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by

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Committee Chair: Dr. David B. Smithrud

Abstract

Given the complex nature of proteins it is often beneficial to examine pieces of their sum total in order to simplify the investigative process. One powerful method is to create and investigate synthetic mimics of proteins. Mimics have the advantage of being more stable and more easily manipulated than true proteins. Herein, the mimetic approach is used to investigate the "hot spot/O-ring" relationship in protein binding domains. Many protein binding domains have been found to incorporate a "hot spot" of three to five amino acids that supply most of the binding energy and an "O-ring" of 20-30 amino acids whose function is not fully understood. In our studies we utilize a type of compound called rotaxanes, which afford us a relatively simple synthetic route toward a dynamic system that incorporates mimics of both the "hot spot" and "o-ring" moieties.

Acknowledgments

I have had the benefit of wonderful support from a wide variety of people in the development of my career. I would like to thank Dr. David Smithrud for serving as the chairperson of my Research Advisory Committee.

I would also like to thank my committee members, Dr. Apryll Stalcup and Dr. R. Marshall Wilson, for their time and critique of this thesis. I would also like to extend my gratitude to my group members, past and present, as well as members of the organic division for their aid and advice over the past few years. In particular, I would like to acknowledge Juris Fotins and Vadims Dvornikovs for being good friends and great people.

Gratitude is also extended to Dr. David J. Hart, my undergraduate advisor, for preparing me for graduate school and being a great guide into the life of chemistry.

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GO BUCKS!!

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Introduction

Most biological processes are controlled by protein interactions. The forces that govern protein complexes are well understood, but how nature employs these forces to obtain highly selective ligand recognition still needs to be fully determined. Another difficult challenge facing those who view protein binding domains as potential targets of interest is that these domains can be quite large and relatively flat. One long range goal of the Smithrud research group is to create small molecules that specifically bind protein surfaces. Investigating this type of compound will provide fundamental information on protein properties. Solving the mysteries of protein binding domains is necessary for the design of small molecules that can disrupt protein-protein interactions and is the goal of my project.

To rationally design small molecules that specifically bind proteins, one must know the relationship between the spatial arrangement of amino acid side chains and their resulting potential energy, which is used for ligand recognition.

Investigation of 20 protein binding domains through crystallography and mutation studies have shown that their binding epitopes are split into a large structural epitope (10-33 amino acids) and a much smaller functional epitope (2-5 amino acids).[1,2] Mutations in the functional epitope weaken protein complexes dramatically, whereas changes in the structural epitope have relatively small effects. For example, approximately 30 amino acids of the human growth hormone receptor (hGH_R) are buried in the complex with the human growth hormone, but only Trp104, Trp169, and Arg43 provide the majority of the binding energy (Figure 1).[3,4]

Structural analysis has so far been unable to identify potential hot spots on protein surfaces.[4] Moreover, it is not known whether an amino acid in a hot spot is important by itself or whether it interacts with other residues in a cooperative manner.

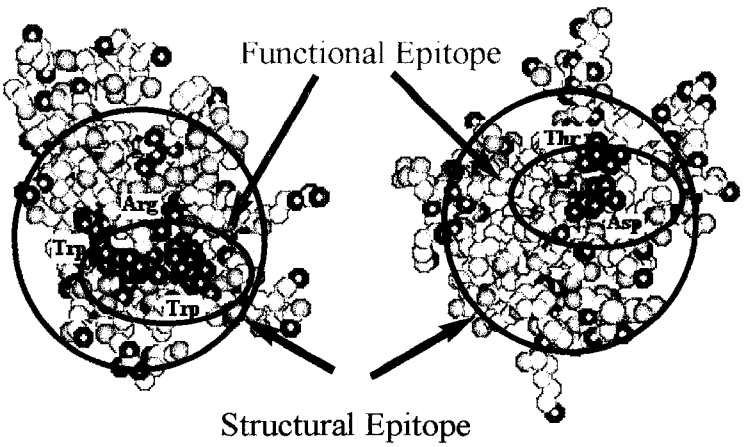


Figure 1: Taken from Clackson T., Science, 1995, 267, 383-386

Investigating these complex domains and exploiting their properties through mimetic chemistry requires a unique system that contains both a functional and a structural epitope. Multiple domains have to be readily combined in order to provide the multiple mimics necessary to discover how nature controls the delicate interplay between amino acid residues to achieve ligand recognition. The use of rotaxanes will fulfill these requirements.[5] Their unique architecture composed of interchangeable parts: tethers, blocking groups, and rings (Figure 2), reduces the synthetic burden of creating many compounds. More importantly, the ring slides on the tether, allowing the complex to self adjust to a more energetically favorable state.

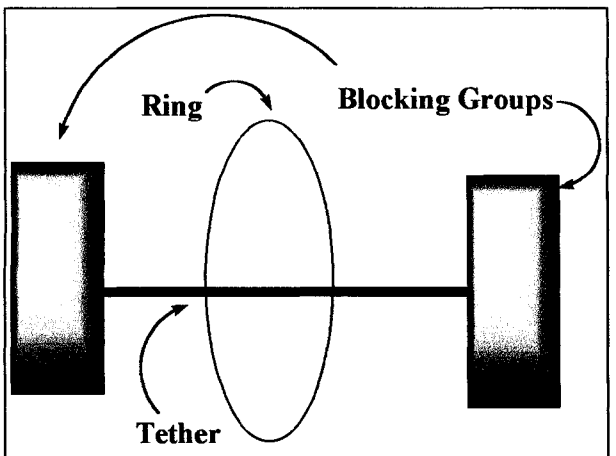


Figure 2: Cartoon of a rotaxane

Having a mobile O-ring can reduce the difficult task of engineering binding domains to within a few angstroms for selective recognition. For our systems, the hot spot is incorporated as a blocking group and the rotaxane's ring acts as the O-ring, whose motion will be controlled by ligand recognition.

Discussion

Previous work in the Smithrud group towards the construction and study of hot spots resulted in the exploration of cationic amino acids (Arg or Lys) covalently linked to scaffolds containing aromatic rings, mimicking the hGH_R.

Templates 1 and 2 were synthesized and studied[6], showing that there is a cation-π interaction between the aromatic rings and the cationic charges of the side chains. Potentiometric titrations were conducted in a variety of solvents to determine the strength of this interaction, and it was determined that the cation-π interaction is strongest in a hydrophobic environment (i.e. the environment found on the interior of the hGH_R).

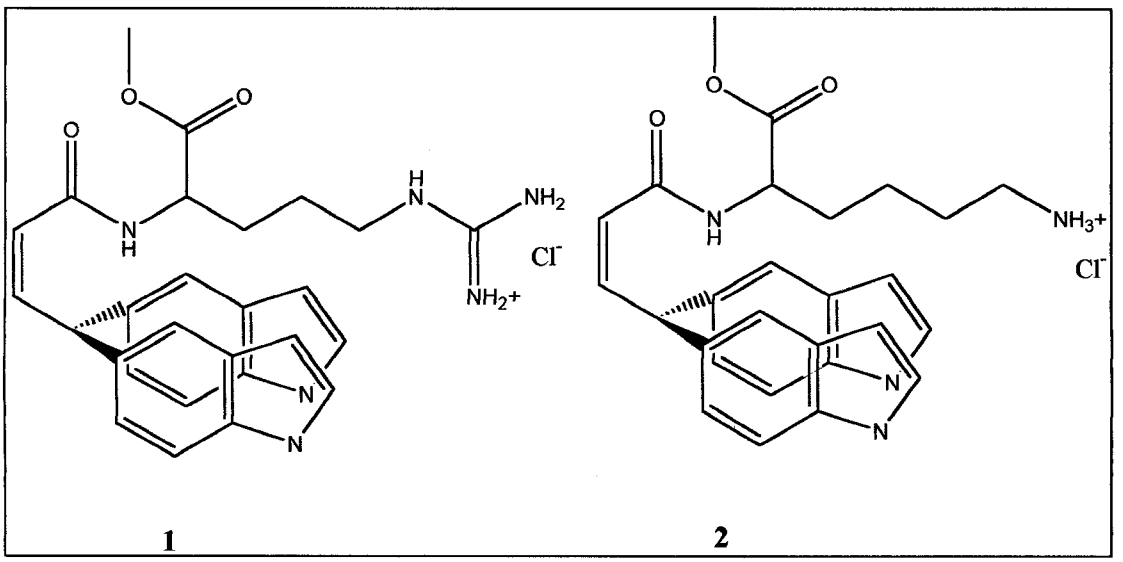
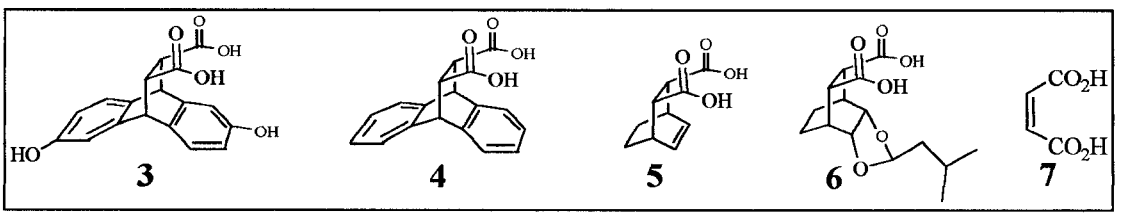


Table 1. pK_a's of the amino acid side chain of compounds	60% H2O/ 40% DMSO	100% DMSO	75% THF/ 25% DMSO
1	6.67 ± 0.06	7.70 ± 0.14	5.72 ± 0.05
2	7.52 ± 0.05	7.33 ± 0.14	4.86 ± 0.02

Another mimic investigated had a stacked arrangement of a carboxylate over an aromatic ring (SACA) that binds the side chain of Arg and Lys.[7] This arrangement was observed in the complex of the antibody HyHEL-5 and lysozyme,[8] hGH_R and its hormone,[9] fibrinogen to the tripeptide Gly-Pro-Arg,[10] and within the salivary α-amylase protein (Glu27-Arg387).[11] To determine the driving force for salt-bridge formation, a series of templates (**3-7**) were synthesized, and NMR and potentiometric titrations were performed (Table 2).



