\Delta G°$ and n for a homologous series. A plot of *ln* k' versus *n* at a constant temperature would have the slope:

$$\frac{d\ ln\ k'}{d\ n} = \frac{-\overline{\Delta G}}{RT}$$

As long as there was only a single process in partitioning, the slope would be constant and linear. The addition of secondary processes would create a non-linear relationship. Host/guest complexation is a desired secondary process associated with calixarene stationary phases. By reducing all other possible secondary equilibria, the presence of peak tailing and a non-linear relationship of *ln* k versus *n* would indicate host/guest complexation of solutes with calixarene bound stationary phases.

Chapter II

Literature Review

In 1942, Alois Zinke and Erich Ziegler took phenol-formaldehyde chemistry to the next level by looking at *p*-substituted phenols in condensation reactions with formaldehyde. These scientists, along with Joseph Hiederl and his coworker Heinz Vogel performed elemental analysis of the product and proposed a cyclic tetrameric structure.[1]

In the early 1970's, C.D. Gutsche was a researcher in bioorganic chemistry studying the new area of enzyme mimics. Zinke's cyclic tetramers seemed perfect for this research due to their classification as "cavity-containing substances."[1] These compounds were easily accessible by a one-step synthesis that could be carried out on a variety of *p*-substituted phenols. Gutsche's investigation also brought up the issue of what to call these compounds. Zinke referred to them as "mehrkernmethylenephenolverbindungen,"[1] but this was impractical. It was known that the cyclic oligomer was non-planar and the space-filling molecular model resembled the shape of a Greek vase, known as a calix crater. Gutsche derived a name using "calix" which means "vase" in Greek, and "arene" which indicated the presence of aryl groups, hence the term "calixarene."

Before the use of calixarenes in bound stationary phases, crown ethers and cyclodextrins, two other classes of macrocyclic compounds, were used as stationary phases in gas and liquid chromatography. Once calixarenes were discovered to have the

ability to take the place of these chiral stationary phases, they became increasingly attractive for an array of applications.

Calixarenes have become useful in a variety of separations. These macrocycles have been bonded to stationary phases for gas chromatography, [5-10] and used as mobile phase additives for HPLC and capillary electrophoresis.[11,12,14] Reviews have been published on the uses of calixarenes in ion-selective electrodes, as chromoionophores and fluoroionophores in optical analyses as well as extractants and liquid membranes.[12,13] Calixarenes have been found to separate enantiomers and perform chiral separations.[13-16] The uses for calixarenes are limitless and this leads to their uses as bound stationary phases in HPLC.

W. Xu et al.[17] prepared p-tert-butyl-calix[6]arene bonded stationary phases for HPLC using 3-glycidioxypropyl triethoxysilane as a linker to the silica. The synthesis was performed in toluene under an inert atmosphere. Characterization was done with an elemental analyzer and through Fourier transform infrared (FTIR) spectroanalysis. HPLC analysis was done using isomers of nitroaniline and aminophenol, PAHs and nucleosides.

R. Brindle et al.[18] performed detailed CP/MAS NMR spectroanalysis on two different silica-bonded calixarenes. The first phase was prepared by attaching a calix[4]arene tetraamide by a triethoxysilyl derivative in toluene. The second phase was prepared with a p-allycalix[6]arene hexaester bonded to the silica by dimethoxy methylsilane in toluene. HPLC analysis was not performed on these phases.

A p-tert-butyl-calix[4]arene was synthesized by S. Freibe et al.[19] and immobilized onto silica by an unspecified "short hydrophilic spacer." The procedure was

not specified for the material and synthesis was patented in Germany. ^{1}H NMR and IR were used to analyze the structure and purity of the stationary phase. The carbon content was determined by elemental analysis and electron microscopy. Nitroanilines, nucleosides and nucleobases were used in the HPLC analysis of the stationary phases.

p-Tert-butyl-calix[4]arene was attached to silica using the linker - (ethylenediamino)-propyl-triethoxyl-silane in toluene by X.Z. Xiao et al.[20] ^{13}C and ^{29}Si CP/MAS NMR was used to characterize the silica. HPLC performance was evaluated using PAHs, nucleosides and bases. Y. Zhang et al. [21] published the same work in Chinese.

J. D. Glennon, et al. [22,23] used p-allyl-calix[4,6]arene ester derivatives attached to silica through a triethoxysilane tether. These HPLC phases were used to separate alkali metal chlorides, amino acid ester chlorides and a mixture of benzamide, benzopheone and biphenyl using methanol/ water mobile phases. Glennon et al. also tested calix[4]arene tetradiethylamide stationary phases in a similar manner.[15]

L. O. Healy, et al. [25] synthesized a chiral calixarene by functionalizing the lower rim of a triethoxysilyl-calix[4]arene with L-(-)-ephedrine. Enantiomeric separations of R- and S- 1-phenyl-2,2,2-trifluoroethanol were reported.

T. Sokoließ et al. [26] did comparative studies of commercially made calix[n]arene-bound stationary phases to phenyl and C_{18} phases using acetonitrile, methanol, tetrahydrofuran, isopropanol and 1,4-dioxane as mobile phases. The calixarenes were attached through a "propyl spacer." The surface concentration of the calixarene phases ranged from 0.12 mol/m^2 to 0.33 mol/m^2. Phenols, alkylated and unsubstituted aromatics, benzoic acid esters, PAHs, barbituric acid derivatives and

xanthine derivatives were used to analyze the HPLC performance of these stationary phases.

R.S. Hirschl [27] used p-tert-butyl-calix[4]arenes and p-tert-butyl-calix[6]arenes attached by 1,2-dichlorotetramethyldisilane and 1,7-dichlorooctamethyltetrasiloxane, through synthesis in toluene, to the silica trying to increase the carbon content of the stationary phases. Increasing the carbon content would indicate an improved coverage of the p-tert-butyl-calix[4]arene on the silica. HPLC performance was evaluated using a homologous series of alkyl benzenes and phenyl rings. Characterization of the stationary phases was performed using CP/MAS NMR and fluorescence.

Chapter III

Statement of Problem

Separations by HPLC are possible through changing many variables, but often, the most important variables are the "right" combination of stationary phase and mobile phase. Mobile phases can be easily optimized through changes in solvent strength whereas stationary phases cannot be as easily optimized. More efficient stationary phases require either purchasing new columns or deriving new stationary phases. Unfortunately, there is a relatively limited number of types of stationary phases available for purchase, and they are expensive. Research into the development of new stationary phases is on the increase, in order to make them less expensive and give a wider range of separations.

Recent developments in stationary phase synthesis involve the attachment of macrocyclic molecules, such as cyclodextrins or calixarenes. These molecules are known to separate enantiomers, ions, and neutral solutes, making them very versatile stationary phases. The goal of this research is develop a straightforward method for attaching calixarenes to the silica surface, with the maximum amount of surface coverage. A new synthesis is proposed using an amino-derived silica and an amino-derived calix[4]arene tethered together by a glutaraldehyde linker, to improve the surface coverage of the silica surface.

Spectroscopic and chromatographic characterization is necessary to determine the extent of attachment of the calixarene to the silica surface. Solute probes, such as PAHs and an alkyl benzene homologous series, are used to determine the efficiency of the

calixarene stationary phase for separations in the reversed phase mode of chromatography.

Chapter IV

Materials and Methods

Synthesis of Stationary Phase

3-Aminopropyldimethylethoxysilane Tethered Silica

Five grams of Macherey-Nagel Nucleosil, 7 μm, 1000 Å-pore size (Alltech Associates, Inc., Deerfield, IL) was dried in a vacuum oven at 150°C for 2 hours. The silica was added to 50 mL of 95% ethanol (Pharmco, Brookfield, CT) in a round-bottomed flask. Ten mL of 3-aminopropyldimethylethoxysilane (United Chemical, Bristol, PA) was added to the suspension and left to react for 24 hours. The suspension was filtered through a 0.45 μm Nylon filter and washed with 100 mL of 95% ethanol, 100 mL DI water (in house deionizer) and 100 mL of anhydrous ethanol (Quantum Chemical Corporation, Tuscola, IL). The amino-substituted silica was placed in a vacuum dessicator to dry.

Tetra-t-butyl-tetra [N–(2-amino-ethyl)-2-oxy-acetamide] calix[4]arene

4-*t*-butylcalix[4]arene, O, O', O'', O''' tetraacetic acid tetraethyl ester (0.7 g) [28] was added to 50 mL of ethylene diamine (Aldrich Chemical Co., Milwaukee, WI) and stirred under nitrogen for 4 days. The reaction mixture was extracted with 50 mL of saturated ammonium chloride (J.T. Baker Chemical Co, Phillipsburg, NJ) and 25 mL of tetrahydrofuran (Fisher Scientific, Fairlawn, NJ). The aqueous layer was rotoevaporated to leave a yellowish powder. It was purified through flash chromatography using a starting solvent system of 5:1 1-propanol (Fisher Scientific, Fairlawn, NJ): ammonium

hydroxide (Fisher Scientific, Fairlawn, NJ) and ending with 2:1 1-propanol: ammonium hydroxide to recover a brownish powder, which was the most polar product. (Figure 4.1)

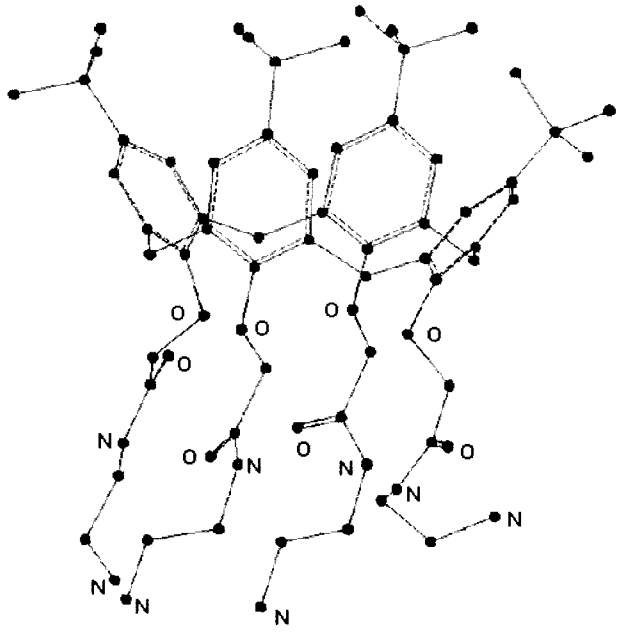


Figure 4.1: Tetra-t-butyl-tetra [N–(2-amino-ethyl)-2-oxy-acetamide] calix[4]arene

Tethering and Attachment

To determine the number of moles of tetra-t-butyl-tetra [N–(2-amino-ethyl)-2-oxy-acetamide] calix[4]arene (amino calix[4]arene) required to cover the surface area of the silica, the area of the amino calix[4]arene and the surface area of the silica were needed. PC Spartan Pro was used to determine the area of the amino calix[4]arene, 1138 $Å^2$. The following calculation was use to determine the minimum number of moles needed to cover the surface of the silica:

$$\frac{1138\ Å^2 \text{ area of amino calix[4]arene}}{\text{molecule}} \times \frac{1 \times 10^{-20}\ m^2}{1\ Å^2} = \frac{1.138 \times 10^{-17}\ m^2}{\text{molecule}}$$

$$\frac{1.138 \times 10^{-17}\ m^2}{\text{molecule}} \times \frac{1}{25\ m^2 \text{ per 1g of silica [29]}} = \frac{3.794 \times 10^{-20} \text{ g of silica}}{\text{molecule}}$$

$$\text{Inverse} = \frac{2.196 \times 10^{18} \text{ molecules}}{\text{g of silica}} \times \frac{1 \text{ mole of amino calix[4]arene}}{6.023 \times 10^{23} \text{ molecules}} = \frac{3.65 \times 10^{-6} \text{ moles}}{\text{g of silica}}$$

$$\frac{3.65 \times 10^{-5} \text{ moles}}{\text{g of silica}} \times \frac{1049.40 \text{ g amino calix[4]arene}}{1 \text{ mole of amino calix[4]arene}} = \frac{0.0038 \text{ g amino calix}}{1 \text{g silica}}$$

The 3-aminopropyldimethylethoxysilane tethered silica (2.04 g) was suspended for 2 hours in 40 mL of 9.4% glutaraldehyde solution made from 15 mL of 25% glutaraldehyde (Aldrich Chemical Co., Milwaukee, WI) and 25 mL of disodium phosphate buffer, pH = 7 (J.T. Baker Chemical Co, Phillipsburg, NJ). The suspension was filtered through a 0.45 μm Nylon filter and washed with 100 mL of 95% ethanol, 100 mL DI water and 100 mL of anhydrous ethanol.

The linked amino silica was immediately transferred (wet) to a solution of 0.0974 g of amino calix[4]arene in 50 mL of chloroform (Fisher Scientific, Fairlawn, NJ) and left to react for 48 hours. The suspension was filtered through a 0.45 μm Nylon filter and washed with 100 mL of 95% ethanol, 100 mL DI water and 100 mL of anhydrous ethanol to give a yellowish-brown product.

The tether was reduced by placing the dry calix-linked silica in 50 mL of anhydrous ethanol and adding 2 g of sodium borohydride to the mixture (Aldrich Chemical Co., Milwaukee, WI) slowly to the solution. It was allowed to react for 2 hours, filtered through a 0.45 μm Nylon filter and washed with 100 mL of 95% ethanol, 100 mL DI water and 100 mL of anhydrous ethanol. The silica was dried in a vacuum dessicator overnight. The complete reaction scheme is drawn out in Figure 4.2.

The calix-linked silica was endcapped after all chromatographic studies were performed to help determine if retention was due to the presence of free silanol groups on the silica surface or, if indeed, calixarenes on the silica surface were forming host-guest complexes.

