Host−[2]Rotaxanes as Cellular Transport Agents

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Abstract: Host−[2]rotaxanes, containing a diarginine-derivatized dibenzo-24-crown-8 (DB24C8) ether as the ring and a cyclophane pocket or an aromatic cleft as one blocking group, are cell transport agents. These hosts strongly associate with a variety of amino acids, dipeptides, and fluorophores in water (1 mM phosphate buffer, pH 7.0), DMSO, and a 75/25 (v/v) buffer to DMSO solution. All peptidic guests in all solvent systems have association constants (K_A's) in the range of 1×10^4 to 5×10^4 M^{-1}, whereas the K_A range for the fluorophores is 1×10^4 to 9×10^5 M^{-1}. Association constants for the cyclophane itself, cyclophane **3**, are smaller. These values are in the 1×10^3 to 5×10^3 M^{-1} range, which shows that the rotaxane architecture is advantageous for guest binding. Cyclophane−[2]rotaxane **1** efficiently transports fluorescein and a fluorescein-protein kinase C (PKC) inhibitor into eukaryotic COS-7 cells, including the nucleus. Interestingly, cleft−[2]rotaxane **2** does not transport fluorescein as efficiently, even though the results from the fluorescence assays show that both [2]rotaxanes bind fluorescein with the same ability.

Introduction

The goals of protein mimicry include identifying the structural elements that give proteins their function and incorporating their essential features into synthetic systems to obtain novel receptors. Extensive research in molecular recognition chemistry has produced compounds that resemble the structures of protein binding domains by positioning amino acids or peptides around a hydrophobic pocket.[1−5] The attachment of peptide loops onto synthetic scaffolds has impressively provided mimetics that recognize large patches on protein surfaces.[6−8] Molecularly imprinted polymers have demonstrated the importance of a convergent arrangement of recognition elements.[9−15] These polymeric pockets are sprinkled with various functional groups, which are locked in a programmed position to strongly associate with the template used to form the pocket or other guests with similar physical properties. Our approach to creating mimics of protein binding domains is to use the rotaxane architecture to arrange multiple functional groups in a convergent fashion.

Now that several methods can be used to construct mimetic pockets, the next challenge facing chemists is the creation of synthetic compounds that provide protein-like function. One important function, highlighted in this paper, is cell transport.[16] Cell transport agents need to bind a guest strongly in the different environments experienced in cells and be soluble in these environments. The agent needs to "cover" any feature of a guest, such as an anionic charge, that prevents membrane passage. In this report we show that host−[2]rotaxanes can satisfy these requirements. The long-term goal of this research is to obtain mimetics that operate in the various biological milieus, including those found within cells.

We have shown that a host−[2]rotaxane, containing an arginine-derivatized DB24C8 ring and a calix[4]arene, binds a variety of guests (with a preference for aromatic carboxylates) in DMSO, water, and mixed solvent systems with large association constants.[17] One unique feature of the rotaxane architecture for pocket formation is that the ring, which carries the recognition elements, can adjust to a guest by changing its position on the axle to maximize the favorable binding free energy.[18] The aromatic rings and polar recognition elements of the DB24C8 ring can both contribute to guest recognition, and

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the importance of an interaction depends on the nature of the guest and the environment. Considering these features, we predicted that host–rotaxanes would be able to transport guests through cellular membranes. We envisioned that in an aqueous environment the pocket would contract, bringing the aromatic moieties of the ring closer to an encapsulated guest. In apolar environments, such as a membrane, the pocket would expand, allowing salt bridges and H-bonds to form between the guest and the recognition elements of the ring. In this paper, we compare the ability of cyclophane–[2]rotaxane **1** and cleft–[2]rotaxane **2** to bind various guests and transport fluorescein and show that, although there was not a demonstrated advantage for guest recognition in the fluorescence assays, there is an advantage afforded a cyclophane pocket for fluorescein transport into a cell. A comparison is also made to cyclophane **3** to show that hosts built on the [2]rotaxane architecture can be better for guest association. Cyclophane **3** could not be tested as a transport agent because of its poor water solubility.

Results

Design of the Host–[2]Rotaxanes. To further test rotaxanes as hosts and transporters, two new host–[2]rotaxanes were created by combining a cyclophane or an aromatic cleft with an arginine-derivatized DB24C8 ring. Association constants were derived for the hosts bound to amino acids, dipeptides, and various fluorophores in water, DMSO, and a 75/25 (v/v) water to DMSO mixture. These solvents were chosen to represent the various biological milieus. The guests presented a wide range of functional groups for recognition, including mono- and dicarboxylates, amides, aromatic rings, and aliphatic moieties. Guests containing dicarboxylates were tested to determine whether both positively charged groups of the ring's recognition elements (guanidinium and ammonium ions) could form noncovalent bonds. To measure [2]rotaxanes' abilities to associate with aliphatic moieties, dipeptides containing Ala, Leu, and Ile were used as guests. Cellular transport was investigated with fluorescein and a fluorescein–PKC inhibitor conjugate[19] as the guests, because fluorescein is strongly bound by the [2]-rotaxanes and its location within a cell can be easily monitored through fluorescence microscopy.

A cyclophane-pocket was chosen as a blocking group for one [2]rotaxane, because cyclophanes usually interact strongly with aromatic guests, especially in aqueous solutions through the hydrophobic effect.[20] Moreover, their rigid, preformed pockets contribute favorable binding energy by reducing the desolvation and entropic penalty that can occur upon complexation.[21–25] The chosen cyclophane is similar to ones developed by Diederich, which bind mono and para-disubstituted aromatic rings in water with K_A values in the 10^3 M^{-1} range.[20] Clefts have also been used as hosts,[26–34] and cleft–[2]rotaxane **2**

contains half of the cyclophane of [2]rotaxane **1** with additional benzylic groups to give it a greater aromatic surface area (Chart 1). The DB24C8 ring used in these studies is similar to the one used in previous studies,[17,18] except that the arginine recognition elements are fully deprotected.

Construction of host–[2]rotaxanes **1** and **2** is based on the DCC–[2]rotaxane method, which involves a reaction between an amine with a DCC–[2]rotaxane.[35] The piperidinylamine of the Diederich cyclophane initially appeared to be a convenient site for the attachment of an alkylamine chain to react with the DCC–[2]rotaxane. However, molecular modeling results suggested that the depth of the ring could prevent the arginine residue on the ring from reaching the pocket or cleft. Therefore, one piperidine ring was replaced with an alkylamine. The methoxy groups were also eliminated from the same side of the hosts to reduce possible steric congestion with the ring's arginine residues.

Synthesis of the [2]Rotaxanes. Because synthetic routes have already been developed to construct the cyclophane portion of the host–[2]rotaxanes,[36] new methods were only needed to attach the axle to the cyclophane and cleft. 4,4′-Dihydroxybenzophenone **4** was chosen as the commercially available precursor of the derivatized end of the hosts (Scheme 1). The phenolic oxygen atoms were protected as benzyl ethers, and the carbonyl group was reduced to give the diphenylcarbinol **5**. Formation of a carbocation, through dehydration of the secondary dibenzyl alcohol, was accomplished upon exposure to trifluoroacetic acid (TFA). Allyltrimethylsilane was added to react with the carbocation to form a C–C bond; however, the initial product is ether **6**, which exists in equilibrium with the carbocation. After extended reaction times (24 h), the carbocation is fully consumed through the irreversible reaction with allyltrimethylsilane to give cleft **7**. Conventional deprotection of the benzylic groups with H$_2$/Pd was not used, because the olefin would be reduced as well. Instead, the single-electron reducing agent lithium di-*tert*-butylbiphenylide was used to remove the benzylic groups in high yields. However, complete deprotection required as many as 16 equiv of this expensive reagent. Later, we found that employing boron trichloride is a more economical way to deprotect cleft **7**, albeit in a slightly lower yield. Bisphenol **8** was coupled with dibromide **9** to furnish cyclophane **10** in a 16–20% yield, which is typical for this type of macrocyclization.[20] Hydroboration of the olefin gave alcohol **11**, which was brominated by treating the alcohol with carbon tetrabromide and triphenylphosphine. The resulting bromide **12** was displaced with azide to give cyclophane **13**, and the azidoalkane was reduced with H$_2$/Pd on carbon to give aminocyclophane **14**. A similar synthetic route was used to construct the cleft framework (compounds **4–8**, **15**, **17–19**, Scheme 1).

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Chart 1

1

2   **3**
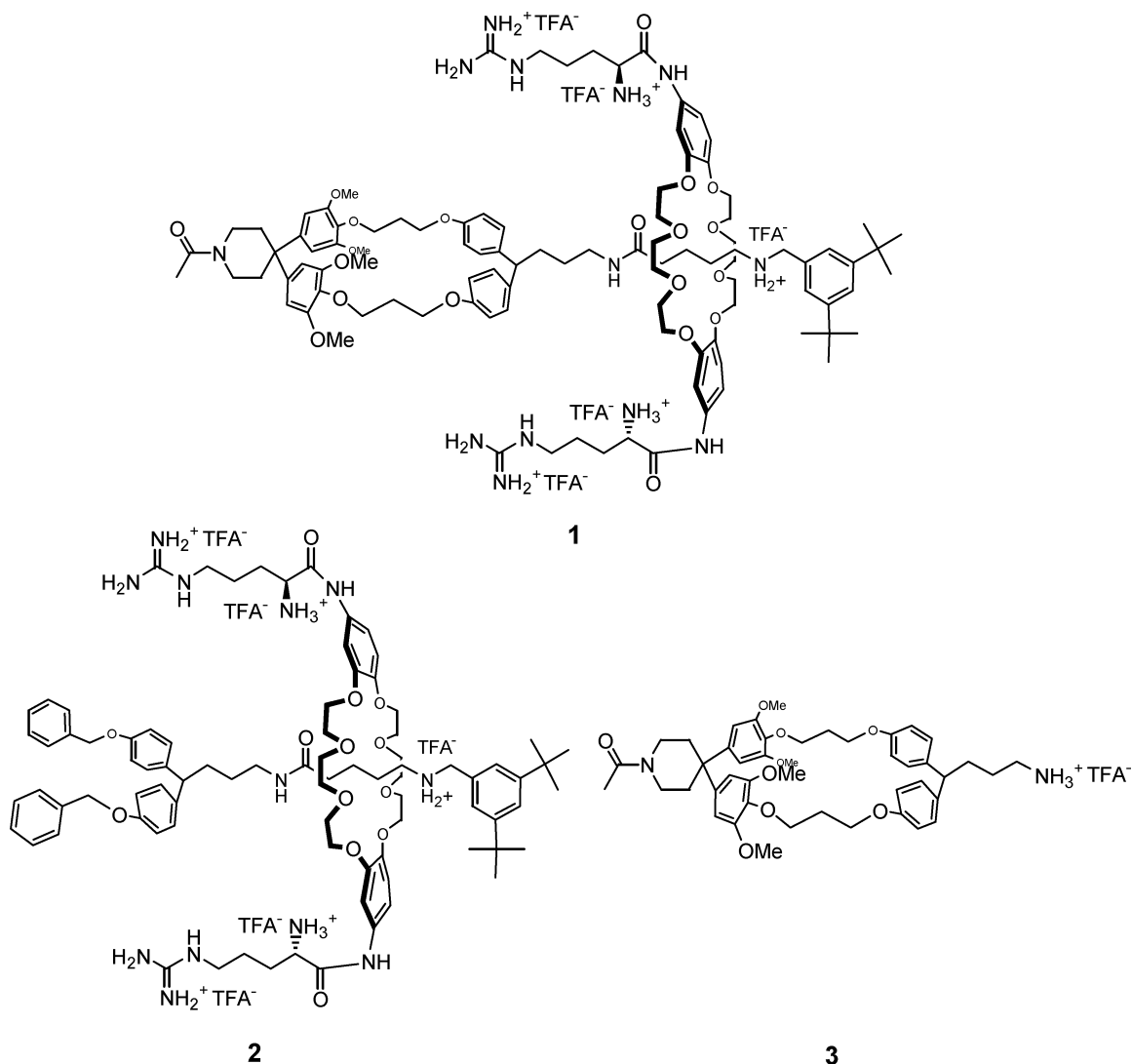
An alternative route was developed that gave higher yields of cyclophane. The primary alcohol was created on the cleft (**15**) prior to macrocyclization. This approach allows the more routine H_2/Pd reduction method to be used to remove the benzyl groups, which gives bisphenol **16**. By using bisphenol **16**, the yield of macrocyclization was improved as well from 16–20% to 25–30%. These reactions were performed several times.

