JOURNAL OF PHYSICAL ORGANIC CHEMISTRY, VOL. 5, 649–655 (1992)

ALLOSTERIC EFFECTS IN A SYNTHETIC RECEPTOR FOR ALKALI METAL IONS AND DINITROBENZENE

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A novel host is described which contains three potential binding sites, viz. a cleft and two crown ether rings. The molecule can adopt three different conformations. One of these is able to form a complex with two alkali metal ions. The binding of potassium ions is shown to be a cooperative process. The association constant of the second ion is 128 times larger than that of the first. The compound also binds 1,3-dinitrobenzene at the third site by $\pi-\pi$ interactions. The complex with two potassium ions binds 1,3-dinitrobezene more strongly than does the free compound by a factor of 2–6, depending on the solvent used. This allosteric effect is caused by the conformational change which is induced by the binding of the potassium ions.

INTRODUCTION

Many biological processes and functions in nature are regulated by molecular systems which respond to changes in the environment or to outside stimulants. Very often these systems contain receptors or enzymes whose binding properties are controlled by interaction with light, small molecules or ions. Mimicking such systems is one of the most challenging goals in host–guest chemistry. Moreover, understanding the underlying principles of these interactions will be an important step towards the construction of novel molecular devices.

A number of examples of 'responsive hosts'[1] have been described. Crown ethers that change the binding strength of ions on irradiation with light are probably the best studied examples within this class of molecules.[2] These crown ethers contain a photoreactive functionality, e.g. a diaza group, which induces a change in conformation of the host by interaction with light and hence a change in affinity of the binding site. Beer[1] and others have prepared redox-responsive hosts which contain an electrochemically switchable functionality, such as a quinone or a ferrocenyl group. Other examples are pH-responsive[3] hosts and temperature-responsive[4] hosts. Some of these molecules have been used to realize ion transport across liquid membranes, induced by, e.g., light[2] or a pH gradient.[3a,5]

A very important way of regulating binding in receptors and proteins in nature is the allosteric effect.[6] In an allosteric protein more than one binding site is present. Binding of a guest (substrate in biochemical terminology) at one site influences the binding strength at a remote site. The paradigmatic example of an allosteric protein is haemoglobin, a tetrameric protein, that can bind four molecules of oxygen. Binding of the fourth molecule of oxygen is stronger than binding of the first three.[7] If the binding sites in an allosteric protein are identical, the allosteric effect is described as cooperative binding between homologous sites. Depending on whether the binding of successive guests is increasingly stronger or weaker, it is called positive or negative cooperativity.

Few synthetic receptors displaying allosteric effects have been described. One of the first successful ones in this respect was reported by Rebek, *et al.*,[8] who synthesized bis-crown ethers capable of binding two alkali metal ions or two mercury complexes such as $Hg(CN)_2$. For the latter guest the second binding constant was shown to be about ten times larger than the first, whereas for the alkali metal ions mutual electrostatic repulsion prevented cooperative binding of the second guest.

Only recently have organic chemists succeeded in developing synthetic receptors that show allosteric effects or cooperative binding for organic guests. A tetracarboxylic acid has been reported by Ebmeyer and Rebek[9] which binds two diamines in a cooperative fashion. Schneider and Ruf[10] developed a water-soluble host which binds aromatic guests 10–100 times more strongly when copper ions are present.

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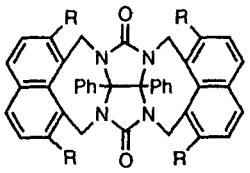
0894–3230/92/100649–07$08.50

Received 4 February 1992



1 aR = OH
1b R = OMe

Elsewhere we have reported on the conformational properties of host compounds of type **1**.[11] These molecules are receptors for aromatic guests. In solution, compound **1** exists in three conformations, *ss*, *sa* and *aa* (Figure 1). The most predominant conformations, *ss* and *sa*, are non-binding. When an aromatic guest is added the equilibrium shifts to *aa*, and the guest is bound in a sandwich-like fashion between the naphthalene walls of the host. As an extension of this work, we describe here a bis-aza-crown ether derivative of **1** which displays allosteric binding properties (compound **2**).

Space-filling models indicate that only the *aa* conformer of **2** is able to bind an alkali metal ion. We expected that addition of alkali metal ions would promote binding of an aromatic guest, as depicted schematically in Figure 2. We also expected that the binding of the alkali metal ions would be a cooperative process, because conversion of the receptor to the *aa* conformation by the uptake of one ion would facilitate the binding of the second ion. Experiments will be presented that confirm these ideas.