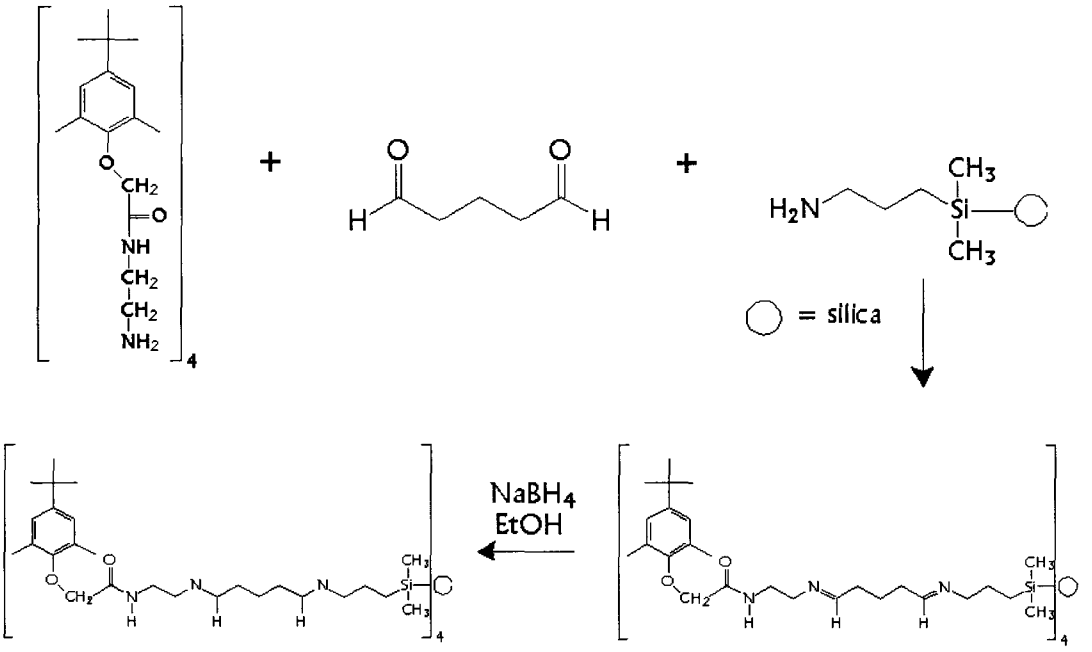


Figure 4.2: Reaction scheme

The stationary phase was washed with 100 mL of anhydrous ethanol and placed in a vacuum oven at 120°C for 30 minutes to get rid of any residual solvent. The silica was then placed in a 500 mL round-bottom flask containing 200 mL of dry methylene chloride (Fisher Scientific, Fairlawn, NJ). Five mL of trimethylchlorosilane (TMCS) (Aldrich Chemical Co., Milwaukee, WI) was injected into the flask and allowed to react for 2.5 hours. 200 mL of 95% ethanol was added to the reaction to deactivate any extra TMCS. The suspension was filtered through a 0.45 μm Nylon filter and washed with 100 mL of 95% ethanol, 100 mL DI water and 100 mL of anhydrous ethanol. It was dried under vacuum. The silica was then repacked for chromatographic comparisons.

Column

An Alltech air-driven slurry column packer was used to pack the stationary phase into a 4.5 mm x 50 mm threaded stainless steel column (Alltech Associates, Inc., Deerfield, IL). 2 g of stationary phase was suspended in 15 mL of trichloroethylene

(Mallinckrodt, Paris, KY) and added to the column. HPLC grade methanol (Fisher Scientific, Fairlawn, NJ) was pumped at 3000 psi by nitrogen into the column until 200 mL had passed through it.

Chromatography

The mobile phase used in the experiments consisted of HPLC grade methanol (Fisher Scientific, Fairlawn, NJ) and in-house deionized water. All phases were filtered through 0.45 μm Nylon filters before use.

PAH Series

Benzene, naphthalene, (J.T. Baker Chemical Co, Phillipsburg, NJ) and anthracene (Eastman Kodak Co., Rochester, NY) were dissolved in methanol qualitatively. Individual solutions as well as a mixture of the PAHs were made. Runs were made at 30% methanol / 70% water.

Alkyl Benzene Series

Methyl, ethyl, propyl, and butyl benzene (Alltech Associates, Inc., Deerfield, IL) were dissolved in methanol qualitatively. Individual solutions, as well as a mixture of the alkyl benzenes were made and run. Chromatograms were obtained using 15% methanol / 85% water as the mobile phase.

ENE Series

1-aminonaphthalene, 2-aminonaphthalene, 1-aminoanthracene and 2-aminoanthracene (Aldrich Chemical Co., Milwaukee, WI), naphthalene and anthracene were dissolved in methanol qualitatively. Individual solutions as well as a mixture of the ENE series were made and run. Chromatograms were obtained using 15% methanol / 85% water as the mobile phase.

Reversed Phase Series

Uracil, (Eastman Kodak Co., Rochester, NY) N,N-diethyl-*m*-toluamide, (Aldrich Chemical Co., Milwaukee, WI) toluene, (Alltech Associates, Inc., Deerfield, IL) and phenol (Mallinckrodt Chemical Works, St. Louis, MO) were dissolved in methanol qualitatively. Individual solutions as well as a mixture of the reversed phase series were made and run. Chromatograms were obtained using 15% methanol / 85% water and 10% methanol/ 90% water as the mobile phase.

Knox Test

The Knox test is used to determine the extent of free silanol interaction with a solute molecule before and after endcapping the stationary phase. The test required dry hexane (Pharmco, Brookfield, CT) as a mobile phase and dry methanol as the solute. If the solute retains on the stationary phase, this indicates the presence of free silanols on the surface of the silica.

HPLC System

HPLC analyses were performed with the Beckman System Gold Software (ver. 5.10), Varian 9010 pump, and Beckman System Gold Diode Array Detector Module 168. Injections of 5 µL were made using a 10 µL syringe into a 10 µL loop with a Rheodyne injector, at a flow rate of 1 mL/min at an absorbance of 254 nm.

UV Spectra

All UV spectra were obtained on a HP 8453 UV-Diode Array Spectrophotometer. Samples were suspended in ethylene glycol (J.T. Baker Chemical Co, Phillipsburg, NJ) and placed in quartz sample cuvettes. Spectra were collected over a range from 190 nm to 500 nm in order to find all possible maxima.

Fluorescence

Fluorescence spectra were recorded with a Shimadzu Instruments Model RF 5000, Scanning Spectrofluorophotometer. Samples were prepared in the same fashion as the UV-VIS samples.

Mass Spectrometry

All mass spectra were obtained on a Bruker Daltonik Esquire ~LC with HP 1100, HPLC Mass Spectrometer. Samples were dissolved in methylene chloride (Fisher Scientific, Fairlawn, NJ) at a concentration of 1 mg/mL. Conditions of instrument are stated with each mass spectrum.

Nuclear Magnetic Resonance

^{1}H and ^{13}C spectra were recorded on a Varian Gemini 2000, 400 MHz Spectrometer. Deuterated chloroform with 0.1% tetramethylsilane (Aldrich Chemical Co., Milwaukee, WI) was used to dissolve the samples for analysis.

Elemental Analysis

Samples were run on a Carlo Erba Instrument NA 1500 Nitrogen/ Carbon/ Sulfur Analyzer. An acetanilide standard (Carlo Erba Strumentazione, Rodano, Milano, Italy) was used to calculate the K factor to determine the percentage of elements, where K is:

$$K = \frac{\text{(percent theoretical standard) x (weight of standard in mg)}}{\text{Peak area for standard}}$$

Samples and standards were weighed on a Cahn 29 Microbalance and placed in tin sample holders (Costech Analytical Technologies Inc., Valencia, CA). Determination of the percent of each element was determined by:

$$\text{Elemental Percentage} = \frac{\text{(K factor from standard) x (Sample Area)}}{\text{Weight in mg of sample}}$$

Chapter V

Results and Discussions

Spectroscopic Characterization

Tetra-t-butyl-tetra [N–(2-amino-ethyl)-2-oxy-acetamide] calix[4]arene

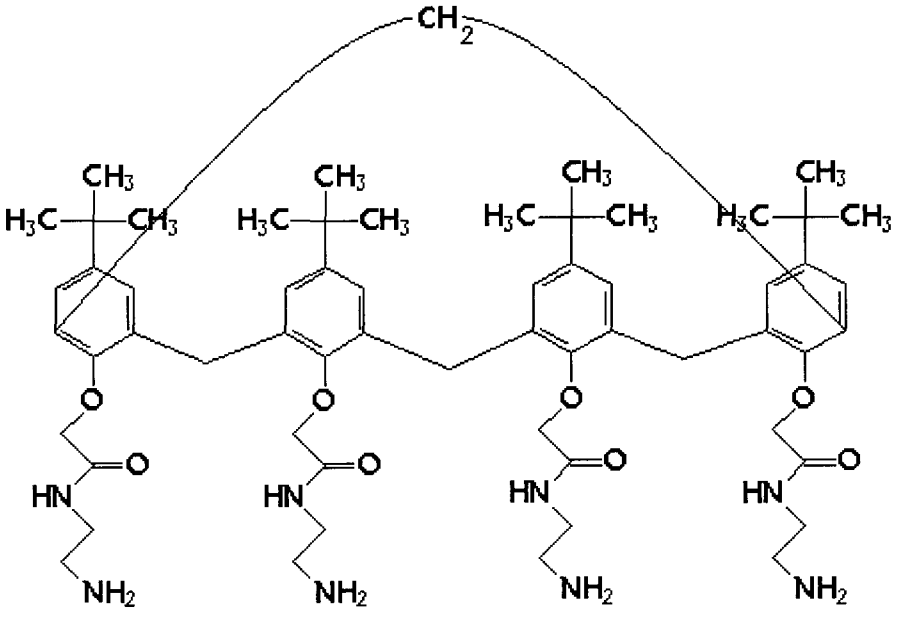


Figure 5.1: Amino derived calix[4]arene

Mass Spectrometry

The crude amino derived calix[4]arene was spotted on a TLC plate and run in 5:1 and 2:1 mixtures of 1-propanol:ammonium hydroxide. The 5:1 mixture yielded five spots, with R_f values of 0.96, 0.80, 0.49, 0.29 and zero. By changing the solvent system to a 2:1 mixture, the spot with an R_f of zero moved to a value of 0.39. Three products were separated using the 5:1 eluent and one was collected using the 2:1 eluent via flash chromatography. The products were analyzed by mass spectrometry and the 2:1 eluent fraction had a $[M + H]^+$ peak of 1049.77 (Figure 5.2). This corresponds to the formula weight of the amide calix[4]arene that was predicted, $C_{60}\ H_{88}\ N_8\ O_8$, 1048.7 g/mol. The

moderately strong peak at 1031.76 m/z was confirmed by MS/MS to be a loss of ammonia from the structure. The other minute peaks could be the cyclization of the tethered groups upon fragmentation. Table 5.1 shows the acquisition parameters used on the LCMS for the amide calix[4]arene.

APCI Temp	454 °C	**Polarity**	Positive
Capillary Current	190 nA	**Skim 1**	52.5 V
Scan Range	15.00-2000.00 m/z	**Trap Drive**	59
Corona Current	10274 nA	**Dry gas Temp**	352 °C
Corona Voltage	2600 V	**Octopole**	2.4 V

Table 5.1: Acquisition parameters of Esquire LCMS for Tetra-t-butyl-tetra [N–(2-amino-ethyl)-2-oxy-acetamide] calix[4]arene

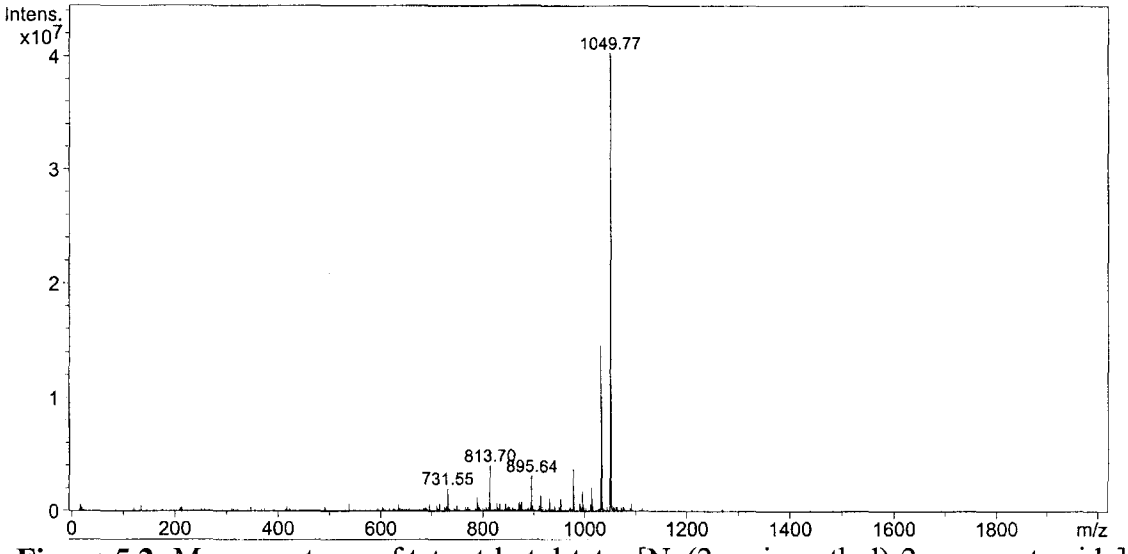


Figure 5.2: Mass spectrum of tetra-t-butyl-tetra [N–(2-amino-ethyl)-2-oxy-acetamide] calix[4]arene

Nuclear Magnetic Resonance Spectroscopy

An NMR spectrum of the amide-calix[4]arene was obtained. Figure 5.3 is the ^{1}H spectrum for the amide calix[4]arene in $CDCl_3$. A ^{13}C spectrum was not collected due to a lack of product after immobilization on the silica. The ^{1}H spectrum had a strong singlet at 1.04 ppm that corresponded to the tert-butyl groups (1). A broad peak at 2.31 ppm (2)

