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A Cyclopeptide-Derived Molecular Cage for Sulfate Ions That Closes with a Click

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Dedicated to Professor Günter Wulff on the occasion of his 75th birthday

Abstract: The 2:1 sandwich-type complexes formed between a cyclopeptide with alternating L-proline and 6-aminopicolinic acid subunits and inorganic anions can be stabilized by covalently linking a tris-alkyne and a tris-azide derivative of this peptide through copper-catalyzed azide–alkyne cycloaddition. The resulting triply linked bis-cyclopeptide can interact with anions such as sulfate ions in aqueous solution by including them into the cavity between the two cyclopeptide rings, where they can form hydrogen bonds to amide NH groups, distributed along the inner surface. The binding kinetics of this system differ significantly from those of a bis-cyclopeptide that contains only one linker because the rate of guest exchange is considerably slower. Thermodynamically, the stability of the sulfate complex of the triply linked bis-cyclopeptide approaches a $\log K_a$ value of 6 in H_2O/CH_3OH 1:1 (v/v) which is, however, only approximately one order of magnitude larger than affinity of the more flexible monolinked analogue. Titration calorimetry revealed that this behavior is mainly due to the change in the binding enthalpy from exothermic to endothermic upon increasing the number of linkers. Results from NMR spectroscopy and X-ray crystallography indicate that the mono- and triply linked bis-cyclopeptides adopt similar conformations in their complexes with sulfate ions, but the complex formation is enthalpically unfavorable for the cage. The substantial entropic contribution to sulfate complexation of this receptor more than compensates for this disadvantage, so that the overall sulfate affinity of both bis-cyclopeptides ends up in the same range. These investigations provide important insight into the structure–property relationships of such receptors, thus leading the way to further structural improvement.

Keywords: anions • calorimetry • cycloaddition • molecular containers • supramolecular chemistry

Introduction

A synthetic receptor can achieve substrate binding by holding onto the substrate at only few selected positions, by encircling the substrate, or by completely encapsulating it inside a three-dimensional cavity. The latter probably most closely resembles binding inside the active center of a protein. Early examples of synthetic receptors that encapsulate their substrate are the cryptands developed by Lehn[1a] and subsequently the carcerands developed by Cram[1b] and the cryptophanes developed by Collet et al.[1c]

A carcerand has a closed surface that completely surrounds the guest molecule, thus preventing every possible way of escape.[1b] Although carcerand complexes (or carceplexes) are fascinating systems that still attract attention, for example, in the form of endohedral fullerenes,[2] they lack reversibility in guest exchange, thus precluding an evaluation of thermodynamic stability or binding selectivity. Opening a small portal in the surface of a carcerand furnishes a hemicarcerand, the complexes of which must be, by definition, sufficiently kinetically inert at room temperature to be isolable.[3] Receptors that exhibit reversible binding equilibria at ambient temperature can be obtained by increasing

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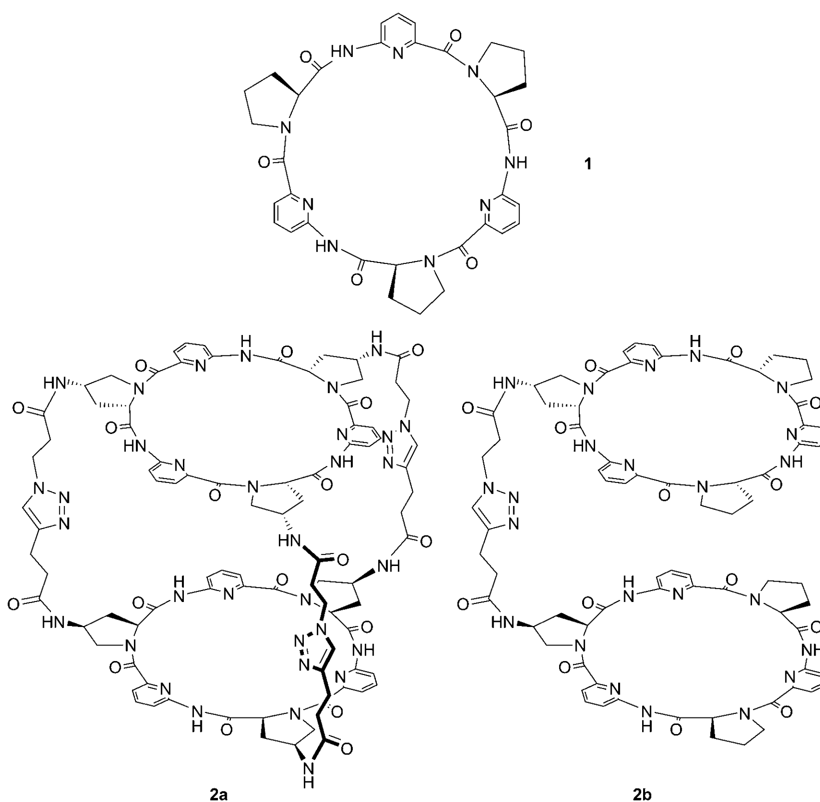
the number and/or the size of the portals or by introducing a gating mechanism that causes opening and closing of larger portals on the receptor surface. Terms such as molecular containers, cages, or capsules are used for such receptors for which the complexation/decomplexation rates are often significantly slower than those of receptors with more exposed cavities.[4a,b] These unique properties and others have made molecular containers attractive research objects in molecular-recognition studies.[4c] Molecular containers have, for example, been shown to stabilize reactive intermediates,[5a,b] act as nanoreactors,[5c] induce guest molecules to adopt conformations unstable outside the container cavity[5d–h] or give rise to unusual forms of isomerism if the mobility of the guest is impaired inside the cavity and/or more than one guest molecule is bound.[5i]

The construction of intricate systems such as molecular containers requires special synthetic approaches that often rely on the presence of molecules in the reaction mixture to act as templates. To assemble containers through covalent irreversible reactions, for example, the template must preorganize the container subunits in a fashion that allows them to be linked intermolecularly—a strategy used for the preparation of carcerands, hemicarcerands, and cryptophanes.[1b–c,3] In contrast to this kinetic approach, thermodynamic templation requires reversible interactions between the container building blocks and is based on the template-induced stabilization of the desired product.[6] Reversible reactions used for the latter approach include boronic ester formation,[7a] imine exchange,[7b–d] disulfide exchange,[7e] metal coordination,[5b,7f–h] or a combination of different covalent reactions.[7i,j] Noncovalent interactions, such as ion-pairing,[7k–m] hydrogen-bonding,[5i,7n,o] or hydrophobic interactions, have also been used.[7p]

Although molecular containers represent a large and structurally diverse family of synthetic receptors, many are synthesized from appropriately functionalized aromatic building blocks, thus rendering their interiors relatively nonpolar. Substrate binding often has an electrostatic component, such as cation–π interactions between a positively charged substrate and the aromatic cavity walls,[8a] or electrostatic interactions of the polar or charged substrate with polar regions along the container portals[8b] or with metal cations used for the assembly of the cage.[8c] Directed attractive interactions between the substrate and binding sites located inside the cavity as in the active centers of enzymes are, however, relatively rare. Notable examples of containers with functionalized interiors are two independently described coordination cages for sulfate ions,[9a,b] an interlocked sulfate-binding capsule,[9c] self-assembled phenylalanine-substituted resorcarene dimers,[9d–f] and a few other systems.[9g–i] In addition, bicyclic and tricyclic ammonium- or amide-based anion receptors possess cavities lined by properly arranged hydrogen-bond donors.[9j,k]

A promising building block, recently described by us, for the construction of container molecules with internal binding sites is cyclic hexapeptide **1**.[10a] This peptide has been shown to bind inorganic anions such as sulfate or halide ions in the form of 2:1 complexes in competitive aqueous solvent mixtures in which the anion is sandwiched between two cyclopeptide rings and is engaged in hydrogen-bonding interactions with six NH groups that point into the cavity. Covalent linkage of two cyclopeptide rings through one linker has furnished bis-cyclopeptides that bind to anions in a 1:1 ratio with considerably higher efficiency than the monotopic analogue **1**.[10b–e] A logical extension of this approach is the introduction of three linkers between the cyclopeptide rings, which should give rise to anion-binding molecular containers. Questions that can be raised in this context are how the increase in the number of linkages influences the binding properties and whether it is possible to construct carcerands with permanently entrapped anions based on such systems.

Connecting two appropriately functionalized analogues of **1** through three linkers can in principle be realized by using



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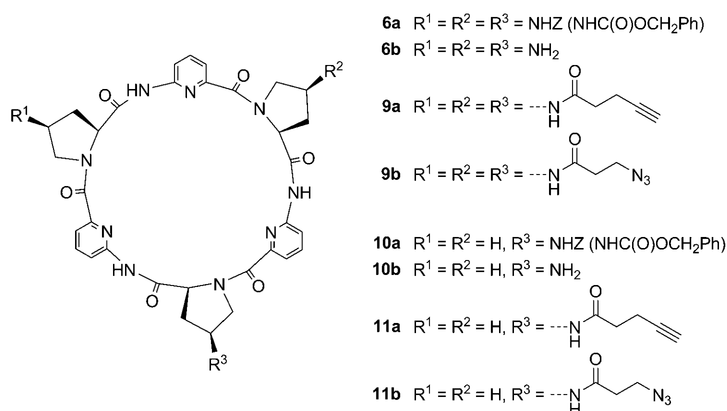
irreversible or reversible reactions in conjunction with the template effects of anions. Both approaches are currently being pursued in our group. Herein, we present the first bis-cyclopeptide **2a** obtained in this context, which was prepared by using a copper-catalyzed 1,3-dipolar cycloaddition to connect tris-alkyne **9a** and tris-azide **9b**.[11a] This reaction, often referred to as a click reaction,[11b–c] has been shown to be immensely useful for a variety of purposes. However, to the best of our knowledge it has only rarely been applied to the synthesis of molecular containers or cages so far.[9c,12]



Our results indicate that **2a** forms a stable complex with sulfate ions in H_2O/CH_3OH mixtures in which the anion is indeed located inside the cavity between the two cyclopeptide rings.[13] Interestingly, the sulfate affinity of **2a** is not significantly higher than that of analogue **2b**, which contains only one linker despite the improved preorganization that the three linkers were expected to induce. Characterization of the thermodynamics of the sulfate complexation of **2a** and **2b** with isothermal titration calorimetry (ITC) provided valuable information about the causes of this unexpected behavior.

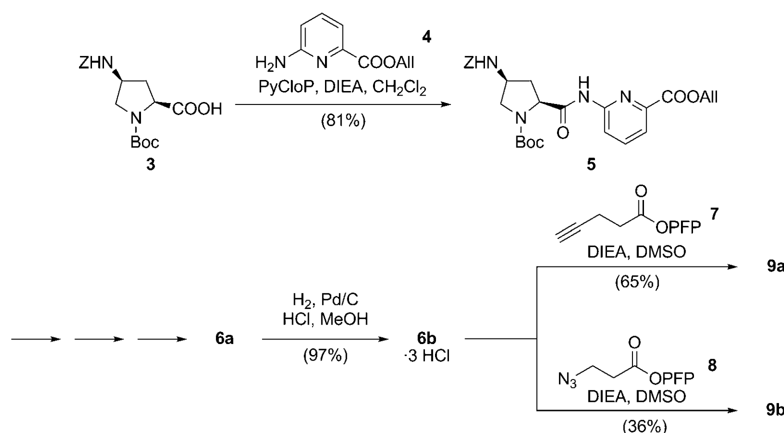
Results and Discussion

Synthesis and structural characterization: Bis-cyclopeptide **2a** contains two subunits of cyclopeptide **6b** with alternating 4*S*-amino-L-proline and 6-aminopicolinic acid subunits. This building block was assembled prior to the introduction of the peripheral functional groups required for the azide–alkyne cycloaddition. The starting point of the synthesis of **6b** was (2*S*,4*S*)-4-(benzyloxycarbonylamino)-1-(*tert*-butyloxycarbonyl)proline (**3**), which was prepared analogously to a known procedure.[14] This acid was coupled with allyl 6-aminopicolinate (**4**), synthesized from 6-aminopicolinic acid and allyl bromide, by using chlorotripyrrolidinophosphonium hexafluorophosphate (PyCloP) as the coupling agent (Scheme 1). The resulting dipeptide **5** was used to assemble the cyclic hexamer **6a** by following established protocols.[15] Hydrogenation of **6a** in the presence of hydrochloric acid gave the trihydrochloride salt of **6b**, which could be converted into the trisubstituted derivatives **9a** and **9b** by treatment with the pentafluorophenol esters of 4-pentynoic acid (**7**) or 3-azidopropanoic acid (**8**), respectively, the latter being obtained by the addition of hydrogen azide to acrylic acid.

