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Published on Web 07/13/2006

Controlling the Biological Effects of Spermine Using a Synthetic Receptor

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Abstract: Polyamines play an important role in biology, yet their exact function in many processes is poorly understood. Artificial host molecules capable of sequestering polyamines could be useful tools for studying their cellular function. However, designing synthetic receptors with affinities sufficient to compete with biological polyamine receptors remains a huge challenge. Binding affinities of synthetic hosts are typically separated by a gap of several orders of magnitude from those of biomolecules. We now report that a dynamic combinatorial selection approach can deliver a synthetic receptor that bridges this gap. The selected receptor binds spermine with a dissociation constant of 22 nM, sufficient to remove it from its natural host DNA and reverse some of the biological effects of spermine on the nucleic acid. In low concentrations, spermine induces the formation of left-handed DNA, but upon addition of our receptor, the DNA reverts back to its right-handed form. NMR studies and computer simulations suggest that the spermine complex has the form of a pseudo-rotaxane. The spermine receptor is a promising lead for the development of therapeutics or molecular probes for elucidating spermine's role in cell biology.

Introduction

Spermine (**1**, Figure 1) is a polycation that plays a key role in many cellular processes[1,2] forming strong interactions with DNA and RNA.[1,3] Spermine has been shown to condense DNA[3,4] and chromatin,[5,6] to facilitate the formation of nucleosomes,[7] to induce[8−10] and to stabilize[8,9] specific DNA conformations, to modulate DNA−protein interactions,[11] and to protect DNA from external agents.[12] These phenomena may underlie spermine's ability to regulate cell proliferation[1] and influence tumor growth[13,14] and apoptosis.[15] The exact mechanism of action of spermine in most of these processes is still under debate. Thus, there is a considerable incentive to develop synthetic molecules that can sequester spermine under physiological conditions. While receptors have been reported,[16,17] designing a water-soluble synthetic receptor with sufficient affinity to interact with spermine in biological systems remains a huge challenge. Binding affinities of synthetic hosts are typically in the millimolar range, while biological systems usually exhibit submicromolar affinities.[18] We now report that a dynamic combinatorial selection approach[19−21] can deliver a synthetic receptor that bridges this gap and binds spermine with nanomolar affinity, sufficient to remove it from its biological host DNA in vitro, causing the nucleic acid to change its conformation from a left-handed to a right-handed helix. NMR studies and computer simulations suggest the spermine complex has the form of a pseudo-rotaxane.

Results and Discussion

As spermine lacks a well-defined geometry in solution, designing a receptor for it is inherently difficult. We therefore took a selection approach, generating mixtures of potential receptors by linking building blocks together using a reversible reaction. The resulting dynamic combinatorial libraries (DCLs)

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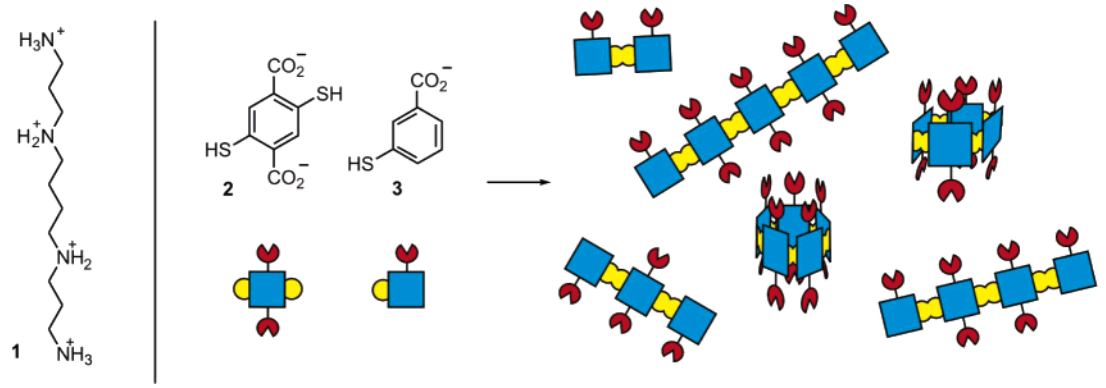