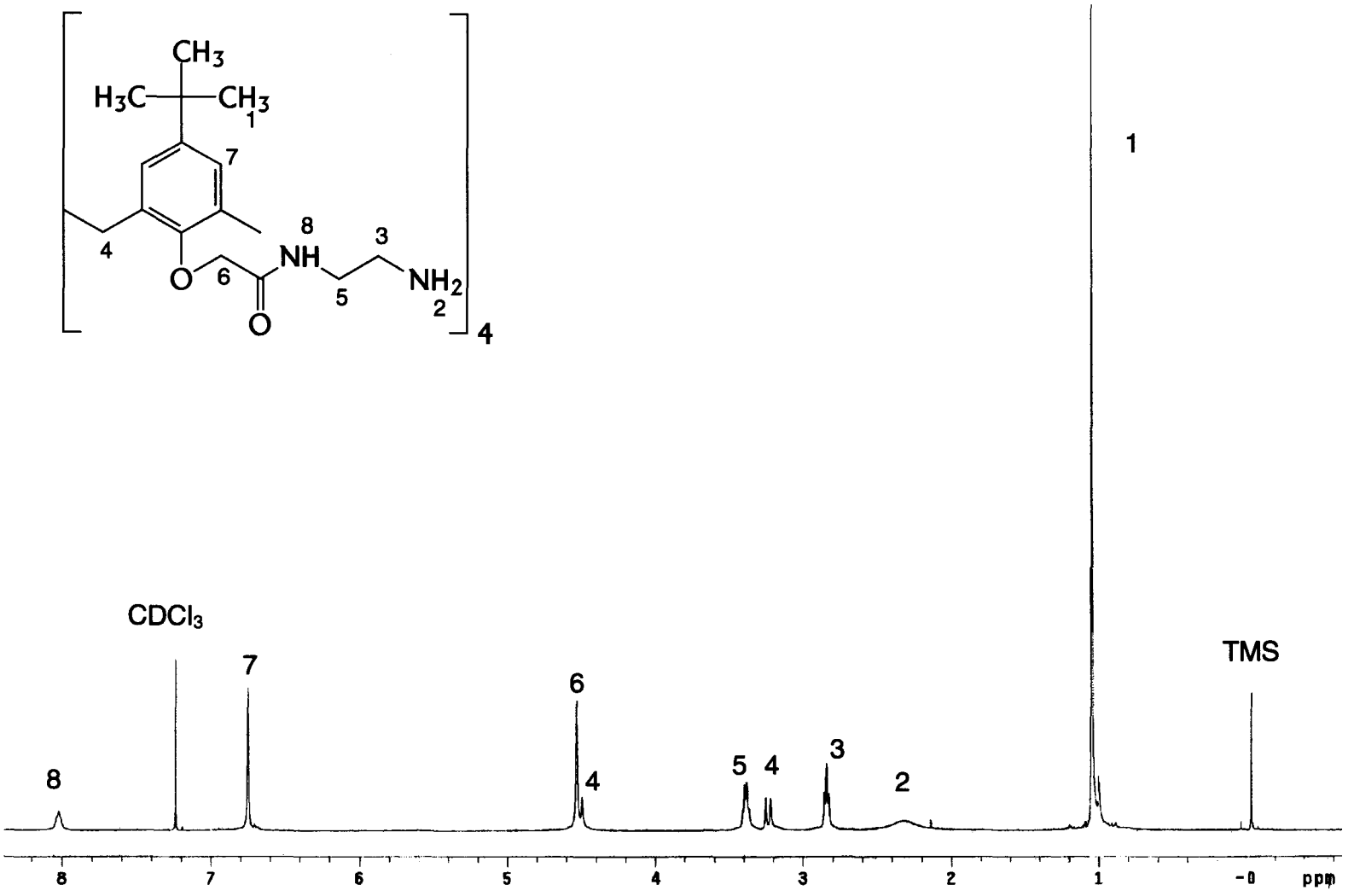


Figure 5.3: ^{1}H NMR spectrum of tetra-t-butyl-tetra [N–(2-amino-ethyl)-2-oxy-acetamide] calix[4]arene

represented the NH_2 group at the bottom of the tethered calixarene. The CH_2-CH_2 groups in the chain appeared as a triplet at 2.84 ppm (3) and a quartet at 3.38 ppm (5). The methylene bridges had 2 sets of doublets (4); one at 3.21 ppm and the other hidden under a peak at 4.53 ppm. The singlet at 4.53 ppm represented the methylene group being shifted downfield due to its placement between the two oxygen groups (6). The singlet at 6.75 ppm was due to the -CH groups in the ring (7) and the broad peak at 8.02 ppm represented the NH group (8).

Synthesis of the Stationary Phase

Elemental Analysis

Elemental analysis was used to determine the attachment of the 3-aminopropyl-dimethylethoxysilane to Nucleosil by comparing carbon and nitrogen content of Nucleosil alone and the tethered Nucleosil. The tethered Nucleosil glutaraldehyde-linked amide-calix[4]arene was also compared. An increase in carbon and nitrogen content would verify that there was a successful attachment to the Nucleosil. Table 5.2 shows the percent carbon and nitrogen determined.

Sample	N%	C%
Nucleosil	0.00492%	0.0862%
3-aminopropyldimethylethoxysilane attached Nucleosil	0.0681%	0.297%
Amino calix[4]arene bonded Nucleosil	0.141%	0.988%
Endcapped amino calix[4]arene bonded Nucleosil	0.136%	0.955%

Table 5.2: Elemental analysis results

There was an increase in N and C content after each attachment step. The carbon content of 0.988% for the amide calix[4]arene attachment correlates to percent carbon coverage of 1% determined for a calixarene-bound silica by R.S. Hirschl [27]. To determine if free silanols on the surface are present by elemental analysis, the stationary

phase was endcapped with trimethylchlorosilane (TMCS). The TMCS would react with any free OH groups and add to the carbon content of the stationary phase. There was not a significant increase in carbon content after endcapping.

Loading of the calixarene-bonded stationary phases are reported as the concentration of the surface coverage in μmoles/m^2. The calculation to determine the concentration of the surface coverage from the percent carbon of the stationary phase is as follows [31]:

$$N_{(\mu mol/m\hat{}2)} = \frac{(1.0 \times 10^6) \times P_c}{1200n_c - P_c(m-1)} \times \frac{1}{S}$$

where P_c is the C% of the bonded stationary phase, n_c is the number of carbon atoms bonded to the silica, m is the molar mass of the molecules attached to the silica and S is the surface area of the silica in m^2/g.

$$N = \frac{(1.0 \times 10^6)\ mol/mol \times (0.988\%)}{1200\ g/mol\ (66) - [(0.988\%)(1209.11\ g/mol - 1)]} \times \frac{1}{25\ m^2/g} = 0.51\ \mu mol/m^2$$

This concentration is higher than reported concentrations of 0.12 to 0.33 μmol/m^2 for calixarene-bound stationary phases[26]. This could be due to the larger pore volume of 1000 Å Nucleosil used in this synthesis, compared to the reported pore volumes of 100 Å. The calixarenes are able to "fit" into the pores of the Nucleosil better than with smaller pore volumes. Therefore, the increase in percent carbon would be expected with Nucleosil because it has a large pore volume.

Fluorescence and UV-VIS Spectroscopy

The benzene rings in the calixarene structure are a conjugated system that fluoresces when excited by ultraviolet light. Silica, glutaraldehyde, and 3-amino-propyldimethylethoxysilane do not fluoresce, nor do they significantly absorb UV

radiation. Therefore, the fluorescence and UV spectra of the silica-bound calixarene should be due only to the calixarene.

Samples of the bound calixarene phases were washed exhaustively to ensure that the spectra would be due only to bonded calixarenes and not to free or adsorbed calixarenes. The samples were suspended in ethylene glycol and the UV-VIS spectrum was collected at a wavelength range of 200 nm to 600 nm. Peaks at 204 nm and 275 nm were observed for the free amide calix[4]arene (Figure 5.4). In UV spectrum for the silica-bound amide calix[4]arene, peaks at 201 nm and 284 nm were observed (Figure 5.5). These are very close in respect to the free amide calix[4]arene and would indicate that calixarenes were present on the silica surface.

The UV sample was used for the fluorescence sample as well. The fluorescence spectrum for the free amide calix[4]arene was collected at an excitation of 250 nm over an emission range of 250 nm to 750 nm (Figure 5.6). Peaks at 308 nm, 418 nm and 600 nm in the fluorescence spectrum were observed. The fluorescence spectrum for the silica-bound calixarene was shifted to an excitation of 225 nm with peaks at 296 nm, 366 nm and 571nm (Figure 5.7). These peaks are shifted in comparison to the peaks for the free calixarene due to the immobilization of the calixarene to the silica. The peaks are close to each other and indicate that there is calixarene on the silica surface.

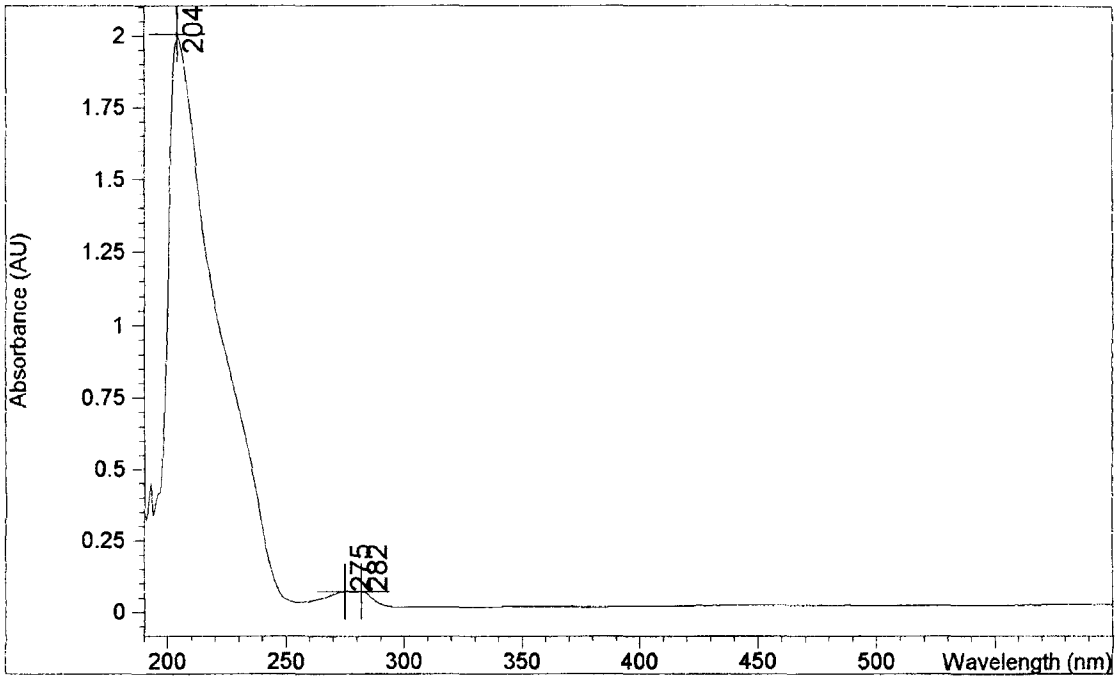


Figure 5.4: UV spectrum of tetra-t-butyl-tetra [N–(2-amino-ethyl)-2-oxy-acetamide] calix[4]arene

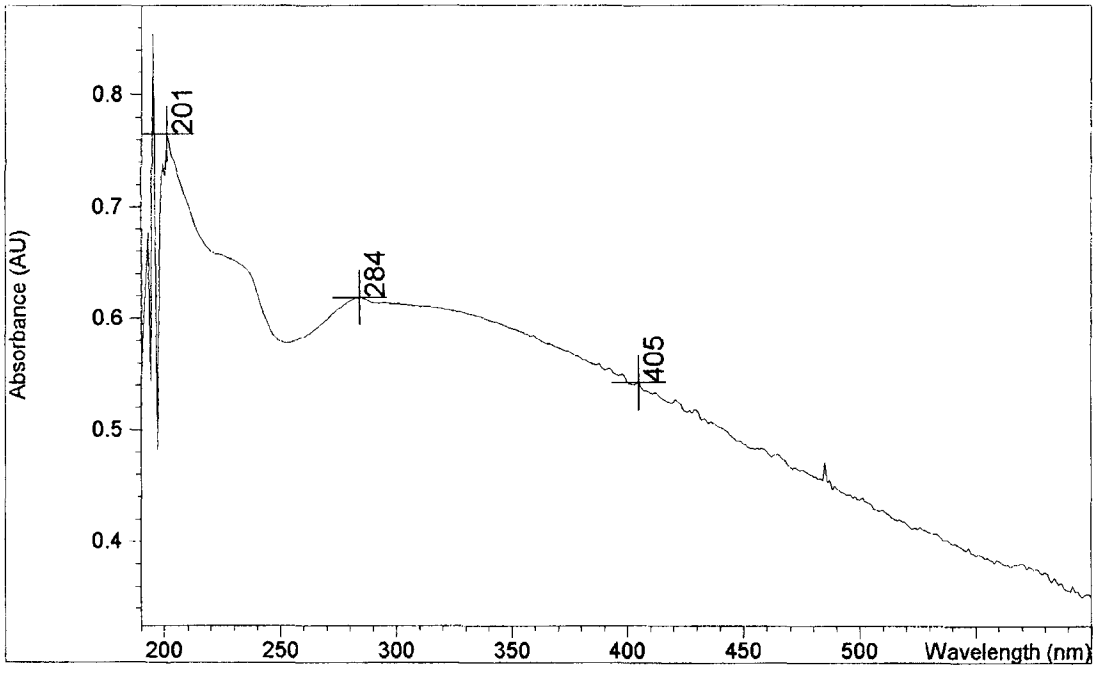


Figure 5.5: UV spectrum of Tetra-t-butyl-tetra [N–(2-amino-ethyl)-2-oxy-acetamide] calix[4]arene bound Nucleosil

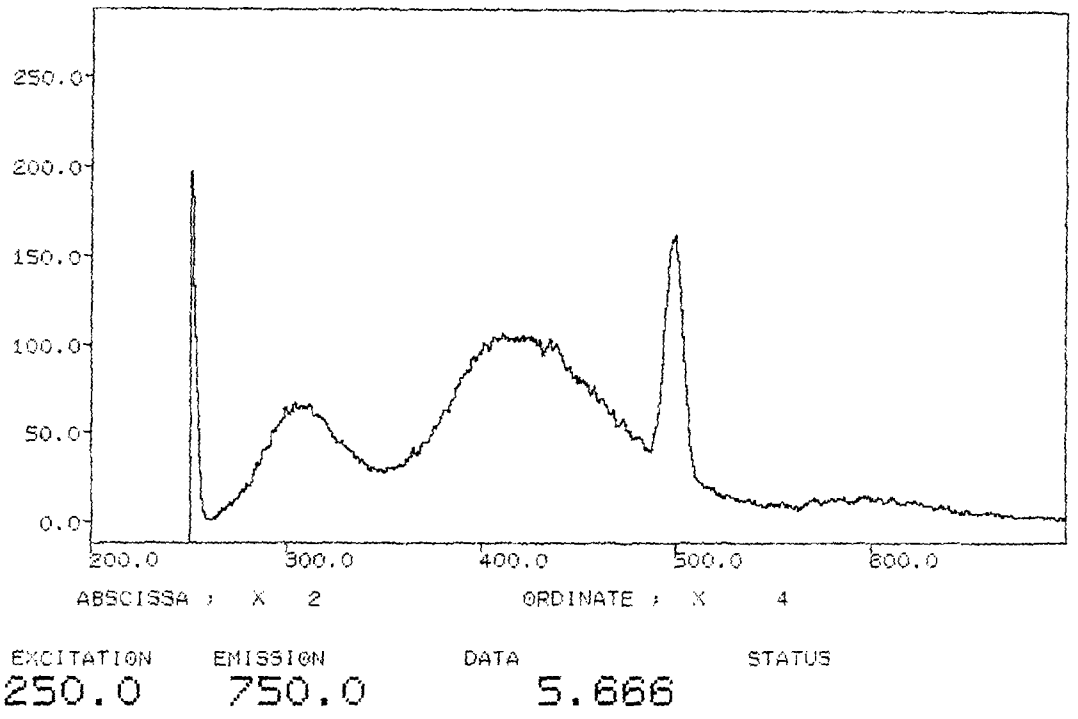


Figure 5.6: Fluorescence spectrum of tetra-t-butyl-tetra [N–(2-amino-ethyl)-2-oxy-acetamide] calix[4]arene