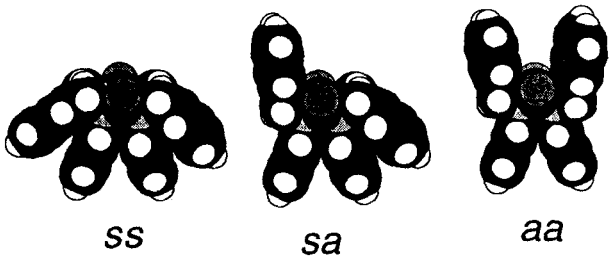


Figure 1. Computer-generated structures of the three conformers of **1**

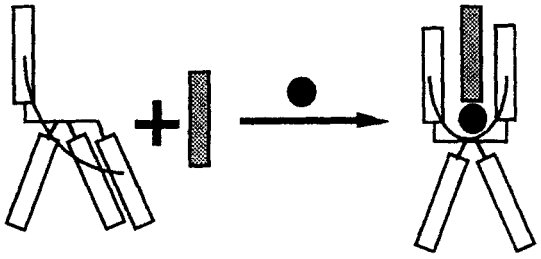


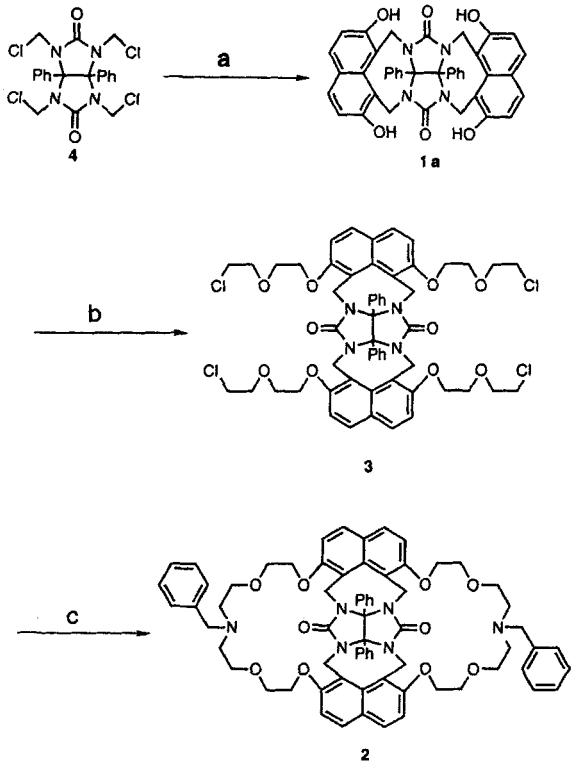
Figure 2. Induced binding of a guest in **2** by the addition of a metal ion

RESULTS AND DISCUSSION

Synthesis and characterization

The bis-aza-crown ether derivative **2** was synthesized via the route in Scheme 1. Starting compound **4** was refluxed in 1,2-dichloroethane with 2,7-dihydroxynaphthalene using $SnCl_4$ as a catalyst to give **1a** (64% yield). Compound **1a** was alkylated with the tosyl ester of chloroethoxyethanol to give **3** in 41% yield. Intermediate **3** was converted into **2** by reaction with 2·2 equiv. of benzylamine in acetonitrile in the presence of sodium iodide using sodium carbonate as a base. After column chromatography, pure **2** was isolated in 91% yield.

In $CDCl_3$ solution, both **2** and **3** are present as a mixture of *sa*, *ss* and *aa* conformers. For **3**, the ratio of these conformers is 82 : 12 : 6. In the solution of **2** the ratio of *sa* and *ss* conformers is 88 : 12. The *aa* con-



Scheme 1. (a) 2,7-Dihydroxynaphthalene, $SnCl_4$, $C_2H_4Cl_2$, reflux; (b) $TosO(CH_2)_2O(CH_2)_2Cl$, DMSO, KOH, 20 °C; (c) $PhCH_2NH_2$, Na_2CO_3, NaI, acetonitrile, reflux

Table 1. ^{1}H NMR signals (ppm) and coupling constants (Hz, in parentheses) in the ^{1}H NMR spectrum of the *ss*, *sa* and *aa* conformers of **2** and **3** in $CDCl_3$