It was determined that the aromatic ring raised the pKa's of the carboxylates and partially desolvated them, making them more reactive for ligand binding. The alkyl portion of the amino acid side chain of *N*-acetyl arginine methyl ester was necessary for complex formation, since there was no binding observed in the cases of the simple cations guanidinium (GH^+) and K^+. It was concluded that interactions between the alkyl chain and aromatic rings, and not aliphatic chains below carboxylates (e.g., template **6**), are important in water. Salt bridge formation is

stabilized by the more hydrophobic environment provided for by the aromatic ring and alkyl chain and the raise in pKa. These salt bridges may also play a role in protein folding.

Table 2. Changes in pKas and free energy values for diacids at 298 K as determined by potentiometric titrations.

Cmpd	Cond	pK_{a2}[a]	ΔG° (kcal/mol)
3	Neat	6.1	-
	1M KCl	5.3	-0.9
	1M GH^+	5.3	-0.9
	0.05M Arg	5.1	-3.1 (-1.1)[b]
4	Neat	6.4	-
	1M KCl	5.4	-1.3
	1M GH^+	5.3	-1.6
	0.05M Arg	5.7	-2.6 (-1.1)
5	Neat	6.6	-
	1M KCl	5.7	-1.2
	1M GH^+	5.5	-1.5
	0.05M Arg	6.5	-1.2 (NB)[c]
6	Neat	5.2	-
	1M KCl	4.9	0.1
	1M GH^+	4.6	-0.6
	0.05M Arg	5.1	-0.8(NB)
7	Neat	6.0	-
	1M KCl	5.3	-0.8
	1M GH^+	5.2	-0.9
	0.05M Arg	5.9	-1.1(NB)

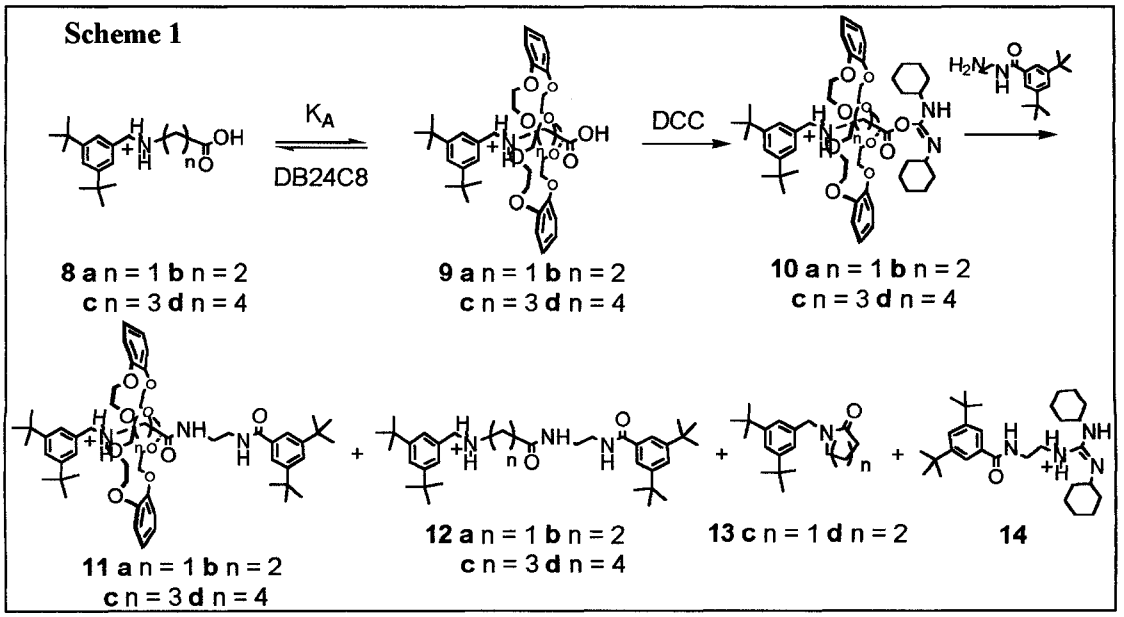
[a]errors are ± 0.1, [b]values in parentheses were obtained form NMR titrations, [c]NB means no binding detected

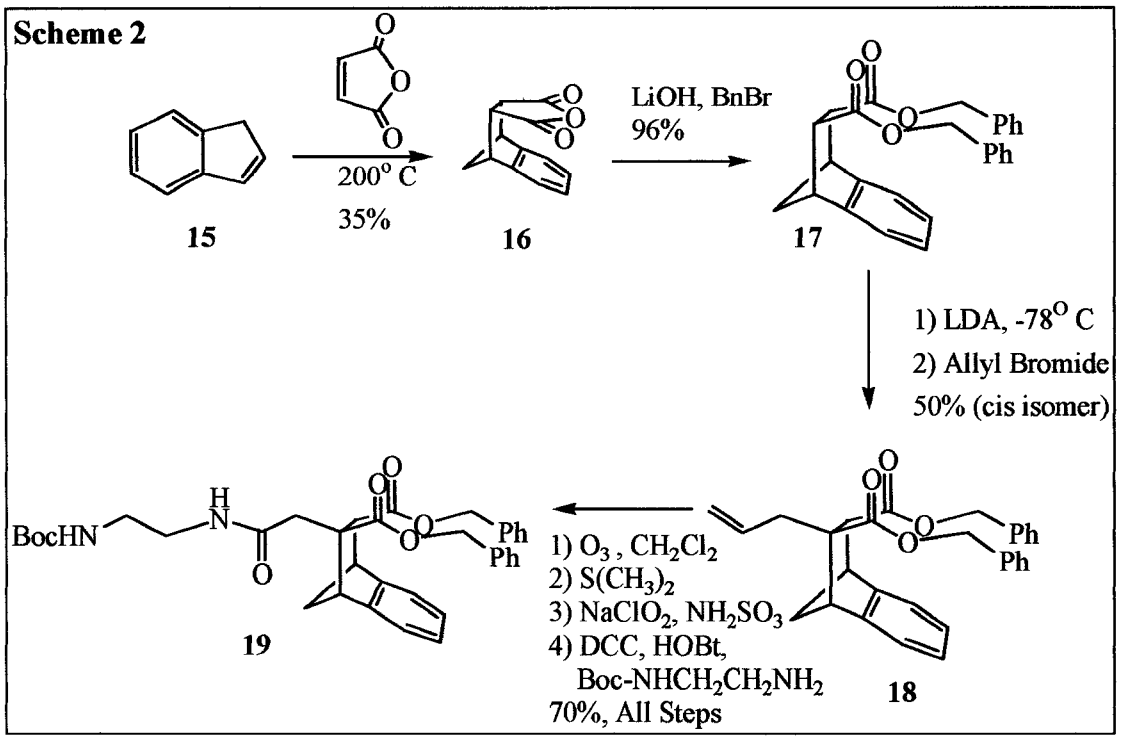
Results

Utilizing this information and incorporating the SACA template as our hot-spot, we could begin to work towards our goal of elucidating the role of the O-ring. To accomplish this we decided to employ rotaxane technology.

Previously in our group methodology was developed to easily produce rotaxanes in good yields. We have created novel DCC-rotaxanes that can be added to amino groups to make rotaxanes in a single, high yielding step (50-70%) (Scheme 1).[12] The DCC-rotaxane contains the blocking group, tether, and ring. They are formed through ring threading, which is driven by cation-dipole interactions between the tether's ammonium group and the oxygen atoms of the crown ether. DCC is added to lock the ring onto the tether. DCC-rotaxane **10d** was stable to purification via column chromatography and HPLC. We can now incorporate the SACA template as one of our blocking groups (displacing DCU) provided we have the requisite amino group available.