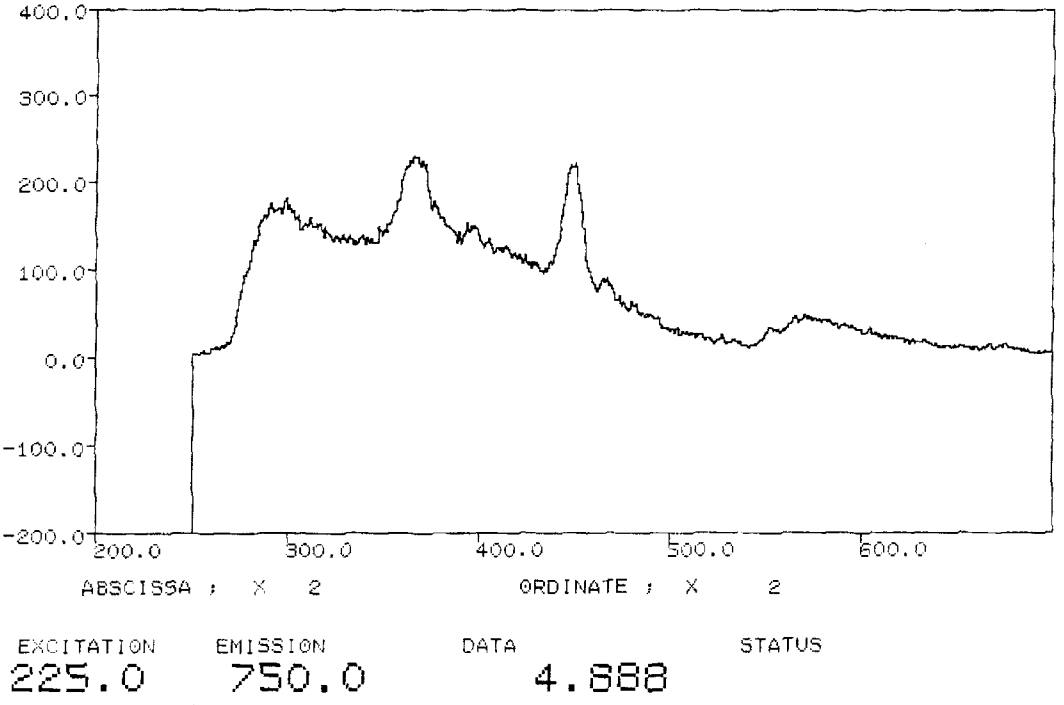


Figure 5.7: Fluorescence spectrum of Tetra-t-butyl-tetra [N–(2-amino-ethyl)-2-oxy-acetamide] calix[4]arene bound Nucleosil

Chromatographic Characterization

Solutes used to probe the silica surface were chosen based on their interaction with the calixarenes. A homologous series of PAHs, alkyl benzenes and amino-functionalized PAHs was used to determine if host-guest complexation occurred between the calixarenes and the solutes. The Knox test was also performed to establish if retention of the solutes were due to free silanols on the surface.

PAH homologous series

Retention characteristics of a homologous series can determine what types of intramolecular interactions are occurring on the surface of the stationary phase. PAH's are known to interact strongly with free silanol groups on the silica surface, as well as forming host/guest complexes with calixarenes. Performing PAH runs on the calixarene-bound stationary phase before and after endcapping can aid in indicating the retention mechanisms affecting peak shape. Plotting the log k' vs. number of rings in the series can also indicate retention characteristics by comparing the non-linearity of the curve to the linear results of a C_{18} column.

	Benzene	Napthalene	Anthracene
Before endcapping	1.25	1.38	3.13
After endcapping	2.75	1.05	2.07

Chart 5.3: Asymmetry factors for PAH series

Asymmetry factors are calculated by measuring the distance of each side of the peak from a central line extrapolated from the top of the peak at half height. They are used to determine the degree of skewness of a peak in a chromatogram. A perfectly Gaussian peak will have an asymmetry factor equal to one. If the asymmetry factor is < 1, the peak is fronting, if >1, the peak is tailing. Before endcapping there is tailing of all peaks in the chromatogram. This could be due to a number of reasons. PAH's are

known to interact strongly with free silanol groups, which result in tailing peaks. Tailing is also a result of interactions of the solute with the calixarenes. To determine if the peaks are tailing due to host/ guest complexation, endcapping of the stationary phase was done. The asymmetry factors decrease after endcapping the stationary phase, which means the PAH's must have been interacting with free silanols on the surface (Table 5.3). The benzene peak shows an increase in tailing after endcapping, but since the peak is at the dead time of the column, the significance of the results are minimal. The naphthalene peak is almost Gaussian after endcapping, meaning that there are no other secondary interactions causing peak tailing. The anthracene peak still significantly tails and the

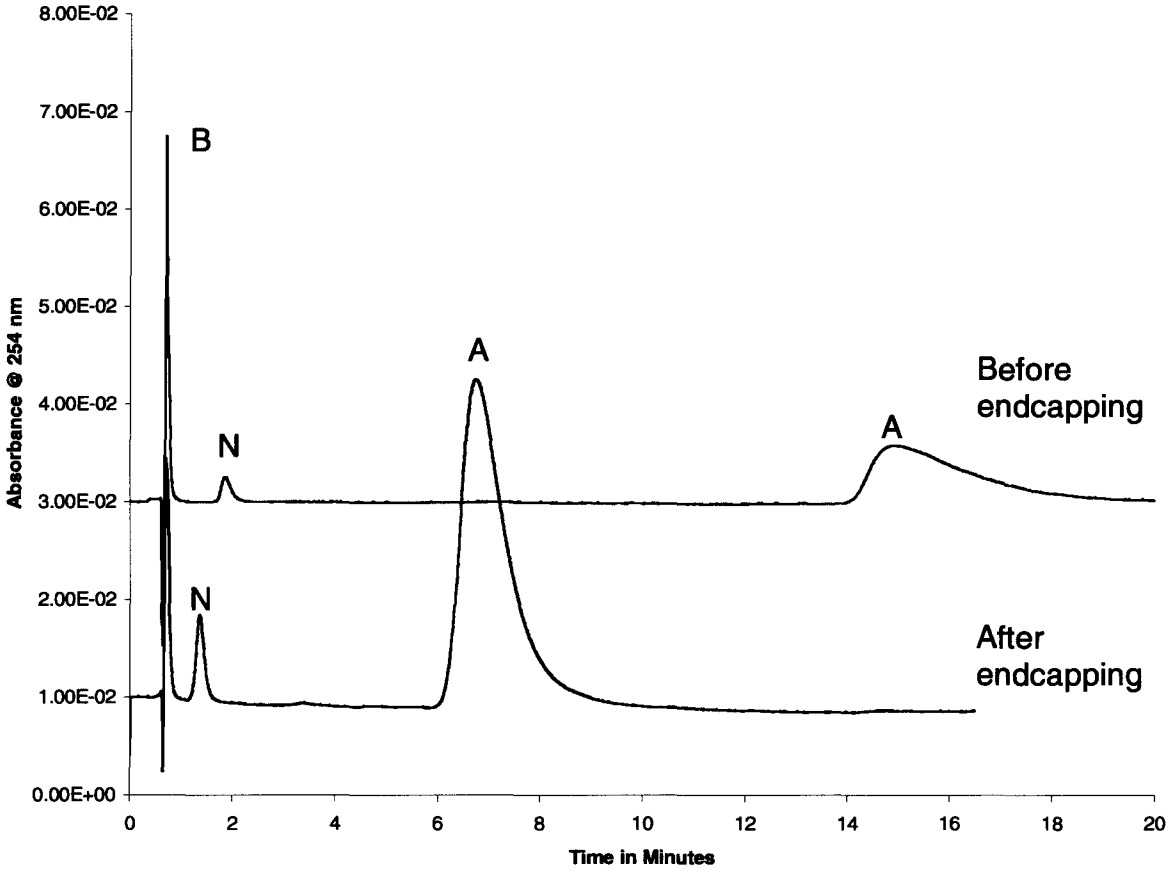


Figure 5.8: PAH series at 30% methanol: 70% water
B = benzene; N = naphthalene; A = anthracene

retention time significantly decreased (Figure 5.8). This indicates that there was an interaction with the free silanols, but any tailing after endcapping should be due to host guest complexation. To further indicate host/guest complexation a plot of log k' vs. the number of rings was made (Figure 5.9). There is a linear relationship with PAH's and the interaction of a C_{18} stationary phase. If any other interactions were occurring, such as host/guest complexation, there will be a non-linear relationship. The graph shows that there is a non-linear relationship, meaning host/guest complexation may be occurring. The error bars represent two standard deviations.

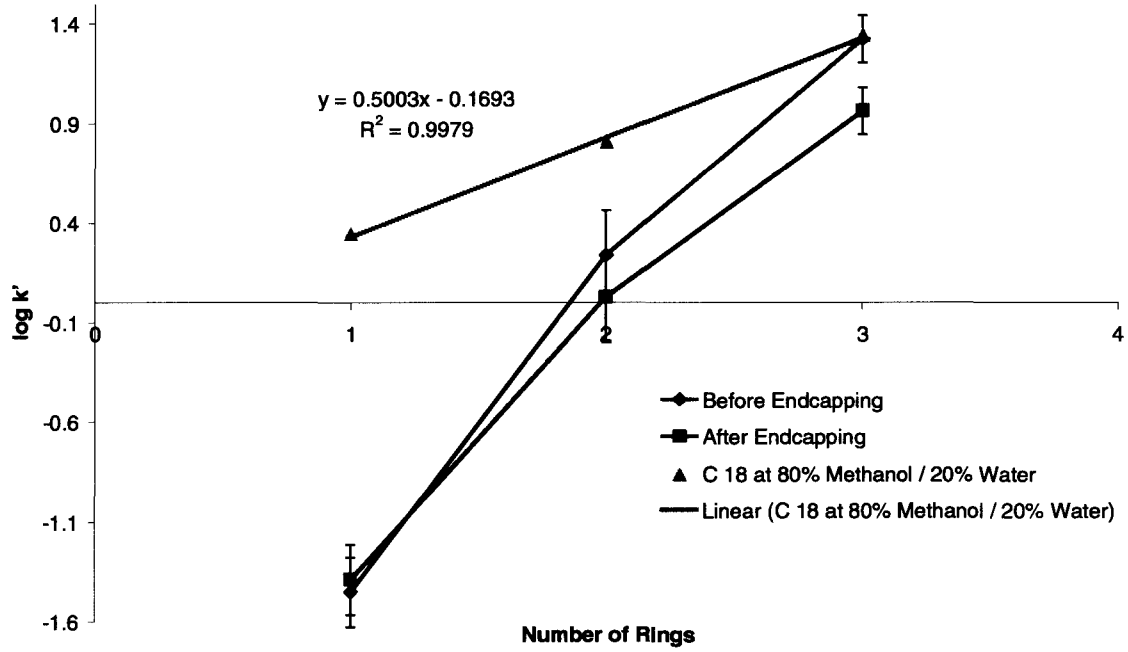


Figure 5.9: Comparison of linearity of log k' vs. number of rings for PAH series

Alkyl benzene homologous series

The alkyl benzene series does not interact with free hydroxyls the way PAH's do. Retention due to the free hydroxyl groups is minimal due to the non-polarity of the molecules. Figure 5.10 is the comparison of the alkyl benzene series before and after endcapping with TCMS. The retention of the series lengthens after the stationary phase

has been endcapped. By covering the free silanols of the surface, the stationary phase becomes less polar. The alkyl benzene series is relatively non-polar and retains longer after endcapping due to the stationary phase becoming more non-polar.

The tailing peaks are still present after endcapping, and the log k' vs. the number carbon atoms seem to have a non-linear relationship (Figure 5.11). The error bars of two standard deviations in this graph are so small the point hides them. This indicates that host/guest complexes may be forming between the alkyl benzenes and the calixarenes.