Formation of the [2]rotaxanes was accomplished using DCC– [2]rotaxane **20**[18] (Scheme 2), which exists as a mixture of syn and anti constitutional isomers; the syn isomer is shown throughout the text. The addition of DCC–[2]rotaxane **20** to cleft **19** and to cyclophane **14** resulted in good yields of [2]-rotaxanes **24** and **21**, respectively. Formation of the [2]rotaxanes can be conveniently followed by ^{1}H NMR spectroscopy. The key signal is the approximately 0.4 ppm downfield shift of the benzylic protons of the axle, which occurs upon threading (Figure 1). Removal of the ring's Boc protecting groups proceeded smoothly with TFA in CH_2Cl_2. Fully Boc protected arginines were added to the ring through DCC coupling with a catalytic amount of HOBt. In a previous paper,[18] we mentioned that $(Boc)_3$–Arg should not be used in the construction of [2]-rotaxanes, because removing all six protecting groups is difficult. However, we subsequently found that the Boc groups can be

removed readily using a 1:1 ratio of trifluoroacetic and acetic acids (or a 1:1:1 TFA/CH_3CO_2H/CH_2Cl_2 solution). The desired [2]rotaxanes **1** and **2**, each bearing five positive charges, were purified on silica gel (CH_2Cl_2/MeOH/TFA).

Determining Acidity Constants. An accurate comparison of association constants of molecular complexes requires knowledge of the ionization states of the components, which requires deriving the acidity constants (pK_a's) of acidic functional groups. This is especially important in DMSO and DMSO/ water mixtures, wherein carboxylic acids can become less acidic than alkylammonium ions. The pK_a values were derived through potentiometric titrations and used to set the pH of the buffered assay solutions to a value that minimized the amount of proton exchange.

The pK_a values of host–[2]rotaxane **1** were derived, and we assume that the pK_a values of host–rotaxanes **1** and **2** are equal. For potentiometric titrations in DMSO, the plot of [2]rotaxane **1** showed only two end points with the addition of over 3 equiv of base. The first pK_a (8.1 ± 0.2) was assigned to both ammonium ions of the ring's side chains, and pK_{a2} (9.5 ± 0.2) was assigned to the ammonium ion in the axle. We have previously found that the axle's ammonium ion generally has a large pK_a value.[18] This reduction in acidity is apparently caused

Scheme 1
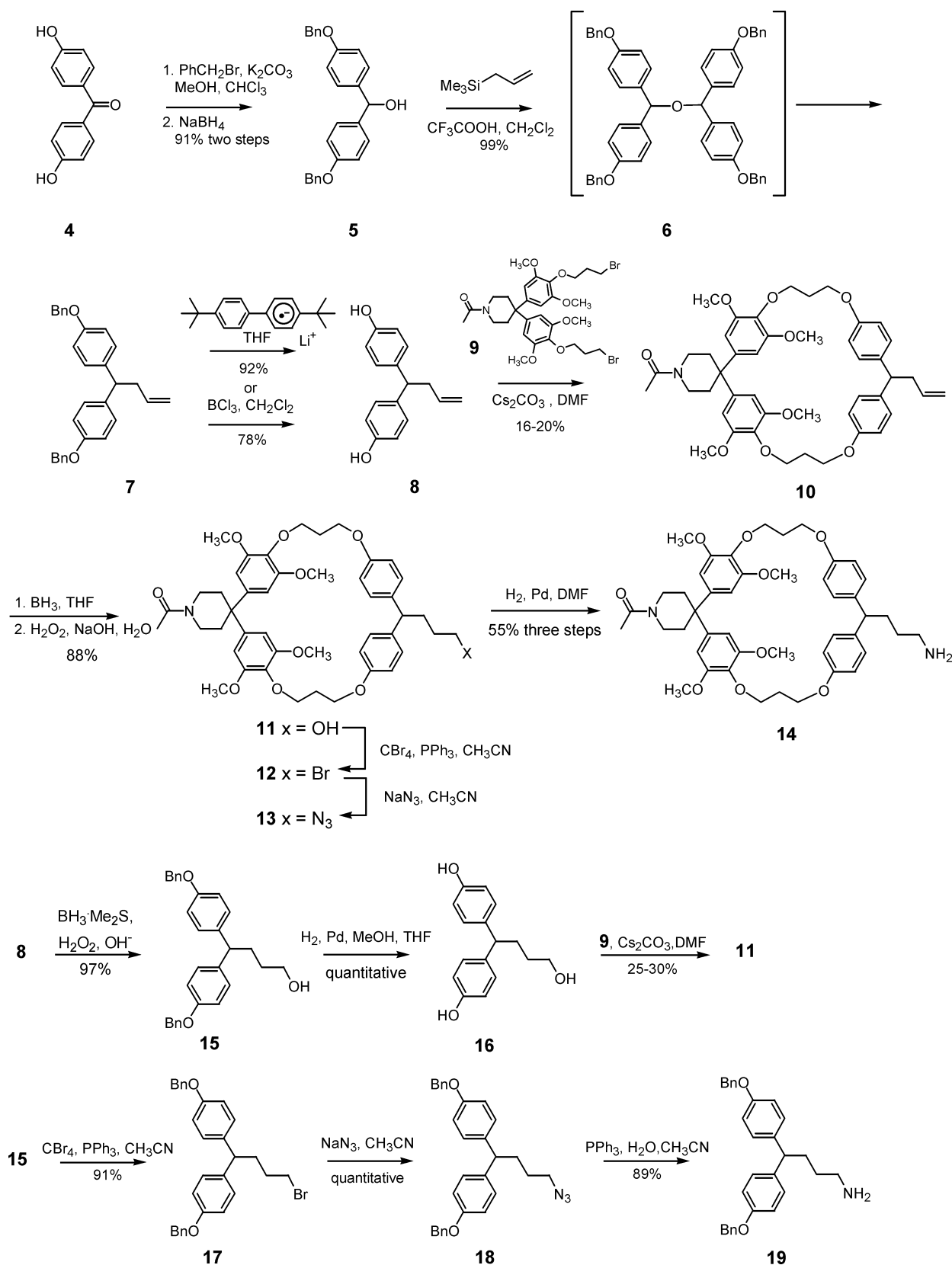
by favorable interactions between the oxygen atoms of the ring and the ammonium ion. Because of the limited solubility of [2]rotaxane **1** (and [2]rotaxane **2**) in a 75/25 (v/v) water to DMSO solution, (the assay solution was equal to or greater than 1 mM), a direct measure of their pK_a values could not be easily obtained. Thus, a suitable compound was needed to obtain representative pK_a's. Di-Arg-DB24C8 was not chosen, because intermolecular or intramolecular interactions could exist between the ammonium ion and the electron-rich cavity of the crown ether, resulting in an unrepresentative pK_a. Instead, arginine

Scheme 2
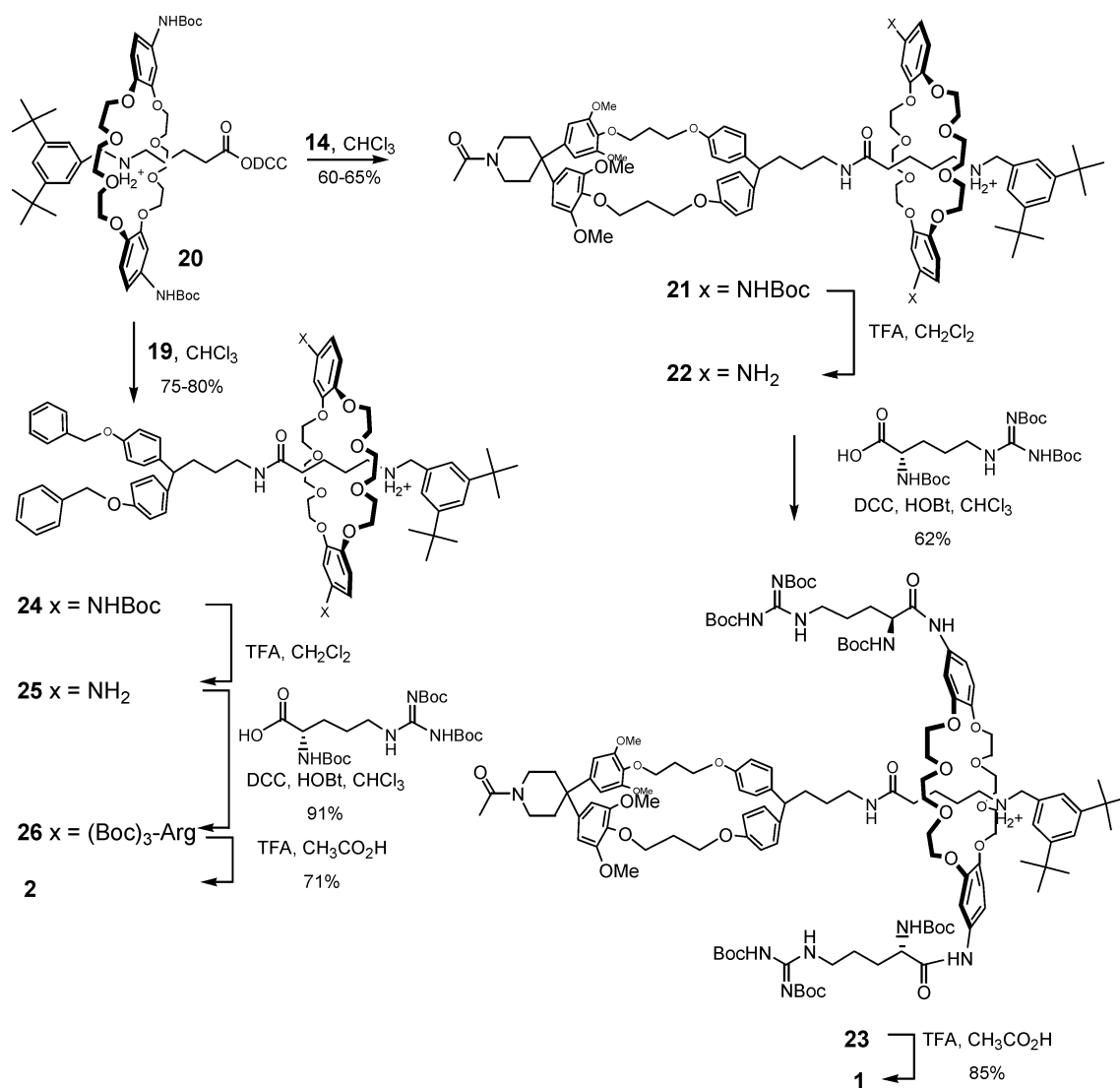
amide was chosen to represent the [2]rotaxanes, which has a pK_a of 7.3 ± 0.1. As discussed above, the pK_a of the axle's ammonium ion is usually greater than that of a free amine, so it is assigned a pK_a value greater than 7.3.