Scheme 1





The synthesis of the SACA template is outlined above (Scheme 2). Starting with commercially available indene, which underwent a Diels-Alder reaction with maleic anhydride, forming compound **16**. Indene is a non-obvious diene in the reaction. By heating indene to 198° C a thermal isomerization takes place, where indene becomes iso-indene (Figure 4).[13]

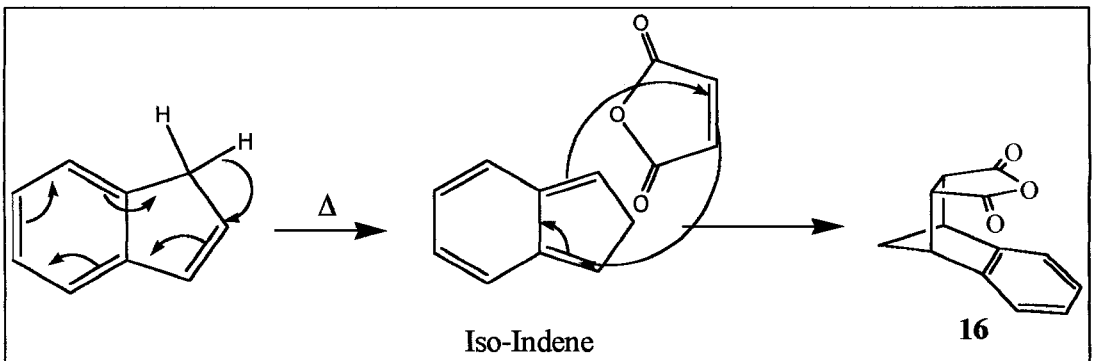
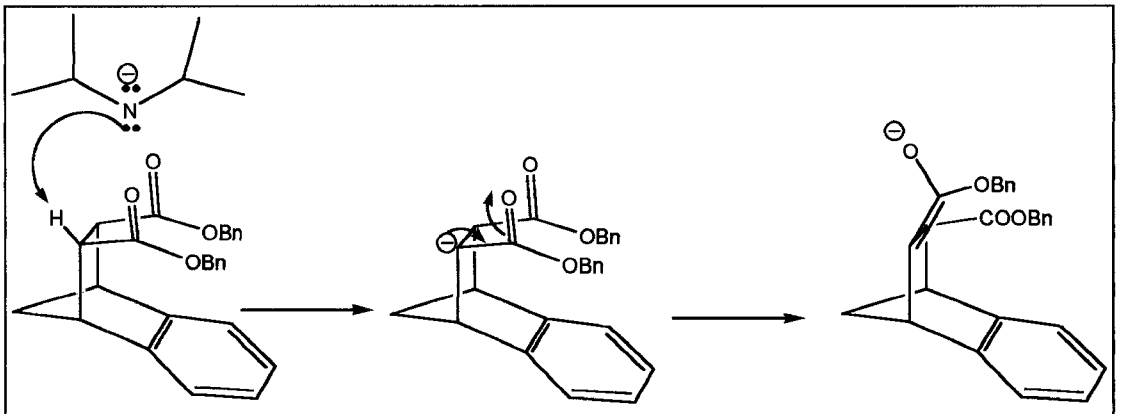


Figure 4: Mechanism of the thermal isomerization of indene

The anhydride **16** was then opened and protected as benzyl esters (**17**). Upon treatment with LDA, an enolate is formed (Figure 5). This enolate could then be allylated with allyl bromide. Attack from either side of the enolate is possible. The

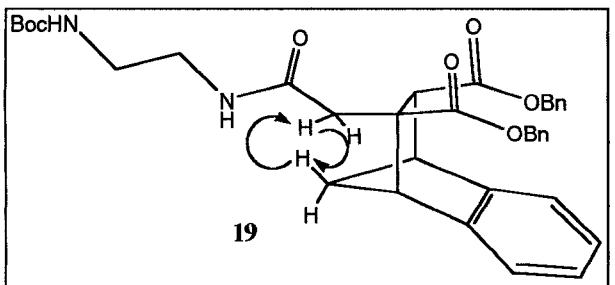
isomer with cis-carboxylate groups was formed preferentially due to partial blocking of the "endo" face from the steric congestion of the template.



17 17a

Figure 5: Formation of enolate **17a**

Alkene **18** was then oxidized by ozonolysis, the ozonide was reduced to the aldehyde with dimethyl sulfide, and finally the aldehyde was oxidized to the carboxylic acid. Ethylene diamine (EDA) was mono-protected with di-*tert*-butyl dicarbonate.[14] The carboxylic acid was activated with DCC and allowed to react with the mono-Boc-EDA, giving compound **19**.



19

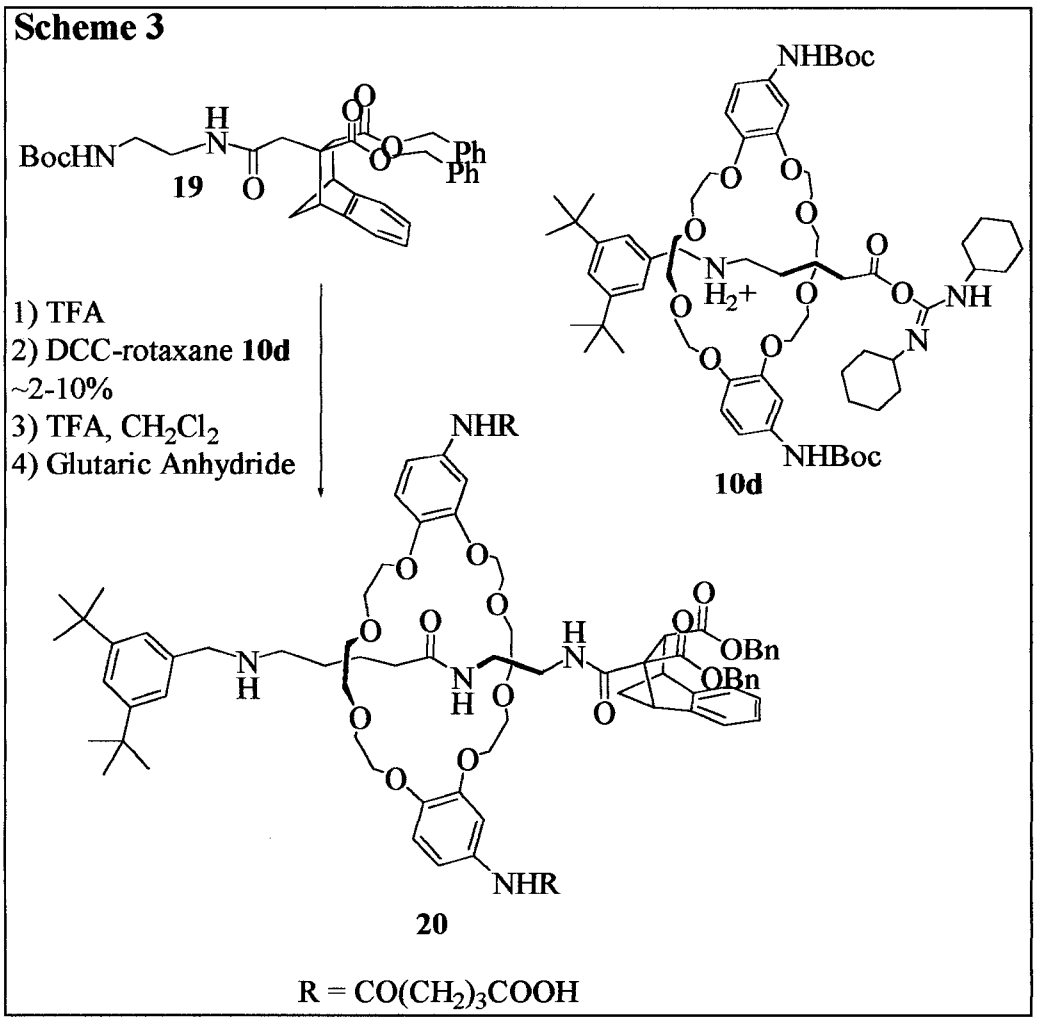
Figure 6: A unique NOE was observed showing the conformation of **19** to be the isomer with cis-carboxylate groups.

We were concerned that a mixture of diastereomers could have been formed in the synthesis of template **19**. To verify our compound's conformation, a series of 2D NMR experiments were performed. Through a series of COSY and NOESY experiments a unique NOE (See appendix A) was found between the hydrogens α-to the carbonyl of the amide side chain and the hydrogens on the

backside of the template (Figure 6). This NOE shows that the conformation of template **19** is indeed the cis-carboxylate isomer.

After confirming the conformation of **19**, we were then able to deprotect the amine, making it available for reaction with the DCC-rotaxane **10d** (Scheme 3).

Scheme 3



This step has proven to be a significant synthetic hurdle in the final rotaxane formation, manifesting as low, inconsistent yields. One possible explanation for this difficulty could arise from the unprotected amine folding back on the template, forming hydrogen bonds with the benzyl esters. To investigate this possibility, Monte

Carlo simulations were conducted using the PC Spartan computational program. Theses computations showed that in the lowest energy conformation of the unprotected template **19a**, there was indeed a hydrogen bond between the terminal amine and the carbonyl oxygen of one of the benzyl esters (Figure 7).

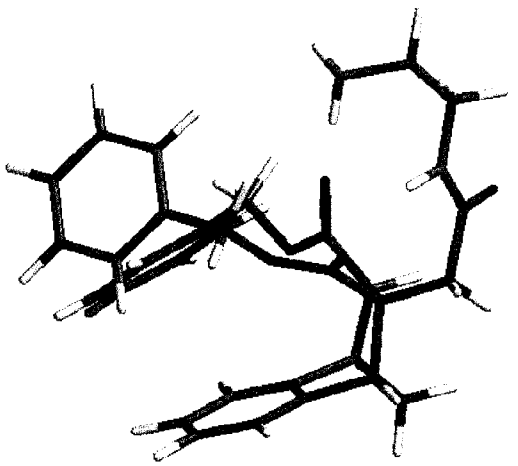


Figure 7: Template **19a;** Energy minimized conformation of the deprotected amine

To combat this problem, it was theorized that three approaches could be taken. At first glance, one could try to manipulate the environment of the template, specifically changing solvent (DMSO perhaps), to disrupt the hydrogen bonding. However, the same types of electronic interactions that are hindering us are also being utilized in the DCC-rotaxane to hold the crown ether over the ammonium ion of the tether. If the crown ether is free to move along the tether, there may be too much steric hindrance to allow our activated carboxylic acid cite to be reactive. Due to this concern, solvent manipulations were not investigated.

