Structural Control of Kinetics for Macrocycle Threading by Fluorescent Squaraine Dye in Water

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S Supporting Information

ABSTRACT: While the general concept of steric speed bumps has been demonstrated in rotaxane shuttles and macrocycle threading systems, the sensitivity of speed bump effects has not been evaluated as a function of structural geometry. Values of $K_a$ and $k_{on}$ for macrocycle threading in water are reported for a series of homologous squaraine dyes with different substituents (speed bumps) on the flanking chains and two macrocycles with different cavity sizes. Sensitivity to a steric speed bump effect was found to depend on (a) structural location, being lowest when the speed bump was near the end of a flanking chain, and (b) macrocycle cavity size, which was enhanced when the cavity was constricted. This new insight is broadly applicable to many types of molecular threading systems.

INTRODUCTION

Pseudorotaxanes are the inclusion complexes formed when a macrocycle is threaded by a long axle molecule. Because of their unique dynamic and supramolecular properties, pseudorotaxanes are under active investigation for a wide range of applications in nanotechnology, materials science, and biomedicine. Structural control of the thermodynamics and kinetics for macrocycle threading is a desirable capability since it greatly facilitates the process of optimizing molecular performance. A recent computational study by Sevick and Williams concluded that threading is a very rare complexation event with a high entropic penalty and that geometrical features, such as length and thickness of the axle, and internal diameter of the macrocycle, greatly affect the rate and extent of threading. The challenge for supramolecular chemists is to design macrocycle/axle pairs with complementary shapes to provide enthalpic interactions between the interlocked components to overcome the inherent entropic barrier for threading. In the simplest case of a [2] rotaxane, the axle structure is usually composed of an internal docking station with appended chains. To reach the docking station, the macrocycle must slide on to the end of one of the flanking chains and translocate over any intervening steric elements within the chain. This mechanistic picture suggests that the rate of threading can be structurally controlled by incorporating steric speed bumps into the chains. To date, most studies of steric speed bumps have examined rotaxane systems that exhibit shuttling behavior, with less attention on macrocycle threading systems. Furthermore, the sensitivity of speed bump effects in macrocycle threading systems has not been explored as a function of geometric variables such as location of the speed bump on the axle structure or relative size of the macrocycle cavity.

In recent years, we have developed a macrocycle threading system called Synthavidin (Synthetic Avidin) that is endowed with favorable optical properties that enable thermodynamic and kinetic measurements. In short, a tetrалactam macrocycle M with anthracene sidewalls is threaded by a deep-red fluorescent squaraine dye S with appended polyethylene glycol (PEG) chains to give the threaded complex $M \supset S$ (Scheme 1). In water, macrocycle threading is very fast, and the association constant ($K_a$) is around $10^9$ M$^{-1}$. Systematic structural studies have measured the rate constant ($k_{on}$) for macrocycle threading.

Scheme 1. Rate of Macrocycle Threading by Squaraine Dye To Give the Complex $M \supset S$ Is Controlled by the Size of the Speed Bump Groups A

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by homologous squaraine dyes that are flanked by amino groups bearing different $N$-substituents. The identity of the $N$-substituents was found to have little influence on $K_w$, but in some cases there was a large effect on the threading kinetics. More specifically, $k_{\text{on}}$ hardly changed when the length of an appended PEG chain was increased by many hundreds of atoms, but $k_{\text{on}}$ was greatly affected by small steric changes to the second substituent ($A$) attached to the nitrogen. In other words, the $A$ group was acting as a steric speed bump that controlled the rate of macrocycle threading. Structural control of the threading kinetics has allowed us to optimize the Synthavidin self-assembly properties for different types of applications. In one set of cases, we have used squaraine dyes with $A = N$-propyl to preassemble threaded fluorescent probes with high mechanical stability for long-term molecular imaging of living subjects. A different Synthavidin project used squaraine dyes with $A = N$-methyl to ensure rapid capture of the dye after it was liberated by an enzyme-catalyzed cleavage process.25

In order to optimize supramolecular performance, we have to learn how much the kinetics for Synthavidin threading depends on changes in molecular structure, and here we address two questions that are fundamental to any macrocycle threading system. Does the magnitude of a steric speed bump effect change with spatial location on the squaraine axle? Does sensitivity to a speed bump effect increase when the macrocycle cavity is constricted? To answer these questions, we synthesized the squaraine dyes and macrocycles shown in Scheme 2 and measured $K_w$ and $k_{\text{on}}$ for macrocycle threading in water.