N-Ac-Trp-Asp was chosen as the representative guest for pK_a determination. Their carboxylates, being close together, may interact in DMSO, giving them a second pK_a value that is larger than the other carboxylic guests. Thus, we expect that the pK_a values of the other carboxylic guests to lie within the pK_a range of *N*-Ac-Trp-Asp. For *N*-Ac-Trp-Asp, a single end point was observed in the potentiometric titration plot with the addition of over 2 equiv of base. Apparently, the carboxylates do not interact significantly, resulting in only a single pK_a of 7.5 ± 0.1. A single end point was also observed for *N*-Ac-Trp-Asp in a 75/25 (v/v) water to DMSO solution, which resulted in a pK_a of 4.8 ± 0.1. For both *N*-Ac-Trp-Asp and arginine amide in 98% water, pK_a values were assumed to be equal to their corresponding values obtained in 100% water.

Determining Association Constants. Association constants were derived for complexes formed between various rotaxanes and guests (Table 1). Fluorescence quenching assays were performed in the DMSO, 75% water, and 98% water solutions.[37] A typical calculated structure of a [2]rotaxane **1** associated with

a guest is shown in Figure 2. The cyclophane pocket is large enough to accommodate a single side chain (in Figure 2, Leu is positioned within the pocket) and one Arg moiety of the ring forms a salt bridge with the carboxyl terminus of the dipeptide. We should note that the same complex energies were obtained for rotaxanes having either isomer of the ring, i.e., the arginine moieties positioned syn or anti to each other.

For studies in DMSO, amino acids containing carboxylic acids were converted to carboxylates by adding an equal molar amount of Me_4NOH to the stock solutions. Fluorescein and the fluorescein−PKC conjugate were used as the disodium salts, and 1,5-DNS was investigated as the sodium chloride salt. [2]-Rotaxanes **1,2** and cyclophane **3** were onium salts with trifluoroacetate counterions. The pK_a values of guests and hosts are close in DMSO, and thus, a small percentage of the components may have undergone proton exchange upon mixing.

Association constants were also derived for complexes formed in aqueous solutions. These solutions were buffered at pH values that ensured that the carboxylic acids remain deprotonated and the [2]rotaxane−amines remain protonated during association. Thus, according to the derived pK_a values, the 75/25 (v/v) water

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(37) For a representative titration plot, see ref 17, Figure 3 for cyclophane **2**.
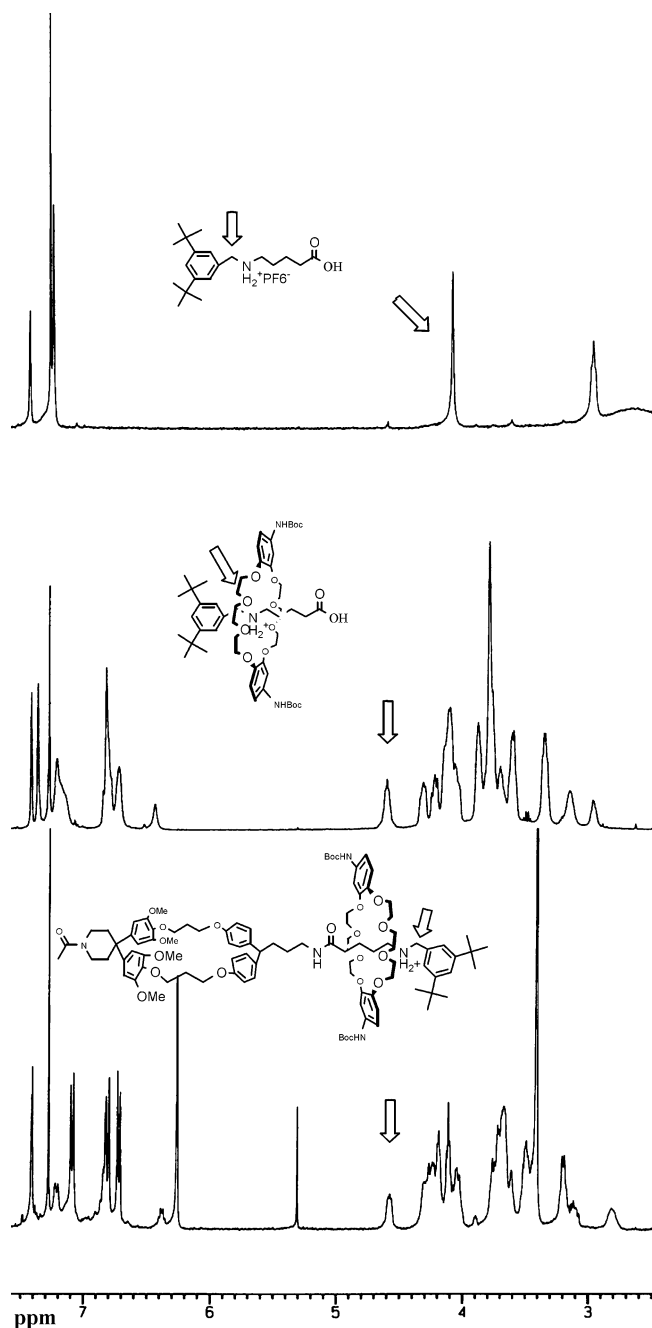
Figure 1. Stacked ^{1}H NMR plots demonstrate the large downfield shift of the benzylic protons of the axle (indicated by arrow) that occurs when the crown ether is threaded onto the axle.

Table 1. Association Constants of Various Host–Guest Complexes Measured at 25 °C^a

guest	% H$_2$O in a H$_2$O/DMSO solution	$K_A \times 10^{-4}$ (M^{-1}) for host–guest complexes		
		1	2	3
Ac-Trp	98	2.9	2.4	NS^b
	75	2.1	2.1	0.6
	0	2.5	2.2	0.1
Ac-Trp-NH$_2$	98	2.1	4.8	NS
	75	2.4	1.8	0.4
	0	1.6	1.7	0.5
indole	98	2.3	2.2	NS
	75	3.0	2.3	0.6
	0	1.7	1.6	0.5
In(CH$_2$)$_2$CO$_2$H^c	98	2.1	1.5	NS
	75	2.0	1.5	0.5
	0	2.3	2.0	0.5
Ac-Trp-Gly	98	1.2	1.3	NS
	75	1.8	1.6	0.5
	0	2.0	2.4	0.4
Ac-Trp-Glu	98	1.3	1.5	NS
	75	2.0	1.9	0.5
	0	1.8	2.0	0.5
Ac-Trp-Asp	98	2.1	2.6	NS
	75	2.0	2.2	0.4
	0	2.9	2.5	0.4
Ac-Trp-Ala	98	2.2	2.2	NS
	75	1.9	1.8	0.4
	0	3.5	2.5	0.5
Ac-Trp-Leu	98	2.0	2.8	NS
	75	2.2	1.8	0.5
	0	2.7	2.2	0.6
Ac-Trp-Ile	98	2.2	2.2	NS
	75	2.0	1.9	0.4
	0	2.6	2.4	0.5
fluorescein	98	6.6	5.3	NS
	75	3.1	2.8	0.9
	0	86	77	0.5
pyrene	98	8.0	4.1	NS
	75	2.7	1.8	0.1
	0	2.2	1.7	0.4
1,5-DNS^d	98	3.4	5.8	NS
	75	2.3	3.9	0.6
	0	2.0	1.5	0.4

^{a} Determined from analysis of fluorescence spectra, water solutions were buffered with 1 mM phosphate (pH 7.0) for the 98% water studies and 0.1 mM phosphate (pH 6.5) for the 75% water studies. Standard deviations are less than 5% of the reported values, except for the fluorescein study in 0% buffer and studies of host **3**, where the standard deviations are less than 10%. ^{b} NS means not soluble. ^{c} 3-Indolepropionic acid. ^{d} 1-(Dimethylamino)-5-naphthalenesulfonate.

to DMSO solution was buffered with 0.1 mM phosphate at pH 6.5. In this solution, a small amount of precipitate formed at the highest concentration of cyclophane **3** used in the assays. The titration curves, however, look consistent throughout the concentration range, and the derived association constants have reasonably low standard deviations. For binding assays performed in the 98% water solution, the water was buffered with 1.0 mM phosphate at pH 7.0. Besides minimizing proton exchange, this pH value was chosen because it is similar to the pH generally found in biological solutions. The poor solubility of cyclophane **3** in water prevented us from testing its ability to form complexes, whereas the [2]rotaxanes were soluble in the 98% water solution to at least a 0.1 mM concentration. This relatively high water solubility of the host–[2]rotaxanes, as compared to other large cyclophanes, is an important feature for cell transport agents.

Cellular Transport Studies. The demonstrated ability of the [2]rotaxanes to form tight complexes in a variety of solvents suggested to us that they could act as artificial transporters by carrying a guest through the many environments encountered during cell penetration. Fluorescein was chosen as the guest to test this hypothesis, because it is not cell permeable in the anionic state and its strong fluorescence makes monitoring its cellular location convenient. COS-7 cells were plated on slides, placed in 1 mL of buffer (1.0 mM phosphate, pH 7.0), and exposed to fluorescein or mixtures of fluorescein and [2]-rotaxanes **1** or **2**. As expected, fluorescein does not noticeably penetrate the cells (Figure 3A). Fluorescein, however, is noticeably transported when the cells are exposed for ca. 30 min to the same concentration of fluorescein (0.4 μM) in the presence of 6 μM of [2]rotaxane **1** (Figure 3B). [2]rotaxane **2** at 6 μM did not noticeably transport fluorescein; however, increasing its concentration to 60 μM did result in observable
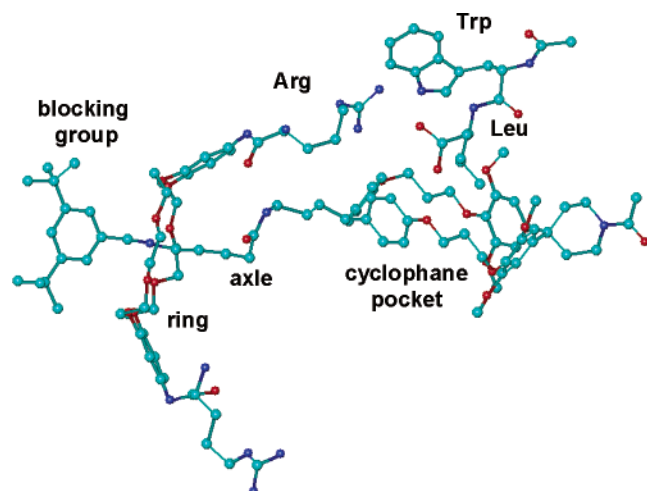
Figure 2. A low-energy conformation of the complex between [2]rotaxane **1** and *N*-Ac-Trp-Leu (molecular mechanics calculations,[18] MM+ force field as presented by Hyperchem[38]). Several components of the [2]rotaxane and guest are labeled and the H-atoms are removed for clarity.

transport (Figure 3E). Note that a longer film exposure time was needed to observe the fluorescence (15 s for the assays containing 60 μM [2]rotaxane **2** and 6 μM [2]rotaxane **1** versus 3 s for assays with 60 μM of [2]rotaxane **1**). Increasing the concentration of [2]rotaxane **1** to 60 μM produced strongly fluorescent cells, showing clearly the various organelles, including the nucleus (Figure 3C,D). The cells appeared to be healthy, and we observed at least one cell undergoing mitosis (Figure 3D). A single outer membrane contained two nuclei, which is consistent with the cell being in the telophase of mitosis. These results show that [2]rotaxane **1** and fluorescein are not immediately toxic to the cells.