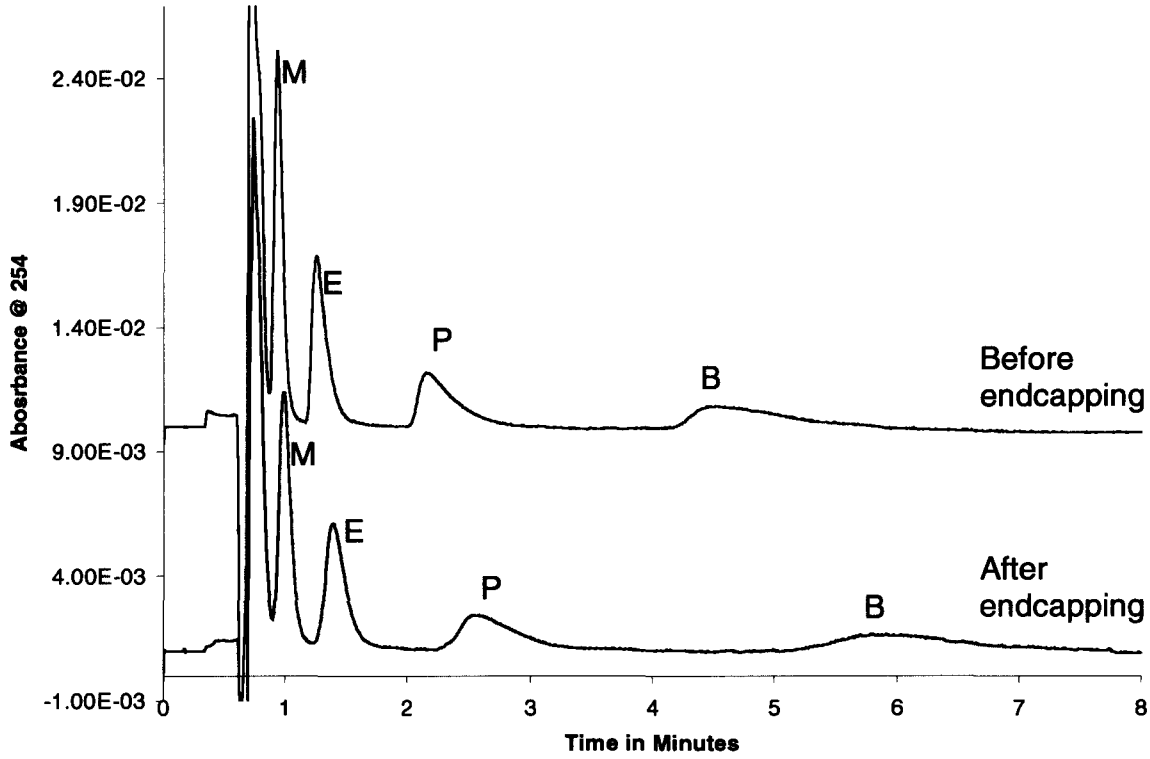


Figure 5.10: Alkyl benzene series at 15% methanol: 85% water
M = methylbenzene; E = ethylbenzene; P = propylbenzene; B = butylbenzene

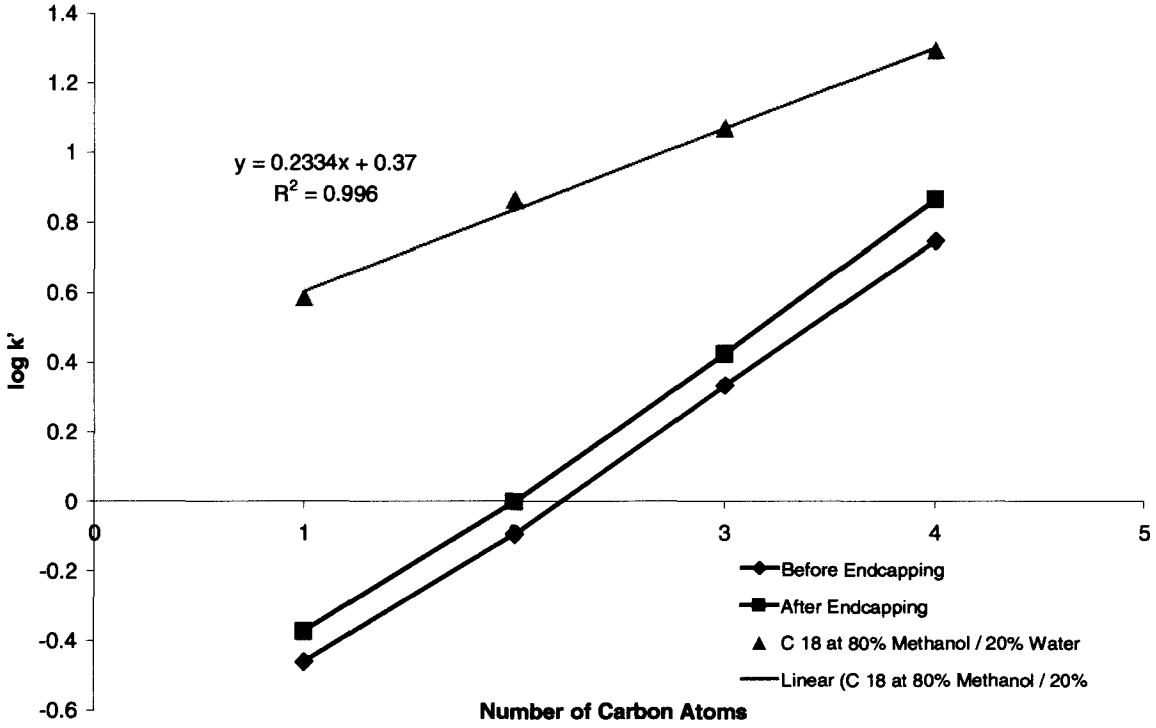


Figure 5.11: Comparison of linearity of log k' vs. number carbon atoms for alkyl benzene series

ENE series

The ENE series has a unique retention that is difficult to explain. The series contains naphthalene and anthracene along with its amino derivatives. It would be expected that the PAH's should elute faster off the column due to the minimized interaction of the –OH groups after endcapping. This series shows, however, an increase in retention time for all solutes after endcapping.(Figure 5.12 and 5.13) This can be explained in different ways. First, if the PAH's are able to interact with polar groups, such as surface silanols, the PAH's should also interact with the amino groups on the other solutes. This increased interaction could interfere in the retention of the solute on the column and cause longer than expected retention times.

The order of elution is also interesting. The position of the amino groups plays a role in retention. The 1-amino functionalized PAHs elute before the 2-amino

functionalized PAHs. The 2-amino functionalized PAHs are apparently able to fit inside the calixarene better than the 1-amino functionalized PAHs. The tailing peaks also suggest that this host/guest complexation is occurring.

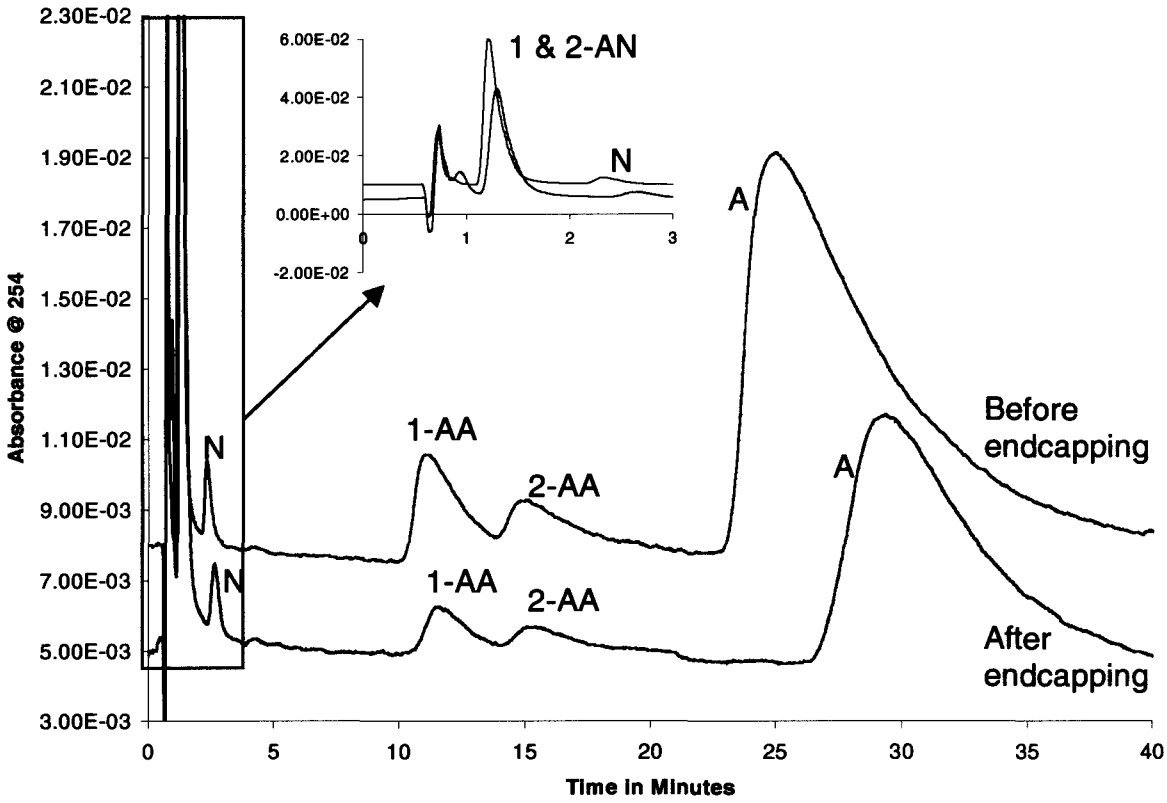


Figure 5.12: ENE series at 15% methanol: 85% water
1-AN = 1-amino naphthalene; 2-AN = 2-amino naphthalene; N = naphthalene
1-AA = 1-amino anthracene; 2-AA = 2-amino anthracene ; A = anthracene

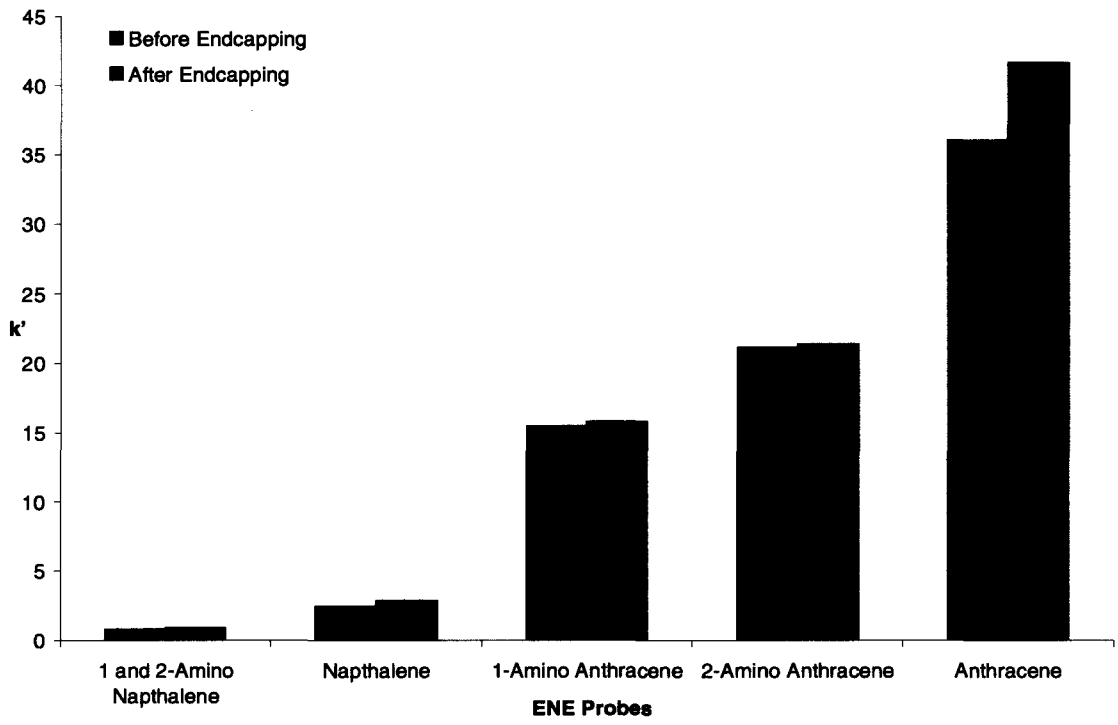


Figure 5.13: Comparison of k' values for ENE series

Knox Test

The Knox test is used to determine the extent of free silanol interaction with a solute molecule. By using a non-polar mobile phase with a non-polar stationary phase, any polar solute should not interact and elute at the dead time. If the polar solute does retain on the column, there must be a polar surface on the stationary phase interacting with the solute, which indicates the presence of free silanol groups. Figure 5.13 shows the results of the Knox test. The methanol retained longer before endcapping than after

	Capacity factor, k'
Before Endcapping	0.263
After Endcapping	0.140

Table 5.4: Comparison of k' from Knox Test

endcapping. This indicates that free hydroxyl groups, which are polar, must be present on the surface before endcapping. After endcapping the retention shortened, therefore the

endcapping must have been successful. Table 5.4 shows the capacity factors before and after endcapping of the stationary phase.

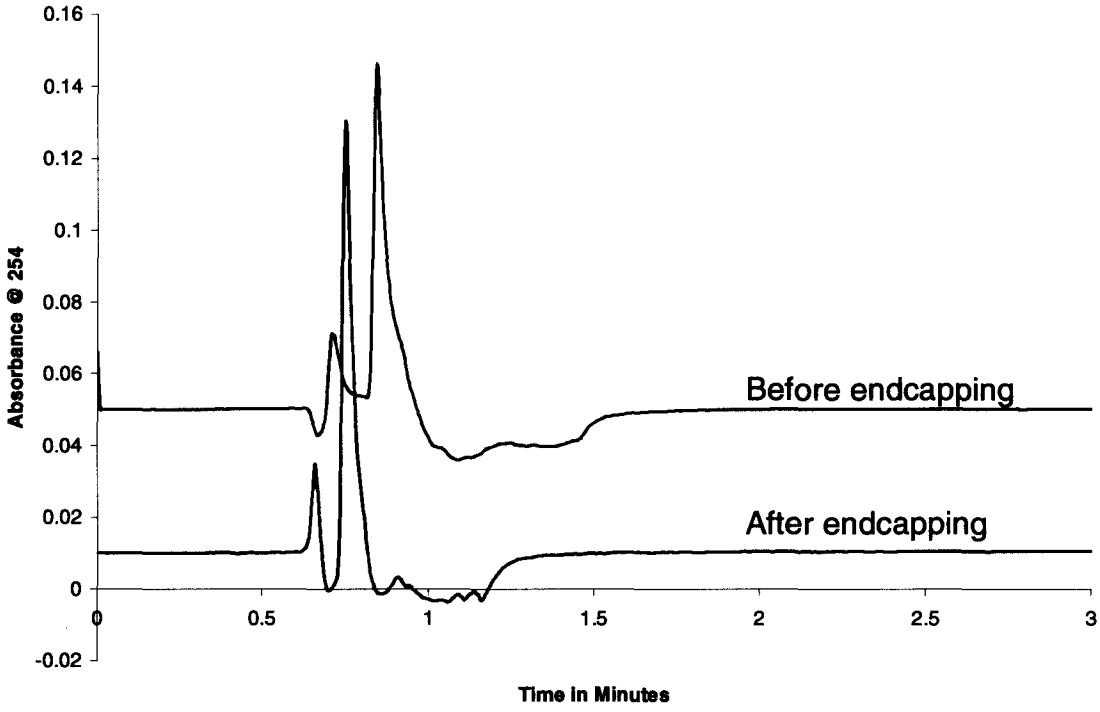


Figure 5.14: Comparison of Knox Test results

Isolated Products

Mass Spectrometry

Three other amide-calix[4]arene products were separated by flash chromatography and characterized. The products are identified by the test tube fraction number they were collected in. Fraction 9-12, 15-16 and 20-25 were collected from the 5:1 1-propanol:ammonium hydroxide column, rotoevaporated and characterized by ^{1}H, ^{13}C NMR and mass spectrometry. The Table 5.5 shows the $[M + H]^{+}$ peak found for each compound. All conditions for the LCMS and the spectra for the compounds are in the appendix (Figures and Tables A.1-A.3).