Synthetically, one solution to our problem could be to provide some rigidity to the tether and making it unable to fold back and form this hydrogen bond. Another synthetic option could be to elongate the tether, giving it more degrees of freedom and making it too energetically costly to form the hydrogen bond. To that end, a series of diamines were chosen based on either commercial or synthetic[15] availability. Hence, 1,4-xylene diamine, 1,5-diaminopentane, 1,4-diaminobutyne, and 1,4-diaminobutene were examined. Calculations showed that incorporating these diamines would indeed eliminate the issue of the problematic hydrogen bond. However, not all diamines would result in similar conformations (Figures 8-11).

Examination of the 1,4-xylene diamine showed that there was an edge to face or t-stack arrangement between the aromatic ring of the template and that of the xylene. This may result in the amine being too sterically congested to be reactive, and thus, was not investigated. The lowest energy conformation of the 1,4-diaminobutene showed a similar folding around the template, however without any aromatic-aromatic interactions, the terminal amine may be available to react. The most promising templates appeared to be the 1,4-diaminobutyne and the 1,5-diaminopentane, both of which showed the terminal amine to be well free of the template.

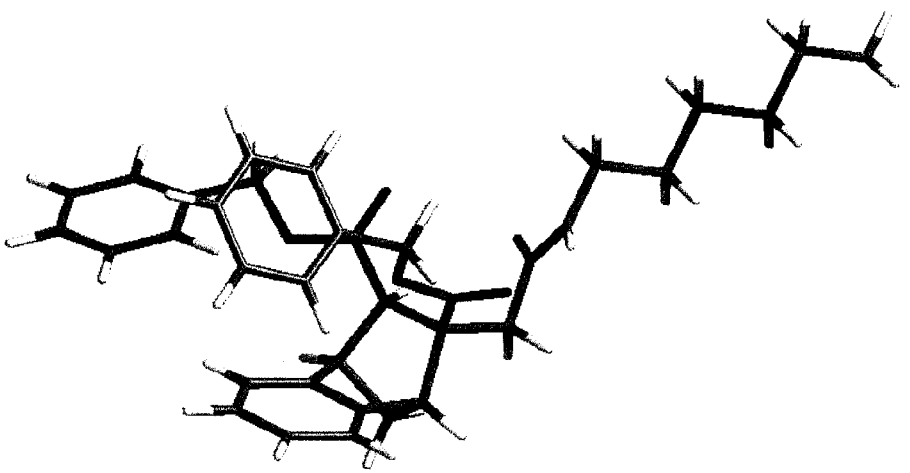


Figure 8: SACA scaffold attached to diaminopentane

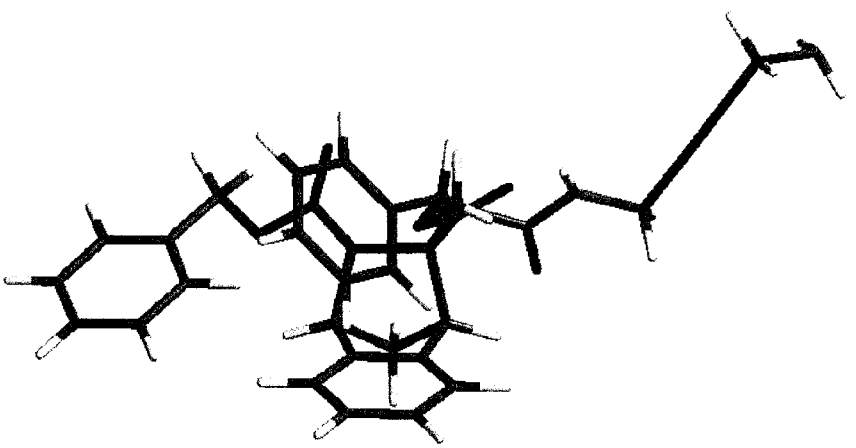


Figure 9: SACA scaffold attached to diaminobutyne

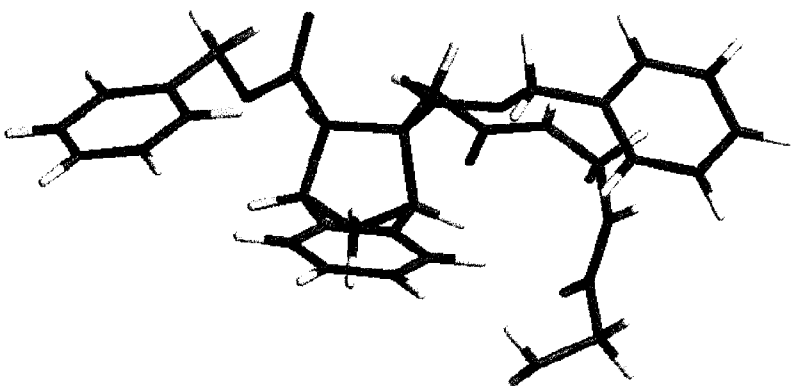


Figure 10: SACA scaffold attached to diaminobutene

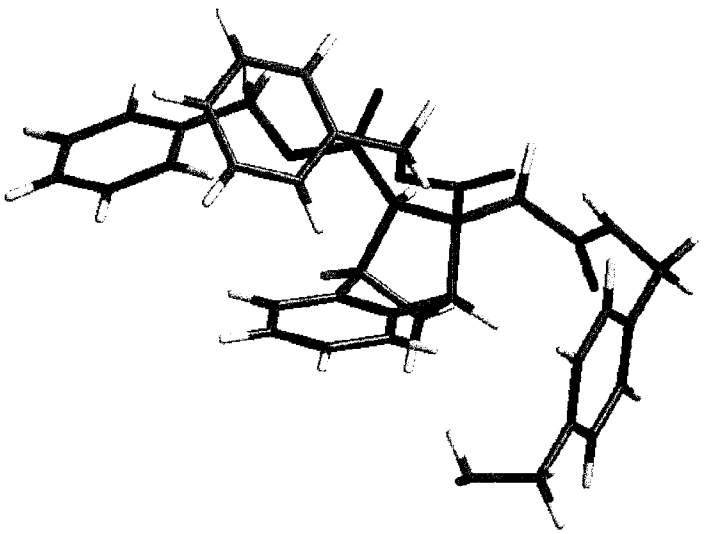
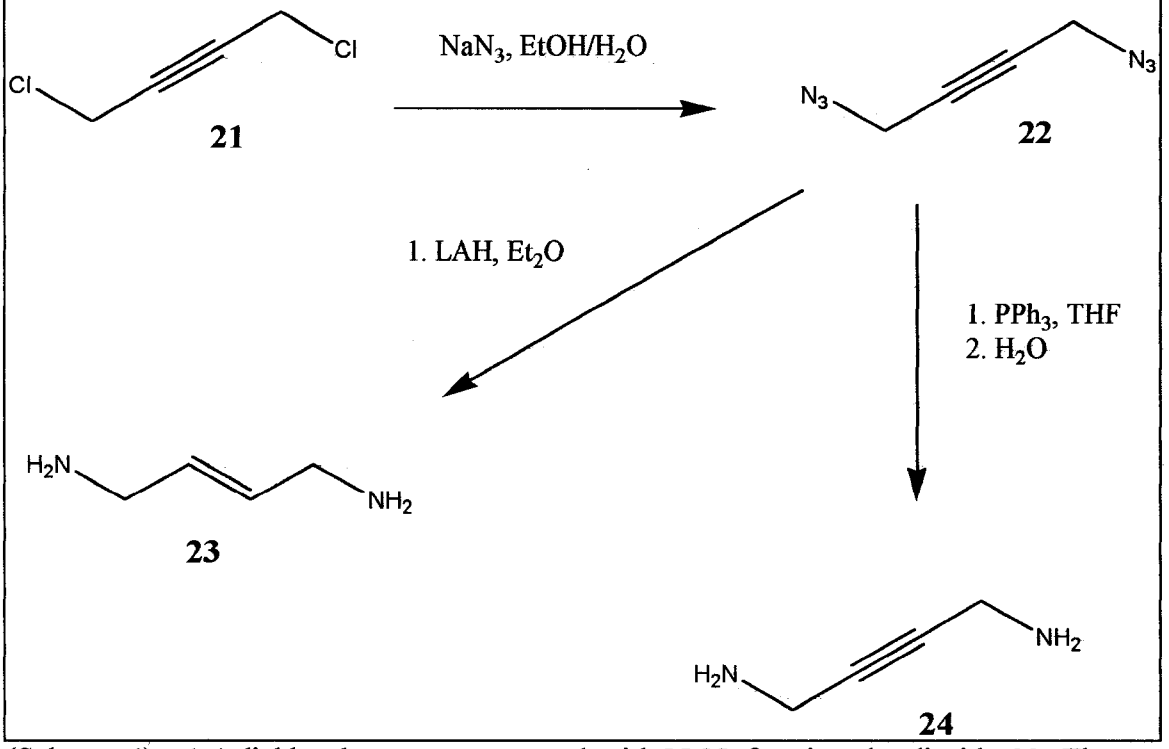