To further investigate the potential of rotaxanes as transport agents, the ability of [2]rotaxane **1** to transport fluorescein–PKC inhibitor conjugate **27** into COS-7 cells was determined.
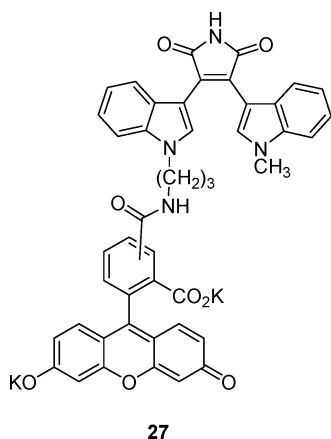
27

Conjugate **27** is transported into acidic solutions (pH < 6.5) and used to locate PKC within cells.[19] [2]Rotaxane **1** strongly binds conjugate **27** in the 98% buffer solution ($K_A = 1.2 \pm 0.2 \times 10^5$ M^{-1}) and in DMSO ($K_A = 2.0 \pm 0.2 \times 10^4$ M^{-1}). Conjugate **27** is transported into COS-7 cells by [2]rotaxane **1** in a pH 7.5 solution using the same experimental conditions as discussed above (0.4 μM conjugate exposed to 60 μM [2]-rotaxane **1** for 30 min, Figure 3F). Without the rotaxane, no fluorescence was observed. These results demonstrate that host–

[2]rotaxanes can efficiently deliver fluorescein and some fluorescein-derivatized agents through the various membrane barriers into the cytoplasm and the nucleus and uniformly deliver these agents throughout the entire population of cells independent of cellular differentiation.

Discussion

The association constants for complexes formed with cyclophane–[2]rotaxane **1** and cleft–[2]rotaxane **2** are quite large in the 98% aqueous solution (Table 1). K_A values in the 10^4 M^{-1} range compare well with current hosts designed to bind small peptides.[39–43] A comparison to cyclophane **3** cannot be obtained, because this host is insoluble in the 98% aqueous solution at the concentration needed to obtain complexes. With an increase in DMSO from 2% to 25%, cyclophane **3** is sufficiently soluble to observe association. A 4–6-fold enhancement in K_A is observed for the hosts built on the [2]rotaxane architecture, as compared to cyclophane **3**. One exception is the complexes formed with pyrene, which are preferred by the [2]rotaxanes by 10–20-fold. This result suggests that the aromatic surfaces supplied by the rotaxane ring contribute significantly to the binding free energy. Favorable binding energy could arise through dispersion interactions, aromatic stacking interactions, or the hydrophobic effect. Having a large hydrophobic pocket that is reasonably soluble in water is an important feature for compounds that mimic protein function.

Host–[2]rotaxanes **1** and **2** also form more stable complexes in DMSO than cyclophane **3**. In this solvent, interaction energies caused by aromatic and aliphatic groups are diminished in importance, whereas interactions between polar and charged groups become more important. For cyclophane **3**, there is very little change in the association constants going from 25% to 100% DMSO. The weak association of *N*-Ac-Trp in DMSO is an anomaly. The degree of association of the similar guest *N*-Ac-Trp-NH$_2$, which lacks the negative charge, is consistent with the other guests. Possibly burying the indole ring of *N*-Ac-Trp into the pocket may position the carboxylate too close to the apolar host. There is a 3–30-fold increase in K_A for complexes formed with [2]rotaxanes in DMSO as compared to cyclophane **3**, except for fluorescein. The strong preference of the [2]-rotaxanes for fluorescein (ca. 200-fold larger association constants) is consistent with our previous rotaxane study.[17] The fact that all guests investigated (with apolar and polar residues) are bound more strongly by the [2]rotaxanes than cyclophane **3** shows that the ring of the rotaxane contributes favorable binding free energy through a variety contacts, e.g., salt bridges, H-bonds, and dispersion interactions, with the guests. The strongest evidence for participation of the ring's functional groups is the 40-fold preference of the [2]rotaxanes for fluorescein, which has a carboxylate available for salt bridge formation, as compared to pyrene in DMSO.

Host–[2]rotaxanes **1** and **2** bind the guests with similar affinities in the solvent systems investigated. Apparently, the

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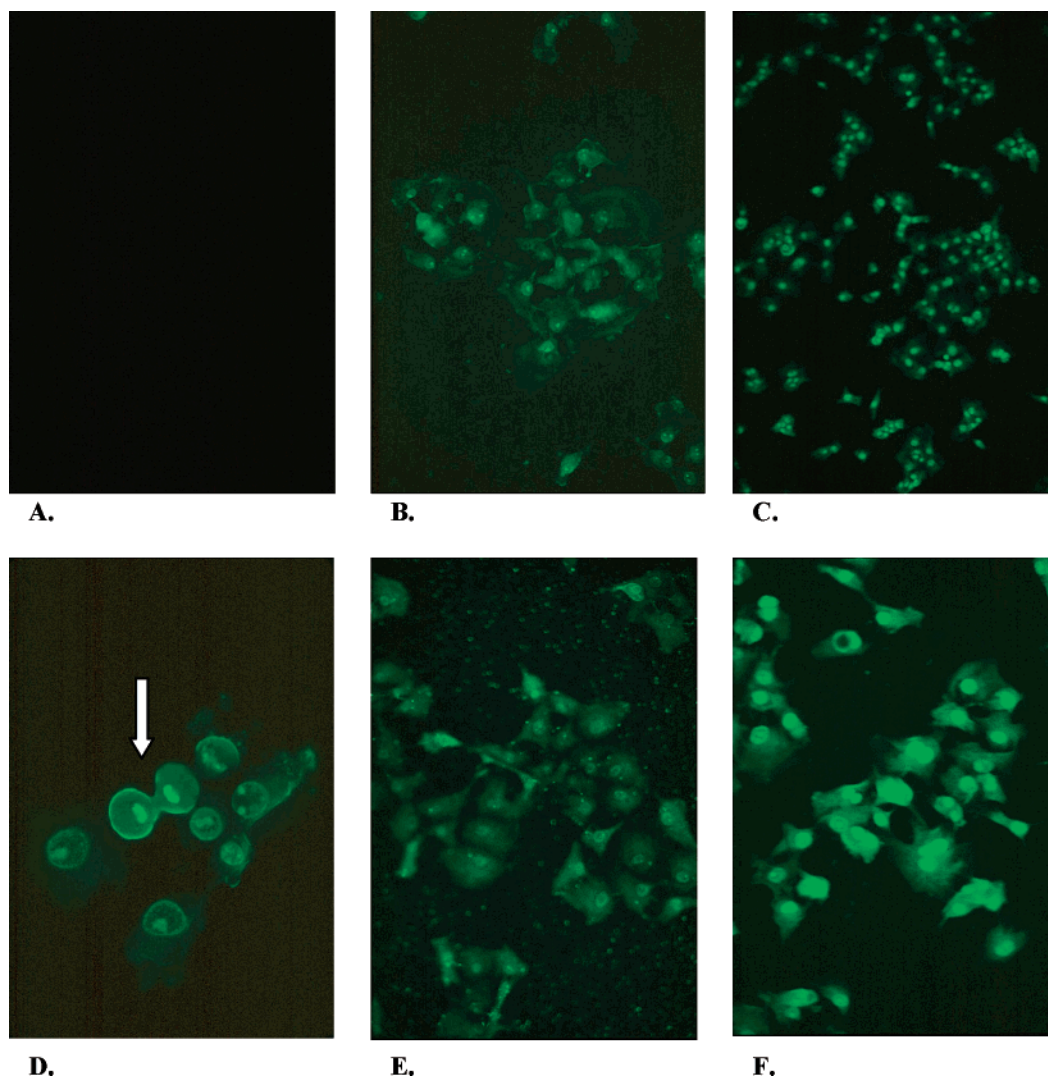
Figure 3. Fluorescence microscopy experiments showing the transport of fluorescein (0.4 μM) or a fluorescein−PKC inhibitor (0.4 μM) into COS-7 cells by the host−[2]rotaxanes. The cells were grown on slides, placed in 1 mL of phosphate buffer (pH 7), and exposed to the agents for ca. 30 min. (A) Fluorescein does not penetrate the cell membranes (30 s film exposure time). (B) Fluorescein is transported into the cells in the presence of 6 μM [2]rotaxane **1** (40× magnification, 15 s film exposure time). (C) In the presence of 60 μM of [2]rotaxane **1** the cells are highly fluorescent (20× magnification, 3 s film exposure time). This picture also shows that fluorescein is uniformly delivered throughout the entire population of cells. (D) An example of the cells undergoing mitosis (indicated by the arrow), which shows that [2]rotaxane **1** and fluorescein are not immediately toxic to the cells. Interestingly, transport of fluorescein was not observed with 6 μM of [2]rotaxane **2**. (E) At a higher concentration of [2]rotaxane **2** (60 μM), transport was observable (40× magnification, 15 s film exposure time). **F.** [2]Rotaxane **1** also efficiently transports fluorescein−PKC inhibitor **27** (40× magnification, 3 s film exposure time).

pocket of [2]rotaxane **1** does not provide a large advantage for association compared to the cleft. We thought that the aliphatic dipeptides would be more favorably sequestered into the pocket of [2]rotaxane **2** (Figure 2). There is a slight preference for the aliphatic dipeptides associating with [2]rotaxane **1** in DMSO, but this preference is lost with the addition of water to the solution. A partial collapse of the cyclophane pocket in an aqueous environment could explain the loss in guest affinity. For larger guests [1-(dimethylamino)-5-naphthalenesulfonate (1,5-DNS), pyrene, and fluorescein], the slight preference for the pocket maintains throughout the solvent systems. The larger preorganized aromatic surface provided by [2]rotaxane **1** may be responsible for the greater affinity, as compared to the open cleft of [2]rotaxane **2**. Larger association constants are observed for complexes formed with the large aromatic guests than for the amino acids and dipeptides in the aqueous solutions. The additional binding energy, most likely, stems from a greater hydrophobic effect.

Interactions between the aromatic and aliphatic surfaces appear to be the dominant source of favorable binding free energy for the smaller guests as well. Other interactions, such as a salt bridge, seem to provide a small amount of favorable binding energy. For example, [2]rotaxane **1** binds *N*-Ac-Trp more favorably than *N*-Ac-Trp-NH$_2$ and indole in DMSO and in the 98% buffered solution. Among the negatively charged peptidic guests, there is a slight preference for smaller guests. By comparing *N*-Ac-Trp to *N*-Ac-Trp-Glu, one can see that extending the carboxylate further from the aromatic ring weakens association. This effect is also observed by comparing the association constants of the dicarboxylate guests *N*-Ac-Trp-Asp and *N*-Ac-Trp-Glu in DMSO and the 98% buffer solution. The fact that *N*-Ac-Trp-Asp has a larger K_A than *N*-Ac-Trp-Gly in all solvent systems, and especially in the 98% buffered solution, suggests that the two cationic groups of the ring (ammonium and guanidinium ions) can favorably interact with guests having multiple anionic charges.