Scheme 2. Squaraine Dyes and Macrocycles Used in This Study

![Scheme 2](image)

RESULT AND DISCUSSION

Does Sensitivity to a Steric Speed Bump Effect Change with Spatial Location on the Squaraine Axle?

The speed bump effect was determined at three squaraine locations, labeled in Scheme 3 as $A$, $B$, and $C$. This was achieved by preparing the new squaraine dyes S3–S7 using the sequence of conjugation reactions shown in Scheme 4. Since each dye had the same squaraine core there was little difference in the deep-red absorption and emission properties. Values of $K_w$ and $k_{\text{on}}$ for threading of macrocycle M1 by each squaraine dye in water were measured using the same titration and stopped-flow methods employed in previous studies. In each case, macrocycle threading was indicated by a diagnostic ~30 nm red-shift in squaraine fluorescence maxima (Table 1). In addition, the threaded complex ($M\sigma S$) exhibited efficient internal fluorescence energy transfer; that is, when the anthracene sidewalls of the surrounding macrocycle ($M$) were excited with UV light there was greatly enhanced deep-red fluorescence emission from the encapsulated squaraine ($S$). Entries 1 and 2 in Table 2 provide a point of calibration for the previously observed steric speed bump effect at location $A$.22 These studies used uncharged squaraine dyes S1 and S2. When the $A$ substituent was an $N$-ethyl group (S1, entry 1), $k_{\text{on}}$ was measured to be $(1.2 \pm 0.1) \times 10^7 \text{ M}^{-1} \text{s}^{-1}$. Decreasing the substituent size by one carbon atom to $N$-methyl (S2, entry 2) increased $k_{\text{on}}$ by a factor of 100, indicating high sensitivity to the size of the speed bump at location $A$.

A common structural feature of the new squaraines used to measure the steric speed bump effect at locations $B$ and $C$ is an anionic carboxylate at the end of each flanking chain. This terminal carboxylate was included in the molecular designs to ensure that the dyes maintained high solubility in water and did not self-aggregate. Since M1 has a $-6$ charge, we suspected that the terminal carboxylate group would induce an electrostatic effect on the threading process,27 and this was measured by comparing $K_w$ and $k_{\text{on}}$ for neutral S1 with a terminal hydroxyl (entry 1) against its isosteric analogue S3 (entry 3) with a terminal carboxylate. The values of $K_w$ for each system were within a factor 6, a relatively small difference that was expected since the terminal carboxylate in S3 is ~20 atoms away from the binding interface of the M1$\sigma$S3 complex. But $k_{\text{on}}$, for
Scheme 4. Synthesis of Squaraine Dyes

![Scheme 4](image)

Table 1. Absorption and Emission Maxima Wavelengths

<table>
<thead>
<tr>
<th>compd</th>
<th>λ\text{abs} (nm)</th>
<th>λ\text{em} (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S3</td>
<td>660</td>
<td>674</td>
</tr>
<tr>
<td>M1\text{a}s3</td>
<td>676</td>
<td>706</td>
</tr>
<tr>
<td>S4</td>
<td>645</td>
<td>665</td>
</tr>
<tr>
<td>M1\text{a}s4</td>
<td>665</td>
<td>711</td>
</tr>
<tr>
<td>S5</td>
<td>663</td>
<td>676</td>
</tr>
<tr>
<td>M1\text{a}s5</td>
<td>676</td>
<td>708</td>
</tr>
<tr>
<td>S6</td>
<td>662</td>
<td>676</td>
</tr>
<tr>
<td>M1\text{a}s6</td>
<td>676</td>
<td>706</td>
</tr>
</tbody>
</table>

*In H₂O (5 μM).