Fraction Number	Molecular Formulas (g/mol)	$[M + H]^+$ (m/z)
9-12	928.54	929.64
15-16	988.60	989.73
20-25	988.60	989.73

Chart 5.5: MS data for isolated products

Nuclear Magnetic Resonance

NMR samples were run in $CDCl_3$ for all fractions and with the combined data, proposed structures were made. Figure 5.15 represents the proposed structures for the isolated compounds in three-dimensional representation. Figures 5.16-5.18 are the

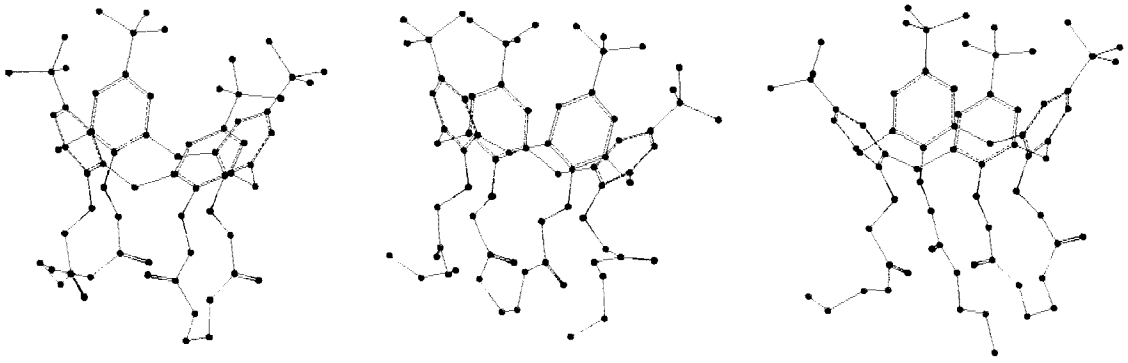


Figure 5.15:
Fraction 9-12 Fraction 15-16 Fraction 20-25

two dimensional representations and are included for clarity. Fraction 9-12 is proposed to be a "stirrup" calix[4]arene. There are two possible ways that the amide-calix[4]arene can have a "stirrup" structure, Figure 5.16 shows how the "stirrups" are linked by an adjacent amide group. Another possible structure is to have the "stirrups" form from opposite amide groups. These two structures are of the same molar mass, 928.54 g/mol, and have similar properties. Separation of these two compounds would be difficult, if not impossible.

The ^1H spectrum for fraction 9-12 (Figure 5.19) had a singlet at 1.06 ppm (1) that represented the t-butyl groups that are on the phenyl ring. There are two sets of

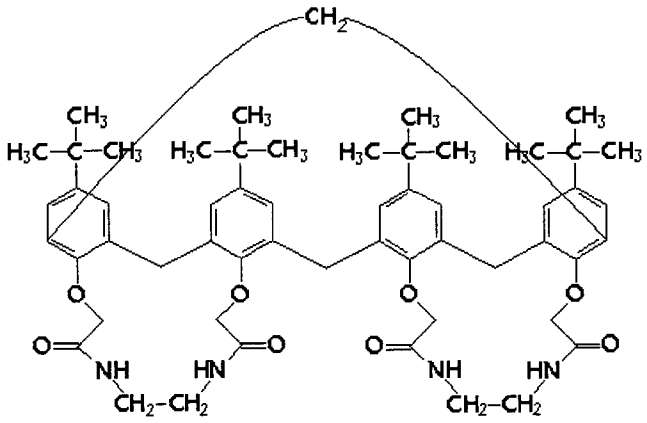


Figure 5.16: Fraction 9-12

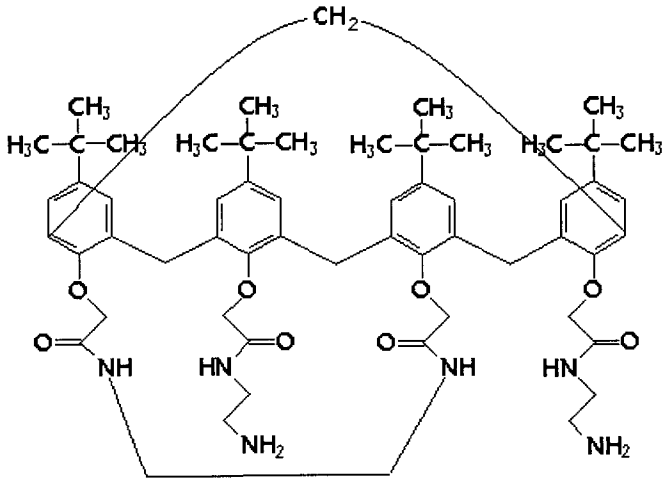


Figure 5.17: Fraction 15-16

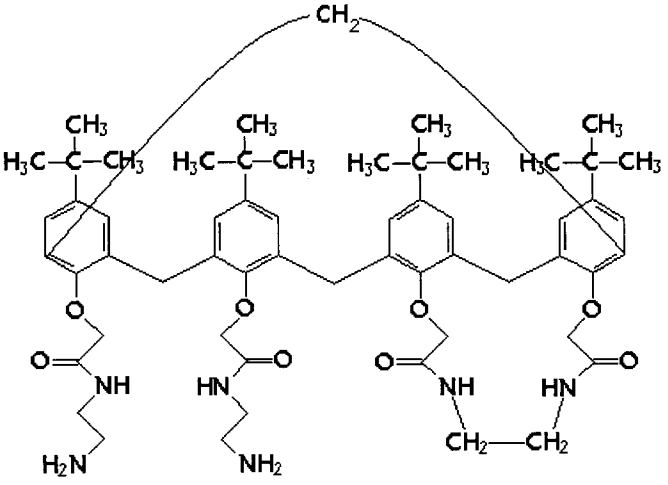


Figure 5.18: Figure 20-25

doublets of doublets at 3.17 ppm and 4.19 ppm (2) that represented the methylene bridges linking each ring. The additional set could represent the mixture of adjacent-linked and opposite-linked calixarenes. The multiplet at 3.62 ppm (3) corresponded to the CH_2 groups that link the nitrogen groups together. This multiplet signified the mixture of calixarenes. The CH_2 group between the oxygen groups showed as a doublet (4). There are two sets of these doublets, one at 3.91 ppm and one at 4.73 ppm, one for each structure. The CH group in the ring also has two peaks, each representing each structure, at 6.78 ppm (5). The broad singlet at 7.41 ppm (6) represented the NH groups in the stirrup.

The ^{13}C spectrum was more straightforward (Figure 5.20). The methylene group appeared at 32.2 ppm (A), this was shifted due to the ring structure and was confirmed in an APT spectrum. The t-butyl groups appeared as a peak at 32.6 ppm (B) and the C atom connecting them appeared at 35.2 ppm (C). The CH_2 groups that linked the stirrups appeared at 39.0 ppm (D) and the CH_2 between the oxygen groups appeared down field at 75.8 ppm (E). The CH in the ring is represented by the peak at 126.7 ppm (F) and the C groups in the ring are represented by peaks at 33.4 ppm, 146.7 ppm and 153.4 ppm (G, H, I). The carbonyl group is shifted downfield at 171.3 ppm (J).

Fraction 15-16 was determined to have a structure of one "stirrup" connected through opposite amide bonds and two free amide groups. The molar mass was determined to be 988.60 g/mol and agreed with the mass spectra. The ^{1}H and ^{13}C NMR spectra did confirm the structure. (Figure 5.21 and 5.22) The ^{1}H spectrum for fraction 15-16 had quite a few similarities to the characterized amide-calix[4]arene. The ^{1}H spectrum showed a strong singlet at 0.80 ppm and 1.30 ppm. These corresponded to the

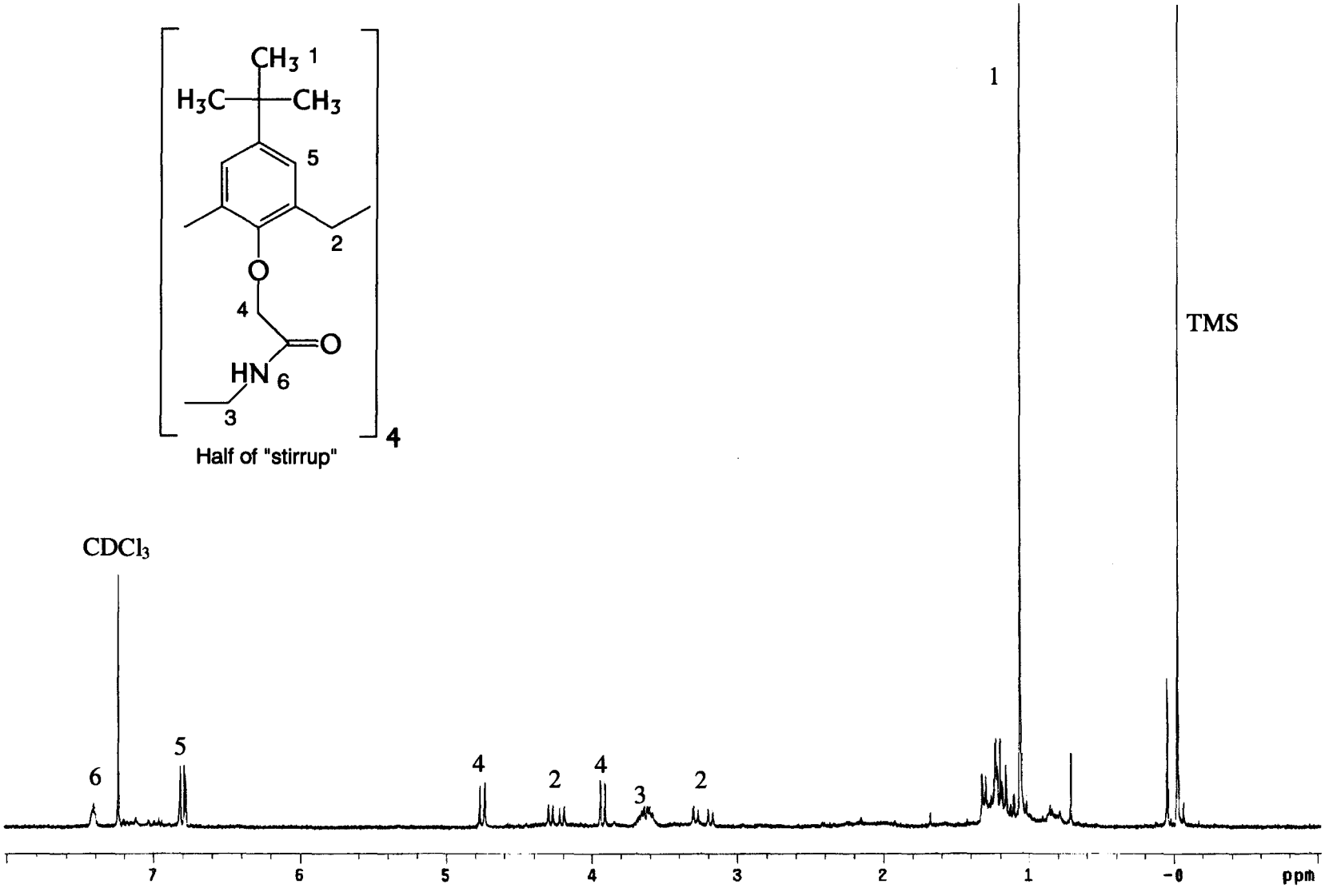


Figure 5.19: ^{1}H NMR spectrum of fraction 9-12

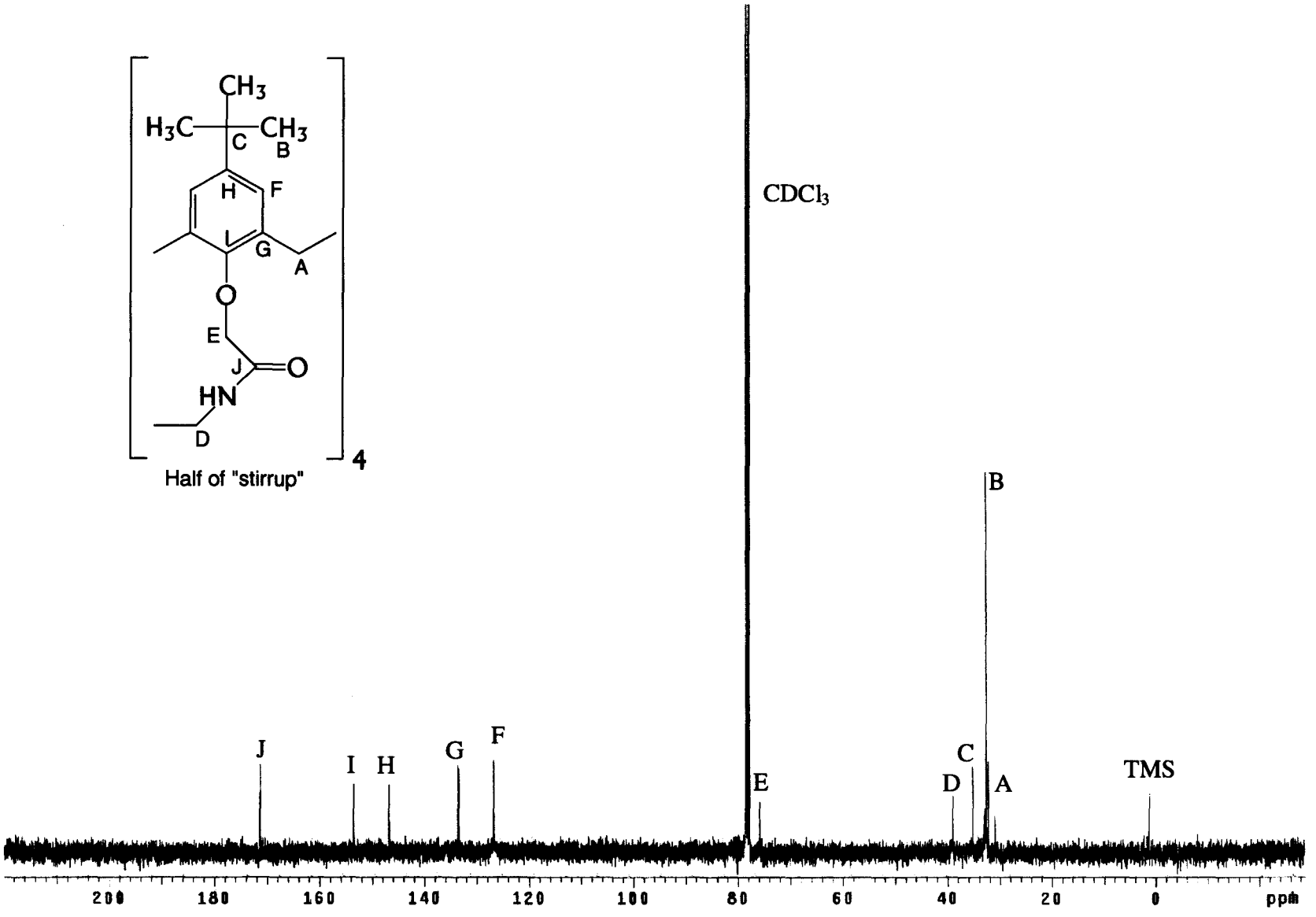


Figure 5.20: ^{13}C NMR spectrum of fraction 9-12

tert-butyl groups in different environments, one set in the "stirrup" and one set in the free tethered groups (1). The broad singlet at 2.76 ppm is indicative of the electron rich NH_2 group (2). The triplet at 2.96 ppm (3) and the multiplet at 3.47 ppm (5) were the methylene groups in the free amide chain. The methylene bridge between the phenol groups appeared as two sets of doublets at 3.23 ppm and one hidden at 4.41 ppm (4). The multiplet at 3.47 ppm (6) was the CH_2 group in the stirrup that connected the NH groups together. The CH_2 group next to the carbonyl in the stirrup showed as a singlet at 4.26 ppm (7) and in the free amide tether was a singlet at 4.38 ppm (8). These groups were shifted more downfield due to the surrounding oxygens. The protons in the phenyl groups show as a singlet at 6.47 ppm (9) for the non-cyclized group and a singlet at 7.10 ppm (10) for the cyclized group. The NH groups appeared as broad singlets at 8.19 ppm (11) for the free amide group and 8.63 ppm (12) for the cyclized group.