Compound	NCH_2Ar *syn*	NCH_2Ar *anti*	Napht-*H* *syn*	Napht-*H* *anti*	Crown CH_2N	Benzyl CH_2N	Napht-OCH_2	Benzyl-*H*
2-*sa*	4·79	4·10	7·23	7·75	3·00	4·00	4·60	7·43
	6·12	6·08	6·80	7·23				7·26
	(14·8)	(16·8)	(8·9)	(8·9)				7·20
								(7·2)
2-*ss*	5·18		6·83		3·14	[a]	4·35[b]	[a]
	6·33		7·26					
	(14·8)		(8·9)					
3-*sa*	4·79	4·11	7·23	7·73				
	6·03	6·01	6·86	7·27				
	(14·8)	(16·8)	(8·9)	(8·9)				
3-*ss*	5·13		7·25					
	6·20		6·87					
	(14·8)							
3-*aa*		3·92		7·34				
		5·78		6·95				
		(16·8)		(8·9)				

[a] No assignment could be made.
[b] Tentative assignment.

former is not detectable in the ^{1}H NMR spectrum and hence constitutes less than 2% of the population. Because of the presence of the conformers, the spectra of **2** and **3** are complicated, but most peaks could be assigned by comparison with the spectra of **1b** and with the aid of 2D NOESY and COSY spectra. Apart from cross-peaks due to the nuclear Overhauser effect, the NOESY spectra of **2** and **3** also contained cross-peaks due to conformational exchange processes between the conformers. The positions of the most important peaks are given in Table 1.

Complexation

Alkali metal ions

The effect of alkali metal ions on the conformational equilibrium of **2** was investigated by ^{1}H NMR in $CDCl_3$–DMSO-d_6 (9:1,v/v) using 2 mM solutions of host and 8 mM potassium thiocyanate or sodium thiocyanate. Under these circumstances virtually all molecules of **2** were found to be in the *aa* conformation (Figure 3). The spectrum of the sodium thiocyanate complex is different from that of the potassium thiocyanate complex, as can be seen in Figure 3 and Table 2. In the complex with sodium ions the signals of the naphthyl groups and those of the benzyl groups have moved upfield compared with their positions in the complex with potassium. The signal of the CH_2N-protons in the crown ether moieties of **2** is a triplet in the potassium complex, but is split into two multiples in the sodium complex. These differences suggest that in the latter complex, the crown ether moieties are folded

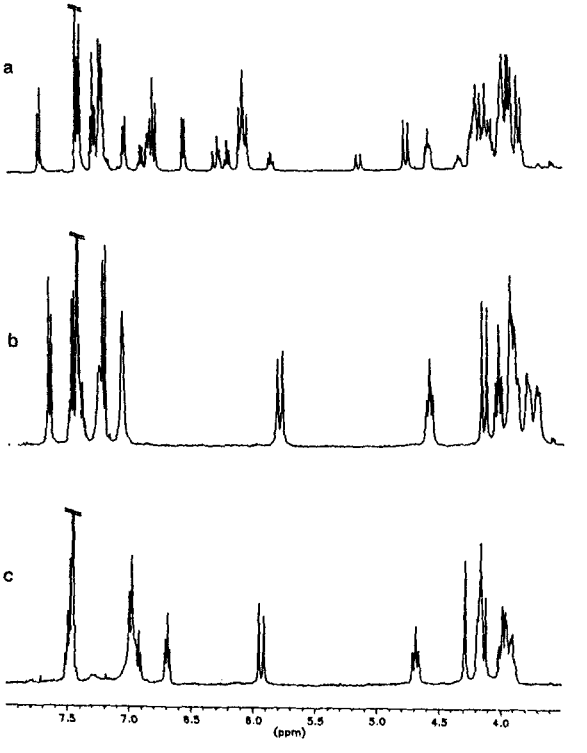


Figure 3. ^{1}H NMR spectra of 2 mM solutions of **2** in $CDCl_3$–DMSO-d_6 (9:1, v/v) (a) in the absence of salt, (b) in the presence of 8 mM KSCN and (c) in the presence of 8 mM NaSCN

Table 2. Relevant signals (ppm) and coupling constants (Hz, in parentheses) in the ^{1}H NMR spectra of the potassium and sodium thiocyanate complexes of **2** in $CDCl_3$–DMSO-d_6 (9 : 1, v/v)