The speed bump effect at terminal position C was assessed by comparing \(K_C \) and \(k_{\text{on}} \) for the two squaraines S4 and S5 which have the same squaraine core but different groups near the very end of the flanking chains. The N-acylalanine group in S4 is much smaller than the N-acylphenylalanine group in S5, and a comparison of entries 4 and 5 in Table 2 shows little variation in \(K_C \) as expected, but surprisingly, there was also no difference in \(k_{\text{on}} \). This means that the rate of macrocycle threading is quite insensitive to the size of a speed bump group at squaraine location C very near the end of a flanking chain. The speed bump effect at midpoint position B was assessed by comparing the measured values of \(K_C \) and \(k_{\text{on}} \) for squaraines S6 and S7 whose structures have the same squaraine core and terminal carboxylates but differ by having alanine or phenylalanine units inserted near the midpoint of the flanking chains. A comparison of entries 6 and 7 in Table 2 shows that the smaller alanine unit in S6 did not hinder threading of M1, but the effect of the larger phenylalanine unit in the case of S7 was profound in that it completely blocked macrocycle threading. This conclusion is based on the following experimental observations. Unlike the threading process that formed M1\text{a}s6 with a significant squaraine red-shift effect (Figure 1a), there was very little change in the squaraine absorption band when M1 and S7 were mixed in equal molar amounts (Figure 1b). In addition, an equal molar sample of M1 and S6 exhibited efficient energy transfer from excited M1 to S6, whereas an equal molar sample of M1 and S7 produced very weak energy transfer (Figure 2). Furthermore, a competitive energy transfer experiment showed that the presence of S7 did not inhibit the threading of M1 by a subsequent addition of S1 (Figure 2). It was not possible to monitor the interaction of M1 and S7 by \(^1\)H NMR because S7 self-aggregates at millimolar concentrations in water and the NMR signals are very broad. However, independent verification that M1 is not threaded by S7 was gained by conducting an insightful TLC experiment. We have previously shown that the presence of M1 completely prevents elution of S1 up a TLC plate due to formation of a very polar M1\text{a}s1 complex. Using the same TLC conditions, we find that the presence of M1 does not alter the \(R_f \) of S7, indicating that a threaded complex is not formed (Figure S61). Taken together, the results in Table 2 show that the presence of substituents on the chains appended to the squaraine dyes can lower \(k_{\text{on}} \) for macrocycle threading but sensitivity to the substituent’s steric size (speed bump effect) depends on the location. There is high steric sensitivity at locations A and B, but very low steric sensitivity at location C. It is worth emphasizing that this conclusion focuses on the sensitivity to a speed bump effect at different locations. It is not valid to compare absolute values of \(k_{\text{on}} \) for speed bumps at two different locations. An explanation for the difference in sensitivities is provided by

Table 2. Association Constants (\(K_C \)) and Threading Rates (\(k_{\text{on}} \)) for Association of Squaraine and Macrocycle at 20 °C