The monosubstituted analogues **11a** and **11b** were obtained in a similar manner from the hydrochloride salt of **10b**. The Z-protected precursor of **10b**, namely, cyclopeptide **10a**, was assembled from dipeptide **5** and two equivalents of an unsubstituted dipeptide, the synthesis of which has been described previously.[15]

The copper(I)-catalyzed azido–alkyne cycloaddition to give 1,4-disubstituted 1,2,3-triazoles is widely used in organic synthesis, but optimization of the reaction conditions is usually necessary.[16] Our attempts to identify suitable reaction conditions initially concentrated on the monofunctionalized cyclopeptides **11a** and **11b**, the coupling of which should be straightforward because it does not strictly require the template effect of anions to bring the two cyclopeptide subunits together. Two aspects important for the subsequent synthesis of the desired cage **2a** had to be considered: First, reactions should be carried out in aqueous solvent mixtures, which favor the anion-promoted self-assembly of two cyclopeptide rings, and second they should proceed at or below room temperature to make sure that most binding partners are involved in complex formation.

Systematic variation of the conditions showed that by performing the reaction with equimolar amounts of **11a** and **11b** in H_2O/CH_3OH (2:3, v/v), two equivalents of copper(II) sulfate as a source of copper(I) ions and the anionic template,[17] sodium ascorbate as a reducing agent, and 2,6-luti-



Scheme 1. Synthesis of cyclopeptides **9a** and **9b**. All = allyl, Boc = *tert*-butoxycarbonyl, DIEA = diisopropylethylamine, DMSO = dimethyl sulfoxide, PFP = pentafluorophenol, Z = benzyloxycarbonyl.

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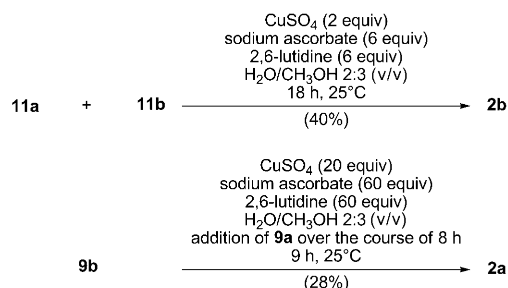
dine as a ligand to stabilize the copper(I) ions and decrease the risk of coordination of the aminopicolinic acid subunits in the cyclopeptides to the metal ions leads to complete conversion after approximately 18 h at room temperature (Scheme 2). Due to the demanding workup, pure **2b** could only be isolated in 40% yield, which was nevertheless sufficient for the following binding studies.



Scheme 2. Syntheses of bis-cyclopeptides **2a** and **2b**.

In the presence of catalytic amounts of $CuSO_4$ or two equivalents, as used for the coupling of **11a** and **11b**, the reaction between **9a** and **9b** proved to be very slow and was still incomplete after approximately one day. Because performing the reaction at higher temperature was undesirable, the reaction rate was accelerated by increasing the amount of reagents. Indeed, in the presence of 20 equivalents of $CuSO_4$ and the corresponding amounts of sodium ascorbate and 2,6-lutidine, complete disappearance of the starting materials was observed after 18 h. Moreover, MALDI-TOF mass spectrometric analysis of the reaction mixture indicated the formation of a product with the *m/z* ratio expected for **2a**, but an insoluble material was also formed under these conditions, which most probably represents a cross-linked polymer containing both of the cyclopeptide building blocks. To suppress uncontrolled polymerization, the reaction was carried out under higher dilution conditions and cyclopeptide **9a** was added very slowly (over the course of 8 h) to a solution containing **9b** and the other reagents (Scheme 2). The formation of a precipitate was much less pronounced under these conditions and HPLC analysis of the reaction mixture indicated the formation of a soluble product with the *m/z* ratio expected for **2a**. This compound was isolated after precipitation of the sulfate ions from the reaction mixture as $BaSO_4$ and two chromatographic steps, of which the final step was semipreparative HPLC, to give the analytically pure form in 28% yield.

The fact that the *m*/*z* ratios of the signals observed in the MALDI-TOF mass spectrum of the product obtained from the coupling of **9a** and **9b** can be assigned to a bis-cyclopeptide containing both building blocks is no sufficient proof that all three linkages between the cyclopeptide rings are indeed closed because a bis-cyclopeptide unit with residual alkyne or azide moieties would have the same mass. Evidence that the structural assignment of **2a** is correct comes from the following results:

1) No azide or alkyne band can be detected in the FTIR spectrum of the isolated product.
2) A characteristic fragmentation of cyclopeptide azides, such as **9b**, upon collision-induced decay of the molecular ion in the ESI-MS/MS spectrum corresponds to loss of nitrogen atoms. Corresponding fragments were absent in the spectrum of **2a**.
3) The relatively simple ^1H and ^{13}C NMR spectra of the product are consistent with a C_3 symmetrical structure containing three intact linkers (the two cyclopeptide rings are indistinguishable in the NMR spectrum because directionality of the relatively remote triazole units in the linkers does not translate into measurable differences in the chemical shifts of the cyclopeptide protons).
4) Finally, the molecular structure of **2a**, determined from single crystals obtained by slow evaporation of solutions of **2a** in H_2O/CH_3OH or H_2O/CH_3CN with and without added Na_2SO_4, clearly demonstrated that all three linkers of the cage are fully formed, and that the three triazole moieties exhibit the expected 1,4-disubstitution pattern. The result of the crystallographic analysis is depicted in Figure 1.

Figure 1 shows that **2a** adopts an overall C_2 symmetrical conformation in the crystal with the crystallographic symmetry axis intersecting the triazole ring of one tightly folded linker. Because the C_2 symmetrical conformation is not compatible with the three triazole linkers oriented similarly with respect to one of the cyclopeptide rings, we have to assume that molecules in which the N1 and C4 atoms of the triazole rings are interchanged are superimposed in the crystal.

Interestingly, the individual cyclopeptide rings in **2a** adopt an almost ideal C_3-symmetrical conformation despite the decreased symmetry in the crystal. The two cyclopeptide rings are tilted by 19(1)° to one another and not by 0° as expected for an overall C_3-symmetrical conformation of the cage. The receptor crystallizes with 21 water molecules, which appear to have a very structured arrangement (Figure 1, bottom). Indeed, apart from one oxygen atom of one water molecule, which lies outside the cavity and is disordered over two positions, all the water molecules, including the hydrogen atoms, could be located on difference Fourier syntheses, thus suggesting that the water molecules are tightly bound. All six N−H groups of the two cyclopeptides and four C=O groups in the linkers are involved in hydrogen bonding to water molecules in the cavity. It is worthy of note that there are no direct N−H···O=C hydrogen bonds between molecules of **2a** in the crystal, thus indicating that water is an important factor in stabilizing the crystal.

Qualitative evaluation of the binding of sulfate ions: The first qualitative information on the binding properties of receptors **2a** and **2b** came from ESI mass spectrometric and

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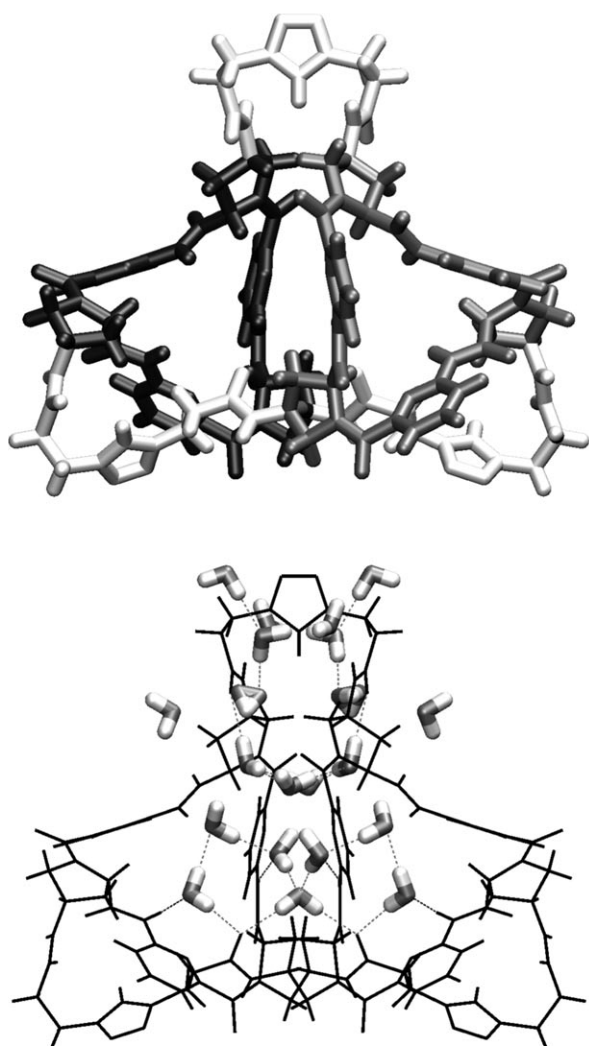

Figure 1. Molecular structure of **2a**·21 H_2O obtained by slow evaporation of a solution of this bis-cyclopeptide in H_2O/CH_3OH (1:1, v/v) in the presence of one equivalent of Na_2SO_4. Top: the mutual orientation of the two cyclopeptide rings and the conformations of the linkers in **2a**; bottom: the arrangement of the 21 water molecules including the corresponding hydrogen-bonding pattern (crystallographic C_2 axis is vertical).

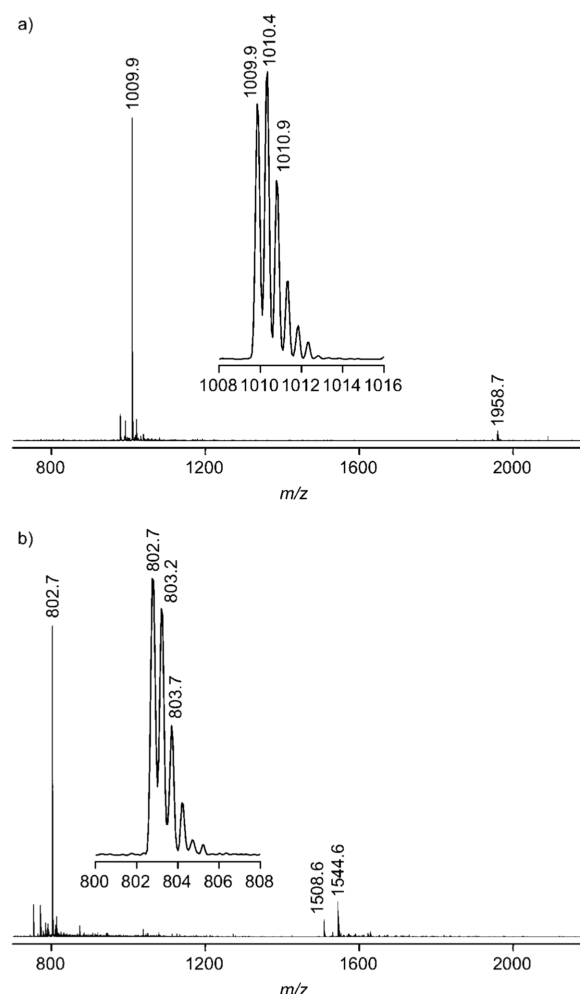

Figure 2. ESI mass spectra (negative mode) of a) **2a** (0.01 mM) in the presence of one equivalent of Na_2SO_4 and b) **2b** (0.01 mM) in the presence of three equivalents of Na_2SO_4 in H_2O/CH_3OH (1:1, v/v).