The ^{13}C spectrum contains all the peaks for the carbon groups in the cyclized and non-cyclized groups as well. It was more difficult to determine which peak was in the cycle conformation; therefore the groups were identified together. The methylene bridges were the first peaks at 31.9 ppm (A) and the set of different t-butyl groups appeared at 33.2 ppm and 32.9 ppm (B). There are 2 peaks at 34.9 ppm and 35.4 ppm that also represented the different C atom that connect them (C). The CH_2 groups that connect the stirrup and appeared in the non-cyclized group between the amino groups appeared as two peaks at 39.0 ppm and 42.0 ppm.(D). The peaks at 76.2 ppm and 76.6 ppm were the CH_2 groups between the oxygen groups.(E) There are 2 peaks that represented each C and CH atom in the ring (F, G, H, I). The carbonyl appears at 170.6 ppm and 171.4 ppm (J).

There were problems obtaining a NMR spectrum of fraction 20-25. It could be that the spectrum for this calixarene is too complicated to assign, for it is a less symmetric molecule. It could also be a mixture of compounds. The time and effort required to characterize this sample was beyond the scope of this research, and the NMR spectra are included in the appendix.(Figures A.4 and A.5)

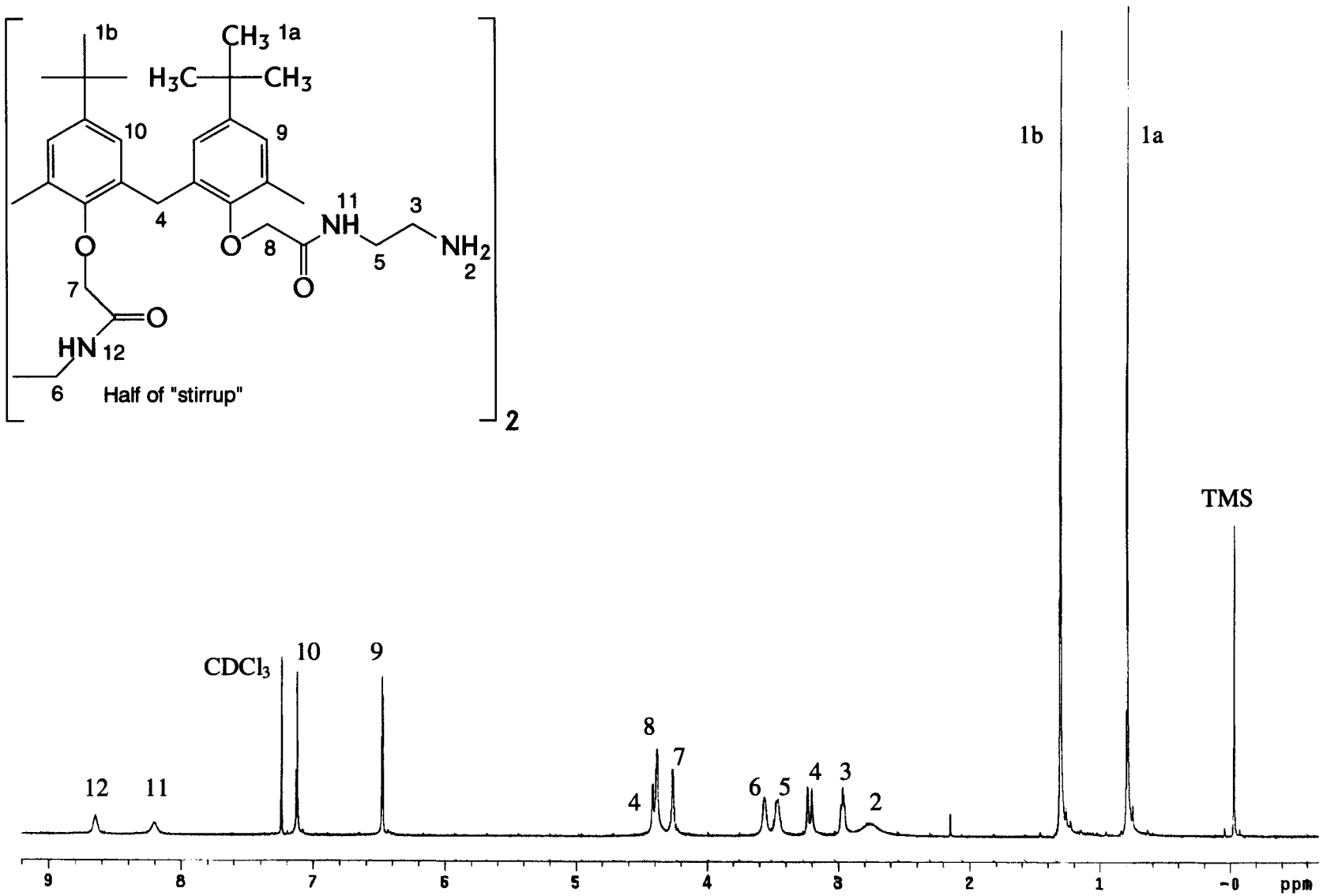


Figure 5.21: ^{1}H NMR spectrum of fraction 15-16

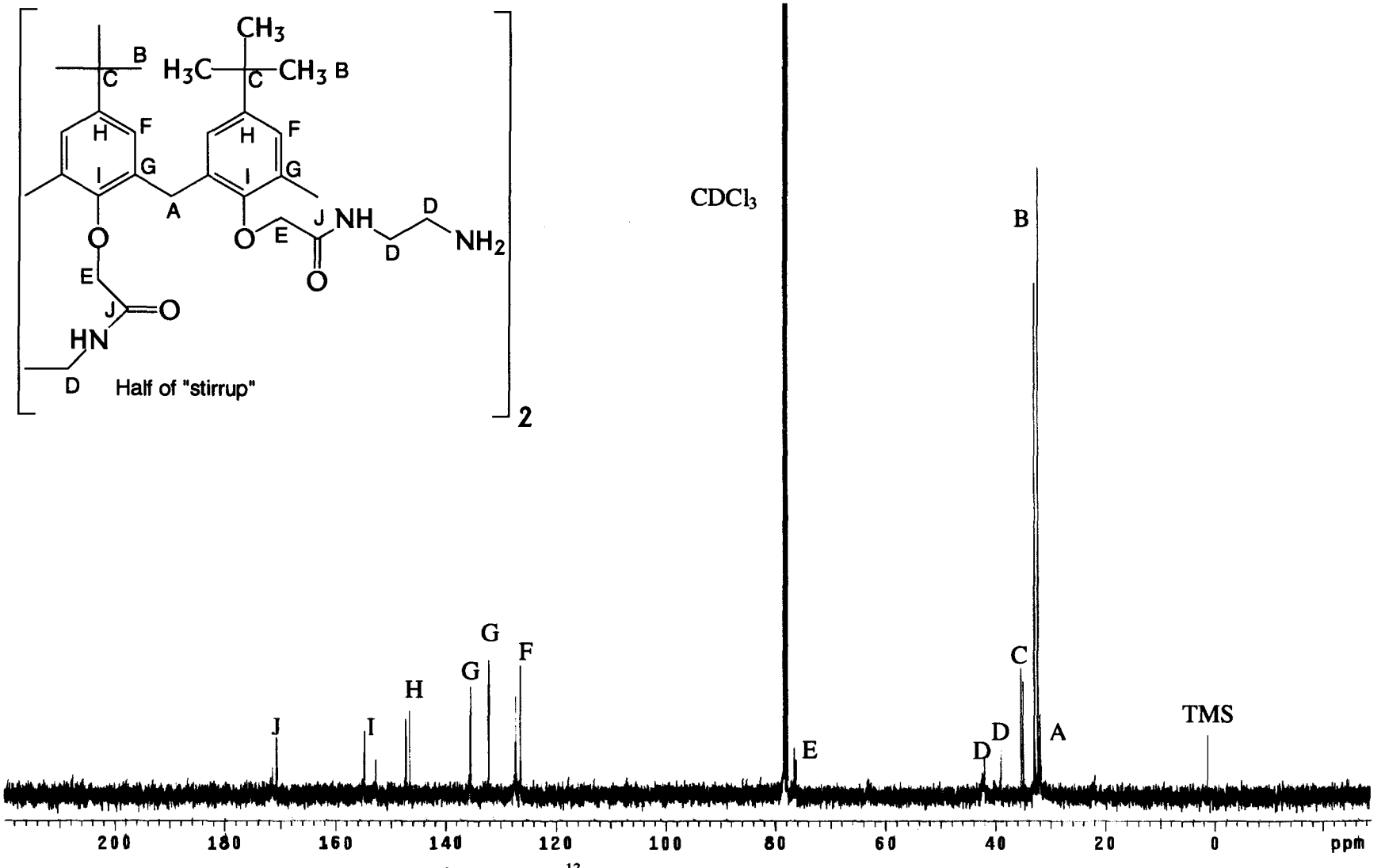


Figure 5.22: ^{13}C NMR spectrum of fraction 15-16

Chapter VI

Conclusions

The spectroscopic characterization of tetra-t-butyl-tetra [N–(2-amino-ethyl)-2-oxy-acetamide] calix[4]arene determined that there were four free amino tethers available for attachment. The amide-calix[4]arene bound stationary phase was washed extensively to show that the spectra obtained was due to bound calixarenes and not to adsorbed or absorbed calixarenes. The fluorescence and UV spectra were similar to that of the free tetra-t-butyl-tetra [N–(2-amino-ethyl)-2-oxy-acetamide] calix[4]arene. Elemental analysis further determined that there was attachment of the 3-aminopropyldimethylethoxysilane, glutaraldehyde and the tetra-t-butyl-tetra [N–(2-amino-ethyl)-2-oxy-acetamide] calix[4]arene to Nucleosil. It was established that the surface coverage of the amide-calix[4]arene on the Nucleosil was 0.507 mol/m^2, a higher value than of other reported calixarene stationary phases. This could be due to the larger pore size of the Nucleosil.

The chromatographic characterization indicated that the free silanols were present on the silica surface and that endcapping the stationary phase is necessary to eliminate the secondary interaction. After endcapping, peak tailing and non-linear relationships of log k' vs. number of homologues implied that host/guest complexes were forming with the solutes and the calixarenes. It is concluded that the attachment of the tetra-t-butyl-tetra [N–(2-amino-ethyl)-2-oxy-acetamide] calix[4]arene was successful and that it is useful as a reversed-phase stationary phase.

FUTURE WORK

Three other amide-calix[4]arenes were isolated from the synthesis by flash chromatography. The original synthesis was stirred under nitrogen for five days. If the synthesis was to be repeated, refluxing the reaction during this time may give more of the tetra-t-butyl-tetra [N–(2-amino-ethyl)-2-oxy-acetamide] calix[4]arene and less of the other products.

The isolated products should be further characterized and considered for use in chromatography. The double stirruped calixarene may be useful as a mobile phase additive.

The synthesized column should have further chromatographic studies performed on it. The ENE series should be analyzed further and rerun to determine the cause of lengthened retention times after endcapping. Napthols and naptholamines should also be investigates because they are soluble in alcohols and are known to bind with calixarenes. It would be interesting to see if these results differ from the ENE series in retention behavior.

CP/MAS NMR studies should also be done on the amide-calix[4]arene stationary phase. This should further show that attachment of the calixarene to the silica was successful. One study should be a host/guest complexation comparison of a C_{18} phase and the calix[4]arene phase with anthracene. The anthracene should have no interaction with the C_{18} phase but should complex with the calix[4]arene. This study would further support the complexation of solutes on a calixarene stationary phase.

