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Synthesis and inclusion ability of a bis-β-cyclodextrin pseudo-cryptand towards Busulfan anticancer agent

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Abstract—The synthesis of a C_2-symmetric receptor including two β-cyclodextrins connected by urea linkers to a chiral diaza-crown ether organising platform is reported. This molecular system, long thought to be a potent selective carrier for chiral/achiral organic/inorganic guests at the supramolecular level, was found to be an efficient complexing tool towards the Busulfan anticancer agent.

1. Introduction

Cyclodextrins (CDs), a class of natural cyclic oligosaccharides with six, seven or eight D-glucose units linked by α-1,4-glucose bonds, are known to accommodate various guest molecules into their hydrophobic cavity in aqueous solution.[1] If natural CDs are themselves of great interest as molecular hosts, much of their utility in supramolecular chemistry derives from their structural modification.[1b] On the other hand, unmodified CDs may be considered as molecular scaffolds on which functional groups and/or other substituents of increasing sophistication can be assembled with controlled geometry.[2] Enhancing the binding abilities of CDs first introduced by Breslow's work[3] has been a permanent challenge for three decades and different strategies have been proposed to reach a high level of reaction rates. Increased binding and catalytic power was achieved with capped, dimeric or tetrameric CDs coupled by different linkers. For example, bridged CDs are known to exhibit greatly enhanced binding abilities as a result of cooperative binding of one guest molecule by the two hydrophobic cavities located in close vicinity.[4] Much effort has been devoted to the design and synthesis of bis-CDs with a large panel of structures and the investigation of their inclusion behaviour with model guests.[5] Metal ions have also been introduced as additional recognition sites to enhance the binding selectivity of chromogenic bis-CDs and CD derivatives.[6] Particularly, coupling of a diaza-crown ether with a CD was early investigated by Pikramenou et al.[7] to develop light emitter chemosensors upon recognition of a guest.[8] Some rare examples of enantiopure *C*-substituted-aza-crown ethers have been reported over three decades,[9] but there is so far no contribution in which a CD is covalently associated with a chiral diaza-crown ether in a heterotopic co-receptor. The underlying idea was to use the known high propensity of CDs to form stable inclusion complexes with hydrophobic guests, notably in the case of CD dimers. This feature was expected to give a pseudo-cryptand framework by closing together the CD side arms through the formation of a 1:1 inclusion complex with a suitable organic guest. As illustrated in Figure 1, the novelty of our approach is supported by the symmetry of the chiral aza-crown organising platform and functionalisation of the latter with ureido-β-CD cavities. More precisely, such a podand architecture associates in a close spatial proximity: (i) one metallic complexing site (aza-crown cavity), (ii) two polar pockets (threonyl and tertiary nitrogen moieties) and (iii) two hydrophobic cavities (β-CDs).

Keywords: β-Cyclodextrin; Diaza-crown ether; Pseudo-cryptand; Busulfan; Inclusion; Molecular modelling.
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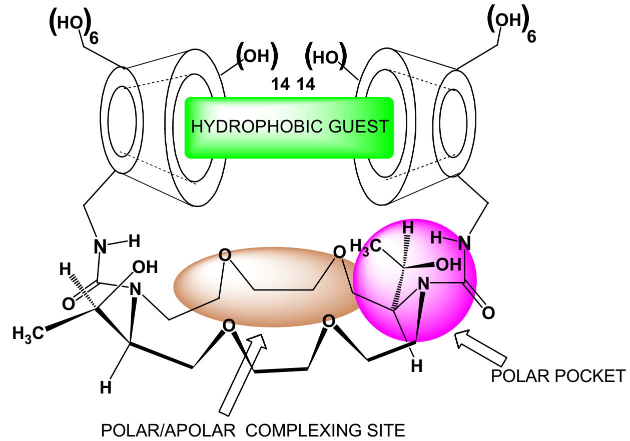


Figure 1. Schematic representation of the hetero tritopic co-receptor concept.