^{1}H NMR spectroscopic measurements. The sulfate ion was used as model substrate in these investigations because it is usually bound by our bis-cyclopeptides with the highest affinity.[10b,c] Figure 2 shows the ESI mass spectra of solutions of **2a** and **2b** in H_2O/CH_3OH (1:1, v/v) containing sodium sulfate (negative mode). In both spectra, the major peak has an *m/z* ratio and isotopic pattern that can be assigned to the 1:1 sulfate complexes of both bis-cyclopeptides, namely, **2a**·SO_4^{2-} and **2b**·SO_4^{2-} (*m/z* 1009.9 and 802.7, respectively). Minor signals are attributable to the chloride complexes of **2a** and **2b** (**2a**·Cl^-: *m/z* 1958.7 and **2b**·Cl^-: *m/z* 1544.6, in Figure 2a and b, respectively). The peak at *m/z* 1508.6 in spectrum (b) can be assigned to the deprotonated receptor. Both spectra, thus, strongly suggest that the major complex species of both receptors possess a 1:1 stoichiometry.

A characteristic effect of anion binding in the ^{1}H NMR spectra of our bis-cyclopeptides is usually the pronounced downfield shift of approximately $\Delta\delta = 1$ ppm of the proline Hα) signals, which is caused by the spatial proximity of the corresponding protons to the anion in the complex.[10b–c] Because the rate of complex formation of most systems studied so far is fast on the NMR timescale, averaging of the signals of free and complexed bis-cyclopeptides has usually been observed.

Figure 3 shows a series of ^{1}H NMR spectra of **2a** and **2b** in D_2O/CD_3OD (1:1, v/v) containing increasing amounts of sodium sulfate. The strong downfield shift of the Hα signals upon sulfate complexation is clearly visible, thus indicating that both receptors behave like other structurally related bis-cyclopeptides and bind the anion in the space between the two cyclopeptide rings. Additional changes in the aliphatic regions of the ^{1}H NMR spectra of **2a** and **2b** in which the remaining proline protons and protons in the aliphatic parts of the linkers absorb are attributable to a conformational reorganization of the receptors upon complex

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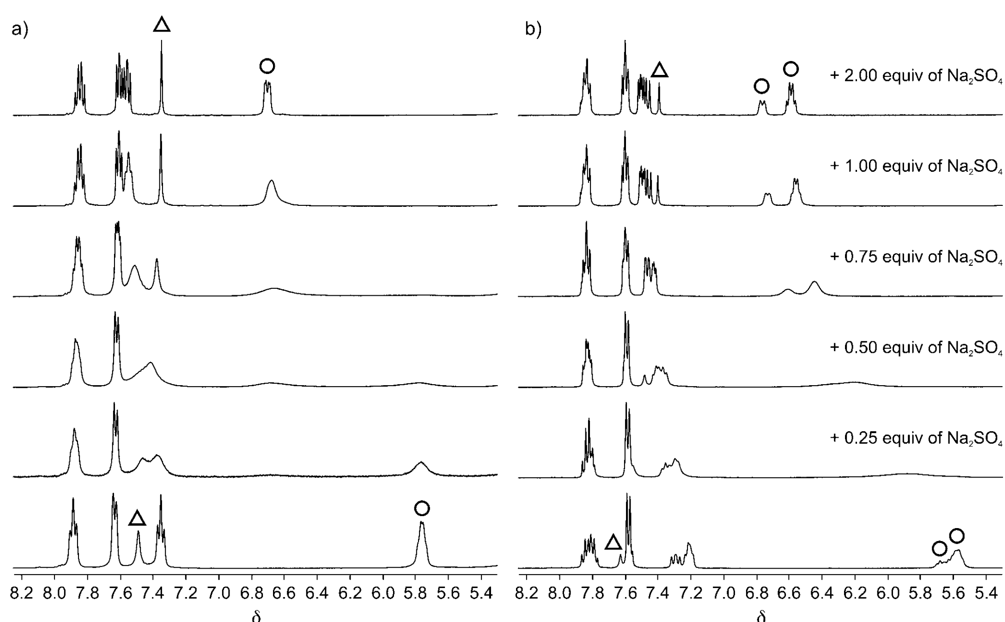

Figure 3. ^{1}H NMR spectra of a) **2a** and b) **2b** in D_2O/CD_3OD (1:1, v/v) (**2a**: c = 1.6 mM, **2b**: c = 2.8 mM) containing varying amounts of Na_2SO_4. The triazole CH and the proline Hα signals are marked as triangles and circles, respectively.

formation (see the Supporting Information). The signal of the triazole protons experiences an upfield shift, thus suggesting that these protons are not directly involved in the interaction with the anion in contrast to other triazole-containing anion receptors.[18]

Differences in the spectra of **2a** and **2b** (Figure 3) provide important information about the kinetics of complex formation. In the case of **2b**, there is a continuous shift of the Hα signals, which is accompanied by pronounced line broadening in the intermediate region between the free and fully complexed receptor. The underlying complexation/decomplexation equilibrium is thus still fast on the NMR timescale, although it clearly approaches a rate that can be resolved with the 400 MHz instrument used to record the spectra. In contrast, clearly separated Hα signals are observed for the free and complexed **2a**, with the signals for the free receptor decreasing in intensity as the substrate concentration rises and the signal for the complex concomitantly increasing, thus showing that sulfate exchange is slower than for **2b**. Thus, closing the cage affects complexation kinetics in a similar fashion as observed for other container-type molecules.[4a,b] Interestingly, the triazole signal exhibits a different behavior as it progressively shifts from the uncomplexed to the fully complexed form. Therefore, it appears that complex formation and conformational mobility of the linkers proceed on different timescales, the latter being faster, which leads to averaging of the linker protons.

To obtain an idea about the structure of the sulfate complex of **2a**, molecular modeling studies based on the known preferred conformation of the cyclopeptide ring and its mode of interactions with anions were performed.[10a,d] These calculations yielded the structure depicted in Figure 4 as the global minimum.[19] Seven structurally, closely related conformations were found within an energy window of 5 kJ mol^{-1} above the global minimum. In all of these structures, the cyclopeptide moieties adopt conformations very similar to that found in the crystal structure of the free macrocycle.[10a] Moreover, the mutual arrangement of both cyclopeptide rings allows short hydrogen-bonding interactions with the included sulfate anion with N···O distances that range between 2.6 and 3.1 Å. The hydrogen-bonding pattern (indicated as dotted lines in Figure 4) comprises direct hydrogen bonds between two sulfate oxygen atoms and one NH group in each cyclopeptide ring. The other two sulfate oxygen atoms are sandwiched between pairs of NH groups from both rings to which they form bifurcated hydrogen bonds. A molecular dynamics simulation indicated that the linkers retain considerable flexibility even in the complex, which is consistent with the NMR spectroscopic result that the triazole signal exhibits a continuous shift in the NMR spectrum upon complex formation.

Thermodynamics of sulfate binding: The sulfate affinity of **2a** and **2b** was evaluated quantitatively by using isothermal titration calorimetry (ITC). This method has the advantage of providing the full thermodynamic signature of complex formation in one measurement.[20a] Changes in complex stability induced by varying, for example, the structure of the receptor, the nature of the substrate, or the solvent can thus be traced to whether they are enthalpic or entropic in origin. Because ITC measures the overall heat change of a solution upon complex formation, it does not enable the effects that result from direct receptor–substrate interactions and from, for example, the desolvation of the binding partners to be distinguished. Information in this regard can sometimes be obtained by using systematic trend analyses

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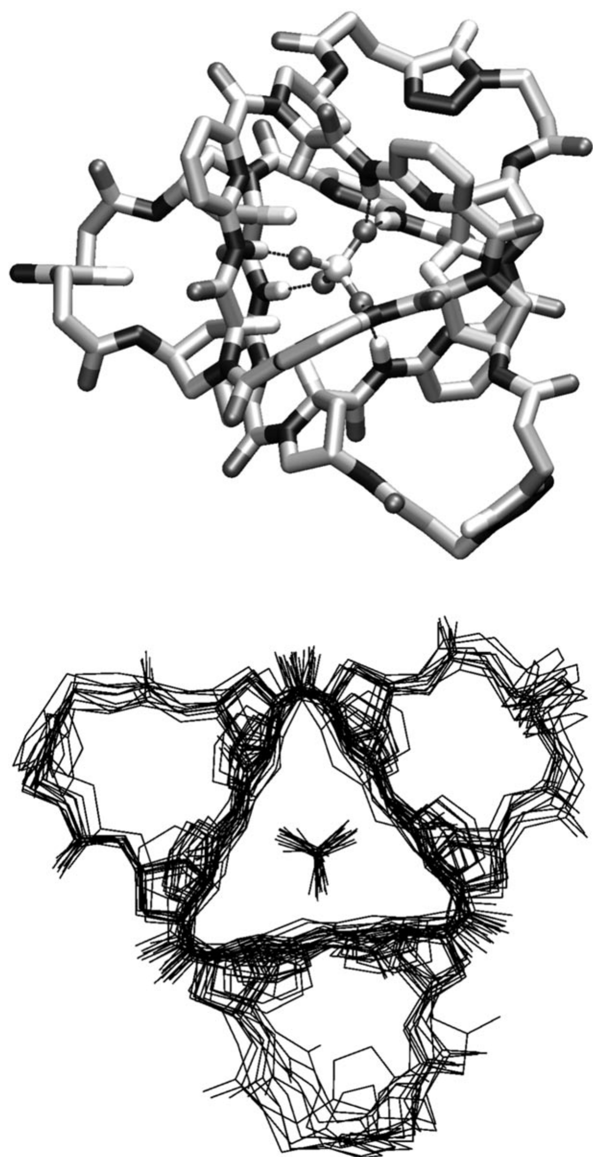

Figure 4. Calculated conformation of the sulfate complex of **2a** according to a Monte Carlo simulation (top) and its conformational mobility according to a subsequent molecular dynamics simulation (bottom). Hydrogen bonds are indicated by dotted lines. (Macromodel 9.0 with Maestro 7.0 interface, Schrödinger, Inc.; MMFF94S force-field with GB/SA water model, 5000 steps, dynamics: 100 ps, 25 °C; the bottom picture shows an overlay of 10 snapshots taken after intervals of 10 ps).

Table 1. Association constants $\log K_a$, Gibbs energies ΔG, enthalpies ΔH, and entropies $T\Delta S$ of binding of Na_2SO_4 to receptors **2a** and **2b** in H_2O/CH_3OH of varying ratios at 298 K.