APPENDIX

APCI Temp	454 °C	Polarity	Positive
Capillary Current	203 nA	Skim 1	62.5 V
Scan Range	15.00-2000.00 m/z	Trap Drive	54
Corona Current	10159 nA	Dry gas Temp	351 °C
Corona Voltage	2500 V	Octopole	2.4 V

Table A.1: Acquisition parameters of Esquire LCMS for Fraction 9-12

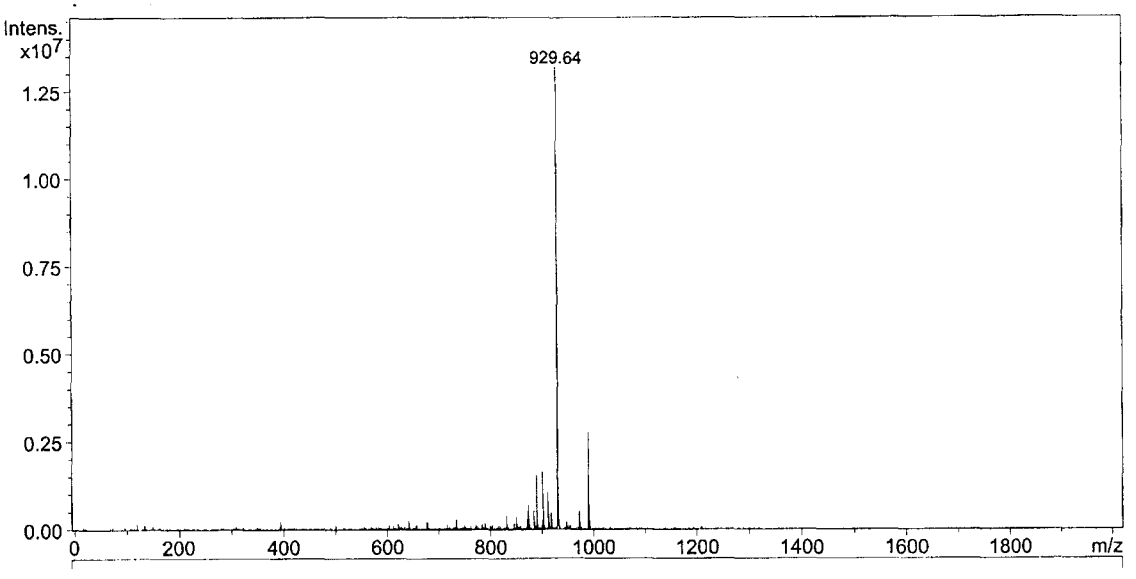


Figure A.1: Mass spectrum of Fraction 9-12

APCI Temp	457 °C	Polarity	Positive
Capillary Current	166 nA	Skim 1	66.5 V
Scan Range	15.00-2000.00 m/z	Trap Drive	59
Corona Current	10868 nA	Dry gas Temp	351 °C
Corona Voltage	2800 V	Octopole	2.4 V

Table A.2: Acquisition parameters of Esquire LCMS for Fraction 15-16

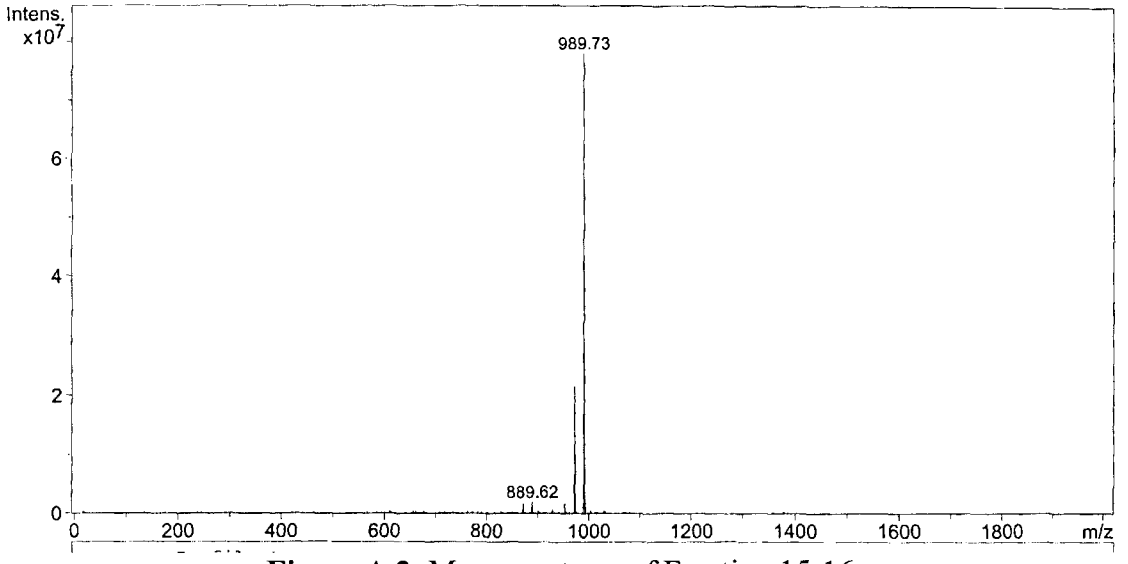


Figure A.2: Mass spectrum of Fraction 15-16

APCI Temp	458 °C	**Polarity**	Positive
Capillary Current	162 nA	**Skim 1**	52.5 V
Scan Range	15.00-2000.00 m/z	**Trap Drive**	59
Corona Current	10390 nA	**Dry gas Temp**	352 °C
Corona Voltage	2800 V	**Octopole**	2.4 V

Table A.3: Acquisition parameters of Esquire LCMS for Fraction 20-25

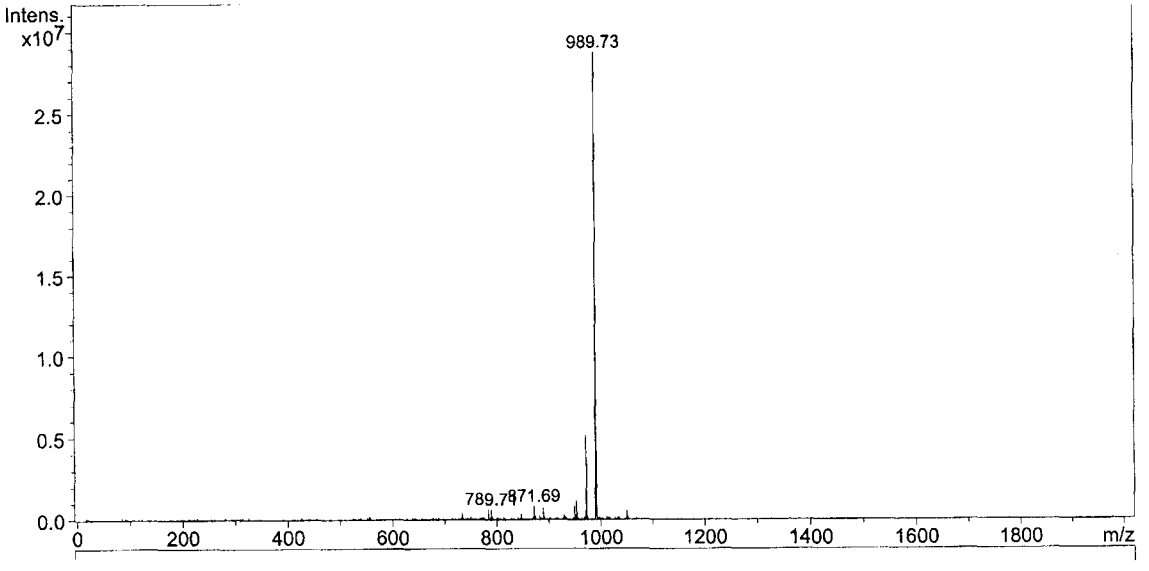


Figure A.3: Mass spectrum of Fraction 20-25

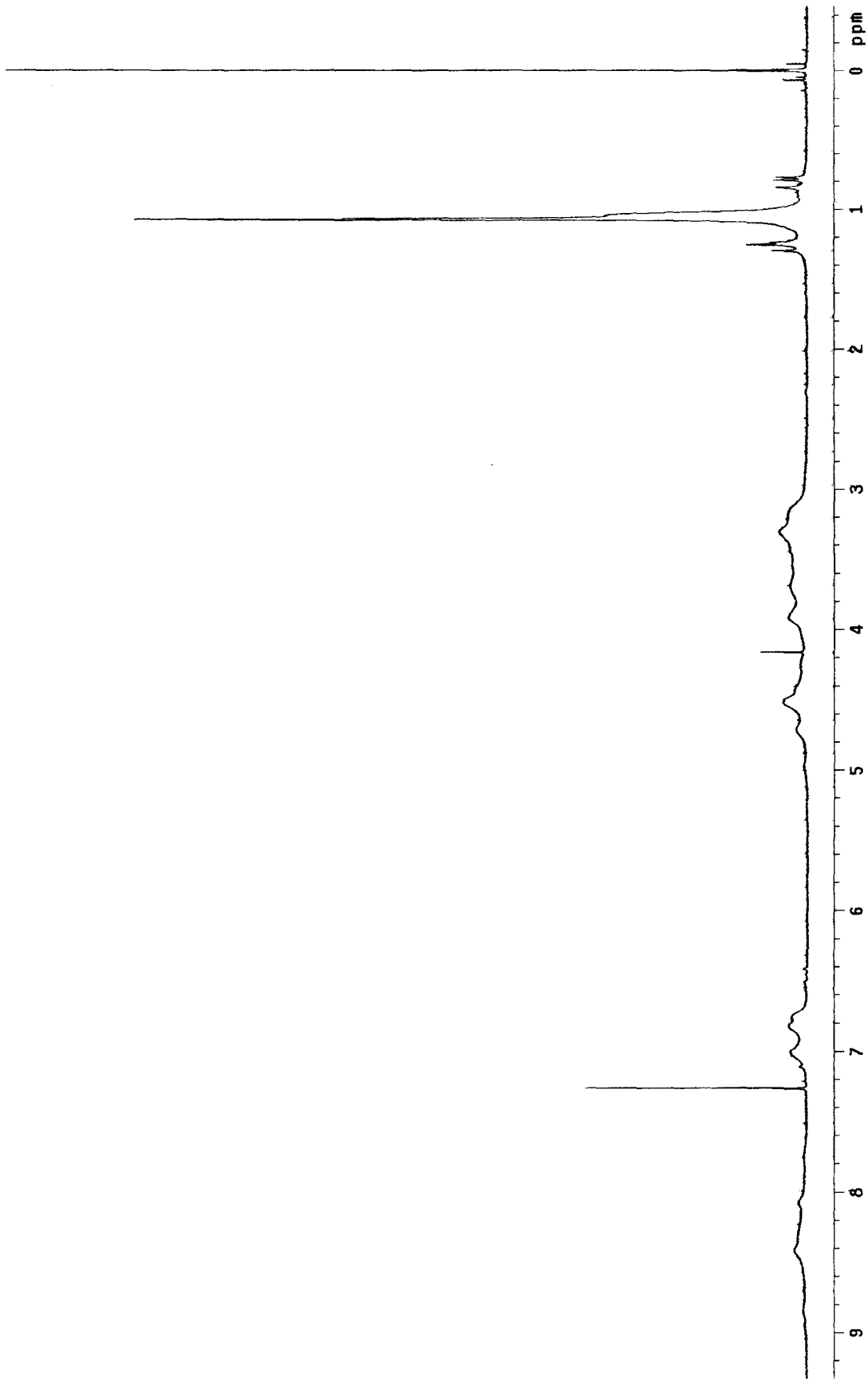


Figure A.4: ^{1}H NMR spectrum of fraction 20-25

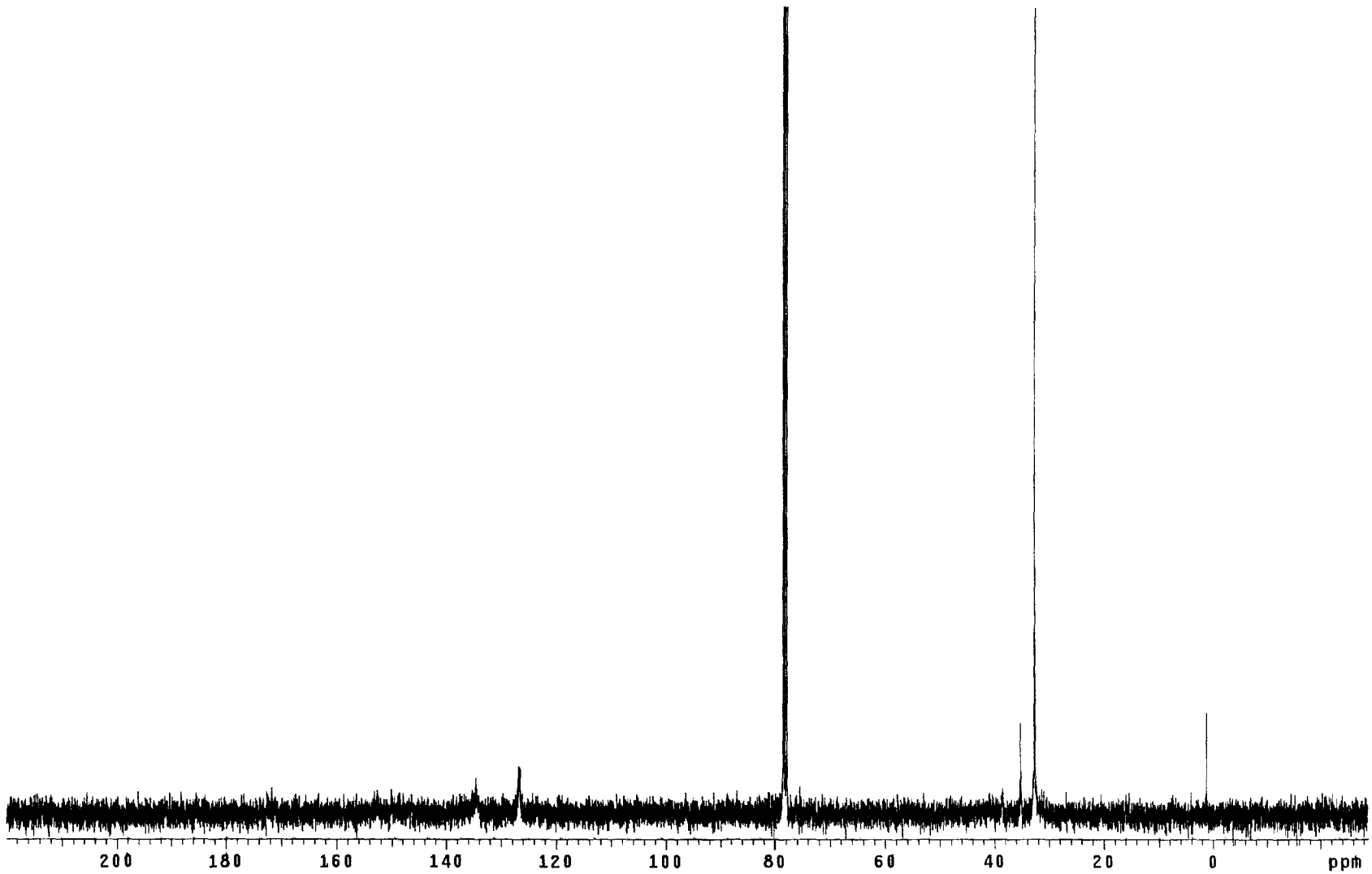


Figure A.5: ^{13}C NMR spectrum of fraction 20-25

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