Figure 11: SACA scaffold attached to diaminoxylene

Synthesis of a series of diamino-templates:

1,5-diaminopentane is commercially available from Sigma-Aldrich. 1,4-diaminobutyne and 1,4-diaminobutene were prepared by literature methods[15]

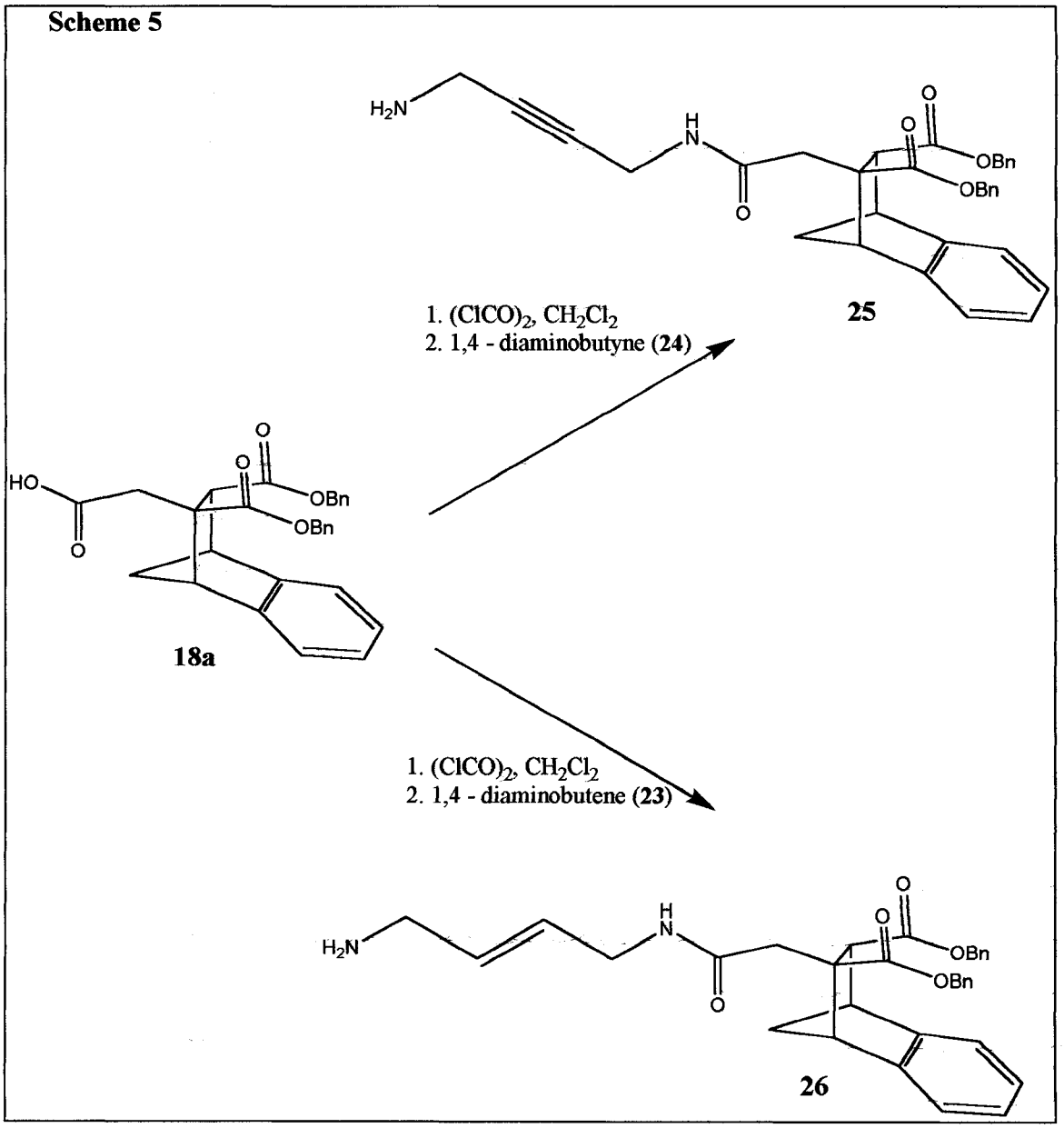
Scheme 4



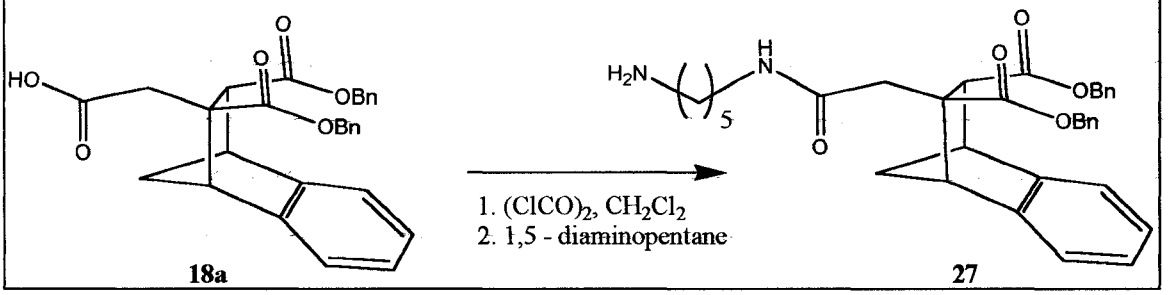
(Scheme 4). 1,4-dichlorobutyne was treated with NaN_3 forming the diazide **22**. The diazide was reduced by LAH giving **23**. Diazide **22** was also reduced by the Stautinger reduction, giving **24**. Once the diamines were available, a series of coupling reactions were performed to yield templates **25-27** (Schemes 5 and 6).

Once these templates were available, their ability to form rotaxanes was investigated. Each was exposed to the DCC-rotaxane giving consistent yields as determined by ^{1}H-NMR integrations (see Appendix B). Each reaction yielded approximately 50-60% of the desired rotaxane (Scheme 7).

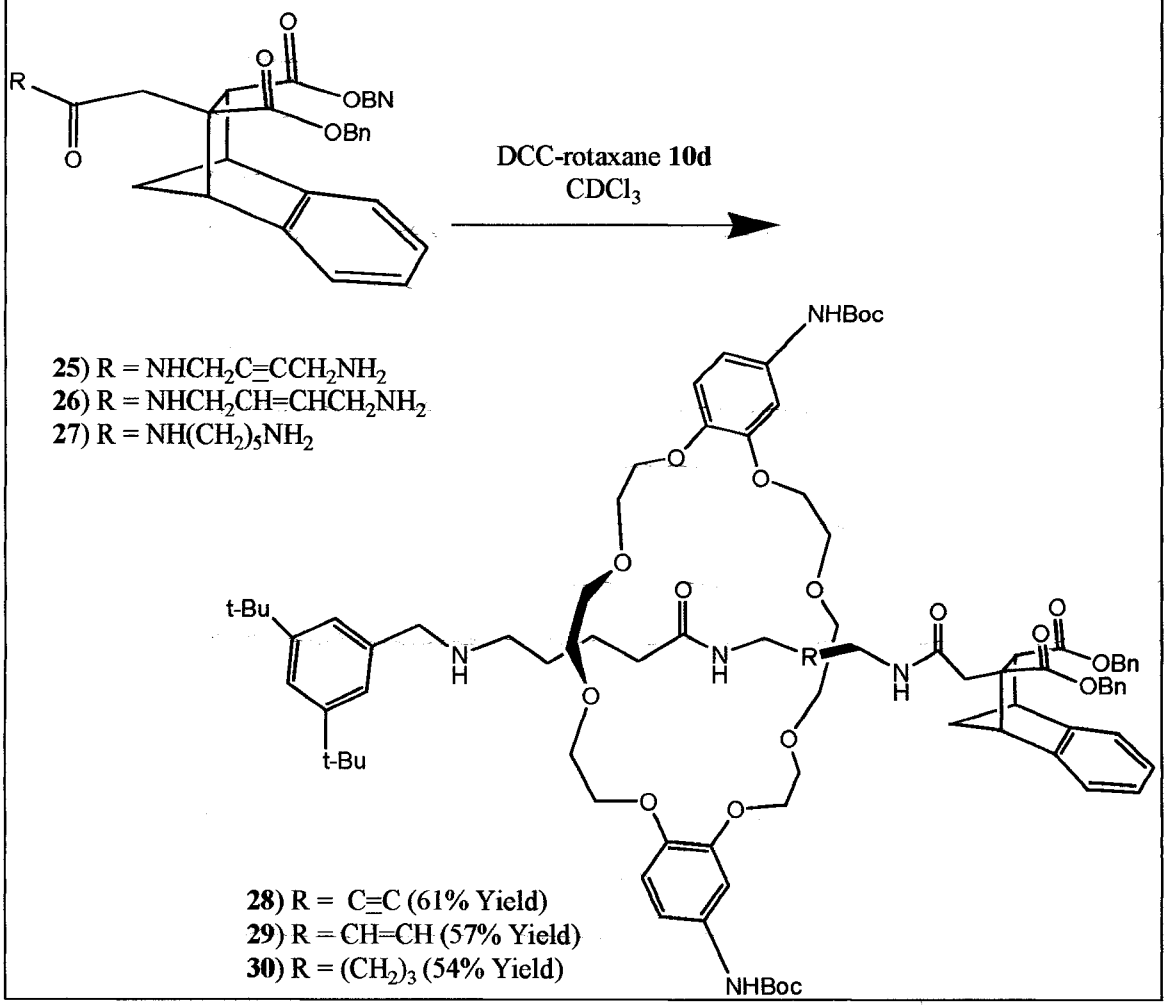
Scheme 5



Scheme 6



Scheme 7



Future Work

Now that the synthetic hurdles of the rotaxane formation have been overcome, the deprotection and functionalization of the rotaxane needs to be completed. Optimization of the conditions can result in determination of the ideal linker to use, each having its own advantages. The butyne and butene linkers have rigidity and less steric hindrance that could be useful, specifically in regard to facilitating ring movement. However, the added functionality of the alkyne or alkene could undergo chemical transformation under certain conditions. To eliminate this functionality, both the alkene and alkyne could be removed by catalytic reduction, which would also deprotect the benzyl esters of the SACA template. The pentane linker has the advantage of being longer, which can provide for a more dramatic sliding motion of the ring. The specific properties of the linker can be suited to the needs of the designed experiment.