	2a			**2b**		
$H_2O:CH_3OH$ (v/v)	35:65	50:50	65:35	35:65	50:50	65:35
n[a]	0.89 (±0.01)	0.92 (±0.01)	0.75 (±0.14)	0.88 (±0.01)	0.87 (±0.05)	0.85 (±0.01)
$\log K_a$	6.34 (±0.02)	5.70 (±0.03)	5.19 (±0.05)	5.67 (±0.02)	4.96 (±0.05)	4.26 (±0.02)
ΔG [kJ mol^{-1}][b]	−36.2 (±0.1)	−32.6 (±0.2)	−29.6 (±0.3)	−32.3 (±0.1)	−28.4 (±0.3)	−24.3 (±0.1)
ΔH [kJ mol^{-1}][b]	13.3 (±0.1)	10.1 (±0.1)	6.9 (±0.3)	−12.4 (±0.1)	−13.5 (±0.3)	−11.8 (±0.2)
$T\Delta S$ [kJ mol^{-1}][b]	49.5 (±0.1)	42.7 (±0.2)	36.5 (±0.1)	19.9 (±0.2)	14.9 (±0.5)	12.5 (±0.3)

[a] Interaction stoichiometry factor obtained from the ITC titrations. [b] Standard deviations of at least three independent measurements are specified in brackets.

that involve changing one parameter, for example, the structure of the receptor, and keeping all the other parameters invariable.[20b–e]

The results of the ITC measurements (Table 1) show that with a $\log K_a$ value between 4 and 6 the monolinked bis-cyclopeptide **2b** possesses very high sulfate affinity in the solvent mixtures studied, albeit an approximately one order of magnitude lower affinity than the previously described analogue containing a 2,2′-(1,3-phenylene)diacetic acid linker, which binds sulfate in H_2O/CH_3OH (1:1, v/v) with a $\log K_a$ of 5.97.[10e] As for other bis-cyclopeptides investigated previously, complex formation in H_2O/CH_3OH is exothermic and has a favorable entropic contribution.[10b,e] Interestingly, increasing the water content of the solvent mixture from 35 to 65 % has almost no effect on the enthalpy of the complex formation. The decrease in complex stability observed upon increasing the water content is therefore only due to the entropic term, which becomes smaller in solutions containing greater concentrations of water.

A comparison of the sulfate affinity of the triply linked bis-cyclopeptide **2a** with that of the monolinked derivative **2b** reveals that affinity improves upon introduction of the additional linkers. The increase of only approximately one order of magnitude is, however, disappointingly small when considering the much better preorganization **2a** should have for anion binding.

Insight into the cause of the unexpected low sulfate affinity of **2a** can be obtained from Table 1 by comparing the individual thermodynamic parameters associated with binding of **2a** or **2b** to sulfate ions. The most important difference in the behavior of these bis-cyclopeptides is that sulfate binding of **2b** is exothermic, whereas it is endothermic for **2a**, thus being enthalpically disfavored. Thus, increasing the number of linkers between the cyclopeptide rings clearly causes profound changes in the thermodynamics of sulfate binding. The strongly favorable entropy of complex formation observed for **2a** is cancelled out, to a large extent, by a highly unfavorable enthalpic term that causes complex stability to be only slightly larger than that of **2b**.

The question arises as to why the enthalpy of binding changes so strongly upon increasing the number of linkers between the cyclopeptide rings. Endothermic sulfate binding has been previously reported for other receptors and was attributed to the fact that the favorable binding enthalpy associated with the direct receptor–substrate interactions cannot compensate for the high enthalpy required for desolvation of the sulfate anion,[21] but this explanation does not correlate structural parameters of a receptor with the thermodynamics of binding. Because knowledge of the relevant structural parameters that influence the overall affinity of **2a** is

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of great importance for the design of such cage-type receptors, we tried to obtain more detailed insight into the underlying structure–property relationship. In the concrete case of **2a**, the following assumptions could provide plausible explanations for the endothermic sulfate binding:

1) The introduction of three linkages prevents the two cyclopeptide rings from approaching each other efficiently, thus enthalpically weakening the interactions with an included anion relative to **2b**.
2) Complex formation induces energetically unfavorable linker conformations, an adverse effect on the enthalpy of binding that becomes the more pronounced the higher the number of linkers.
3) The energy required for desolvation of the binding sites prior to sulfate binding is significantly higher in the case of **2a**.

Unfortunately, calorimetry alone could not reveal how these factors might play a role. Therefore, additional evidence from independent control experiments was required. NMR spectroscopic analysis was used to obtain information about the conformational reorganization of **2a** and **2b** during complex formation.

Comparing the 2D NOESY NMR spectrum of monolinked bis-cyclopeptide **2b** with its sulfate complex revealed crosspeaks between signals of substituted and unsubstituted proline units in the spectrum of the complex absent in the spectrum of uncomplexed **2b** (see the Supporting Information). Such crosspeaks have been previously observed for other bis-cyclopeptides and they are a result of a folded conformation of the receptor in the complex, which arranges the two cyclopeptide rings in close proximity and allows the anion to efficiently bind to all six NH groups simultaneously.[10b] An additional indication of the proximity of the substrate to protons located inside the receptor cavity, in particular the Hα protons, is the strong deshielding these proton experience upon sulfate binding that causes their signals to shift in the ^{1}H NMR spectrum with respect to uncomplexed bis-cyclopeptide by up to $\Delta\delta = 1.08$ ppm to lower field.

The downfield shift of the Hα signals is slightly smaller when sulfate ions are included in the cavity of **2a** ($\Delta\delta = 0.94$ ppm), thus suggesting that the distance between the anion and corresponding protons is on average larger in this complex than in **2b**. The effect is so small, however, that it is unlikely to be the sole reason for the differences in the thermodynamics of sulfate complexation of both receptors. Unfortunately, 2D NOESY NMR spectroscopic analysis does not provide information about the mutual orientation of the cyclopeptide rings in the sulfate complex of **2a** because the two rings are NMR spectroscopically indistinguishable. In comparison with the spectra of **2b**, there are stronger crosspeaks in the 2D NOESY NMR spectra of **2a** between the triazole protons and protons in the ethylene subunits of the linkers, particularly to the protons in the methylene group at N1. These crosspeaks account for folded compact linker conformations, but because there are no significant differences between the spectra of the free and complexed **2a** these spectra provided no information about a conformational rearrangement of the linkers upon complex formation. Therefore, NMR spectroscopic analysis indicated that weaker receptor–substrate interactions most probably do not explain the endothermic reaction observed during sulfate binding of **2a**, but it did not allow us to derive conclusive evidence about the conformation of the linkers in the complex.

Additional information about the structural factors that might influence the anion binding of **2a** was obtained from crystal-structure analyses. Seven crystals of **2a**, obtained from both salt-free and solutions containing Na_2SO_4, were investigated, and in each case the salt-free form was obtained, thus indicating that the hydrated receptor forms more stable crystals (Figure 1). It is worth noting that the larger and better formed crystals were found in the samples containing Na_2SO_4, and this outcome may be a result of salting out.[22] Because there are no direct intermolecular hydrogen bonds between the receptor molecules in the crystals of **2a**·21 H_2O, all the hydrogen-bonding interactions of the receptor are with solute water, and the arrangement indicates that the water in the cavity is strongly stabilized by cooperative hydrogen bonding, i.e., N−H···O(H)H···O=C, C=O···HOH···O=C, ···O(H)H···O(H)H···, and branched ···O-(H···O(H)H···)H···O(H)H··· hydrogen bonding).[23] Nine of the water molecules form a cluster in the center of the receptor. This cluster is made up of three four-membered rings and two six-membered rings, but each of these water molecules forms hydrogen bonds with the receptor, in contrast to the endohedral 'molecular ice' recently observed in a hydrophobic pocket of a self-assembled cage.[24] Removal of the nine water molecules from one receptor in the crystal results in a void of 274 Å^3,[25] which is almost an order of magnitude larger than the 33 Å^3 calculated by removing the sulfate anion from the crystal structure of the tetrabutylammonium sulfate complex of a monolinked bis-cyclopeptide,[10d] thus indicating that the conformation of **2a** in the crystal and the arrangement of the hydrogen-bond donors is far from optimum for sulfate anion binding.

It is not unproblematic to use structural information derived from crystal structures to explain behavior in solution, but because the crystal packing in the case of **2a**·21 H_2O involves strongly "solvated" receptor molecules with a mutual arrangement that does not rely on directed intermolecular interactions a cautious analysis should be feasible. Two factors appear to be relevant: First, **2a** prefers to crystallize from aqueous solution without included sulfate anions in a conformation that is different from that expected from molecular modeling of the sulfate complex and derived from NMR spectroscopic analysis. This behavior may be a result of energetic differences in the conformations of free and complexed receptor. The enthalpically more favorable conformations of the bis-cyclopeptide in solution appear to be uncomplexed ones, which are most probably similar to the conformations found in the crystal. Complex formation induces a pronounced structural reorganization, consistent

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with the NMR spectroscopic results, but leads to conformations that may be less stable, presumably because the arrangement of the two cyclopeptide rings induces strain in the three tightly folded linkers. Thus, receptor **2a** has to pay an enthalpic penalty during complex formation, which causes the interaction with sulfate ions in H_2O/CH_3OH to be endothermic overall. In the crystal, in which entropic factors play a minor role, the sulfate complex of **2a** is unstable, thus explaining why this bis-cyclopeptide prefers to crystallize without included sulfate ions, even if the anion is present in solution. Second, the solvate water in the crystal appears to be tightly bound. Additional contributions to the endothermicity of complex formation could, therefore, come from desolvation effects if **2a** binds water molecules in solution as tightly as in the crystal. Whether and to what extent this effect contributes substantially to binding is difficult to quantify experimentally. Measurement of solvent isotope effects (binding in D_2O vs. H_2O) can provide information about the contribution of solvation effects to intermolecular interactions, but because the enthalpic gain of the stronger hydrogen bonds in deuterated solvents is usually compensated to a large extent by a more unfavorable entropic term, we do not expect such measurements to provide clear-cut information with respect to sulfate binding in the case of **2a**.[26] Therefore, we tentatively conclude that enthalpically unfavorable conformations of this bis-cyclopeptide in its sulfate complex possibly in conjunction with the need to remove tightly bound water molecules from the host upon anion binding are responsible for the endothermic complex formation. Evidently, the high stability of the sulfate complex of **2a** in H_2O/CH_3OH is solely a consequence of the very large entropic factor, which most probably arises in part from the release of the ordered water molecules from bis-cyclopeptide and anion upon binding. Because only entropy allows **2a** to adopt the conformations necessary for anion binding, this particular system illustrates a structure-determining effect of entropy in a synthetic receptor.

It is worth noting that evaluation of solvent dependence of sulfate affinity revealed the decrease in complex stability observed for **2a** and **2b** upon increasing the water content of the solvent to be entirely due to a decrease in the favorable entropy term. In the case of **2a**, binding enthalpy becomes even less unfavorable (producing a positive effect on complex stability) as the water content increases. A similar effect has been observed previously for a monolinked bis-cyclopeptide.[10d] At first sight this trend seems counterintuitive since one would have most probably predicted the opposite attributing the weaker binding in solvents containing more water to weaker interactions between the receptor and the substrate. This view, however, focuses solely on the direct receptor–substrate interactions and neglects energetic contributions from desolvation that, as previously pointed out, cannot be separated from the binding event by calorimetry.

To rationalize how desolvation causes the observed solvent-composition-dependent trends in binding enthalpy and entropy, we previously proposed that sulfate ions have the tendency in H_2O/CH_3OH mixtures to be preferentially solvated by water molecules.[10d] Thus, decreasing the amount of water should cause the water–sulfate ions interactions to become enthalpically more favorable (less polar medium) and entropically more costly (less water available). As a consequence, desolvation of sulfate ions upon complex formation will be entropically more favorable as the amount of water decreases, but increasingly endothermic. The same arguments hold when making the reasonable assumption based on the crystal structure that the receptor cavity of **2a** is preferentially solvated by water molecules.