<table>
<thead>
<tr>
<th>entry</th>
<th>squaraine</th>
<th>macrocycle</th>
<th>(K_C ) (M⁻¹)</th>
<th>(k_{\text{on}} ) (M⁻¹ s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>S1</td>
<td>M1</td>
<td>(6.0 ± 1.2) × 10⁷</td>
<td>(1.2 ± 0.1) × 10⁷</td>
</tr>
<tr>
<td>2</td>
<td>S2</td>
<td>M1</td>
<td>(1.2 ± 0.5) × 10⁷</td>
<td>(1.1 ± 0.1) × 10⁷</td>
</tr>
<tr>
<td>3</td>
<td>S3</td>
<td>M1</td>
<td>(1.0 ± 0.6) × 10⁷</td>
<td>(1.9 ± 0.2) × 10⁴</td>
</tr>
<tr>
<td>4</td>
<td>S4</td>
<td>M1</td>
<td>(5.8 ± 0.1) × 10⁴</td>
<td>(2.0 ± 0.1) × 10⁴</td>
</tr>
<tr>
<td>5</td>
<td>S5</td>
<td>M1</td>
<td>(6.5 ± 0.2) × 10⁴</td>
<td>(2.0 ± 0.1) × 10⁴</td>
</tr>
<tr>
<td>6</td>
<td>S6</td>
<td>M1</td>
<td>(3.5 ± 0.1) × 10⁴</td>
<td>(6.6 ± 0.2) × 10⁴</td>
</tr>
<tr>
<td>7</td>
<td>S7</td>
<td>M1</td>
<td>no threading</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>S1</td>
<td>M2</td>
<td>(2.1 ± 0.2) × 10⁸</td>
<td>(2.6 ± 1.2) × 10³</td>
</tr>
<tr>
<td>9</td>
<td>S2</td>
<td>M2</td>
<td>(1.5 ± 0.6) × 10⁸</td>
<td>(5.4 ± 0.8) × 10⁶</td>
</tr>
</tbody>
</table>

*aData for this entry taken from ref 22.

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inspecting Scheme 3. At locations A and B there is high sensitivity because the macrocycle cavity is already partially occupied by a segment of the oxyethylene chain which makes it harder for the macrocycle to translocate over the speed bump. In contrast, the speed bump group at location C has the option of passing through the macrocycle before the oxyethylene chain which makes it easier to avoid steric congestion. Knowing the sensitivity to speed bump sterics at each location along the squaraine structure will greatly help our future efforts to design cleavable squaraine systems for in situ capture applications such as turn-on fluorescent enzyme assays.25 These applications require a squaraine structure with blocking groups that ensure no macrocycle threading until the groups are cleaved. At position C a blocking group would have to be relatively large, but at positions A and B a blocking group could be substantially smaller.

Does Sensitivity to Speed Bump Effect Increase when the Macrocycle Cavity Is Constricted? In principle, an alternative way to enhance the magnitude of a steric speed bump effect is to reduce the internal diameter of the macrocycle cavity. Previously, we and others have shown that the 1,3-benzene dicarboxamide29−31 X-ray crystal structures show that the pyridine nitrogen atom forms hydrogen bonds with the two adjacent NH residues which reduces the angle between the two carboxamides and shortens the distance $d$ between the two parallel aromatic sidewalls (Figure 3).32 Furthermore, the internal hydrogen bonding rigidifies the macrocycle and makes it harder for the cavity to transiently expand its size by adopting a conformation that directs an NH residue out of the cavity.29

Figure 1. Absorption spectra for 5 μM samples of: (a) S6 or (b) S7 before and after addition of aliquots of M1 in H2O at 20 °C.

Figure 2. Change in fluorescence spectra (ex: 390 nm) caused by addition of S1 (5 μM) to a mixture of S7 (5 μM) and M1 (5 μM) in H2O at 20 °C. The decrease in anthracene emission at 420 nm and concomitant increase in squaraine emission at 715 nm indicates formation of M1⊂S1 with enhanced fluorescence energy transfer from excited M1 to S1.

Figure 3. Calculated cavity sizes for macrocycles with 1,3-benzene dicarboxamide (left) and 1,3-pyridine dicarboxamide (right) bridging units with the red lines representing intramolecular hydrogen bonds. The cavity sizes were calculated using the PM7 method and match the trend seen with X-ray structures of squaraine rotaxanes where $d$ values for analogues of M1 (6.91−7.18 Å) are longer than $d$ values for analogues of M2 (6.61−6.78 Å).32
M2, the difference in free energies of activation ($\Delta G^\ddagger$) is 2.7 kcal mol$^{-1}$, and for M2, $\Delta G^\ddagger$ is 4.6 kcal mol$^{-1}$. Thus, threading of the constricted M2 is not only slower but $\Delta G^\ddagger$ is more sensitive to changes in speed bump steric. One of the planned applications with these threaded complexes is biological imaging, and it is helpful if the preassembled complexes have high mechanical stability in biological fluids. As a preliminary test, we measured the stability of the preassembled complexes M2 ⊂ S2 and M2 ⊂ S1 in fetal bovine serum (FBS), and as shown in Figure 4 there was no fluorescence evidence for complex unthreading after 15 h.