Once these rotaxanes are properly functionalized and deprotected, their properties need to be examined. The role of the O-ring can be elucidated by comparing the binding strength and selectivity of the SACA template alone to that of the SACA-rotaxane. A series of studies can be done to examine if the O-ring acts to desolvate the hot spot, or if the O-ring can aid in selectivity or specificity of the overall binding domain.

Previously, it has been shown that rotaxanes can act as cell transport agents.[17] The cell permeability of these rotaxanes can be investigated. The possibility lies that with further development, rotaxanes could be used as drug delivery agents.

One last possibility could be that the SACA rotaxane itself could lead to anti-

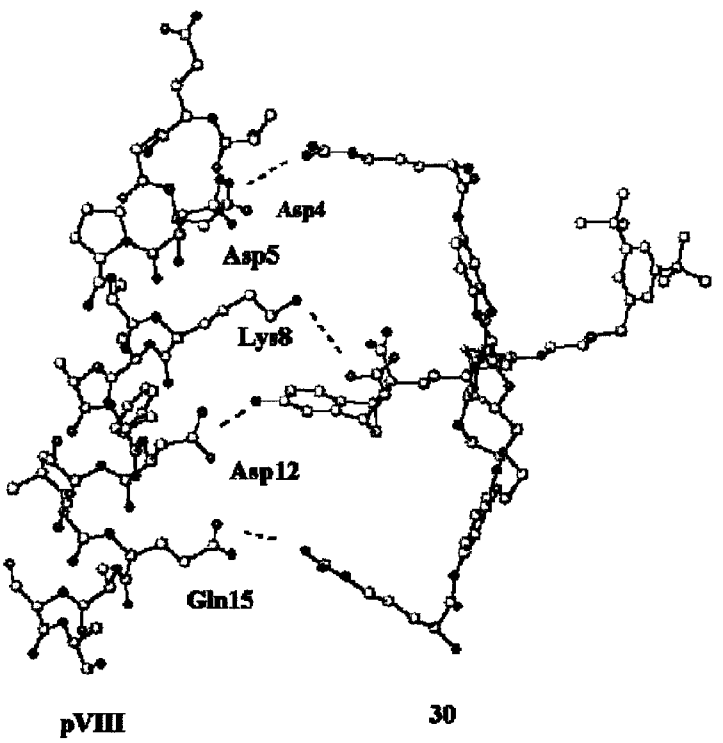


Figure Energy minimized structure of the SACA rotaxane positioned next to the X-ray crystal structure of the pVIII coat protein of the M13 bacteriophage

viral agents. Figure **X** shows the energy minimized structure of the SACA rotaxane forming a complex with the pVIII coat protein of the M13 bacteriophage (molecular modeling calculations).

Experimental

General procedure

All experiments were carried out in oven-dried glassware under ambient atmospheric conditions unless otherwise stated. Solvents were purchased from Fischer Scientific or Aldrich. THF was distilled over sodium and degassed with argon. All other reagents were purchased from commercial sources and were used without further purification. All NMR spectra were recorded on either a Bruker 250-MHz or a Bruker 400-MHz spectrometer in deuterated chloroform and referenced to TMS at 0.00 ppm. All mass spectra information was provided by the facility at the University of Cincinnati.

The Diels-Alder product of maleic anhydride and indene was prepared by literature methods.[13] Preparation of the PF_6 tether **8d** and the di-boc-protected DB24C8 was performed in accordance with procedures published previously by our research group.[12, 16] Procedures for the preparation of compounds **22**, **23**, **24**, and mono-boc-ethylenediamine were taken from literature.[14, 15]

Synthesis of Template 17. Template **16** (15.6 g, 0.0729 mol) was dissolved in DMF and LiOH (6.185 g, 0.1473 mol) was added. The solution was heated (80 °C) and stirred for 5 h. Benzyl bromide (24.93g, 0.1458 mol) was added and the solution stirred overnight. The solvent was removed by vacuum and the residue was dissolved in CH_2Cl_2 and extracted with water and brine. The organic layer was concentrated in vacuum and the residue was recrystallized (EtOAc) yielding white crystals of **17** (28.4 g, 94.7%). (^{1}H NMR, δ 7.31-7.06 (m, 14H), 4.90 (d, J=12 Hz, 2H), 4.68 (d, J=12 Hz, 2H), 3.64 (s, 2H), 3.53 (s, 2H), 1.77 (dd, J=9 Hz, 2H); ^{13}C NMR, 170.67,

143.54, 135.52, 128.05, 127.93, 127.63, 125.69, 122.88, 65.65, 49.31, 47.21; ESMS *m/z* calcd for $C_{27}H_{24}O_4Na^+$ 435.1572, found 435.1543.)

Synthesis of Template 18. Template **17** (1.1882 g, 2.8804 mmol) was dissolved in 20 mL THF and cooled to –78 °C. Lithium diisopropyl amine (1.45 mL, 2M in heptane/THF) was added and the solution stirred for 20 minutes. Allyl bromide (1.0454 g, 8.6397 mmol) was added and the solution stirred for two hours warming from –78° C to –10° C. The solution stirred for an additional two hours warming from –10° C to RT. The solution was allowed to stir at RT overnight. The THF was removed by vacuum and the oil was extracted with methylene chloride and 5% HCl, sat. $NaHCO_3$, and brine. The organic layer was dried over Na_2SO_4. The solution was concentrated by vacuum and purified by flash chromatography (10% ether/petroleum ether. To remove any trans product, the oil was recrystallized from 1:1 ether/petroleum ether to yield **18** (0.6517 g, 50%). (^{1}H NMR, δ 7.41-6.99 (m, 14H), 5.96-5.82 (m, 1H), 5.06-4.62 (m, 6H), 3.588 (s, 1H), 3.36 (s, 1H), 3.09 (s, 1H), 2.81 (q of d, J=5 Hz, 2H), 1.90 (d of d, J=10 Hz, 2H); ^{13}C NMR, δ 172.19, 171.68, 144.93, 144.55, 136.12, 134.42, 128.69, 128.35, 128.18, 127.80, 126.24, 125.82, 124.97, 121.60, 117.59, 66.58, 66.07, 59.58, 54.47, 53.21, 47.47, 47.34, 45.07)

Synthesis of Template 18a. Template **18** (1.04 g, 2.301 mmol) was dissolved in dichloromethane and cooled to –78 °C. The flask was purged with oxygen (x3) and ozone was bubbled into the solution (10 min. @ 4L/h). Dimethyl sulfide (4 mL) was added and the solution was allowed to warm to RT while stirring overnight. Solvent was removed by vacuum. The resulting oil was dissolved in 4:1 THF:water and $NaClO_2$ (0.8588 g, 9.4895 mmol) and NH_2SO_3H (0.4684 g, 4.9305 mmol) was

added to the solution. After stirring for one hour at rt, the THF was removed by vacuum, and the solution was extracted with dichloromethane and 5% HCl, saturated $NaHCO_3$, and brine. The organic layer was dried over Na_2SO_3 and concentrated by vacuum. The oil was purified by flash chromatography (CH_2Cl_2 to 5% MeOH/ CH_2Cl_2) to yield **18a** (0.78 g, 72.2%). (^{1}H NMR, δ 7.42-6.92 (m, 14H), 4.86-4.78 (m, 2H), 4.63-4.54 (m, 2H), 3.63 (s, 1H), 3.48 (s, 1H), 3.20 (s, 2H), 2.78 (d, J=15 Hz, 1H), 1.97-1.85 (m, 2H); ^{13}C NMR, δ 175.89, 171.41, 171.20, 144.53, 143.99, 136.47, 135.43, 128.92, 128.47, 128.43, 128.29, 127.99, 126.84, 126.22, 125.21, 122.01, 67.22, 66.15, 57.05, 54.55, 53.15, 47.50, 47.37, 44.42; ESMS *m/z* calcd for $C_{29}H_{25}O_6^-$ 469.1651, found 469.1646.)