Unfortunately, only Gibbs energies for the transfer of sulfate ions from water to H_2O/CH_3OH mixtures are available,[27] but not the individual enthalpic and entropic contributions. Therefore, appropriate data is currently missing to confirm our interpretation and verification requires further systematic binding studies. The characteristic effect of the structures of receptors **2a** and **2b** on the thermodynamics of sulfate binding is evident in the different extent to which the complexation enthalpy for each receptor varies upon increasing the water content of the solvent from 35 to 65 %, with almost no change in the case of **2b**, whereas the complexation enthalpy decreases by approximately 6 $kJ\,mol^{-1}$ for **2a**.

Kinetics of sulfate binding: The NMR spectroscopic changes observed upon sulfate complexation of bis-cyclopeptides **2a** and **2b** qualitatively indicate that the rate of guest exchange is slower for the receptor containing three bridges between the cyclopeptide rings. Temperature-dependent NMR and quantitative ^1H–^1H NOESY NMR spectroscopic measurements (exchange spectroscopy, EXSY) were carried out to gain more quantitative insight into complexation/decomplexation kinetics.

The simplest way to spectroscopically estimate the rate of exchange in a dynamic system by using ^1H NMR spectroscopic techniques is to determine the distance $\Delta\nu$ in s^{-1} between two peaks of exchanging protons in the slow-exchange regime and the temperature at which these peaks coalesce. The rate constant at this temperature k_c is then $\pi\Delta\nu/2^{0.5}$. Although this correlation strictly only applies to dynamic equilibria in which the interconverting species are equally populated, are transformed into one another through first-order reactions, and possess peaks in the NMR spectra that do not exhibit coupling, this correlation has also been shown to provide a rough estimate of reaction rates that do not fulfill all of these requirements, including binding equilibria.[28] The problems associated with studying binding equilibria are: 1) only complex dissociation is usually a first-order reaction, whereas complex formation follows a second-order rate law and 2) temperature variation not only affects binding kinetics but also the ratio of free and complexed binding partners, namely, the thermodynamics.

Aware of these drawbacks, we studied the effect of temperature on the NMR spectra of solutions of **2a** and **2b** in D_2O/CD_3OD (1:1, v/v) in the presence of 0.5 equivalents of Na_2SO_4. In the case of **2b**, increasing the temperature caused the expected sharpening of the Hα signals, whereas

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decreasing the temperature caused the signal to become broader until two peaks emerged in the spectral region of the Hα signals at temperatures below 10 °C, which can be assigned to free **2b** and its sulfate complex (see the Supporting Information). Both signals are rather broad even at −10 °C, but further lowering of temperature caused the formation of a precipitate in the sample, thus precluding measurements below this temperature. By using the chemical shifts of the Hα signals of free receptor and those of the receptor in the presence of two equivalents of Na_2SO_4 to calculate $\Delta\nu$, a rate constant k_c at the coalescence temperature of 10 °C between 1400 and 1500 s^{-1} was calculated. The range derives from the fact that the Hα signals of the substituted proline units in **2b** experience a slightly larger shift in the NMR spectra upon complex formation than the signals of the unsubstituted proline units. Analogously, a comparable k_c value of 1200 s^{-1} can be calculated from the corresponding spectra of **2a**. For this receptor, however, it was impossible to determine the coalescence temperature because the ^{1}H NMR spectrum of **2a** in D_2O/CD_3OD (1:1, v/v) in the presence of 0.5 equivalents of Na_2SO_4 still showed two, albeit very broad, Hα signals even at 60 °C and a further temperature increase was not feasible. Therefore, these measurements only allow the conclusion that for **2a** to approach a similar rate of guest exchange as observed for **2b** at 10 °C, the temperature must be more than 50 °C higher.

To obtain quantitative data for the rate of guest exchange for **2a**, we performed 2D EXSY NMR measurements.[29] This method has been proven to be very useful to evaluate the rate of binding equilibria.[4b] However, it can only be applied to reactions with equilibria that are slow on the NMR timescale and associated with two separated signals of exchanging protons in the spectrum. The optimal mixing time for the measurement was estimated by recording ^{1}H–^{1}H NOESY NMR spectra of **2a** in D_2O/CD_3OD (1:1, v/v) in the presence of 0.5 equivalents of Na_2SO_4 at different mixing times (0.03, 0.045, 0.06, 0.08, 0.1, 0.15, 0.25, and 0.5 s) and determining under which conditions the volumes of the crosspeaks of the exchanging Hα protons were largest. The first-order magnetization rate constants k^*_{in} and k^*_{out} were calculated from the volumes of the cross and diagonal peaks of the Hα signals by using the program EXSYCalc.[36]

The spectrum of **2a** in D_2O/CD_3OD (1:1) in the presence of 0.5 equivalents of Na_2SO_4 recorded with the optimum mixing time τ_m of 0.06 s thus furnished a first-order rate constant for the complexation of sulfate ions by **2a** at 22 °C of $k^*_{in} = 25.4\ s^{-1}$ and a value for the corresponding decomplexation of $k^*_{out} = 3.8\ s^{-1}$. As dissociation of the complex can be considered to be a first-order reaction $k^*_{out} = k_{out}$. Thus, these values confirm that even at 22 °C, the complexation/decomplexation equilibrium of the triply linked bis-cyclopeptide is considerably slower than that of the more flexible analogue **2b** at 10 °C. Moreover, they compare favorably with the rate constants determined for the binding equilibria of other receptors with a similarly confined cavity, for example, the hexameric resorcarene-derived capsule described by Rebek and co-workers.[4b,30]

Conclusion

These investigations demonstrate that click chemistry can be successfully used to construct a molecular cage from two cyclopeptides, which efficiently interacts with sulfate ions in highly competitive aqueous solvent mixtures with stability constants in the range of 10^5–$10^6\ M^{-1}$. Thus, this compound enlarges the family of anion cages by another member; other examples are macrobicyclic polyammonium recaptors,[31a] the macrotricyclic receptors described by Schmidtchen and co-workers,[31b] the coordination cages described by the groups of Custelcean,[9a] Wu,[9b] and Severin,[31c] and a number of anion-templated capsules,[9c,31d] including the self-assembling receptors described by Rebek and co-workers.[31e] The characteristic structural feature of this cage is the polar interior caused by the peptide NH groups that converge inside the cavity. The binding kinetics of the cage differ significantly from those of an analogue containing only one linker between the cyclopeptide rings in that the rate of guest exchange is considerably slower. This finding can be explained by the greater difficulty a substrate experiences when entering or leaving the cavity of a molecular cage, a notion for which Cram et al. introduced the term "constrictive binding".[32] Thermodynamically, the sulfate complex of the triply linked bis-cyclopeptide is only approximately one order of magnitude more stable than the one of the more flexible analogue containing only one linker. Titration calorimetry showed that this behavior is partly due to the change in the binding enthalpy from exothermic to endothermic upon increasing the number of linkers. Thus, interactions between sulfate ions and the triply linked bis-cyclopeptide cannot compensate the energy required for desolvation of the binding partners. As a result, complex stability is entirely due to entropy. Structural investigations indicate that this behavior has most probably structural reasons (the receptor conformations in the complex are energetically unfavorable) possibly in combination with solvation effects (desolvation of the receptor in aqueous solution is enthalpically costly).

Therefore, these investigations show that closing a cage does not necessarily lead to a significant improvement in binding if this causes pronounced changes in the energetics of complex formation. They also demonstrate how important knowledge of the thermodynamics of complex formation is for an understanding of binding behavior. Further investigations will address strategies to alleviate the enthalpic disadvantage of the anion binding of **2a**, for example, by structurally optimizing the linkers. In addition, binding studies will be undertaken to determine whether the mutual arrangement of the two cyclopeptide rings in **2a** allows complexation of anions normally not bound by our bis-cyclopeptides, such as more sizeable anions including di- or triphosphates. Investigations in both directions are currently underway.

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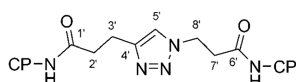
Experimental Section

General: Analyses were carried out as follows: melting points, Müller SPM-X 300; NMR, Bruker Avance 600 and Bruker DPX 400; the chemical shifts were referenced against the shifts of the residual solvent protons: δ([D$_6$]DMSO) = 2.54 ppm, δ([D$_4$]methanol) = 3.34 ppm; MALDI-TOF MS, Bruker Ultraflex TOF/TOF; ESI quadrupole MS, Bruker Esquire 3000; IR, Perkin–Elmer FTIR Spectrometer, Spectrum 1000; elemental analysis, Perkin–Elmer 2400 CHN; ITC, Microcal VP-ITC; HPLC, Dionex P680 HPLC Pump, ASI-100 Autosampler, TCC-100 Column Oven, UVD 170U UV/Vis detector, Chromeleon V6.70 Software, Chromolith Sem*i*Prep 100–10 mm RP-18 endcapped (for **2a**), Purospher STAR RP-18 endcapped (5 µm; for **2b**). The following abbreviations are used: Pro = L-proline, Apro = 2*S*,4*S*-aminoproline, APA = 6-aminopicolinic acid, PA = 4-pentynoic acid, AzP = 3-azidopropanoic acid, PyCloP = chlorotripyrrolidinophosphonium hexafluorophosphate; EDC = *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride. ^{1}H and ^{13}C NMR spectroscopic assignments of protons and carbon atoms in the linker:



Allyl 6-aminopicolinate (4): A mixture of 6-aminopicolinic acid[33] (6.90 g, 50 mmol) and $NaHCO_3$ (8.40 g, 100 mmol) was suspended in DMF p.a. (300 mL). 3-Bromopropene (13.1 mL, 150 mmol) was added to the reaction mixture, which was stirred for seven days at room temperature. The solvent was evaporated in vacuo and the residue was suspended in ethyl acetate. The resulting mixture was washed twice with 10% aqueous Na_2CO_3, washed with water (3×), dried, and evaporated to dryness. The remaining crude product was purified by chromatography (SiO_2, pentane/ethyl acetate, 1:1) and crystallizes as a light yellow solid upon tituration with hexane (4.90 g, 55%). M.p. 72–73 °C; ^{1}H NMR (500 MHz, $CDCl_3$, 22 °C): δ = 4.78 (s, 2H; NH), 4.89 (d, ^3J(H,H) = 5.7 Hz, 2H; All CH$_2$), 5.31 (dd, ^2J(H,H) = 1.3, ^3J(H,H) = 10.4 Hz, 1H; All H$_{cis}$), 5.43 (dd, ^2J(H,H) = 1.3, ^3J(H,H) = 17.2 Hz, 1H; All H$_{trans}$), 6.07 (m, 1H; All H$_{vic}$), 6.69 (dd, ^3J(H,H) = 8.2, ^4J(H,H) = 0.9 Hz, 1H; APA H3), 7.51 (dd, ^3J(H,H) = 7.3, ^4J(H,H) = 0.9 Hz, 1H; APA H5), 7.56 ppm (t, ^3J(H,H) = 7.8 Hz, 1H; APA H4); MS (EI): *m/z* (%): 177.8 (21) [*M*]$^+$; elemental analysis calcd (%): for $C_9H_{10}N_2O_2$: C 60.66, H 5.66, N 15.72; found: C 60.60, H 5.72, N 15.47.