Figure 1. Oxidation of thiol building blocks **2** and **3** produces a DCL of linear and macrocyclic disulfides that contains potential receptors for protonated spermine (**1**).

are equilibrium mixtures that are responsive to influences that result in the selective stabilization of specific library members. Thus, introducing a guest into a DCL of potential hosts will shift the equilibrium, amplifying strong binders at the expense of their inferior counterparts.[20,21] Amplification allows new synthetic receptors to be identified and, in small DCLs, even provides a method for their preparation.[22−24]

We have set up a DCL from building blocks **2**[25] and **3** equipped with carboxylate groups (Figure 1) tailored to recognize the protonated amine groups of spermine through hydrogen-bonding and cation−anion interactions. We deliberately introduced a large number of carboxylate groups as each amine group of spermine is able to interact with several of these units. An excess of carboxylate groups also ensures the solubility of the library members in water by preventing neutralization of all negative charges by the cationic spermine. The thiol functionality allows the building blocks to be oxidatively linked together to form disulfide bonds, which can subsequently undergo disulfide exchange to generate the DCL.[26]

As it was not clear whether linear or cyclic architectures would be more suitable for spermine recognition, we designed our libraries to contain both architectures (Figure 1). Thus, while the oxidation of dithiol **2** will give macrocyclic species of various ring sizes, the presence of monothiol **3** will enable the additional formation of linear species.

Equimolar amounts of the building blocks **2** and **3** were allowed to oxidize and equilibrate in aqueous solution at pH 8.25 in the presence of air following standard protocols.[26] The composition of the ensuing DCL was analyzed by HPLC and mass spectrometry (Figure 2a). Linear tetramer **4** was the major compound in the absence of spermine. When spermine was introduced into the DCL, the cyclic tetramer **6** was amplified, alongside linear dimer **5** built up from two units of monothiol building block **3**. The strong affinity of spermine for **6** (vide infra) drives the conversion of dithiol **2** into this receptor and leaves monothiol **3** with no alternative but to dimerize.

A biased DCL made from only building block **2** and spermine gives macrocycle **6** in more than 90% yield allowing straightforward isolation by preparative HPLC (Figure 2c and d).

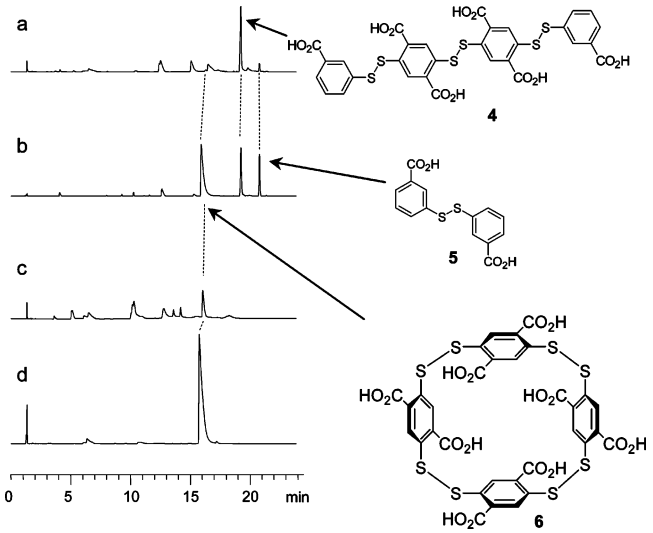

Figure 2. HPLC analyses of the libraries made from thiols **2** and **3** (10 mM each) in the absence of any template (a), and in the presence of spermine (2.5 mM) (b). HPLC analyses of the libraries made from thiol **2** only (10 mM) in the absence of any template (c),[27] and in the presence of spermine (2.5 mM) (d).