Another important consideration when comparing K_A's is the differences in the solvent−guest interactions. In this study, complexation describes a guest being removed from bulk solvent into an apolar pocket of a host. Although changing the solvent can have a profound effect on the degree of complexation,[25] a somewhat surprising, small solvent effect is observed. For example, fluorescein and pyrene have similar interaction energies with the rotaxanes in the aqueous solutions, even though fluorescein is highly water soluble and pyrene is not. The close similarity in the magnitude of the K_A values of most guest complexes (pyrene, 1,5-DNS, amino acids, and dipeptides) suggests that the combining sites of the [2]rotaxanes can accommodate all the functional groups that interact weakly with solvent molecules. Furthermore, since similar K_A values were obtained for guests that have dissimilar properties, the combining sites appear to adjust to maximize the number of favorable contacts between the various functional groups. The ability of the host−[2]rotaxanes to change their conformations to maintain a maximum in the favorable binding free energy can explain the efficient transport of fluorescein and fluorescein−PKC conjugate **27**. In aqueous environments, the hydrophobic effect keeps the complex together, and within membranes, the combining sites adjust, making salt bridges and H-bonds more important sources of favorable binding free energy.

These studies demonstrate that knowledge gained from binding assays performed in different solvents can indicate the likelihood of cell transport. The observed differences in the transport efficiencies of [2]rotaxane **1** with a change in its concentration is consistent with the K_A values obtained from the fluorescence assays. Assuming that the association constants for cellular and fluorescence assays are the same and with fluorescein at 0.4 μM, a change of concentration of [2]rotaxane **1** from 6 to 60 μM increases the percentage of fluorescein in a complex to free in solution from approximately 25% to 75%. Results obtained from the fluorescence assays also suggest that [2]rotaxane **2** should transport fluorescein as well as [2]rotaxane **1**. However, [2]rotaxane **2** is a less efficient transporter than [2]rotaxane **1**, requiring the higher concentration of rotaxane (60 μM) to produce noticeable transport. The cyclophane pocket of [2]rotaxane **1** is not beneficial for complex formation in DMSO and buffered solutions, but it is apparently beneficial for cell transport. Another interesting observation is that [2]-rotaxane **1** transports fluorescein to a greater extent into the nucleus, whereas [2]rotaxane **2** delivers fluorescein more uniformly throughout a cell.

Conclusion. Two new host−[2]rotaxanes were readily assembled through the key step of combining a DCC−[2]rotaxane with a cyclophane or an aromatic cleft. These hosts form tighter complexes with amino acids, dipeptides, and fluorophores than a more traditional cyclophane in water, DMSO, and 75/25 (v/ v) water to DMSO solution. The similarity in the K_A values of the complexes formed with cyclophane−[2]rotaxane **1** and cleft−[2]rotaxane **2** suggests that the unique assembly of the recognition elements—held together through threading—allows them to adjust to maintain a maximum in the favorable binding free energy. Although both [2]rotaxanes transport fluorescein into COS-7 cells, the transport ability of cyclophane−[2]-rotaxane **1** is substantially better. This result is surprising considering that the [2]rotaxanes bind fluorescein to the same extent in the fluorescence assays. Apparently, the cyclophane

pocket is beneficial for cell transport. Successful transport of the fluorescein−PKC inhibitor conjugate suggests that other fluorescein-linked agents can also be transported. These studies are currently being pursued.

Experimental Section

General. Solvents, reagents, and starting materials for the hosts were purchased from Aldrich. The derivatized amino acids are available commercially (Sigma or CHEM-IMPEX). ^{1}H NMR and ^{13}C NMR spectra were obtained in CDCl$_3$ using a Bruker AMX400 spectrometer operating at 400.14 MHz for proton and 100.23 MHz for carbon nuclei or a Bruker AC250 operating at 62.9 MHz for carbon nuclei and 250 MHz for proton. Chemical shifts are in ppm and are referenced using an internal TMS standard. A MEL-TEMP (Laboratory Devices) apparatus was used to determine the melting points of the compounds; these values are given uncorrected. HPLC analysis was performed on a Shimadzu 10A series HPLC. Solvents were purified by drying over a suitable drying agent and then distilled. Water for potentiometric titrations and HPLC assays was purified on a Millipore water purification system.

Potentiometric Titrations. 5.0 mM solutions of the templates were prepared from freshly distilled DMSO and boiled water. These solutions were titrated under Ar with solutions containing 0.10 M of *t*-BuOK for experiments performed in DMSO and 0.105 M of KOH for the experiments performed in 75% and 100% water. The pK_a values of the hosts and amino acids were calculated using the BEST program.[44]

Monitoring Association. Fluorescence binding assays were performed on the hosts and fluorescent guests. A known amount of guest, dissolved in a particular solution, was added to a 3.5 mL cuvette (maintained at 25 °C), and aliquots from a concentrated host stock solution in DMSO were added. The change in volume caused by the addition of a host was less than 2% for [2]rotaxanes **1** and **2** and less than 10% for cyclophane **3**. A fluorescence spectrum was recorded and analyzed after each addition of host. The amino acids and dipeptides were held at 1×10^{-5} M, and the concentration of [2]rotaxanes **1** and **2** was increased in a 10^{-5} M increment from 1×10^{-5} to 8×10^{-5} M. The fluorophores were held at 3×10^{-6} M and the hosts were added at various concentrations from 1×10^{-6} to 3×10^{-5} M. For studies of cyclophane **3**, the host concentration was increased from 1×10^{-5} to 5×10^{-4} M, and the guests were held at a constant concentration of 3×10^{-5} M. Hosts were added to the assay solutions until saturation began to be observable, giving an experimental maximum change in fluorescence equal to 65−90% of the calculated value (ΔF_{max}). Plots of the changes observed in the binding titrations were fitted using a nonlinear least-squares procedure to derive K_A and ΔF_{max} values.[45] Most assays were repeated using different experimentalists with different stock solutions. The deviation in the results of these repeated assays was equal to or below the standard deviation (given in Table 1) obtained from the mathematical analysis of the data.

Compound Synthesis. Dipeptides were constructed by mixing an amino acid with *N*-Ac-Trp-CO$_2$H, which was activated with ethylchloroformate. Pure product was obtained through HPLC separation, and its authenticity was verified via ^{1}H NMR and mass spectral analysis.

Bis(4-benzyloxyphenyl)methanol (5). A 25.0 g (117 mmol) sample of 4,4′-dihydroxybenzophenone **4** was dissolved in the mixture of 300 mL of chloroform and 300 mL of methanol. Benzyl bromide (36.3 mL, 304 mmol), followed by 48.3 g (350 mmol) of potassium carbonate, was added. The reaction mixture was refluxed for 2 days. Solvents were evaporated, and the residue was partitioned between 2.0 L of chloroform and 0.5 L of water. The organic layer was dried over sodium sulfate. The solvent was evaporated and the residue was washed with 3×50 mL of hexane in order to remove the residual benzyl bromide.

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White crystals were produced (44.6 g, 11.3 mmol, 97%). The spectral data and the melting point matched the published information about the compound.[46] A 35.6 g (90.3 mmol) sample of the resulting 4,4′-dibenzyloxybenzophenone was dissolved in 400 mL of a 50/50 CH_2Cl_2/EtOH solution, and 3.43 g (90.3 mmol) of $NaBH_4$ was added. After stirring of the reaction mixture at room temperature for 24 h, the solvents were removed in vacuo. The residue was partitioned between 200 mL of methylene chloride and 200 mL of water. The aqueous layer was extracted twice with 50 mL of methylene chloride, and the combined organic extracts were dried over sodium sulfate. Removal of the solvent in vacuo gave 35.5 g (89.4 mmol) of alcohol **5** in a 99% yield, as a white solid (mp = 115−117 °C). ^{1}H NMR ($CDCl_3$): 5.05 (4H, s), 5.77 (1H, s), 6.94 (4H, d, J = 8.3 Hz), 7.28 (4H, d, J = 8.4 Hz), 7.32−7.43 (10H, m). ^{13}C NMR ($CDCl_3$): 69.9, 75.2, 114.7, 127.4, 127.7, 127.9, 128.5, 136.6, 136.9, 158.1. MS: found 419.1648, calcd for $C_{27}H_{24}O_3Na$ [M + Na] 419.1623.

3,3-Bis(4-benzyloxyphenyl)propene (7). A solution of trifluoro-acetic acid (0.72 g, 6.3 mmol) in 1 mL of CH_2Cl_2 was added dropwise to a 30 mL solution of CH_2Cl_2 containing 5.00 g (12.6 mmol) of alcohol **5** and 2.89 g (25.3 mmol) of allyltrimethylsilane. The reaction mixture was stirred at room temperature for 24 h. The solvent and volatile byproducts were evaporated under reduced pressure to give 5.09 g (12.1 mmol) of alkene **7** in a 96% yield, as an off-white solid (mp = 93−95 °C). ^{1}H NMR ($CDCl_3$): 2.75 (2H, t, J = 7.3 Hz), 3.91 (1H, t, J = 7.7 Hz), 4.91−5.05 (6H, m), 5.69 (1H, m), 6.89 (4H, d, J = 8.5 Hz), 7.13 (4H, d, J = 8.5 Hz), 7.29−7.43 (5H, m). ^{13}C NMR ($CDCl_3$): 40.4, 49.6, 70.0, 114.8, 116.2, 127.5, 127.9, 128.6, 128.9, 137.1, 137.3, 137.4, 157.2. MS: found 443.2013, calcd for $C_{30}H_{28}O_2Na$ [M + Na] 443.1987.

3,3-Bis(4-hydroxyphenyl)propene (8). A 154 mg (22.0 mmol) sample of lithium metal was added in small pieces to a solution of 5.32 g (20.0 mmol) of 4,4′-di-*tert*-butylbiphenylene dissolved in 44 mL of anhydrous THF. The reaction mixture was stirred under argon for 24 h until all of lithium was consumed. Alkene **7** (700 mg, 1.7 mmol) was dissolved in 2 mL of THF and added dropwise to the freshly prepared solution of LDDB. To this solution was added 2 mL of methanol followed by 1 M of HCl until the pH = 3. The mixture was extracted with 3 × 50 mL of CH_2Cl_2. The combined organic layers were washed with brine and dried over Na_2SO_4. Evaporation of the solvents under reduced pressure gave a yellow solid. Purification of the crude material by column chromatography (5:100 of EtOH/CH_2Cl_2) yielded 370 mg (1.5 mmol) of alkene **8** in a 92% yield, as pink crystals (mp = 111−113 °C). ^{1}H NMR ($CDCl_3$): 2.72 (2H, t, J = 7.0 Hz), 3.88 (1H, t, J = 7.3 Hz), 4.93−5.04 (2H, m), 5.65−5.71 (1H, m), 6.74 (4H, d, J = 8.4 Hz), 7.06 (4H, d, J = 8.4 Hz). ^{13}C NMR ($CDCl_3$): 40.3, 49.5, 115.2, 116.0, 128.8, 136.8, 137.1, 154.1. MS: found 263.1058, calcd for $C_{16}H_{16}O_2Na$ [M + Na] 263.1048.