Synthesis of Template 19. Template **18a** (.200 g, 0.4251 mmol) was dissolved in dichloromethane. 1-Hydroxybenzotriazole (HOBt) (0.0066 g, 0.0488 mmol), mono-Boc-EDA (0.1415 g, 0.8844 mmol), and DCC (0.0877 g, 0.4250 mmol) were added and the solution stirred at rt overnight. The solution was diluted with 50 mL of acetonitrile, and the DCU was filtered off. The solution was concentrated by vacuum and the oil was extracted with dichloromethane, 5% HCl, saturated $NaHCO_3$, and brine. The organic layer was dried over Na_2SO_4 and solvent was removed by vacuum yielding compound **19** (0.2264 g, 87%) which was carried on without further purification. (^{1}H NMR, δ 7.40-7.15 (m, 14H), 5.08 (m, 1H), 4.95 (m, 2H), 4.80 (m, 2H), 4.75 (m, 1H), 3.70 (m, 1H), 3.39 (s, 2H), 3.31 (m, 1H), 3.26 (m, 1H), 3.22 (m, 2H), 3.04 (m, 2H), 1.97 (d of d, J=3, 2H), 1.43 (s, 9H); ESMS *m/z* calcd for $C_{36}H_{40}N_2O_7Na^+$ 635.2733, found 635.2748.) To deprotect the amine, **19** was stirred in 10% TFA for 1.5 hours. The solution was concentrated by vacuum,

extracted with dichloromethane and 2M NaOH, and the organic layer was dried over Na_2SO_4.

Formation of SACA Rotaxane 20 with Template 19. Tether **8d** (0.0702 g, 0.1536 mmol) and crown ether **31** (See appendix C) (0.1072 g, 0.1579 mmol) were dissolved in deuterated chloroform and stirred for 20 minutes. The solution was cooled to -10° C and DCC (0.0338 g, 0.1641 mmol) was added and the solution stirred for 1.5 hours yielding DCC-rotaxane **10d**. The template **19** (0.048 g, 0.0767 mmol) was added as the free amine (see above for deprotection) and the solution stirred at rt overnight. The solution was diluted with acetonitrile and the DCU was removed by filtration. The solution was concentrated by vacuum and the oil was dissolved in a minimal amount of dichloromethane and 30 mL ether was added to remove the *N*-(3,5-di-*tert*-butylbenzyl)valerolactam byproduct as a precipitate. The ether was decanted and concentrated by vacuum. The residue was purified by chromatatron (dichloromethane to 5% MeOH in dichloromethane) to yield **20**. Variable low yield (2-10%). (^{1}H NMR, δ 7.49-6.68 (m, 23H), 4.89-4.27 (m, 10H), 4.25-3.69 (m, 24H), 3.68-3.01 (m, 17H), 2.12-1.98 (m, 2H), 1.62 (s, 9H), 1.49 (s, 9H), 1.45-1.16 (m, 19H); ESMS *m/z* calcd for $C_{85}H_{114}N_5O_{18}^+$ 1492.8159, found 1492.8077.)

Synthesis of Template 25. To a flask of fresh CH_2Cl_2 was added template **18a** (0.100 g, 0.2125 mmol) and triethylamine (0.0214 g, 0.2119 mmol). Oxalyl chloride (0.0270 g, 0.2123 mmol) was added and the solution stirred at RT for 15 minutes. The solution was added to a dilute solution (0.1 M) of **24** in CH_2Cl_2 (22 mL) and the solution stirred for 5 minutes. The solution was extracted with 5% HCl, sat.

$NaHCO_3$, and brine. The organic layer was dried over Na_2SO_4 and concentrated by vacuum to afford **25** (0.070g, 61%). (^{1}H NMR, δ 7.70-6.88 (m, 14H), 6.42 (m, 1H), 5.20-4.45 (m, 4H), 3.99 (m, 4H), 3.61-3.31 (m, 5H), 2.04-1.93 (m, 2H), 1.26 (m, 2H).)

Synthesis of Template 26. To a flask of fresh CH_2Cl_2 was added template **18a** (0.100 g, 0.2125 mmol) and triethylamine (0.0214 g, 0.2119 mmol). Oxalyl chloride (0.0270 g, 0.2123 mmol) was added and the solution stirred at RT for 15 minutes. The solution was added to a dilute solution (0.1 M) of **23** in CH_2Cl_2 (22 mL) and the solution stirred for 5 minutes. The solution was extracted with 5% HCl, sat. $NaHCO_3$, and brine. The organic layer was dried over Na_2SO_4 and concentrated by vacuum to afford **26** (0.066g, 58%). (^{1}H NMR, δ 7.71-6.79 (m, 14H), 5.42-4.56 (m, 7H), 4.10-3.88 (m, 4H), 3.82-2.94 (m, 5H), 2.08-1.75 (m, 2H), 1.26 (m, 2H).)

Synthesis of Template 27. To a flask of fresh CH_2Cl_2 was added template **18a** (0.100 g, 0.2125 mmol) and triethylamine (0.0214 g, 0.2119 mmol). Oxalyl chloride (0.0270 g, 0.2123 mmol) was added and the solution stirred at RT for 15 minutes. The solution was added to a dilute solution (0.1 M) of 1,5-diaminopentane in CH_2Cl_2 (22 mL) and the solution stirred for 5 minutes. The solution was extracted with 5% HCl, sat. $NaHCO_3$, and brine. The organic layer was dried over Na_2SO_4 and concentrated by vacuum to afford **27** (0.080g, 68%). (^{1}H NMR, δ 7.70-6.79 (m, 14H), 6.26 (m, 1H), 5.20-4.48 (m, 4H), 4.00-2.51 (m, 15H), 2.05-1.81(m, 2H), 1.25 (m, 2H).)

Formation of SACA Rotaxane 28 with Template 25. To a solution of DCC-rotaxane **10d** (0.2501g, 0.2075 mmol) was added template **25** (0.05 g, 0.0932

mmol). The solution stirred overnight at rt. The solution was diluted with acetonitrile and the DCU was removed by filtration. The solution was concentrated by vacuum and the oil was dissolved in a minimal amount of dichloromethane and 30 mL ether was added to remove the *N*-(3,5-di-*tert*-butylbenzyl)valerolactam byproduct as a precipitate. The ether was decanted and concentrated by vacuum. The residue was purified on silica (dichloromethane to 5% MeOH in dichloromethane) to afford **28** (0.0869 g, 61%). (^{1}H NMR, δ 7.72-6.82 (m, 23H), 5.66-4.60 (m, 12H), 4.22-2.87 (m, 38H), 2.23-1.99 (m, 4H), 1.89-1.06 (m, 36H).)

Formation of SACA Rotaxane 29 with Template 26. To a solution of DCC-rotaxane **10d** (0.2493 g, 0.2068 mmol) was added template **26** (0.05 g, 0.0929 mmol). The solution stirred overnight at rt. The solution was diluted with acetonitrile and the DCU was removed by filtration. The solution was concentrated by vacuum and the oil was dissolved in a minimal amount of dichloromethane and 30 mL ether was added to remove the *N*-(3,5-di-*tert*-butylbenzyl)valerolactam byproduct as a precipitate. The ether was decanted and concentrated by vacuum. The residue was purified on silica (dichloromethane to 5% MeOH in dichloromethane) to afford **29** (0.0810 g, 57%). (^{1}H NMR, δ 7.76-6.89 (m, 23H), 5.49-4.67 (m, 14H), 4.32-3.66 (m, 24H), 3.54-3.21 (m, 9H), 3.09-2.55 (m, 6H), 2.33-2.16 (m, 4H), 2.06-1.12 (m, 36H).)

Formation of SACA Rotaxane 30 with Template 27. To a solution of DCC-rotaxane **10d** (0.2421g, 0.2008 mmol) was added template **27** (0.05 g, 0.0902 mmol). The solution stirred overnight at rt. The solution was diluted with acetonitrile and the DCU filtered off. The solution was concentrated by vacuum and

the oil was dissolved in a minimal amount of dichloromethane and 30 mL ether was added to remove the *N*-(3,5-di-*tert*-butylbenzyl)valerolactam byproduct as a precipitate. The ether was decanted and concentrated by vacuum. The residue was purified on silica (dichloromethane to 5% MeOH in dichloromethane) to afford **30** (0.0747 g, 54%). (^{1}H NMR, δ 7.39-6.79 (m, 23H), 5.53-4.60 (m, 12H), 4.20-3.65 (m, 24H), 3.60-3.16 (m, 18H), 2.63-2.49 (m, 5H), 2.23-1.99 (m, 2H), 1.89-1.15 (m, 36H).)