The structure of the complex between **6** and spermine was investigated using proton NMR spectroscopy and computer modeling.

Upon binding of spermine to **6** a strong ring-current-induced upfield shift was observed for the central protons d and e of spermine, while protons a, b, and c near the termini of the molecule are hardly shifted (Figure 3). These observations suggest that the central fragment of spermine is located within the cavity of the receptor and imply the formation of a pseudorotaxane in which spermine is threaded through the cyclic receptor.[16,17]

The presence of multiple signals for the host−guest complex is most likely a result of stereoisomers resulting from different conformations of the disulfide linkages. The preferred 90° torsional angle of the disulfide bond makes this linkage inherently chiral as it can exist in a P or M configuration. In most disulfides, rapid rotation around the S−S bond leads to fast exchange between stereoisomers. However, when spermine is threaded through macrocycle **6**, this rotation is likely to be hindered, thus freezing out up to four different diastereomers that arise from the fact that the four disulfide bonds can have different configurations. The NMR spectrum of the complex is dominated by a singlet for the host protons, indicating that the

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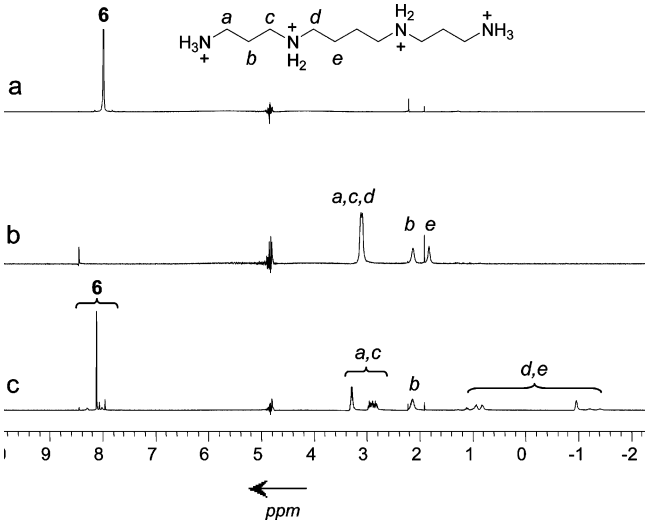

Figure 3. ^{1}H NMR (500 MHz, cryoprobe) analyses of receptor **6** (a), spermine (b), and receptor **6** (1.3 mM) in the presence of 1 equiv of spermine (c). Spectra were recorded at 300 K in 89 mM phosphate buffer (pH 7.4, 11% D_2O) with water presaturation.

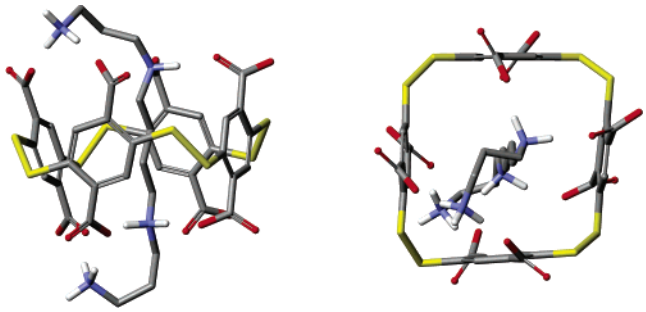

Figure 4. Side and top views of the energy minimized complex of receptor **6** and spermine obtained by computer modeling.[32] Some hydrogen atoms have been omitted for clarity.

major stereoisomer is highly symmetric with all four disulfide bonds having the same configuration.

Computer modeling studies on the free receptor **6** and on its complex with spermine provide further support for these conclusions. In the absence of spermine, the four diastereomers of the receptor were found to be similar in energy. Modeling of the four different spermine−receptor complexes showed that the diastereomer with the highest symmetry was significantly lower in energy than the three others. Furthermore, the energy barrier for the interconversion of the diastereomers was found to be substantially higher in the spermine complex than in the free receptor (see Supporting Information).