Complex	NCH_2Ar	Napht-*H*	Crown-CH_2N	Benzyl-CH_2N	Napht-OCH_2	Benzyl-*H*
$2 \cdot 2K^+$	5·80; 4·14 (16·5)	7·66; 7·22 (8·9)	2·92 (t)	3·92	4·59	7·26 (br) 7·08 (br)
$2 \cdot 2Na^+$	5·93; 4·14 (16·8)	7·45; 6·97 (8·9)	2·93 (m) 2·98 (m)	4·29	4·68	7·0–7·3 (br) 6·92 (t) 6·68 (t)

around the sodium ions in such a way that the benzyl groups are in the shielding zone of the naphthyl groups, or perhaps are complexed in the cleft of the host.

As host **2** has two binding sites for alkali metal ions, it was expected that these ions would be bound in a 1 : 2 host–guest stoichiometry. In order to ascertain this and to find out if **2** showed cooperative binding towards these ions, a ^{1}H NMR titration with potassium picrate in $CDCl_3$/DMSO-d_6 (3 : 1, v/v) was performed. During this titration the fraction of molecules in the *aa* conformation was monitored as a function of the number of equivalents of salt added (Figure 4).

The titration data were evaluated with the help of a computer program that determines the goodness of fit[12] (expressed as χ^2/ν) of a theoretical binding curve to the experimental data for any combination of K_1 and K_2. The latter constants K_1 and K_2 are defined as

$$K_1 = \frac{[\mathbf{2} \cdot M^+]}{[M^+]\,[\mathbf{2}]} \qquad K_2 = \frac{[\mathbf{2} \cdot 2M^+]}{[\mathbf{2} \cdot M^+]\,[M^+]}$$

In order to obtain intrinsic binding constants from these thermodynamic constants, a statistical correction must be applied which takes into account the number of binding sites in the receptor.[13] For a receptor with two binding sites, K_1 must be divided by 2 and K_2 must be multiplied by 2: $K_1{}^i = 1/2K_1$; $K_2{}^i = 2K_2$.

In the calculations the assumption was made that only the *aa* conformer of **2** binds ions. The fact that **2** is completely converted into the *aa* conformer by addition of alkali metal salts shows that this assumption is valid.

For a number of values of $K_2{}^i/K_1{}^i$, the value of $K_1{}^i$ that gives the best fit to the experimental data was calculated. The results of the calculations are shown in Figure 4. For independent binding sites the best fit curve [Figure 4(a)] has a curvature which is too strong and at higher saturation the calculated values of the *aa* fraction are too low. A much better fit is obtained when a strongly cooperative binding process is assumed [Figure 4(b)]. The lowest value of χ^2/ν is obtained for $K_2{}^i/K_1{}^i = 128$ ($K_1{}^i = 43$; $K_2{}^i = 5440$).

A number of models have been proposed to explain cooperative binding by allosteric interactions, one of the first being that of Monod, Wyman and Changeux

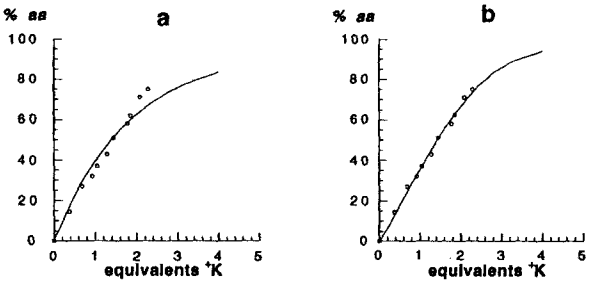
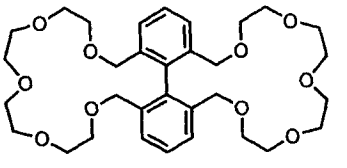


Figure 4. Best fit curves to data points of titration of **2** with potassium picrate. (a) Two independent binding sites, $K_1{}^i = k_2{}^i = 140$; (b) strongly cooperative binding, $K_1{}^i = 43$, $K_2{}^i/K_1{}^i = 128$

(MWC).[14] In this model, the following properties are considered to be required to obtain positive cooperativity: the receptor system should contain identical binding sites at identical, symmetry-related positions; the receptor should have more than one conformation, and these conformations are in equilibrium and differ in their affinity to the substrate; and the symmetry of the receptor should be preserved on going from one conformation to another.

The present system is an excellent example of cooperative binding according to the MWC model. It displays all the properties which are required for the cooperative binding of potassium ions.