An alternative deprotection procedure involves mixing 10.0 g (23.8 mmol) of alkene **7**, dissolved in 200 mL of CH_2Cl_2, and 50 mL (50 mmol) of 1 M boron trichloride, which was introduced via a syringe. After stirring the reaction mixture for 1 h, the solution was washed with water and dried over Na_2SO_4, and solvent was removed under reduced pressure. The crude material was washed with hexane to remove benzyl chloride and then subjected to purification by column chromatography (10:90 EtOH/CH_2Cl_2) to give 4.48 g (18.7 mmol) of alkene **8** in a 78% yield.

Cyclophane Alkene (10). A 1.04 g (1.54 mmol) sample of dibromide **9**[36] and a 1.83 g (7.1 mmol) sample of 3,3-bis(4-hydroxyphenyl)propene **8** were dissolved in 150 mL of anhydrous DMF. The reaction mixture was degassed three times by keeping it under vacuum for ca. 2 min and then introducing dry Ar. To this mixture was added 2.00 g (6.16 mmol) of finely powdered $CsCO_3$. The reaction mixture was stirred under Ar at 80−100 °C for 24 h. After removal of the solvent under reduced pressure, and the residue was partitioned between 50 mL of CH_2Cl_2 and 50 mL of water. The aqueous layer was washed with 2 ×

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25 mL of CH_2Cl_2, and the organic extracts were combined and dried over Na_2SO_4. After the solvent was removed under reduced pressure, the crude material was purified by column chromatography (95/5 CH_2Cl_2/EtOH) to give 0.33 g (0.44 mmol) of host **10** in a 28% yield, as a white foam (mp = 95−97 °C). ^{1}H NMR ($CDCl_3$): 2.10−2.14 (7H, m), 2.25 (4H, m), 2.79 (2H, t, J = 7.2 Hz), 3.42 (12H, s), 3.48 (2H, m), 3.66 (2H, m), 3.84 (1H, t, J = 5.1 Hz), 4.11 (4H, t, J = 5.2 Hz), 4.21 (2H, t, J = 5.2 Hz), 4.93−5.07 (2H, m), 5.6−5.8 (1H, m), 6.24 (4H, s), 6.74 (4H, d, J = 8.4 Hz), 7.06 (4H, d, J = 8.4 Hz). ^{13}C NMR ($CDCl_3$): 21.1, 30.2, 36.1, 36.5, 37.1, 37.6, 38.4, 38.6, 49.5, 55.4, 64.0, 69.5, 104.8, 114.2, 115.8, 127.8, 135.7, 136.7, 137.1, 140.6, 152.5, 157.4, 168.6. MS: found 752.3693, calcd for $C_{45}H_{54}NO_9$ 752.3799.

Cyclophane Alcohol (11). A 200 mg (0.26 mmol) sample of cyclophane **10** was dissolved in 4 mL of THF, and a 0.16 mL (0.32 mmol) sample of 2 M borane−dimethyl sulfide complex in THF was added dropwise. The mixture was stirred for 4 h followed by the slow addition (exothermic!) of 0.3 mL of 30% H_2O_2 and 0.3 mL of 2 M NaOH. After removal of the solvent under reduced pressure, and the residue was partitioned between 10 mL of CH_2Cl_2 and 10 mL of water. The aqueous layer was washed with 2 × 10 mL of CH_2Cl_2, and the organic extracts were combined and dried over Na_2SO_4. After the solvent was removed, the crude material was purified by column chromatography (EtOAc) to give 180 mg (0.23 mmol) of alcohol **11** in a 88% yield, as a white foam (mp = 83−85 °C). ^{1}H NMR ($CDCl_3$): 1.57 (2H, m), 2.10−2.12 (9H, m), 2.24 (4H, m), 3.41 (12H, s), 3.48 (2H, m), 3.64−3.67 (4H, m), 3.75 (1H, t, J = 6.1 Hz), 4.11 (4H, t, J = 5.0 Hz), 4.21 (4H, t, J = 5.1 Hz), 6.25 (4H, s), 6.74 (4H, d, J = 8.3 Hz), 7.01 (4H, d, J = 8.3 Hz). ^{13}C NMR ($CDCl_3$): 21.3, 30.4, 30.7, 31.2, 36.1, 36.3, 36.7, 37.1, 37.3, 37.7, 38.7, 43.7, 55.6, 62.6, 64.2, 69.7, 104.9, 114.4, 127.9, 135.8, 137.8, 140.7, 152.7, 157.6, 169.0. MS: found 792.3754, calcd for $C_{45}H_{55}NO_{10}Na$ [M + Na] 792.3724.

An alternative route for the formation of cyclophane **11** involves mixing a 4.8 g (7.1 mmol) sample of dibromide **9** and a 1.83 g (7.1 mmol) sample of 3,3-bis(4-hydroxyphenyl)propanol **16** (vide infra) in 708 mL of anhydrous DMF. The reaction mixture was degassed three times by keeping it under vacuum for ~2 min and then introducing dry Ar. To this mixture was added 9.24 g (28.4 mmol) of finely powdered $CsCO_3$. The reaction mixture was stirred under Ar at 80−100 °C for 72 h. Following the same purification procedure as used above yielded 32% of cyclophane **11**.

Cyclophane Bromide (12). A 660 mg (0.86 mmol) sample of alcohol **11** and 298 mg (1.14 mmol) of triphenylphosphine were dissolved in 5 mL of CH_3CN. To this solution was added 377 mg (1.14 mmol) of CBr_4, dissolved in 1.6 mL of CH_3CN. The reaction mixture was stirred for 4 h, and the solvent was removed in vacuo. The residue was separated by column chromatography (gradient elution with EtOAc to 5:100 MeOH/EtOAc) to give 620 mg (0.74 mmol) of bromide **12** in an 87% yield, as a white foam (mp = 95−97 °C). ^{1}H NMR ($CDCl_3$): 1.86 (2H, quintet, J = 7.6 Hz), 2.10−2.26 (13H, m), 3.41 (14H, m), 3.48 (2H, m), 3.66 (2H, m), 3.76 (1H, m), 4.11 (4H, m), 4.20 (4H, m), 6.25 (4H, s), 6.75 (4H, d, J = 8.4 Hz), 7.06 (4H, d, J = 8.4 Hz). ^{13}C NMR ($CDCl_3$): 21.3, 30.4, 31.4, 33.0, 33.8, 36.1, 36.3, 36.7, 37.1, 37.4, 37.8, 38.7, 43.7, 55.7, 64.3, 69.7, 105.0, 114.5, 127.8, 135.9, 137.3, 140.8, 152.7, 157.7, 168.9. MS: found 832.3093, calcd for $C_{45}H_{55}BrNO_9$ [M + 1] 832.3055.

Cyclophane Azide (13). A 312 mg (4.80 mmol) sample of NaN_3 was added to 500 mg (0.60 mmol) of bromide **12**, dissolved in 10 mL of CH_3CN. The reaction mixture was refluxed for 72 h, cooled to room temperature, and the salts were removed by filtration. After removal of the solvent under reduced pressure, the residue was dissolved in 10 mL of CH_2Cl_2. Insoluble materials were removed by filtration. Removing the solvent in vacuo resulted in 488 mg (0.60 mmol) of azide **13** in a quantitative yield, as a white foam (mp = 89−91 °C). ^{1}H NMR ($CDCl_3$): 1.58 (2H, quintet, J = 7.2 Hz), 2.01−2.15 (9H, m), 2.15−2.30 (4H, m), 3.27 (2H, t, J = 6.4 Hz), 3.41 (12H, s), 3.48 (2H, m), 3.66 (2H, m), 3.76 (1H, m), 4.11 (4H, m), 4.20 (4H, m), 6.26 (4H,

s), 6.75 (4H, d, *J* = 7.8 Hz), 7.06 (4H, d, *J* = 7.8 Hz). ^{13}C NMR (CDCl$_3$): 21.2, 27.2, 30.2, 31.4, 35.9, 36.2, 36.6, 36.9, 37.2, 37.6, 38.5, 43.5, 51.2, 55.5, 64.1, 69.6, 104.7, 114.3, 127.7, 135.7, 137.2, 140.6, 152.5, 157.5, 168.7. MS: found 817.3813, calcd for C$_{45}$H$_{54}$N$_4$O$_9$Na [M + Na] 817.3788.

Cyclophane Amine (14). A 1.09 g (1.37 mmol) sample of azide **13** was dissolved in 20 mL of freshly distilled DMF. To this solution was added 146 mg (0.14 mmol) of 10% Pd/C, and the reaction mixture was hydrogenated under 50 psi of H$_2$ overnight. The suspension was removed by passing the solution through a Celite pad to remove the catalyst. After the solvent was removed under reduced pressure, the residue was separated by column chromatography (CH$_2$Cl$_2$ to 5:100 Et$_3$N/CH$_2$Cl$_2$) to give 660 mg (0.82 mmol) of amine **14** in a 60% yield, as an amorphous glass. ^{1}H NMR (CDCl$_3$): 1.44 (2H, quintet, *J* = 7.1 Hz), 1.99−2.13 (9H, m), 2.24 (4H, m), 2.72 (2H, t, *J* = 6.5 Hz), 3.41 (12H, s), 3.48 (2H, m), 3.66 (2H, m), 3.72 (1H, t, *J* = 7.5 Hz), 4.10 (4H, t, *J* = 5.2 Hz), 4.20 (4H, t, *J* = 5.1 Hz), 6.24 (4H, s), 6.73 (4H, d, *J* = 8.3 Hz), 7.05 (4H, d, *J* = 8.3 Hz). ^{13}C NMR (CDCl$_3$): 21.3, 29.2, 30.3, 31.8, 36.3, 36.7, 37.3, 37.7, 38.6, 49.6, 53.3, 55.6, 64.3, 69.8, 104.8, 114.3, 127.8, 135.8, 137.8, 140.7, 152.6, 157.5, 168.8. MS: found 769.4062, calcd for C$_{45}$H$_{57}$N$_2$O$_9$ [M + 1] 769.4064.

Benzylcleft Alcohol (15). A 21.0 mL (42.5 mmol) sample of 2 M borane−dimethyl sulfide complex in THF was added dropwise to 16.8 g (40.1 mmol) of alkene **8** dissolved in 40 mL of THF. The mixture was stirred for 4 h followed by the slow addition (exothermic!) of 10 mL of 30% H$_2$O$_2$ and 10 mL of 2 M NaOH. After extracting the solution with 3 × 70 mL of CH$_2$Cl$_2$, the combined extracts were washed with brine and dried over Na$_2$SO$_4$. Evaporation of solvents under reduced pressure gave pale yellow oil, which was purified by column chromatography (5:100 EtOH/CH$_2$Cl$_2$) to give 12.68 g (28.9 mmol) of alcohol **15** in a 78% yield, as an oil, which crystallizes upon standing (mp = 60−62 °C). ^{1}H NMR (CDCl$_3$): 1.53 (2H, quintet, *J* = 7.6 Hz), 2.05 (2H, q, *J* = 7.2 Hz), 3.63 (2H, t, *J* = 6.3 Hz), 3.81 (1H, t, *J* = 7.6 Hz), 5.01 (4H, s), 6.89 (4H, d, *J* = 8.5 Hz), 7.14 (4H, d, *J* = 8.4 Hz), 7.30−7.43 (5H, m). ^{13}C NMR (CDCl$_3$): 31.2, 32.1, 49.4, 62.7, 69.9, 114.7, 127.4, 127.8, 128.5, 128.6, 137.1, 137.7, 157.1. MS: found 461.2054, calcd for C$_{30}$H$_{30}$O$_3$Na [M + Na] 461.2093.