In the lowest-energy conformation of the major pseudorotaxane complex, 8 of the 10 amine protons of the guest are involved in hydrogen bonds with the host (Figure 4). In solution, rapid exchange between different hydrogen-bonding arrangements must occur as any long-lived hydrogen bonds would desymmetrize the receptor and no such effect is observed in the proton NMR analysis.

The dissociation constant for binding of spermine to receptor **6** was found to be 22 (±1) nM, as determined by isothermal titration calorimetry (ITC) (see Supporting Information). This affinity is higher than that reported for the binding of spermine to DNA,[8,28−30] suggesting that **6** should be able to inhibit DNA−spermine interactions.

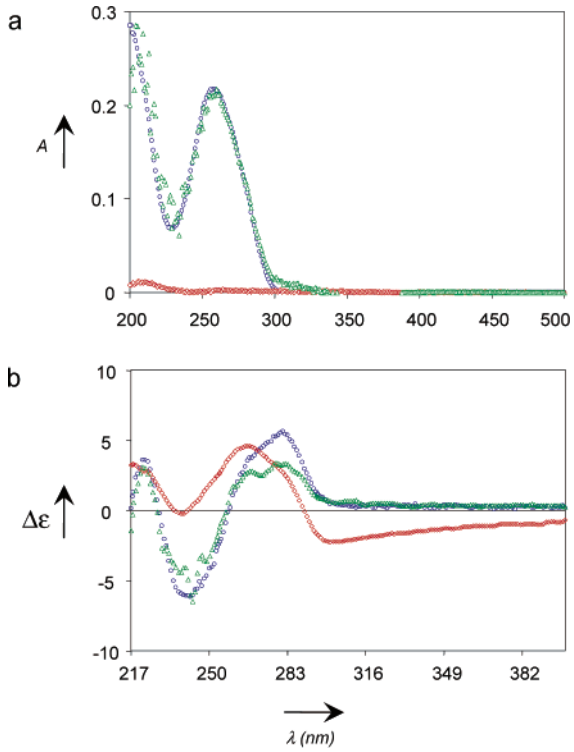

Figure 5. (a) UV spectra of calf thymus DNA (33 μM in 3.0 mM TRIS buffer; pH 7.4; 298 K) without additive (blue ○); in the presence of spermine (2.2 equiv with respect to the number of base pairs) (red ◇); and in the presence of spermine and **6** (2.2 equiv each) (green △). (B) CD spectra of poly(dA-dC)·poly(dG-dT) (97 μM in 3.0 mM TRIS/EDTA buffer; pH 7.4; 1.0 mM NaCl; 298 K) without additive (blue ○); after addition of spermine (0.5 equiv with respect to the number of base pairs) (red ◇); and after addition of **6** (0.5 equiv) (green △). The UV and CD traces correspond exclusively to DNA; contributions from the buffer, spermine, and **6** have been subtracted.

Binding experiments were performed in the presence of DNA to assess the ability of the receptor to (i) inhibit the formation of DNA−spermine complexes, (ii) unbind spermine from DNA, and (iii) control the conformation of DNA.

When an excess of spermine (2.2 equiv with respect to the number of DNA base pairs) was added to a solution of calf thymus DNA, essentially complete precipitation of the biopolymer was observed as was apparent from the disappearance of the UV absorbance of the DNA (Figure 5a, red and blue traces, respectively). However, in the presence of **6** (1.0 equiv with respect to spermine), the addition of spermine does not give any precipitation of DNA and the UV absorbance is unchanged (green trace).