It is of interest to compare our receptor **2** with Ebmeyer and Rebek's bis-crown ether receptor[9] (see formula **5**). In **5** cooperative binding is also the result of binding-induced conformational changes. Ebmeyer and Rebek found that the cooperativity is completely based on an entropy effect: binding of one guest in the



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first binding site freezes the conformational freedom of the second binding site, resulting in increased affinity for the second guest. A similar entropy effect may be operative in our receptor **2**, but enthalpy effects probably also play an important role. The ion-binding conformer constitutes less than 2% of the total mixture of conformers in a solution of the free host **2**. The free energy needed to convert **2** into the binding conformer has to be paid for completely in the first binding step. This fact alone could explain a difference in binding strength of a factor of 50 between the two binding sites. Another important difference is that in Ebmeyer and Rebek's receptor the distance between the two binding sites is smaller than in our receptor. As a result the electrostatic repulsion between the two bound ions in **4** is much larger than in **2**. Consequently, **4** does not show cooperativity in the binding of alkali metal ions, but only in the binding of neutral mercury compounds such as $Hg(CN)_2$.

Ternary complexes

The allosteric effect of potassium ions on the binding of 1,3-dinitrobenzene (DNB) in **2** was qualitatively assessed by determining the complexation-induced shifts of the protons of DNB on the addition of **2**, both in the presence and absence of KPF_6. If 27·5 mM **2** was added to a 19·8 mM solution of DNB in $CDCl_3$–DMSO-d_6, (3 : 1, v/v), the DNB proton signals shifted upfield by 0·03, 0·06 and 0·07 ppm for the H-2, H-4,6 and H-5 protons, respectively. In the presence of an excess of KPF_6 these induced shifts increased to 0·06, 0·14 and 0·19 ppm, respectively. In a separate experiment it was ascertained that the signals of DNB do not shift in the presence of KPF_6 alone.

For a quantitative evaluation of the allosteric effect, a series of UV titrations were performed in two solvent mixtures, viz. $CHCl_3$–DMSO (3 : 1, v/v) and $CHCl_3$–DMSO (9 : 1, v/v). In these titrations the increase in absorption of the charge-transfer band around 400 nm was monitored as a function of the concentration of DNB while the concentration of **2** was kept constant. As both DNB and **2** absorb at the wavelength of the charge-transfer (CT) band of the complex, the absorption due to free DNB and **2** had to be determined separately in each experiment. The molar absorption coefficient (ε) of DNB appeared to be strongly dependent on the concentration of salt present. Titrations were performed with both sodium and potassium thiocyanate salts. The results of the titration experiments are given Table 3.

The association constants are considerably lower in $CHCl_3$–DMSO (3 : 1, v/v) than in $CHCl_3$–DMSO (9 : 1, v/v). The values in Table 3 show that K^+ ions exert an allosteric effect on the binding of DNB. The strength of the effect varies from a factor of less than 2 in $CHCl_3$–DMSO (3 : 1, v/v) to a factor of six in $CHCl_3$–DMSO (9 : 1, v/v). Although sodium ions are bound by **2** [see Figure 3(c)], they are completely ineffective in increasing the binding strength of **2** towards DNB. One possible reason may be that in the sodium complex the benzyl groups are occupying the cleft of the host, thus disfavouring the complexation of DNB. Another explanation may be that in this complex the naphthyl walls are at too close a distance to allow sandwiching of a DNB molecule.

Table 3. Effect of alkali metal ions on the association constants of the complexes between **2** and 1,3-dinitrobenzene in $CHCl_3$–DMSO solvent mixtures ($T = 298$ K)

	K_a (l mol^{-1})	
Host	$CHCl_3$–DMSO (9:1, v/v)	$CHCl_3$–DMSO (3:1, v/v)
2	1·2 ± 0·3	0·3 ± 0·15
2·$2K^+$	7·2 ± 1	0·5 ± 0·12
2·$2Na^-$	1·1 ± 0·3	