Dipeptide Boc-(Z-4S-Apro)-APA-OAll (5): (2*S*,4*S*)-4-(Benzyloxycarbonylamino)-1-(*tert*-butyloxycarbonyl)proline[14] (**3**; 2.19 g, 6.0 mmol), allyl 6-aminopicolinate (**4**; 1.17 g, 6.6 mmol), and PyCloP (2.78 g, 6.6 mmol) were dissolved in CH_2Cl_2 (100 mL). At room temperature, DIEA (2.50 mL, 14.4 mmol) was added dropwise, and the reaction mixture was stirred for 7 days. The solvent was evaporated in vacuo, and the product was isolated from the residue by chromatography on silica gel (pentane/ethyl acetate, 1:1) (2.55 g; 81%). M.p. 68–75 °C; ^{1}H NMR (600 MHz, [D$_6$]DMSO, 80 °C): δ = 1.37 (s, 9H; *t*Bu CH$_3$), 1.93–2.08 (m, 1H; Apro Hβ), 2.54–2.60 (m, 1H; Apro Hβ), 3.28 (dd, ^2J(H,H) = 10.6, ^3J(H,H) = 6.5 Hz, 1H; Apro Hγ), 3.74 (dd, ^2J(H,H) = 10.6, ^3J(H,H) = 6.8 Hz, 1H; Apro Hδ), 4.10–4.13 (m, 1H; Apro Hδ), 4.53 (dd, ^3J(H,H) = 8.3, ^3J(H,H) = 6.5 Hz, 1H; Apro Hα), 4.86 (d, ^2J(H,H) = 5.6 Hz, 2H; Z CH$_2$), 5.03–5.08 (m, 2H; All CH$_2$), 5.31 (dd, ^2J(H,H) = 1.4, ^3J(H,H) = 10.5 Hz, 1H; All H$_{cis}$), 5.43 (dd, ^2J(H,H) = 1.4, ^3J(H,H) = 17.2 Hz, 1H; All H$_{trans}$), 6.02–6.09 (m, 1H; All H$_{vic}$), 6.95 (s, 1H; Z NH), 7.27–7.37 (m, 5H; Z Ph H), 7.78 (d, ^3J(H,H) = 7.4 Hz, 1H; APA H3), 7.97 (t, ^3J(H,H) = 7.9 Hz, 1H; APA H4), 8.27 (d, ^3J(H,H) = 8.3 Hz, 1H; APA H5), 10.61 ppm (s, 1H; NH); ^{13}C NMR (151 MHz, [D$_6$]DMSO, 22 °C): δ = 27.9 + 28.1 (*t*Bu CH$_3$), 35.1 + 35.9 (Apro Cβ), 48.8 + 49.4 (Apro Cγ), 50.9 + 51.6 (Apro Cδ), 58.4 + 58.7 (Apro Cα), 65.5 + 65.7 (Z CH$_2$, all C1), 79.0 + 79.1 (*t*Bu C), 117.4 + 117.6 (All C3), 118.6 (APA C3), 120.7 (APA C5), 127.9 + 128.4 (Z Ph C2–4), 132.4 (All C2), 136.9 (Z Ph C1), 139.7 + 139.9 (APA C4), 145.8 + 145.9 (APA C2), 151.9 (APA C6), 152.8 + 153.3 (*t*Bu CO), 155.6 (Z CO), 164.0 (APA CO), 171.9 + 172.5 ppm (Apro CO); MS (MALDI-TOF): *m/z* (%): 525.2 (100) [*M*+H]$^+$, 547.2 (69) [*M*+Na]$^+$, 563.2 (15) [*M*+K]$^+$; elemental analysis calcd (%): for $C_{27}H_{32}N_4O_7$: C 61.82, H 6.15, N 10.68; found: C 61.64, H 6.34, N 10.55.

Cyclopeptide cyclo[(Z-4S-Apro)-APA]$_3$ (6a): Prior to the synthesis of this cyclopeptide, dipeptide **5** was chain elongated up to the linear hexapeptide Boc-[(Z -4S-Apro)-APA]$_3$-OAll according to a reported procedure.[15] For cyclization, this hexapeptide (1.26 g, 1 mmol) was deprotected at both ends and dissolved in a mixture of degassed DMF (40 mL) and DIEA (1.04 mL, 6 mmol). The resulting solution was added dropwise over the course of 4 h to a solution of (*O*-benzotriazole-1-yl-*N*,*N*,*N'*,*N'*-tetramethyluronium tetrafluoroborate; TBTU (1.60 g, 5 mmol) and DIEA (0.42 mL, 2.4 mmol) in degassed DMF (200 mL) at 80 °C. If necessary, the pH value of the reaction mixture was adjusted afterward to approximately 9 by adding more DIEA, and the reaction mixture was stirred for a further 1 h at 80 °C. The solvent was evaporated in vacuo, and the product was isolated from the residue by chromatography. An initial purification step was carried out by column chromatography on silica gel (acetone). The material recovered was further purified on an RP-8 column. For this purification, the material was dissolved in a small amount of DMF and applied to a column conditioned with 1,4-dioxane/H_2O (1:10). The eluent composition was gradually changed until the pure product was eluted (1,4-dioxane/H_2O, 2:1). The material thus obtained was dissolved in acetone (20 mL), and the resulting solution was poured slowly in diethyl ether (200 mL) with stirring. Stirring was continued for 15 min, the precipitate was filtered off, and dried in vacuo. In case the material thus obtained is still impure, it can be recrystallized from H_2O/ethanol (0.37 g, 34%). M.p. 168 °C; ^{1}H NMR (600 MHz, [D$_6$]DMSO, 100 °C): δ = 2.06 (m, 3H; Apro Hβ), 2.84 (m, 3H; Apro Hβ), 3.52 (m, 3H; Apro Hδ), 3.96 (m, 3H; Apro Hδ), 4.21 (m, 3H; Apro Hγ), 5.08 (s, 6H; Z CH$_2$), 5.54 (m, 3H; Apro Hα), 6.88 (s, br, 3H; Z NH), 7.27–7.34 (m, 18H; APA H3), Z Ph H), 7.48 (d, ^3J(H,H) = 7.6 Hz, 3H; APA H5), 7.73 (m, 3H; APA H4), 9.13 ppm (s, br, 3H; APA NH); ^{13}C NMR (151 MHz, [D$_6$]DMSO, 22 °C): δ = 37.2 (Apro Cβ), 47.9 (Apro Cγ), 52.0 (Apro Cδ), 60.5 (Apro Cα), 65.5 (Z CH$_2$), 115.9 (APA C3), 119.7 (APA C5), 127.8 + 127.9 + 128.4 + 136.9 (Z PhC), 139.1 (APA C4), 148.5 (APA C2), 151.6 (APA C6), 155.8 (Z CO), 166.2 (APA CO), 170.4 ppm (Apro CO); MS (MALDI-TOF): *m/z* (%): 1099.6 (28) [*M*+H]$^+$, 1121.5 (100) [*M*+Na]$^+$, 1137.5 (76) [*M*+K]$^+$; elemental analysis calcd (%): for $C_{57}H_{54}N_{12}O_{12}\cdot 3H_2O$: C 59.37, H 5.24, N 14.58; found: C 59.74, H 5.13, N 14.38.

Triamine (6b): Cyclopeptide **6a** (0.3 g, 0.27 mmol) was dissolved in methanol/dichloromethane (1:1, 80 mL). After the addition of 10% Pd/C (40 mg), 20% Pd(OH)$_2$/C (40 mg), and 1 M HCl (890 µL, 890 µmol, 3.3 equiv), the reaction mixture was hydrogenated at 1 atm for 7 days. Afterward, the catalysts were removed by filtration over celite, washed with methanol, and the filtrate was evaporated to dryness. The product thus obtained was used in the next step without further purification (0.21 g, 97%). MS (MALDI-TOF): *m/z* (%): 697.3 (87) [*M*+H]$^+$, 719.3 (100) [*M*+Na]$^+$, 735.3 (79) [*M*+K]$^+$.

4-Pentynoic acid pentafluorophenol ester (7): Pentafluorophenol (0.74 g, 4 mmol) and 4-pentynoic acid (0.41 g, 4.2 mmol) were dissolved in dry ethyl acetate (40 mL), and EDC (0.81 g, 4.2 mmol) was added in small portions. After stirring overnight, the reaction mixture was washed with water (3×), 10% aqueous Na_2CO_3 (3×), and water (3×) then dried and evaporated to dryness. The product crystallized upon standing at room temperature (0.99 g, 94%). M.p. 51–55 °C; ^{1}H NMR (400 MHz, $CDCl_3$, 22 °C): δ = 2.06 (t, ^4J(H,H) = 2.6 Hz, 1H; H5), 2.65 (td, ^3J(H,H) = 7.3, ^4J(H,H) = 2.6 Hz, 2H; H3), 2.93 ppm (t, ^3J(H,H) = 7.3 Hz, 2H; H2); ^{13}C NMR (151 MHz, $CDCl_3$, 22 °C): δ = 14.3 (C3), 32.6 (C2), 69.9 (C5), 81.0 (C4), 124.9 (Ph C1), 137.0, 138.7, 140.2, 140.4, 142.0 (Ph C2–4), 167.7 ppm (C1); ^{19}F NMR (565 MHz, $CDCl_3$, 22 °C): δ = −162.2 (m, 2F; Ph F3), −157.7 (t, ^3J(F,F) = 21.7 Hz, 1F; PhF4), −152.5 ppm (m, 2F, PhF2); elemental analysis calcd (%): for $C_{11}H_5F_5O_2$: C 50.02, H 1.91; found: C 49.94, H 2.00.

3-Azidopropanoic acid pentafluorophenol ester (8): Pentafluorophenol (0.46 g, 2.5 mmol) and 3-azidopropanoic acid[34] (0.3 g, 2.6 mmol) were

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dissolved in dry ethyl acetate. The solution was cooled in an ice bath, and EDC (0.5 g, 2.6 mmol) was added in small portions. After the mixture had been stirred for 1 h in the cold, the ice bath was removed and stirring was continued for another 4 h. The reaction mixture was washed with water (3×), 10% aqueous Na_2CO_3 (3×), water (2×), 5% aqueous $KHSO_4$ (2×), and water (3×). After drying, the organic solvent was removed and the product, which was obtained as an oil, was dried in vacuo (0.68 g, 97%). ^{1}H NMR (400 MHz, $CDCl_3$, 22°C): δ=2.94 (t, ^3J(H,H)=6.4 Hz, 2H; H2), 3.72 ppm (t, ^3J(H,H)=6.4 Hz, 2H; H3); ^{13}C NMR (151 MHz, $CDCl_3$, 22°C): δ=33.3 (C2), 46.4 (C3), 124.8 (Ph C1), 137.2, 138.9, 140.3, 140.6, 142.0 (Ph C2–4), 167.2 (C1); ^{19}F NMR (565 MHz, $CDCl_3$, 22°C): δ=−162.0 (m, 2F; Ph F3), −157.4 (t, ^3J(F,F)=21.7 Hz, 1F; Ph F4), −152.5 ppm (m, 2F; Ph F2); elemental analysis calcd (%): for $C_9H_4F_5N_3O_2$: C 38.45, H 1.43, N 14.95; found: C 38.43, H 1.44, N 14.76.