At lower concentrations, spermine binding can switch the conformation of DNA from a right-handed (B-form) to a left-handed (Z-form) helix, a change that can be monitored by circular dichroism (CD).[8,10] Thus, the addition of one-half an equivalent of spermine (with respect to the number of DNA base pairs) to a buffered solution of poly(dA-dC)·poly(dG-dT) induced the appearance of a negative CD-band at 294 nm and a shift in the positive band from 280 to 263 nm (Figure 5b).

(27) The complexity of the trace can be explained by the presence of peaks corresponding to linear species in which the terminal thiols have oxidized to sulfinic or sulfonic acids.
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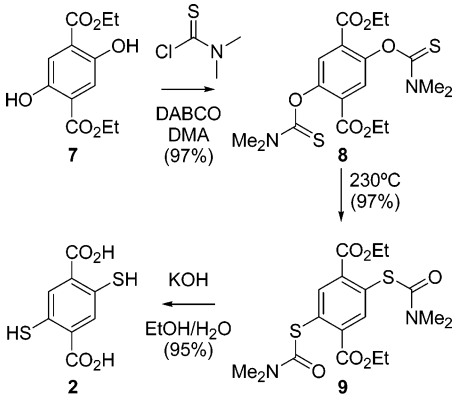
Scheme 1. Synthesis of Building Block **2**



Subsequent addition of one-half an equivalent of **6** to the resulting Z-DNA solution reversed the transition, regenerating the original B-DNA, indicating that the receptor is indeed able to unbind spermine from DNA and thereby control the helicity of DNA.[31]

Conclusions

Our results demonstrate that, after the design of only rudimentary molecular recognition elements, dynamic combinatorial chemistry can be used to guide their assembly into a high-affinity receptor. Thus, starting from a dynamic combinatorial library made from two building blocks that contain carboxylate groups as recognition units, a new macrocyclic receptor for the oligoamine spermine was obtained that binds this guest with a dissociation constant of 22 (±1) nM. Water-soluble synthetic receptors for biologically relevant guests with such high affinities are extremely rare. Analysis of the host−guest complex by NMR and computer simulations indicates a pseudo-rotaxane structure in which spermine is threaded through the macrocyclic host and bound through a series of hydrogen bonds and anion−cation interactions. The host was shown to interfere efficiently with spermine−DNA interactions, being able to prevent and reverse the binding of spermine to DNA and thereby indirectly control the conformation of DNA; while spermine can induce a change in the conformation of DNA from a right-handed (B-form) to a left-handed (Z-form) helix, the addition of the synthetic spermine receptor reverts the DNA back to its original B-form. These results suggest that our spermine receptor may be a promising lead for the development of novel therapeutics or molecular probes that may help unravel the still poorly understood role of oligoamines in cell biology.

Experimental Section

Materials and Methods. Chemicals were purchased from Aldrich or Fluka. Building block **2** was synthesized on the basis of a method described by Field and Engelhardt (Scheme 1).[25] NMR analyses were performed using an Advance 500 Bruker instrument equipped with a standard TCI Cryoprobe. Spectra were recorded at 300 K with water presaturation during relaxation delay and mixing time.

2,5-Bis(dimethylthiocarbamoyloxy)terephthalic Acid Diethyl Ester (8).[25] 2,5-Dihydroxyterephthalic acid diethyl ester **7** (10 g, 39.00 mmol) and DABCO (18 g, 157 mmol) were dissolved in dry DMA (100 mL) under a nitrogen atmosphere and cooled to 0 °C in an ice bath. Dimethylthiocarbamoyl chloride (19 g, 157 mmol) dissolved in dry DMA (50 mL) was added dropwise under nitrogen. The mixture was stirred for 16 h at room temperature. The off-white precipitate was filtered, washed extensively with water (300 mL), and dried under vacuum, yielding compound **8** (16 g, 97%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.73 (s, 2H, CHAr), 4.30 (q, J = 7.1 Hz, 4H, CH_2), 3.46 (s, 6H, CH_3), 3.40 (s, 6H, CH_3), 1.33 (t, J = 7.1 Hz, 6H, CH_3).