EXPERIMENTAL

17b,17c-Dihydro-1,6,10,15-tetrahydroxy-17b,17c-diphenyl-7H,8H,9H,16H,17H,18H-7a,8a,16a,17a-tetraazapentaleno[1",6":5,6,7;3",4":5',6',7']dicyclo-octa[1,2,3-de:1',2',3'-d'e']dinaphthalene-8,17-dione (1a). A mixture of 2·48 g (5·08 mmol) of **4**, 3·2 g (20 mmol) of 2,7-dihydroxynaphthalene and 5·5 ml (44 mmol) of $SnCl_4$ was refluxed for 30 min in 100 ml of $C_2H_4Cl_2$. After refluxing with dilute HCl the product was isolated from the reaction mixture by filtration and was washed with methanol. The product was purified by recrystallisation from DMSO. Yield: 2·1 g (64%) of colourless needles. FAB-MS, m/z 663 $[M + H]^+$. Analysis calculated for $C_{40}H_{30}N_4O_6 \cdot 0{\cdot}5H_2O$, C, 71·53, H 4·65, N 8·34; found, C 71·53, H 4·63, N 8·37%

17b,17c-Dihydro-1,6,10,15-tetramethoxy-17b,17c-diphenyl-7H,8H,9H,16H,17H,18H-7a,8a,16a,17a-tetraazapentaleno[1"6":5,6,7;3",4":5'6'7']dicyclo-octa[1,2,3-de:1',2',3'-d'e']dinaphthalene-8,17-dione (1b). Compound **1a** (1·32 g, 1·99 mmol) was dissolved in 25 ml of DMSO. The solution was degassed and placed under a nitrogen atmosphere. Dimethylsulphate (1·4 ml, 8·3 mmol) and powdered KOH (2·5 g) were added and the mixture was stirred at room temperature for 1 h. The reaction mixture was poured into 250 ml of water. The product was extracted from the aqueous suspension with CH_2Cl_2 and purified by column chromatography [chloroform–methanol (99·5 : 0·5. v/v)]. Yield: 1·09 g (76%) of **1b**. ^1H NMR ($CDCl_3$) (only the signals of the *sa* conformer are given here), δ 7·75, 7·23, 7·20 and 6·84 (4 d, 8 H, napht-H, $J = 8{\cdot}7$ Hz), 7·05–6·30 (m, 10 H, ArH), 6·05, 5·97, 4·81 and 4·14

(4d, 8 H, NC*H*HAr, $J = 15{\cdot}8$ Hz), 4·15 and 3·92 (2s, 12 H, OCH_3); FAB-MS, m/z 719 $[M + H]^+$. Analysis calculated for $C_{44}H_{38}N_4O_6{\cdot}CH_2Cl_2$, C 67·25, H 5·02, N 6·97; found, C 66·93, H 4·96, N 6·94%.

17b,17c-Dihydro-1,6,10,15-tetrakis[2-(2-chloroethoxy)ethoxy]-17b,17c-diphenyl-7H,8H,9H,16H,17H,18H-7a,8a,16a,17a-tetraazapentaleno[1",6":5,6,7;3",4":5',6',7']dicycloocta[1,2,3-de:1',2',3'-d'e']dinaphthalene-8,17-dione (3). Compound **1a** (1·04 g, 1·57 mmol) and 1-{[2-(2-chloroethoxy)ethyl]sulphonyl}-4-methylbenzene (2·0 g, 7·18 mmol) were dissolved in 50 ml of DMSO. The solution was degassed and placed under a nitrogen atmosphere. Powdered KOH (1·7 g, 30 mmol) was added and the mixture was stirred at room temperature for 16 h. The reaction mixture was poured into water and the resulting suspension was acidified and extracted with chloroform. After column chromatography [chloroform–methanol (99 : 1, v/v)], 0·7 g (41%) of **3** was obtained. ^{1}H NMR ($CDCl_3$), see Table 1; FAB-MS, m/z 1087 $[M + H]^+$. Analysis: calculated for $C_{56}H_{58}N_4O_{10}Cl_4$, C 61·77, H 5·37, N 5·15; found C, 61·58, H 5·45, N 5·30%.