Tris-alkyne 9a: Cyclopeptide **6b**·3HCl (81 mg, 0.1 mmol) and DIEA (104 µL, 0.6 mmol, 6 equiv) were dissolved in DMSO (5 mL). A solution of active ester **7** (240 mg, 0.9 mmol, 9 equiv) in dichloromethane (10 mL) was added and the reaction mixture was stirred at room temperature for 4 h. The solvent was removed in vacuo and the product was isolated from the residue chromatographically (dichloromethane/methanol, 5:1). Fractions with pure product were evaporated to dryness, the residue was dissolved in acetone, and the product was precipitated by addition of diethyl ether. After filtration, the product was dried in vacuo (61 mg, 65%). M.p. 188–194°C; ^{1}H NMR (400 MHz, [D$_6$]DMSO, 22°C): δ=1.90 (m, 3H; Apro Hβ), 2.22 (m, 6H; PA H2), 2.28 (m, 6H; PA H3), 2.71 (t, 1H; ^4J(H,H)=2.4 Hz, PA H5), 2.85 (m, 3H; Apro Hβ), 3.37 (m, 3H; Apro Hδ), 3.89 (m, 3H; Apro Hδ), 4.32 (m, 3H; Apro Hγ), 5.60 (m, 3H; Apro Hα), 7.22 (d, ^3J(H,H)=8.2 Hz, 3H; APA H3), 7.43 (d, ^3J(H,H)=7.3 Hz, 3H; APA H5), 7.74 (m, 3H; APA H4), 8.10 (d, ^3J(H,H)=6.4 Hz, 1H; Apro NH), 9.72 ppm (s, br, 3H; APA NH); ^{13}C NMR (100 MHz, [D$_6$]DMSO, 22°C): δ=13.9 (PA C3), 34.0 (PA C2), 37.2 (Apro Cβ), 46.1 (Apro Cγ), 51.8 (Apro Cδ), 60.4 (Apro Cα), 70.9 (PA C5), 83.4 (PA C4), 115.7 (APA C3), 119.5 (APA C5), 138.8 (APA C4), 148.4 (APA C2), 151.4 (APA C6), 165.9 (APA CO), 170.4+170. ppm (PA CO, Apro CO); MS (MALDI-TOF): m/z (%): 937.4 (54) [M+H]$^+$, 959.4 (100) [M+Na]$^+$, 975.4 (80) [M+K]$^+$; elemental analysis calcd (%): for $C_{48}H_{48}N_{12}O_9{\cdot}4H_2O$: C 57.14, H 5.59, N 16.66; found: C 57.23, H 5.33, N 16.57.

Tris-azide 9b: This peptide was prepared analogously to **9a** from **6b**·3HCl and active ester **8**. For the chromatographic purification, dichloromethane/methanol (7:1) was used as the eluent (36 mg, 36%). M.p. 176–186°C; ^{1}H NMR (600 MHz, [D$_6$]DMSO, 22°C): δ=1.95 (m, 3H; Apro Hβ), 2.31 (m, 6H; AzP H2), 2.86 (m, 3H; Apro Hβ), 3.40 (m, 3H; Apro Hδ), 3.43 (t, 6H; ^3J(H,H)=6.3 Hz, AzP H3), 3.89 (m, 3H; Apro Hδ), 4.32 (m 3H; Apro Hγ), 5.60 (m, 3H; Apro Hα), 7.25 (d, ^3J(H,H)=8.3 Hz, 3H; APA H3), 7.43 (d, ^3J(H,H)=7.6 Hz, 3H; APA H5), 7.74 (m, 3H; APA H4), 8.19 (d, ^3J(H,H)=6.4 Hz, 1H; Apro NH), 9.73 ppm (s, br, 3H; APA NH); ^{13}C NMR (151 MHz, [D$_6$]DMSO, 22°C): δ=34.6 (AzP C2), 37.3 (Apro Cβ), 46.3 (Apro Cγ), 46.8 (AzP C3), 51.9 (Apro Cδ), 60.5 (Apro Cα), 115.7 (APA C3), 119.7 (APA C5), 139.1 (APA C4), 148.6 (APA C2), 151.5 (APA C6), 166.1 (APA CO), 169.8 (AzP CO) 170.6 ppm (Apro CO); IR (KBr): $\tilde{\nu}$=2104 cm^{-1} (azide); MS (MALDI-TOF): m/z (%): 954.4 (5) [M−2N$_2$+Na]$^+$, 982.4 (25) [M−N$_2$+Na]$^+$, 998.4 (6) [M−N$_2$+K]$^+$, 1010.4 (100) [M+Na]$^+$, 1026.4 (21) [M+K]$^+$; elemental analysis calcd (%): for $C_{42}H_{45}N_{21}O_9{\cdot}6H_2O$: C 46.03, H 5.24, N 26.84; found: C 46.33, H 5.01, N 26.50.

Triply linked bis-cyclopeptide 2a: $CuSO_4$ (150 mg, 0.6 mmol, 20 equiv) was dissolved in degassed water under argon and solutions of 2,6-lutidine (193 mg, 1.8 mmol, 60 equiv) in methanol (40 mL), sodium ascorbate (357 mg, 1.8 mmol, 60 equiv) in water (30 mL), and **9b** (30 mg, 30 µmol) in methanol (15 mL) were added under stirring. A solution of **9a** (28 mg, 30 µmol) in methanol (15 mL) was added very slowly to the reaction mixture over 8 h at room temperature by using a syringe pump. After stirring overnight, an aqueous solution of $BaCl_2$ (156 mg, 0.75 mmol, 25 equiv) in H_2O (2 mL) was added, and the precipitate was filtered off through a pad of celite. The solvent was removed in vacuo, the residue was dissolved in a small amount of aqueous NH_3 (2.5%)/1,4-dioxane (20:1), and the solution was filtered through a pad of RP-8 silica, which had been preconditioned with aqueous NH_3 (2.5%). Aqueous NH_3 (2.5%) was rinsed through the silica pad until the filtrate was almost colorless. The product was eluted with 1,4-dioxane/water (1:1), the solvent was removed in vacuo, and the residue was purified by using semipreparative HPLC (16 mg, 28%). M.p. >300°C; ^{1}H NMR (400 MHz, [D$_6$]DMSO, 22°C): δ=1.64 (m, 6H; Apro Hβ), 2.38 (m, 6H; H2′), 2.60–2.74 (m, 9H; 6 H7′, 3 H3′), 2.80–2.98 (m, 9H; 6 Apro Hβ, 3H3′), 3.30 (6H; Apro Hδ), 3.92 (m, 6H; Apro Hδ), 4.30–4.57 (m, 12H; 6 Apro Hγ, 6 H8′), 5.56 (m, 6H; Apro Hα) 7.16 (m, ^3J(H,H)=8.4 Hz, 6H; APA H3), 7.42–7.46 (m, 9H; APA H5, H5′), 7.76 (m, 6H; APA H4), 8.14 (d, ^3J(H,H)=6.8 Hz, 3H; AproNH), 8.27 (d, ^3J(H,H)=6.7 Hz, 3H; Apro NH), 9.61 ppm (s, br, 6H; APA NH); ^{13}C NMR (151 MHz, [D$_6$]DMSO, 22°C): δ=20.9 (C3′), 34.5 (C2′), 35.5 (C7′), 37.5 (Apro Cβ), 45.5 (C8′), 46.0+46.1 (Apro Cγ), 51.4 (Apro Cδ), 60.8 (Apro Cα), 116.1 (APA C3), 119.8 (APA C5), 121.9 (C5′), 139.2 (APA C4), 145.6 (C4′), 148.3 (APA C2), 151.4 (APA C6), 166.0 (APA CO), 169.5 (C6′) 170.5 (Apro CO), 171.5 (C1′); MS (MALDI-TOF): m/z (%): 1926.1 (94) [M+H]$^+$, 1947.9 (100) [M+Na]$^+$, 1963.9 (37) [M+K]$^+$; elemental analysis calcd (%): for $C_{90}H_{93}N_{33}O_{18}{\cdot}13H_2O$: 50.07, H 5.56, N 21.41; found: C 49.89, H 5.37, N 21.43.

Crystal structure analysis of 2a: [$C_{90}H_{93}N_{33}O_{18}$]·21[H_2O], M_r=2303.31 g mol^{-1}, colorless prism, crystal size 0.320×0.322×0.862 mm^3, monoclinic, space group C_2, a=30.664(1), 17.7503(6), c=10.5374(4) Å, β=99.197(1)°, V=5661.7(3) Å^3, T=100 K, Z=2, ρ_{calcd}=1.351 g cm^3, λ=1.54178 Å, μ=0.909 mm^{-1}, Gaussian absorption correction (T_{min}=0.50740, T_{max}=0.82840), scaling SADABS, Bruker AXS Proteum X_8 diffractometer, 2.89<θ<66.66, 64165 measured reflections, 9649 independent reflections, 9570 reflections with $I>2\sigma(I)$. Structure solved by charge flipping using Superflip,[35a] and the electron-density map was analyzed with EDMA.[35b] Structure refined by full-matrix least-squares using SHELXL[35c] against F^2 to R_1=0.0256 ($I>2\sigma(I)$), wR_2=0.0682, 783 parameters. The molecular symmetry of the cage is not consistent with the crystal symmetry. Because each molecule sits on a crystallographic two-fold axis passing through C5 of one of the triazole linkers, the N1 and C4 positions for this triazole ring must be disordered. If one triazole ring is disordered, all three triazole rings must be disorderd because the capsule was prepared by the covalent linkage of a tris-alkyne-substituted and a tris-azide-substituted cyclic hexapeptide with the result that the N1 positions of the three triazole rings are similarly orientated. The disorder of the triazole rings was modeled by occupying each N1 and C4 by C and N atoms, each with half-occupancy, and by giving C and N atoms the same atomic displacement parameters. The solute water O atom O20 is disordered over two positions (O20A and O20B). The relative occupancies were refined and converged with an occupancy of 0.820(4) for O20A. All the hydrogen atoms on the solute water molecules were located on a difference Fourier synthesis map calculated by using the cage with riding H atoms and the O atoms of the water molecules. The solute H atoms were first refined isotropically to convergence. They gave an average O−H distance of 0.89(8) Å and an average atomic displacement parameter of 0.06(3) Å^2. To decrease the number of parameters without adversely affecting the model, the atomic displacement parameters of the H atoms on the water molecules were constrained to be 120% of the atomic displacement parameters of the O atoms to which they were attached and the O−H distances were restrained to be 0.84 Å with a standard uncertainty of 0.02. There is a void of 35 Å^3 in a hydrophobic region of unit cell but no electron density was visible in the final difference Fourier synthesis. Absolute structure parameter=0.0(1), S=1.016, residual electron density=+0.20/−0.33 e Å^{-3}. CCDC-763646 (**2a**) contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif. Smaller crystals grown from water/acetonitrile in the absence of Na_2SO_4 gave similar results (CCDC-763645; see the Supporting Information).

Cyclopeptide (10a): This cyclopeptide was prepared from the linear hexapeptide precursor Boc-(Pro-APA)$_2$-(Z-4S-Apro)-APA-OAll analogously to cyclopeptide **6a**. It was eluted from the RP column with 1,4-dioxane/H_2O (1:1) (0.42 g, 53%). M.p. 184–190°C; ^{1}H NMR (600 MHz, [D$_6$]DMSO, 22°C): δ=1.76–1.88 (m, 4H; Pro Hγ), 1.88–1.96 (m, 1H; Pro Hβ), 2.00–2.07 (m, 2H; Pro Hβ, Apro Hβ), 2.52–2.60 (m, 2H; Pro Hβ),

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2.83–2.92 (m, 1H; Apro Hβ) 3.39–3.41 (m, 1H; Apro Hδ), 3.53–3.61 (m, 2H; Pro Hδ), 3.65–3.72 (m, 2H; Pro Hδ), 3.86–3.93 (m, 1H; Apro Hδ), 4.98–5.03 (m, 2H; Z CH_2), 5.47–5.54 (m, 2H; Pro Hα), 5.60–5.62 (m, 1H; Apro Hα), 7.16 (d, ^3J(H,H)=8.1 Hz, 1H; APA H3), 7.21 (d, ^3J(H,H)=8.1 Hz, 2H; APA H3), 7.27–7.35 (m, 5H; Z PhH), 7.39–7.46 (m, 3H; APA H5), 7.52–7.57 (m, 1H; Z NH), 7.69–7.77 (m, 3H; APA H4), 9.54+9.57+9.66 ppm (3×s, 3×1H; NH); ^{13}C NMR (151 MHz, [D$_6$]DMSO, 22°C): δ=22.8+22.9 (Pro Cγ), 32.9+33.0 (Pro Cβ), 37.6 (Apro Cβ), 48.3 (Apro Cγ), 48.5+48.6 (Pro Cδ), 52.5 (Apro Cδ), 61.0 (Apro Cα), 61.9 (Pro Cα), 65.9 (Z CH_2), 115.9+116.3+116.6 (APA C3), 120.1+120.2 (APA C5), 128.2 (Z C2), 128.3 (Z C4), 128.8 (Z C3), 137.4 (Z C1), 139.4+139.5 (APA C4), 148.9+149.0 (APA C2), 152.0+152.3+152.5 (APA C6), 156.2 (Z CO), 166.3+166.5 (APA CO), 170.9 (Apro CO), 171.4+171.5 ppm (Pro CO); MS (MALDI-TOF): *m/z* (%): 801.4 (98) [*M*+H]$^+$, 823.4 (100) [*M*+Na]$^+$, 839.4 (93) [*M*+K]$^+$; elemental analysis calcd (%): for $C_{41}H_{40}N_{10}O_8{\cdot}2.5H_2O$: C 58.22, H 5.36, N 16.56; found: C 58.09, H 5.32, N 16.45.