2,5-Bis(dimethylthiocarbamoylsulfanyl)terephthalic Acid Diethyl Ester (9).[25] Compound **8** (650 mg, 1.51 mmol) was heated under nitrogen at 215 °C for 1 h. The brownish mixture was cooled to 70 °C, and EtOH (20 mL) was added. Pale brown crystals appeared as the sample was slowly cooled to room temperature. The crystals were filtered off, yielding compound **9** (626 mg, 97%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.11 (s, 2H, CHAr), 4.34 (q, J = 7.1 Hz, 4H, CH_2), 3.12 (s, 6H, CH_3), 3.02 (s, 6H, CH_3), 1.37 (t, J = 7.1 Hz, 6H, CH_3).

2,5-Dimercaptoterephthalic Acid (2). A solution of compound **9** (1.3 g, 3.03 mmol) in degassed 1.3 M KOH in EtOH/H_2O (1:1, 40 mL) was refluxed under an inert atmosphere for 3 h. The reaction mixture was cooled in ice, and concentrated HCl (15 mL) was added. A bright yellow precipitate was formed, filtered, and washed extensively with water, yielding compound **2** as a yellow solid (663 mg, 95%). ^{1}H NMR (400 MHz, CD_3OD): δ 8.02 (s, 2H, CHAr).

Dynamic Combinatorial Libraries. In a typical experiment, the thiols (10 mM overall) were suspended in water, and the pH was adjusted to 8.25 by addition of a 1 M solution of NaOH. Where appropriate, spermine (2.5 mM) was added. The mixtures were allowed to oxidize and equilibrate by stirring in an open vial at room temperature. Evaporated water was replenished every day.

Library Analysis. Analyses were performed using an Agilent 1100 series HPLC and Agilent XCT ion-trap mass spectrometer. Solvents and formic acid were acquired from Romil. Analyses were performed using a reversed phase HPLC column (Agilent C8 Zorbax Eclipse XBD, 4.6 × 150 mm, 5 μm), using an injection volume of 10 μL, a flow rate of 1 mL/min, and a gradient (5−95% in 40 min) of acetonitrile in water (both containing 0.1% formic acid) at 303 K. Negative ion mass spectra were acquired in ultrascan mode using electrospray ionization (drying temperature, 350 °C; nebulizer pressure, 55 psi; drying gas flow, 12 L/min; HV capillary, 4000 V; ICC target, 200 000).

Purification of Receptor 6. Receptor **6** was isolated from a dynamic "library" made from thiol **2** (10 mM) and spermine (2.5 mM) in water at pH 8.25. Aliquots of 700 μL of this solution were injected onto a reversed phase preparative HPLC column (Agilent C8 Zorbax Eclipse XBD, 21.2 × 150 mm, 5 μm) at 303 K. Using a flow rate of 5 mL/min and a gradient (5−95% over 30 min) of acetonitrile in water (both containing 0.1% formic acid), the receptor was collected from 14 runs. The solvents were removed under reduced pressure keeping the temperature below 40 °C. The last traces of solvent were removed under high vacuum for 2−3 h. The crude host was dissolved in 12 mL of borate buffer (10 mM pH 9.0) and sonicated for 2 min. The resulting solution was centrifuged and decanted. Hydrochloric acid (3 mL of a 2.0 M solution) was added to the supernatant. The resulting suspension was centrifuged and the pellet resuspended in dilute hydrochloric acid (30 mL of a 40 mM solution) and centrifuged. The solid (6 mg) was dried under vacuum overnight. The LC−MS analysis of **6** is shown in the Supporting Information. ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.34 (s, 8H). Anal. Calcd for **6**·5H_2O: C, 38.32; H, 2.61. Found: C, 38.06; H, 2.76.