Cleft Alcohol (16). A 440 mg (1.0 mmol) sample of alcohol **15** was dissolved in 10 mL of a 50:50 MeOH/THF solution, and 100 mg (0.1 mmol) of 10% Pd/C was added. The mixture was stirred overnight under 50 psi of H$_2$. After the reaction mixture was filtered through a Celite pad and the solvent removed in vacuo, 300 mg (1.0 mmol) of alcohol **16** was obtained in a quantitative yield, as a pale yellow glass. ^{1}H NMR (CDCl$_3$): 1.29 (2H, quintet, *J* = 7.6 Hz), 1.89 (2H, q, *J* = 7.4 Hz), 3.35 (2H, m), 3.65 (1H, t, *J* = 7.7 Hz), 4.33 (1H, t, *J* = 5.1 Hz *OH*), 6.64 (4H, d, *J* = 8.3 Hz), 7.01 (4H, d, *J* = 8.5 Hz), 9.11 (2H,s *OH*). ^{13}C NMR (CDCl$_3$): 31.4, 32.2, 49.0, 60.9, 115.2, 128.5, 136.4, 155.5. MS: found 281.1101, calcd for C$_{16}$H$_{18}$O$_3$Na [M + Na] 281.1154.

Cleft Bromide (17). A 4.87 g (11.1 mmol) sample of 3,3-bis(4-benzyloxyphenyl)propanol **15** and 3.88 g (14.8 mmol) of triphenylphosphine were dissolved in 30 mL of CH$_3$CN. To this solution was added dropwise 4.91 g (14.8 mmol) of carbon tetrabromide, dissolved in 2 mL of CH$_3$CN. The reaction mixture was stirred for 4 h. The solvent was removed under vaccum, and the residue was purified by column chromatography (4:1 hexane/ethyl acetate) to give 5.07 g (10.1 mmol) of bromide **17** in a 91% yield, as pale yellow oil. ^{1}H NMR (CDCl$_3$): 1.81 (2H, quintet, *J* = 7.2 Hz), 2.14 (2H, q, *J* = 7.6 Hz), 3.40 (2H, t, *J* = 6.4 Hz), 3.81 (1H, t, *J* = 7.6 Hz), 5.02 (4H, s), 6.89 (4H, d, *J* = 8.8 Hz), 7.13 (4H, d, *J* = 8.4 Hz), 7.31−7.42 (5H, m). ^{13}C NMR (CDCl$_3$): 31.0, 33.7, 34.2, 48.7, 69.8, 114.7, 127.2, 127.7, 128.3, 128.4, 137.0, 137.1, 157.0. MS: found 523.1310, calcd for C$_{30}$H$_{29}$BrO$_2$Na [M + Na] 523.1249.

Cleft Azide (18). A 3.94 g (60.6 mmol) sample of NaN$_3$ was added to 5.07 g (10.1 mmol) of bromide **17**, dissolved in 20 mL of CH$_3$CN. The suspension was refluxed for 72 h, and the salts were removed

through filtration. Removal of the solvent in vacuo resulted in 4.66 g (10.1 mmol) of azide **18** in a quantitative yield, as a pale yellow oil. ^{1}H NMR (CDCl$_3$): 1.55 (2H, quintet, *J* = 7.1 Hz), 2.05 (2H, q, *J* = 8.0 Hz), 3.26 (2H, t, *J* = 6.5 Hz), 3.80 (1H, t, *J* = 7.8 Hz), 5.02 (4H, s), 6.89 (4H, d, *J* = 8.6 Hz), 7.12 (4H, d, *J* = 8.5 Hz), 7.30−7.42 (5H, m). ^{13}C NMR (CDCl$_3$): 27.1, 32.6, 48.9, 50.9, 69.5, 114.5, 127.1, 127.5, 128.1, 128.3, 136.8, 137.0, 156.9. MS: the azide is unstable to mass spectral analysis.

Cleft Amine (19). A 400 mg (1.6 mmol) sample of triphenylphosphine and 42 mg (2.3 mmol) of water were added to 720 mg (1.6 mmol) of azide **18** in 5 mL of CH$_3$CN. The reaction mixture was stirred for 4 h, and the solvent was removed in vacuo. The residue was separated by column chromatography (successive elutions with 100 mL 1:1 hexane/CH$_2$Cl$_2$, 100 mL of CH$_2$Cl$_2$, 100 mL of 4:100 EtOH/CH$_2$Cl$_2$, 100 mL of 5:10:100 EtOH/Et$_3$N/CH$_2$Cl$_2$) to give 600 mg (1.4 mmol) of amine **19** in a 89% yield, as a yellow solid (mp = 78−80 °C). ^{1}H NMR (CDCl$_3$): 1.43 (2H, quintet, *J* = 7.3 Hz), 1.66 (2H, m), 2.00 (2H, q, *J* = 7.6 Hz), 2.70 (2H, t, *J* = 7.0 Hz), 3.79 (1H, t, *J* = 7.6 Hz), 5.00 (4H, s), 6.88 (4H, d, *J* = 8.3 Hz), 7.12 (4H, d, *J* = 8.4 Hz), 7.30−7.42 (5H, m). ^{13}C NMR (CDCl$_3$): 31.2, 32.7, 41.2, 48.9, 69.3, 114.1, 126.8, 127.2, 127.9, 128.0, 136.6, 137.2, 156.5. MS: found 438.2398, calcd for C$_{30}$H$_{32}$NO$_2$ [M + 1] 438.2433.

BocNH-cyclophane−[2]Rotaxane (21). A DCC−[2]rotaxane **20**[18] solution (0.64 mmol, 1 mL CHCl$_3$) was cooled to 0 °C, and 270 mg (0.35 mmol) of amine **14** in 0.2 mL of chloroform was rapidly added. The reaction was brought to a room temperature and stirred overnight. Then 20 mL of CH$_3$CN was added to the solution, and the resulting precipitated dicyclohexylurea was removed by filtration. After removal of the solvent under reduced pressure, the residue was dissolved in 1 mL of CH$_2$Cl$_2$ and approximately 20 mL of diethyl ether was added to remove the *N*-(3,5-di-*tert*-butylbenzyl)valerolactam byproduct as a precipitate. The solution sat for 4 h until all of the insoluble material precipitated. After decanting the ether layer, the precipitation procedure was repeated. The ether layer was collected, and the solvent was removed in vacuo. Separation of the crude material was achieved using radial centrifugal chromatography (CH$_2$Cl$_2$ to 5:100 MeOH/CH$_2$Cl$_2$) to give 400 mg (0.21 mmol) of [2]rotaxane **1** in a 60% yield, as an off-white foam (mp = 138−140 °C). ^{1}H NMR (CDCl$_3$): 1.27 (18H, s), 1.49−1.51 (18H, 2 × s), 1.74 (4H, m), 1.92 (2H, m), 2.10 (3H, s), 2.25 (10H, m), 2.8 (2H, m), 3.1−3.3 (4H, m), 3.40 (12H, s), 3.48 (2H, m), 3.5−3.8 (18H, m), 4.0−4.3 (17H, m), 4.6 (2H, m), 6.25 (4H, s), 6.71−7.08 (12H, m), 7.20 (2H, m), 7.39 (3H, m). ^{13}C NMR (CDCl$_3$): 172.5, 169.0, 157.5, 153.1, 152.7, 151.1, 147.4, 143.1, 141.0, 138.0, 135.8, 133.1, 128, 124.4, 123.1, 114.5, 113.2, 113.1, 111.8, 105.2, 104.9, 80.1, 70.4, 69.8, 68.2, 64.4, 55.7, 49.5, 48.8, 46.3, 43.7, 39.3, 37.6, 36.6, 35.7, 34.7, 31.3, 30.4, 28.2, 27.9, 25.8, 22.4, 21.3. MS: found 1748.9751, calcd for C$_{99}$H$_{138}$N$_5$O$_{22}$ [M + 1] 1748.9833.

BocNH-cleft−[2]Rotaxane (24). Construction of [2]rotaxane **24** followed the same procedure used to synthesize and purify [2]rotaxane **23** with 0.21 mmol of DCC−[2]rotaxane **20**[18] and 0.14 mmol of amine **19** in 0.5 mL of CDCl$_3$. Purification using radial centrifugal chromatography (CH$_2$Cl$_2$ to 5:100 EtOH/CH$_2$Cl$_2$) gave 150 mg (0.105 mmol) of [2]rotaxane **24** in a 75% yield, as a yellow foam (mp = 115−117 °C). ^{1}H NMR (CDCl$_3$): 1.26−1.28 (18H, 2 × s), 1.49−1.51 (18H, 2 × s), 1.60 (4H, m), 1.86−2.02 (4H, m), 2.72−2.89 (2H, m), 3.08−3.25 (2H, m), 3.40−3.57 (2H, m), 3.57−3.90 (13H, m), 3.90−4.13 (8H, m), 4.13−4.37 (4H, m), 4.57 (2H, m), 4.98−5.03 (4H, 2 × s), 6.47 (2H, br s), 6.74 (2H, s), 6.79−6.91 (6H, m), 7.04−7.22 (6H, m), 7.28−7.43 (13H, m). ^{13}C NMR (CDCl$_3$): 172.3, 156.9, 153.0, 148.5, 147.9, 143.2, 143.1, 137.8, 137.0, 134.6, 134.3, 132.9, 128.5, 128.3, 127.6, 127.2, 124.9, 114.6, 113.0, 106.5, 80.2, 70.3, 69.8, 68.4, 68.1, 52.5, 48.6, 39.2, 35.0, 34.6, 33.0, 31.2, 31.2, 28.1, 25.5, 25.4, 22.3. MS: found 1417.8268, calcd for C$_{84}$H$_{113}$N$_4$O$_{15}$ [M + 1] 1417.8202.

NH$_2$-cleft−[2]Rotaxane (25). A 490 mg (0.31 mmol) sample of BocNH-cleft−[2]rotaxane **24** was dissolved in 3.0 mL of CH$_2$Cl$_2$, To this solution was added 1.5 mL of trifluoroacetic acid, dissolved in 1.0

mL of CH_2Cl_2. The reaction mixture was stirred for 2 h, washed with 2 M KOH, and dried over Na_2SO_4. The organic phase was removed in vacuo to give 410 mg (0.31 mmol) of [2]rotaxane **25** in a quantitative yield, as a brown glass. ^1H NMR ($CDCl_3$): 1.31 (18H, s), 1.25–1.50 (4H, m), 1.85–2.00 (4H, m), 2.48–2.70 (2H, m), 3.36 (4H, m), 3.53–3.58 (2H, m), 3.73–3.86 (16H, m), 3.92–4.13 (9H, m), 4.98–5.01 (4H, 2 × s), 6.18 (2H, m), 6.30 (2H, m), 6.71 (2H, m), 6.88 (4H, m), 7.13 (4H, m), 7.30–7.43 (13H, m). ^{13}C NMR ($CDCl_3$): 157.0, 149.9, 148.1, 142.1, 141.1, 137.7, 137.0, 130.9, 128.6, 128.5, 128.3, 127.6, 127.2, 118.0, 117.5, 114.6, 113.8, 107.3, 102.7, 70.8, 70.3, 67.9, 49.4, 41.8, 34.6, 33.6, 33.2, 32.1, 31.3, 29.4, 24.9, 22.4, 18.9. MS: found 1217.7141, calcd for $C_{74}H_{97}N_4O_{11}$ [M + 1] 1217.7154.