Bis-crown ether compound **2**. Compound **3** (1·45 g, 1·33 mmol), benzylamine (0·143 g, 1·35 mmol), 28 g of NaI and 12 g of Na_2CO_3 were refluxed in 400 ml of acetonitrile. Over a period of 50 h, another 0·29 g (2·7 mmol) of benzylamine in 50 ml of acetonitrile was added. The solvent was removed *in vacuo* and water was added to the residue. The crude product was extracted with chloroform and purified by column chromatography [chloroform–methanol (93 : 7, v/v)]. Yield: 1·42 g (92%) of **2**. ^{1}H NMR ($CDCl_3$), see Table 1; FAB-MS, m/z 1157 $[M + H]^+$. Analysis calculated for $C_{70}H_{72}N_6O_{10}$, C 72·46, H 6·27, N 7·26; found, C 72·16, H 6·34, N 7·21%

2D ^{1}H NMR experiments. EXSY spectra were recorded at 298 K at 400 MHz with the NOESYPH pulse sequence supplied with the Bruker software. Time-proportional phase incrementation (TPPI)[15] was used to obtain phase-sensitive line shapes. A total of 512 t_1 increments were taken with 16 scans at every t_1 value. The mixing time was 0·8 s. The FIDs were multiplied by a $\pi/2{\cdot}5$ shifted sine-bell function in both the F_2 and F_1 domains. The data file was zero-filled to 1K × 1K real data points.

Titration of 2 with potassium picrate. A 3·71 mM stock solution of **2** in $CDCl_3$–DMSO-d_6 (3 : 1, (v/v) was prepared (stock solution A). From this solution, a solution 3·71 mM in **2** and 7·74 mM in potassium picrate was prepared (stock solution B). For each data point in the titration, a precisely weighed amount of solution B was made up to *ca* 0·8 g with solution A. The last data point in the titration was obtained from solution A which had been saturated with solid potassium picrate. For the determination of the fractions of each conformer, the integral of as many non-overlapping peaks as possible was used.

Computer program for the evaluation of the association constants from the ^{1}H NMR titration data. A computer program was written in FORTRAN, which calculates for a range of values of K_1 and K_2/K_1, the fractions of molecules of **2** that are present as the 1 : 1 and 1 : 2 complex at each data point in the titration. The program subsequently evaluates the goodness of fit (χ^2/ν) for each combination of K_1 and K_2/K_1, with the help of the expression

$$\frac{\chi^2}{\nu} = \sum \frac{X_{aa\ \mathrm{obs}} - X_{aa\ \mathrm{calc}})^2}{\sigma^2(n_{\mathrm{exp}} - 2)}$$

where $X_{aa\ \mathrm{obs}}$ is the experimentally observed fraction of molecules in the *aa* conformation, $X_{aa\ \mathrm{calc}}$ is the calculated fraction of molecules in the *aa* conformation, σ is the standard deviation of the measurement, which was taken to be $\pm 0{\cdot}03$, and n_{exp} is the number of data points. By systematic variation of K_1 and K_2/K_1, the lowest value of χ^2/ν is then found.

In the calculations on the titration of **2** with potassium picrate, it was found that the best fit ($\chi^2/\nu = 0{\cdot}9$) was obtained for $K_2{}^i/K_1{}^i = 128$ ($K_1{}^i = 43$; $K_2{}^i = 5440$). For $K_2{}^i/K_1{}^i \gg 128$, the fit was only slightly worse (e.g. $\chi^2/\nu = 1{\cdot}35$ for $K_2{}^i/K_1{}^i = 8{\cdot}2 \times 10^3$). Consequently, the best value of $K_2{}^i/K_1{}^i$ is 128 with a lower limit on $K_2{}^i/K_1{}^i$ of 16, but no upper limit on this ratio can be given.

UV titrations of 2 with 1,3-dinitrobenzene. For the titrations in $CHCl_3$–DMSO (9 : 1, v/v), stock solutions were prepared which contained *ca* 2 mM of **2** and 8 mM of either sodium thiocyanate or potassium thiocyanate (stock solution A). From these stock solutions, new solutions containing also *ca* 1 M of DNB were prepared (stock solution B). Stock solution A (1·7 ml) was placed in a 1 cm cuvette. For each successive data point a 25 μl aliquot of stock solution B was added. For the titrations in the $CHCl_3$–DMSO (3 : 1, v/v) solvent mixture, stock solution A was 0·1 M in **2** and 0·25 M in potassium thiocyanate. From this solution, stock solution B was prepared, containing 0·45 M of DNB. Stock solution A (165 μl) was titrated with 20 μl aliquots of stock solution B in a 1 mm cuvette. K_a values were evaluated with the help of a computer program which evaluates K_a and ε in an analogous manner to that described for the evaluation of association constants from ^{1}H NMR shift titrations.[16] Excellent fits were obtained assuming an experimental error of 0·0003 absorption units. The molar absorption coefficients of free DNB and **2** were determined separately for each solvent mixture and concentration of salt.

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