Isothermal Titration Calorimetry (ITC). The binding studies between **1** and **6** were conducted at three different concentrations using isothermal titration microcalorimetry (MCS-ITC, Microcal LLC, Northampton, MA). Solutions of guest **1** (in 3 mM TRIS buffer pH 7.4) were titrated into solutions of host **6** (in 3 mM TRIS buffer pH 7.4). Binding constants and enthalpies of binding were obtained by curve fitting of the titration data using the one-site binding model available in the Origin 2.9 software as shown in the Supporting Information. The thermodynamic parameters were averaged over three

(31) The amplitude of the CD signals after addition of spermine and after addition of **6** is somewhat lower than the original value as a result of partial precipitation of the DNA−spermine complex. This does not affect the conclusions.

experiments (the enthalpy value for run C [see Supporting Information] was discarded due to an inferior signal-to-noise ratio).

DNA Competition Experiments. Analyses were performed using a Varian Cary 100 Bio UV−visible spectrophotometer and a Jasco J-810 spectropolarimeter with 1 cm path-length cells at 298 K. Calf thymus DNA (highly polymerized), poly(dA-dC)·poly(dG-dT), spermine·4HCl, and buffers were purchased from Sigma and used without further purification. The solutions were prepared by dilution of concentrated stock solutions of the different species (DNA, spermine·4HCl, or **6**) in the desired buffer (3.0 mM TRIS buffer, pH 7.4, or 3.0 mM TRIS-EDTA buffer, 1.0 mM NaCl, pH 7.4). The final concentration in DNA was determined by measuring the absorbance at 260 nm (A_{260}) and assuming that 1 absorbance unit corresponds to 0.15 mM in base pairs. Samples were incubated for at least 30 min at 298 K prior to the spectroscopic measurements. The reported UV and CD traces correspond exclusively to DNA (contributions from the buffers, spermine, or **6** have been subtracted). Where appropriate, the traces have been corrected to compensate dilution.

Theoretical Calculations. Calculations were performed using Macromodel 8.0 and the AMBER* all atoms force field[32] using the continuum water solvent treatment. Conformational searches were carried out using the Monte Carlo Multiple Minimum method (MCMM). For the unbound tetramers, 1000 steps were sufficient to find the 10 lowest energy conformations at least twice each. Between 20 000 and 40 000 steps were calculated for the complexes; this was sufficient for at least 5 of the lowest 10 conformations to be found at least twice. Subsequent molecular dynamics (MD) simulations, also using the AMBER* force field with all atom treatment, were run for 20 ns at a simulation temperature of 300 K, with a 1.5 fs time step and 1 ps initial equilibration time. The variation in the average enthalpy (adjusted to the simulation temperature) during the last 5 ns of each experiment was determined, and in all cases was found to be less than 2.2 kJ mol^{-1}, implying the simulation length was sufficient. The energies of the spermine, free receptors, and complexes as determined by molecular dynamics are given in the Supporting Information. The interconversion barriers between P and M forms of the disulfide bonds were estimated using the dihedral driving function of Macromodel 8.0. Starting from the lowest energy conformer found in the conformational search, the dihedral angle of one disulfide bond was driven, in 1° steps, through 360°, in each direction. The lowest energy barrier for the conversion of the empty (P,P,P,P) host into the (P,P,P,M) conformer was found to be 74 kJ mol^{-1}. In the complex, the energy of interconversion was determined for each disulfide bond, and the lowest barrier was found to be 83 kJ/mol.

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Acknowledgment. We thank J. M. Goodman for advice on the simulations and J. K. M. Sanders for support. This work was supported by EPSRC, the European Union (Marie Curie Intra-European Fellowships), and the Royal Society.

Supporting Information Available: COSY spectrum of the complex between spermine and **6**; LC−MS analysis of **6**; ITC data for binding of spermine to **6**; computer simulation results. This material is available free of charge via the Internet at http://pubs.acs.org.

JA062536B