NH$_2$-cyclophane–[2]Rotaxane (22). A 100 mg (0.064 mmol) sample of BocNH-cyclophane–[2]rotaxane **21** was dissolved in 1.0 mL of CH_2Cl_2. To this solution was added 0.3 mL of trifluoroacetic acid, dissolved in 0.5 mL of CH_2Cl_2. The reaction mixture was stirred for 5 h, washed with 2 M KOH, and dried over Na_2SO_4. The organic phase was removed in vacuo to give 75 mg (0.062 mmol) of [2]rotaxane **22** in a 98% yield, as a pink glass. ^1H NMR ($CDCl_3$): 1.31 (18H, s), 1.39–1.58 (4H, m), 1.81–2.02 (2H, m), 2.10 (3H, s), 2.02–2.15 (6H, m), 2.17–2.32 (6H, m), 2.45–2.61 (2H, m), 3.39 (12H, s), 3.33–3.93 (24H, m), 3.93–4.23 (17H, m), 6.13–6.30 (8H, m), 6.50–6.86 (6H, m), 7.04–7.19 (7H, m). ^{13}C NMR ($CDCl_3$): 172.6, 168.8, 157.4, 152.7, 150.9, 148.1, 142.3, 140.8, 139.7, 137.9, 135.9, 127.9, 127.5, 124.7, 122.9, 114.6, 114.3, 107.2, 105.0, 101.8, 70.1, 69.6, 68.1, 64.3, 55.6, 53.3, 49.4, 48.8, 46.2, 43.6, 39.1, 38.6, 37.5, 36.5, 34.7, 31.2, 30.4, 29.7, 27.9, 25.9, 22.3, 21.1. MS: found 1548.8770, calcd for $C_{89}H_{122}N_5O_{18}$ [M + 1] 1548.8785.

(Boc)$_3$Arg-cleft–[2]Rotaxane (26). A 410 mg (0.31 mmol) sample of the NH$_2$-cleft–rotaxane **25** and 300 mg (0.64 mmol) of (Boc)$_3$ArgOH were dissolved in 3.5 mL of CH_2Cl_2. To this solution was added 9 mg (0.06 mmol) of 1-hydroxybenzotriazole (HOBt), followed by a rapid injection of 132 mg (0.64 mmol) of DCC, dissolved in 0.5 mL of CH_2Cl_2. The reaction mixture was stirred for 2 h and diluted with 40 mL of CH_3CN, and the precipitated DCU was removed by filtration. After collection and removal of the solvent in vacuo, the crude material was separated by means of radial centrifugal chromatography (5:100 MeOH/CH_2Cl_2 to 20:100 MeOH/CH_2Cl_2) to give 609 mg (0.28 mmol) of rotaxane **26** in a 91% yield, as a pink foam (mp = 96–99 °C dec). ^1H NMR ($CDCl_3$): 1.26 (18H, s), 1.40–1.50 (54H, m), 1.56–2.06 (18H, m), 3.09–3.31 (2H, m), 3.34–3.56 (2H, m), 3.56–3.93 (8H, m), 3.93–4.26 (16H, m), 4.41–4.53 (2H, m), 4.96–5.00 (4H, 2 × s), 5.83–6.10 (2H, m), 6.68–6.93 (8H, m), 6.94–7.15 (6H, m), 7.22–7.45 (13H, m), 7.63 (2H, m), 7.77 (2H, m). ^{13}C NMR ($CDCl_3$): 171.8, 171.2, 170.4, 163.1, 163.0, 160.6, 160.5, 157.1, 156.9, 156.1, 155.5, 154.6, 143.4, 137.3, 137.0, 131.5, 131.1, 130.1, 128.5, 128.4, 127.8, 127.7, 127.6, 127.3, 114.5, 114.1, 113.7, 107.0, 84.0, 84.0, 79.7, 71.1, 69.8, 69.7, 68.4, 55.6, 53.4, 49.1, 48.6, 43.8, 41.5, 39.2, 34.8, 34.7, 33.7, 31.4, 30.9, 28.3, 28.2, 27.9, 25.3, 24.9, 24.3. MS: found 2130.2234, calcd for $C_{116}H_{169}N_{12}O_{25}$ [M + 1] 2130.2322.

(Boc)$_3$Arg-cyclophane–[2]Rotaxane (23). [2]Rotaxane **23** was created using the same synthetic and purification procedures used to construct [2]rotaxane **26**, except that 255 mg (0.145 mmol) of [2]rotaxane **22** and 143 mg (0.30 mmol) of (Boc)$_3$ArgOH were dissolved in 0.2 mL. To this solution was added 10 mg (0.075 mmol) of HOBt and 25 mg (0.31 mmol) of DCC dissolved in 1 mL. Purification of the crude material gave 221 mg (0.089 mmol) of rotaxane **23** in a 62% yield, as a pink form (mp =128–130 °C dec). ^1H NMR ($CDCl_3$): 1.29 (18H, s), 1.42–1.49 (58H, m), 1.58–2.03 (10H, m), 2.11 (3H, s), 2.20–2.35 (12H, m), 2.74–2.92 (2H, m), 3.12–3.31 (2H, m), 3.42 (12H, s), 3.47–3.56 (2H, m), 3.56–3.90 (20H, m), 3.97–4.38 (21H, m), 4.37–4.54 (2H, m), 6.27 (4H, s), 6.68–6.86 (8H, m), 7.00–7.28 (6H, m), 7.35–7.51 (3H, m). ^{13}C NMR ($CDCl_3$): 175.7, 172.2, 171.2, 170.5, 168.6, 163.1, 160.5, 159.4, 157.2, 155.4, 154.6,

152.5, 151.0, 146.9, 143.5, 143.4, 140.6, 137.7, 135.7, 127.8, 124.3, 122.8, 122.5, 114.2, 104.8, 84.8, 79.0, 78.7, 70.6, 69.7, 68.4, 64.0, 55.5, 53.2, 49.3, 48.7, 48.4, 46.1, 44.0, 43.5, 39.1, 38.4, 37.1, 36.1, 34.7, 34.6, 33.6, 31.1, 30.2, 28.1, 27.9, 27.7, 25.1, 24.7, 24.2, 21.0, 19.5. MS: found 2461.3928, calcd for $C_{131}H_{194}N_{13}O_{32}$ [M + 1] 2461.3953.

Cleft–[2]Rotaxane (2). A 300 mg (0.20 mmol) sample of (Boc)$_3$Arg-cleft–[2]rotaxane **26** was dissolved in 18 mL of CH_2Cl_2 and 18 mL of acetic acid. Then 18 mL of TFA was added dropwise to this solution. After stirring for 48 h, volatile materials were removed under reduced pressure. The residue was separated using column chromatography (CH_2Cl_2/TFA 100:1 to CH_2Cl_2/TFA/MeOH 50:1:50) to give 210 mg (0.14 mmol) of rotaxane **2** in a 71% yield, as a brown glass. ^1H NMR ($CDCl_3$): 1.19 (18H, s), 1.22–2.03 (18H, m), 3.15 (2H, m), 3.40–3.54 (2H, m), 3.65–3.77 (16H, m), 3.99–4.24 (15H, m), 4.61 (2H, m), 5.02 (4H, m), 6.80–6.90 (8H, m), 7.07–7.22 (6H, m), 7.22–7.46 (13H, m). ^{13}C NMR ($CDCl_3$): 175.1, 167.5, 160.9 (q, J = 40 Hz), 158.6, 158.3, 152.4, 148.6, 146.3, 145.8, 139.2, 138.7, 133.4, 129.7, 129.0, 128.7, 125.1, 124.7, 118.4 (q, J = 290 Hz), 117.1, 116.0, 114.0, 113.9, 107.0, 71.7, 71.1, 70.4, 69.4, 55.7, 54.9, 50.2, 49.7, 41.7, 40.5, 36.0, 35.6, 31.8, 29.3, 28, 26.9, 24.6, 24.4, 23.6. MS: found 1529.9103, calcd for $C_{86}H_{120}N_{12}O_{13}$ [M + 1] 1529.9176.

Cyclophane–[2]Rotaxane (1). A 140 mg (0.055 mmol) sample of (Boc)$_3$Arg-cyclophane–[2]rotaxane **23** was dissolved in 3 mL of CH_2Cl_2 and 3 mL of acetic acid. Then 3 mL of TFA was added dropwise to this solution. After stirring for 24 h, volatile materials were removed under reduced pressure. The residue was separated using column chromatography (CH_2Cl_2/TFA 100:1 to CH_2Cl_2/TFA/MeOH 50:1:50) to give 87 mg (0.047 mmol) of rotaxane **1** in a 85% yield, as a brown glass. ^1H NMR ($CDCl_3$): 1.16 (18H, s), 1.35–1.56 (4H, m), 1.63–1.79 (8H, m), 1.90–2.14 (10H, m), 2.18 (3H, s), 2.25–2.52 (4H, m), 3.10–3.30 (4H, m), 3.34 (12H, s), 3.40–3.90 (18H, m), 3.90–4.30 (23H, m), 4.63 (2H, m), 6.37 (4H, s), 6.74 (4H, d, J = 8.5 Hz), 6.82 (4H, m), 6.95–7.15 (6H, m), 7.15–7.60 (3H, m). ^{13}C NMR ($CDCl_3$): 175.5, 167.4, 160.7 (q, J = 40 Hz), 158.9, 158.5, 154.1, 152.5, 148.6, 145.8, 142.4, 139.2, 137, 132.9, 132.6, 129.3, 125.1, 124.7, 118.9, 117.1 (q, J = 290 Hz), 114.0, 113.9, 107.0, 106.4, 71.8, 71.2, 71.0, 70.8, 69.6, 69.4, 65.6, 56.7, 54.8, 53.9, 50.3, 49.7, 47.3, 41.7, 40.6, 36.0, 35.6, 32.8, 31.8, 31.4, 29.2, 28.8, 26.9, 24.4, 23.8, 20.6. MS: found 1861.0824, calcd for $C_{101}H_{146}N_{13}O_{20}$ [M + 1] 1861.0807.

Cell Studies. COS-7 African green monkey kidney cells (ATCC CRL 1651) were propagated in Dulbecco's modified Eagle's medium with 4 mM glutamine adjusted to contain 1.5 g/L sodium bicarbonate and 4.5 g/L glucose (90%) and fetal bovine serum (10%) (GIBCO/In Vitrogen). Cells were grown at 37 °C in a 5% CO_2 humidified incubator. Cells were subcultured 1:8 twice per week. For analytical studies, cells were plated for 24 h on 11 × 11 mm glass coverslips (Corning) in 12-well culture plates (Fisher). Individual slides were placed in individual wells containing 1 mL phosphate buffer (50 mM, pH 7.0) and exposed to either (i) fluorescein (0.4 μM), (ii) fluorescein (0.4 μM) and cleft–[2]rotaxane **2** (6 μM or 60 μM), (iii) fluorescein (0.4 μM) and cyclophane–[2]rotaxane **1** (6 μM or 60 μM), or (iv) fluorescein–PKC inhibitor conjugate (0.4 μM) and cyclophane–[2]rotaxane **1** (60 μM). After ca. 30 min, the fluorescence intensity of the cells was analyzed via fluorescence microscopy. For weakly fluorescent cells, the film was exposed for 15–30 s, and for strongly fluorescent cells, the film was exposed for 3–5 s. These studies were repeated twice, producing similar results.