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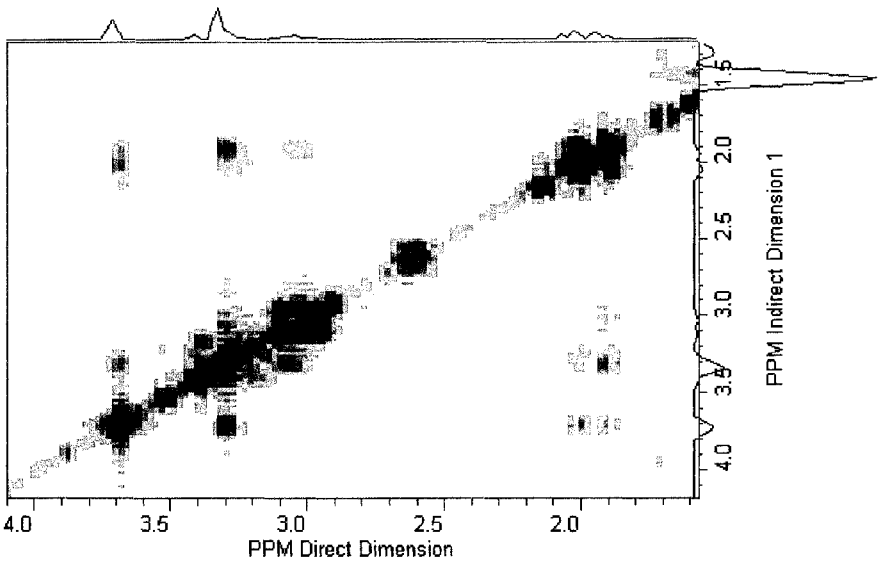
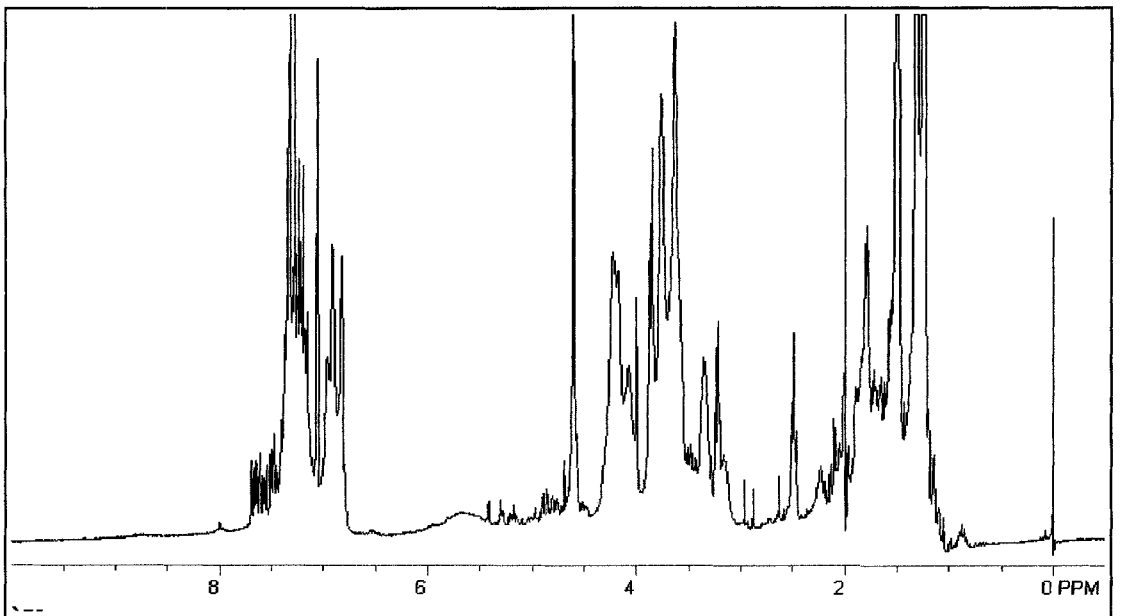
Appendix A

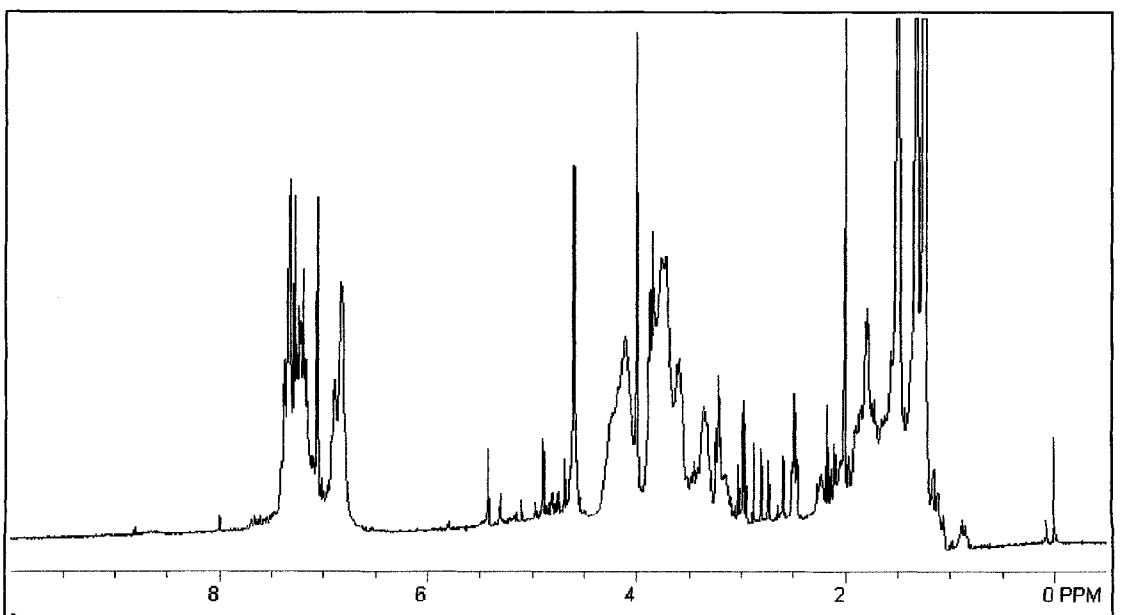
A unique NOE was seen when comparing NOSY and COSY experiments on template **19.** Shown is the NOE between the hydrogens α-to the carbonyl of the amide side chain (3.39 ppm) and the hydrogens on the backside of the template (2.02 ppm). This NOE shows that the conformation of template **19** is indeed the cis isomer.



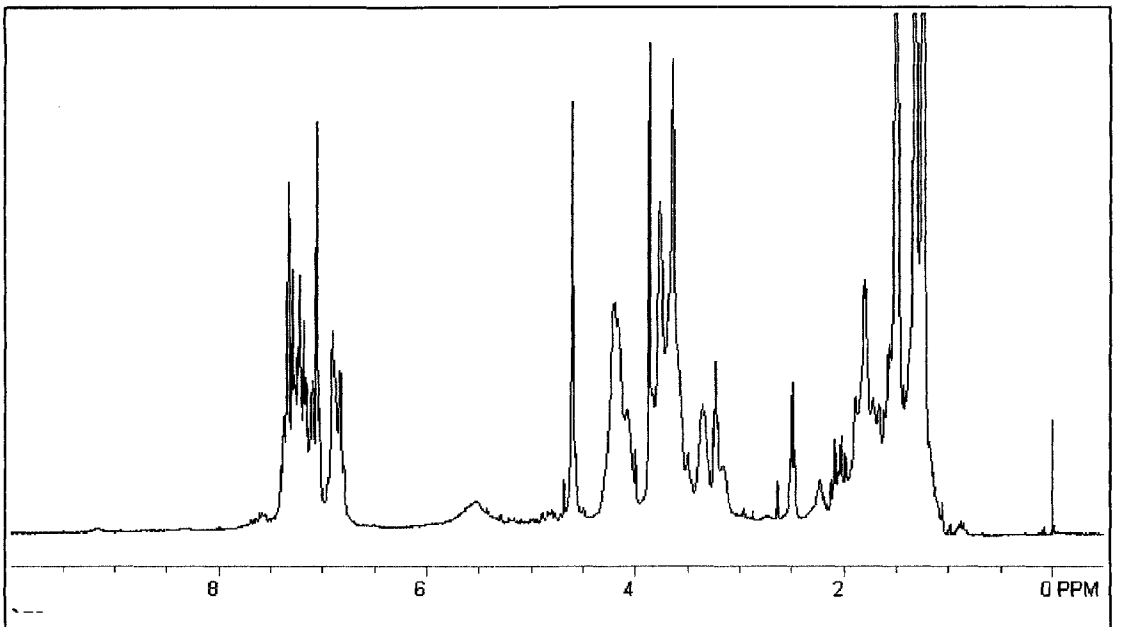
Appendix B



^{1}H NMR spectra of SACA rotaxane 28

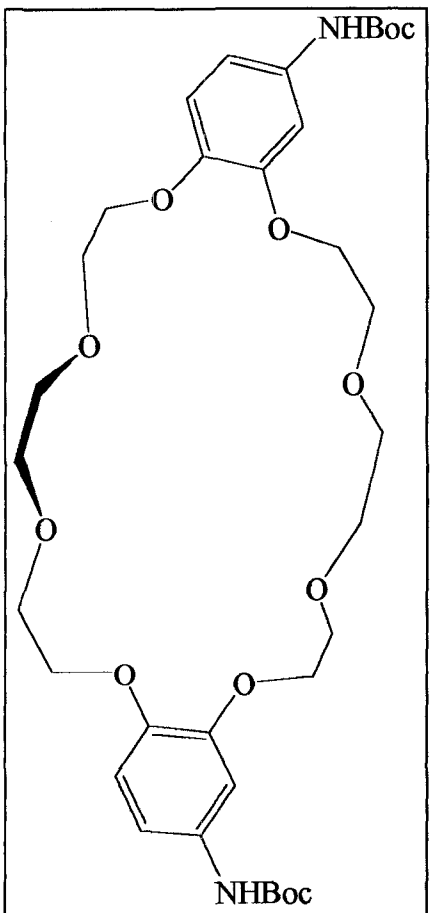


^{1}H NMR spectra of SACA rotaxane 29



^{1}H NMR spectra of SACA rotaxane 30

Appendix C



31

Structure of Crown Ether **31**