![Figure 4. Fluorescence spectra (ex: 620 nm) show no significant dissociation of (left) M2⊂S2 and (right) M2⊂S1 (5 µM) in FBS over 15 h (spectra acquired every 30 min).](Image)

**CONCLUSION**

While speed bump effects have been reported for various threaded molecular systems,14–19 this is the first systematic demonstration that sensitivity to the effect (a) depends on the structural location and decreases in the order of locations $A \sim B > C$ (Scheme 3) and (b) is enhanced when the macrocycle cavity size is constricted (Scheme 6). This new insight is relevant for many types of rotaxane shuttles and pseudorotaxane threading systems and will aid community efforts to optimize the kinetics for dynamic molecular devices such as sensors, actuators, capture agents, and processive catalysts.1–19 It will especially help us to design next-generation Synthavidin pairs for in situ capture of enzyme reaction products and preassembly of fluorescent molecular probes.23–25

**EXPERIMENTAL SECTION**

**Synthesis.** Compounds S6,21 1,33 5,34 and M135 were synthesized using previously reported methods. The synthesis of squaraines S121 and S222 have been reported previously and were confirmed by NMR, UV/vis, and fluorescence spectroscopy. High-resolution mass spectrometry (HRMS) was performed using a TOF mass analyzer.

**Acid Chloride Azide 2.** Compound 1 (100 mg, 0.42 mmol) was dissolved in CH$_2$Cl$_2$ (2 mL). Thionyl chloride (102 mg, 0.87 mmol) was added to reaction mixture. The mixture was stirred overnight at room temperature. The solvent was removed to yield 2 as a brown viscous liquid (104 mg, 96% yield): $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 4.48 (s, 2H), 3.98 (s, 1H), 3.80 ($\sim$ H, 2H), 3.68 ($\sim$ m, 8H), 3.41 (d, $J = 7$ Hz, 2H), 3.33 (m, 2H); $^1$C($^1$H) NMR (125 MHz, CDCl$_3$) $\delta$ 172.0, 71.3, 70.7, 70.6, 70.5, 70.0, 50.6.

**General Procedure for the Synthesis of Compounds 3 and 8.** Compound 2 (1.19 mmol), Et$_3$N (1.42 mmol), and methyl ester derivative (1.42 mmol) were dissolved in CH$_2$Cl$_2$ (3 mL). The reaction mixture was stirred overnight at room temperature. The mixture was dried and dissolved in CHC$_2$H and washed with H$_2$O. The organic layer was evaporated under vacuum to yield the desired derivative as a brown viscous liquid.

**Data for compound 3:** 270 mg, 73% yield; $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.29 (d, $J = 7$ Hz, 2H), 4.59 (quin, $J = 7$ Hz, 1H), 3.98 (s,
**Data for compound 8:** 104 mg, 66% yield; 1H NMR (500 MHz, CDCl3) δ 7.47 (d, J = 7 Hz, 1H), 4.60 (quin, J = 7 Hz, 1H), 4.05 (s, 2H), 3.76–3.63 (m, 10H), 3.39 (s, J = 5 Hz, 2H), 1.47 (d, J = 7 Hz, 3H); 13C NMR (125 MHz, CDCl3) δ 175.2, 170.7, 71.0, 70.5, 70.2, 70.0, 69.5, 59.6, 54.7, 178; HRMS-ESI m/z calcd for 327.1275 [M + Na]+ C11H20N4O6Na+, found 327.1285.