Monoamine 10b: Removal of the Z-protecting group in cyclopeptide **10a** was achieved analogously to the deprotection of **6a**, except that 20% $Pd(OH)_2$/C was not added to the reaction mixture and reaction time was decreased to 24 h (0.18 g, 94%). MS (MALDI-TOF): *m/z* (%): 667.3 (57) [*M*+H]$^+$, 689.3 (100) [*M*+Na]$^+$, 705.3 (22) [*M*+K]$^+$.

Monoalkyne 11a: This peptide was prepared analogously to **9a** from **10b**·HCl and active ester **7** by starting from **10b**·HCl (0.2 mmol), except that the cyclopeptide was dissolved in dichloromethane because of better solubility. For the chromatographic purification dichloromethane/methanol (7:1) was used as eluent (84 mg, 56%). M.p. 196–208°C; ^{1}H NMR (600 MHz, [D$_6$]DMSO, 22°C): δ=1.81 (m, 2H; Pro Hγ), 1.86 (m, 2H; Pro Hγ), 1.91 (m, 1H; Apro Hβ), 2.03 (m, 2H; Pro Hβ), 2.21 (m, 2H; PA H2), 2.28 (m, 2H; PA H3), 2.56 (m, 2H; Pro Hβ), 2.69 (t, ^4J(H,H)=2.5 Hz, 1H; PA H5), 2.88 (m, 1H; Apro Hβ), 3.37 (1H; Apro Hδ), 3.58 (m, 2H; Pro Hδ), 3.69 (m, 2H; Pro Hδ), 3.90 (m, 1H; Apro Hδ), 4.32 (m, 1H; Apro Hγ), 5.55 (m, 1H; Apro Hα), 5.59 (m, 2H; Pro Hα), 7.22 (2×d, ^3J(H,H)=8.2 Hz, 2×1H; APA H3), 7.27 (d, ^3J(H,H)=8.2 Hz, 1H; APA H3), 7.41 (2×d, 2×1H; ^3J(H,H)=7.6 Hz, APA H5), 7.44 (d, ^3J(H,H)=7.6 Hz, 1H; APA H5), 7.70–7.76 (m, 3H; APA H4), 8.12 (d, ^3J(H,H)=6.4 Hz, 1H; Apro NH), 9.69+9.71+9.77 ppm (3×s, 3×1H; APA NH); ^{13}C NMR (151 MHz, [D$_6$]DMSO, 22°C): δ=14.1 (AP C3), 22.4 (Pro Cγ), 32.6 (Pro Cβ), 34.2 (AP C2), 37.4 (Apro Cβ), 46.3 (Apro Cγ), 48.2 (Pro Cδ), 51.9 (Apro Cδ), 60.6 (Apro Cα), 61.5 (Pro Cα), 71.3 (AP C5), 83.6 (AP C4), 115.6+115.7+115.9 (APA C3), 119.7 (APA C5), 139+139.1+139.2 (APA C4), 148.6+148.7 (APA C2), 151.5+152.0+152.1 (APA C6), 166.0+166.1 (APA CO), 170.6+170.7 (Apro CO, AP CO), 171.0+171.1 ppm (Pro CO); MS (MALDI-TOF): *m/z* (%): 747.3 (100) [*M*+H]$^+$, 769.3 (54) [*M*+Na]$^+$, 785.3 (21) [*M*+K]$^+$; elemental analysis calcd (%): for $C_{38}H_{38}N_{10}O_7{\cdot}4H_2O$: C 55.74, H 5.66, N 17.12; found: C 55.59, H 5.79, N 16.67.

Monoazide 11b: This peptide was prepared analogously to **9a** from **10b**·HCl and active ester **8** by starting from **10b**·HCl (0.2 mmol). For the chromatographic purification, dichloromethane/methanol (5:1) was used as the eluent (133 mg, 87%). M.p. 210°C (decomp); ^{1}H NMR (600 MHz, [D$_6$]DMSO, 22°C): δ=1.81 (m, 2H; Pro Hγ), 1.86 (m, 2H; Pro Hγ), 1.95 (m, 1H; Apro Hβ), 2.02 (m, 2H; Pro Hβ), 2.31 (m, 2H; AzP H2), 2.56 (m, 2H; Pro Hβ), 2.88 (m, 1H; Apro Hβ), 3.39 (m, 1H; Apro Hδ), 3.43 (t, ^3J(H,H)=6.3 Hz, 2H; AzP H3), 3.58 (m, 2H; Pro Hδ), 3.69 (m, 2H; Pro Hδ), 3.90 (m, 1H; Apro Hδ), 4.33 (m, 1H; Apro Hγ), 5.58 (m, 1H; Apro Hα), 5.63 (m, 2H; Pro Hα), 7.23 (m, 2H; APA H3), 7.27 (d, ^3J(H,H)=8.2 Hz, 1H; APA H3), 7.41 (d, ^3J(H,H)=7.6 Hz, 1H; APA H5), 7.42 (d, ^3J(H,H)=7.6 Hz, 1H; APA H5), 7.44 (d, ^3J(H,H)=7.6 Hz, 1H; APA H5), 7.71–7.76 (m, 3H; APA H4), 8.20 (d, ^3J(H,H)=6.4 Hz, 1H; Apro NH), 9.74 (s, br, 2H; APA NH), 9.82 ppm (s, br, 1H; APA NH); ^{13}C NMR (151 MHz, [D$_6$]DMSO, 22°C): δ=22.4 (Pro Cγ), 32.6 (Pro Cβ), 34.6 (AzP C2), 37.4 (Apro Cβ), 46.3 (Apro Cγ), 46.8 (AzP C3), 48.2 (Pro Cδ), 51.9 (Apro Cδ), 60.5 (Apro Cα), 61.5 (Pro Cα), 115.5+115.7+115.9 (APA C3), 119.7 (APA C5), 139.1+139.2 (APA C4), 148.6+148.7 (APA C2), 151.6+152.0+152.1 (APA C6), 166.0+166.1 (APA CO), 169.9 (AzP CO), 170.6 (Apro CO), 171.1 ppm (Pro CO); IR (KBr): $\tilde{\nu}$=2102 cm^{-1} (m) (azide); MS (MALDI-TOF): *m/z* (%): 738.6 (100) [*M*−N_2+H_2+H]$^+$, 760.5 (22) [*M*−N_2+H_2+Na]$^+$, 764.6 (41) [*M*+H]$^+$, 776.5 (4) [*M*−N_2+H_2+K]$^+$, 786.5 (26) [*M*+Na]$^+$, 802.5 (17) [*M*+K]$^+$; elemental analysis calcd (%): for $C_{36}H_{37}N_{13}O_7{\cdot}5H_2O$: C 50.64, H 5.55, N 21.33; found: C 50.53, H 5.33, N 21.00.

Monolinked bis-cyclopeptide 2b: $CuSO_4$ (35 mg, 0.14 mmol, 2 equiv) was dissolved in degassed water under argon. Solutions of 2,6-lutidine (45 mg, 0.42 mmol, 6 equiv) in methanol (7 mL), sodium ascorbate (83 mg, 0.42 mmol, 6 equiv) in water (7 mL), **9a** (52 mg, 70 μmol) in methanol (7 mL), and **9b** (53 mg, 70 μmol) in methanol (7 mL) were added, and the resulting mixture was stirred overnight at room temperature. The solvent was removed in vacuo and the product was isolated from the residue chromatographically on an RP-8 column. The residue was dissolved in a small amount of DMF and applied to a column conditioned with 1,4-dioxane/H_2O (1:10). The eluent composition was gradually changed until the pure product was eluted (1,4-dioxane/H_2O, 1:3). In case the material thus obtained was still impure, it was additionally purified by semipreparative HPLC (42 mg, 40%). M.p. 262–284°C; ^{1}H NMR (600 MHz, [D$_6$]DMSO, 22°C): δ=1.76–1.93 (m, 9H; 8 Pro Hγ, 2 Apro Hβ), 2.03 (m, 4H; Pro Hβ), 2.35 (m, 2H; H2′), 2.56 (m, 4H; Pro Hβ), 2.64 (m, 2H; H7′), 2.73 (m, 2H; H3′), 2.85 (m, 2H; Apro Hβ), 3.34 (2H; Apro Hδ), 3.58 (m, 4H; Pro Hδ), 3.69 (m, 4H; Pro Hδ), 3.89 (m, 2H; Apro Hδ), 4.31 (m, 2H; Apro Hγ), 4.43 (m, 2H; H8′), 5.52–5.67 (m, 6H; Apro Hα, Pro Hα), 7.13–7.26 (m, 6H; APA H3), 7.43 (m, 6H; APA H5), 7.66 (s, 1H; H5′), 7.72 (m, 6H; APA H4), 8.05 (d, ^3J(H,H)=6.0 Hz, 1H; Apro NH), 8.18 (d, ^3J(H,H)=6.1 Hz, 1H; Apro NH), 9.63+9.72 ppm (2×s, br, 6H; APA NH); ^{13}C NMR (151 MHz, [D$_6$]DMSO, 22°C): δ=21.1 (C3′), 22.4 (Pro Cγ), 32.5 (Pro Cβ), 34.8 (C2′), 35.5 (C7′), 37.1+37.2 (Apro Cβ), 45.5 (C8′), 46.2 (Apro Cγ), 48.1 (Pro Cδ), 51.9+52.0 (Apro Cδ), 60.6+61.5 (Apro Cα), Pro Cα), 115.8+116.0+116.1 (APA C3), 119.7 (APA C5), 122.0 (C5′), 139.0+139.1 (APA C4), 145.7 (C4′), 148.5+148.6 (APA C2), 151.4+151.5+151.9+152.0 (APA C6), 165.9+166.0+166.1 (APA CO), 169.4 (C6′) 170.6 (Apro CO), 171.0+171.1 (Pro CO), 171.5 ppm (C1′); MS (MALDI-TOF): *m/z* (%): 1510.6 (35) [*M*+H]$^+$, 1532.6 (100) [*M*+Na]$^+$, 1548.6 (46) [*M*+K]$^+$; elemental analysis calcd (%): for $C_{74}H_{75}N_{23}O_{14}{\cdot}8H_2O$: C 53.72, H 5.54, N 19.47; found: C 53.80, H 5.52, N 19.20.

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