**Data for compound 9:** 71 mg, 84% yield; 1H NMR (500 MHz, CDCl3) δ 7.35 (d, J = 8 Hz, 1H), 7.22 (m, 5H), 4.86 (q, J = 7 Hz, 1H), 3.96, 3.92 (ABq, J = 12 Hz, 2H), 3.73–3.46 (m, 12H), 3.35–3.27 (m, 2H), 3.26, 3.04 (ABq, J = 14 Hz, 2H); 13C NMR (125 MHz, CDCl3) δ 173.4, 170.8, 135.9, 129.8, 128.4, 127.0, 71.0, 70.6, 70.4, 70.3, 70.0, 69.5, 68.8, 63.6, 50.8, 43.9, 39.3, 28.0, 18.3; HRMS-ESI m/z calcd for 403.1588 [M + Na]+ C20H25N4O8Na+, found 403.1615.

**General Procedure for the Synthesis of 6 and 10.** Compound 4 or 9 (0.10 mmol), BTB (56 mg, 0.36 mmol), DIPEA (94 mg, 126 μL, 0.73 mmol), and compound 5 (25 mg, 0.24 mmol) were dissolved in CH2Cl2 (3 mL). The reaction mixture was stirred overnight at room temperature. The solvent was removed by vacuum in a rotary evaporator. H2O (20 mL) was added to the residue, and the mixture was extracted with CHCl3 (3 × 20 mL) and dried with anhydrous MgSO4. The organic phases were evaporated by vacuum to give the desired product as a viscous liquid.

**Data for compound 6:** 32 mg, 55% yield; 1H NMR (500 MHz, CDCl3) δ 6.73 (d, J = 8 Hz, 1H), 6.77 (br s, 1H), 4.47 (quin, J = 7 Hz, 1H), 4.07–3.92 (m, 4H), 3.74–3.42 (m, 2H), 1.49 (s, 9H), 1.36 (d, J = 7 Hz, 1H); 13C NMR (101 MHz, CDCl3) δ 172.0, 169.6, 169.4, 168.9, 168.7, 160.9, 70.0, 70.6, 70.4, 70.0, 69.6, 68.9, 68.3, 50.6, 49.2, 39.3, 28.0, 18.3; HRMS-ESI m/z calcd for 530.3083 [M + Na]+ C20H20N4O8Na+, found 530.3101.

**Data for compound 10:** 35 mg, 62% yield; 1H NMR (500 MHz, CDCl3) δ 7.41–7.28 (m, 1H), 7.27–7.09 (m, 5H), 6.55 (s, 1H), 4.68 (q, J = 7 Hz, 1H), 4.00–3.83 (m, 4H), 3.72–3.40 (m, 20H), 3.37–3.25 (m, 4H), 1.21–1.98 (s, 9H); 13C NMR (101 MHz, CDCl3) δ 170.4, 169.7, 169.6, 166.9, 129.4, 129.3, 128.4, 128.0, 81.6, 70.6, 70.6, 70.0, 70.4, 70.4, 70.1, 70.0, 69.5, 68.9, 65.4, 50.9, 39.3, 28.0, 18.3; HRMS-ESI m/z calcd for 626.3396 [M + Na]+ C20H25N4O8Na+, found 626.3385.

**General Procedure for Compounds 7 and 11.** Compound 6 or 10 (0.06 mmol) was dissolved in CH2Cl2 (2 mL), and TFA (1 mL) was added to the mixture. The reaction mixture was stirred overnight at room temperature. The solvents were removed by vacuum in a rotary evaporator. NaOH (1 M) was added dropwise until pH 10, and the solution was washed with ethyl acetate. The aqueous phase was acidified with HCl (1 M) until pH 4, and the product was extracted with CHCl3 (3 × 10 mL). The organic phase was dried over MgSO4, and the solvent was removed by vacuum to give the desired product as a viscous liquid.

**Data for compound 7:** 23 mg, 53% yield; 1H NMR (500 MHz, CDCl3) δ 7.58 (d, J = 8 Hz, 1H), 7.17 (br s, 1H), 4.72–4.63 (m, 1H), 4.18 (s, 2H), 4.03 (s, 2H), 3.80–3.33 (m, 24H), 1.39 (d, J = 7 Hz, 3H); 13C NMR (101 MHz, CDCl3) δ 172.3, 172.2, 170.5, 71.0, 71.0, 70.6, 70.4, 70.3, 70.1, 70.0, 69.6, 68.5, 50.6, 48.4, 39.3, 18.4; HRMS-ESI m/z calcd for 516.2276 [M + Na]+ C19H32N3Na2O8, found 516.2280.
The amine 14 (500 mg, 0.99 mmol), sodium bicarbonate (339 mg, 4.00 mmol), and copper sulfate (8 mg, 0.05 mmol) were dissolved in H2O (5 mL). Triethylmethanesulfonyle azide (696 mg, 4.00 mmol) in MeOH (2 mL) was added. The mixture was stirred overnight. The solvents were removed, and the crude material was purified by column (0–10% MeOH/CHCl3) to give the product 15 as viscous liquid (470 mg, 89% yield): 1H NMR (500 MHz, CDCl3) δ 7.30 (t, J = 6 Hz, 6H), 1.48 (s, 9H), 8.07 (s, 4H), 8.05 (s, 2H), 7.67 (t, J = 6 Hz, 12H), 1.43 (s, 5H). 13C{1H} NMR (125 MHz, CDCl3) δ 170.7, 167.2, 150.6, 140.5, 130.3, 129.4, 126.6, 124.3, 112.1, 80.7, 70.7, 67.9, 67.4, 36.2, 29.8, 28.2; HRMS-ESI m/z calcd for C22H15NO4S2 [M + Na]+ C22H15NO4S2Na2+, found 597.9552. Selected data of the newly synthesized compound 15.

Macrocycles M2. Protected macrocycle 16 (35 mg, 0.02 mmol) was dissolved in CHCl3 (10 mL) in an ice bath under Ar. TFA (2 mL) was added dropwise until pH = 7. The solution was passed through a column (0–10% MeOH/CHCl3) to get the product 16 as light yellow solid (33 mg, 30%): mp 178–180 °C; 1H NMR (600 MHz, CDCl3) δ 8.26 (dd, J = 7, 3 Hz, 8H), 8.07 (s, 4H), 8.05 (s, 2H), 7.61 (t, J = 6 Hz, 8H), 5.75 (s, 4H), 3.95 (s, 12H), 3.67 (t, J = 6 Hz, 12H), 2.45 (t, J = 6 Hz, 12H), 1.43 (s, 5H). 13C{1H} NMR (125 MHz, CDCl3) δ 170.7, 167.2, 150.6, 140.5, 130.3, 129.4, 126.6, 124.3, 112.1, 80.7, 70.7, 67.9, 67.4, 36.2, 29.8, 28.2; HRMS-ESI m/z calcd for C24H25NO4S2Na2+, found 795.9552. Selected data of the newly synthesized compound 16.

Acknowledgments
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References
26. The thermodynamics and kinetics of association were determined from the changes in fluorescence or absorption. As shown in Figures S55–S60, each kinetic profile was fitted using first-order and second-order kinetic equations, and in each case, the second-order fit was equal or better than the first-order fit. Therefore, we assume a second-order threading model with translaction of the macrocycle over the speed bump as the rate-determining step.

Notes
The authors declare no competing financial interest.
A reviewer makes the reasonable point that when a steric speed bump group is located near the very end of a flanking chain (such as location C in $S_4$ and $S_5$) it is less clear what group is acting as the chain and what group is the speed bump. In this case it is simpler to consider both groups as a single unit (i.e., treat the combined groups as a stopper unit). From this perspective, the $N$-acylalanine stopper in $S_4$ is a much smaller than the $N$-acylphenylalanine stopper in $S_5$; nonetheless, there is no difference in $k_{on}$. 