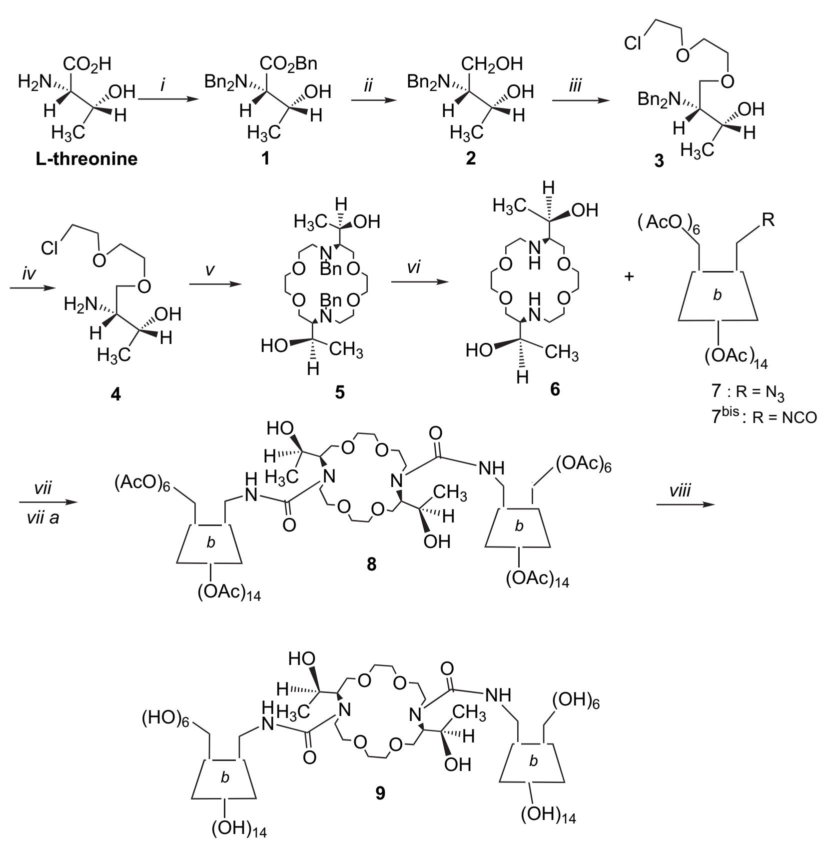
We expected this structure to form *in fine* supramolecular complexes with convenient chiral or achiral organic/inorganic ligands.

2. Results and discussion

2.1. Synthesis

An unprecedented scale-up chiral diaza-crown ether **5** five-step preparation (~4.6 g)[10] starting from L-threonine amino acid followed by a reductive deprotection (H_2, Pearlman's catalysts) and one-pot phosphine imide polymer-supported coupling reaction[11] with 6^A-azido-6^A-deoxy-per-*O*-acetylated-β-CD **7** (method A) afforded the desired bis-ureido-CD chiral co-receptor **8** in a 24% moderate yield.

A higher yield (50%) in **8** was achieved using the direct coupling of the diaza-crown ether **6** with the 6^A-isocyanato-6^A-deoxy-peracetylated-β-CD **7**^{bis} (method B). The target compound **9** was isolated from the peracetylated precursor **8** after a deacetylation step using Zemplén conditions (Scheme 1). All the new compounds were fully characterised by FTIR, ^1H and ^{13}C NMR, 2D NMR, elemental analysis and/or MS. The spectroscopic data are in perfect agreement with the assigned structures. For instance, the FTIR spectrum of **8** shows a medium absorption band at 1675 cm^{-1} characteristic of an urea carbonyl bond, also corroborated by the ^{13}C NMR corresponding signal at 161 ppm (C=O urea) indicating that the CDs have been coupled with the diaza-crown. The MS of **8** displays the partially deacetylated monocharged ion fragment at m/z=3998.19 $[M-9CH_3CO]^+$ and the partially deacetylated discharged ion at m/z=2067.26 $[M-5CH_3CO+5H^+]^{2+}$. The ^{13}C NMR signal of the urea carbonyl bond in the final deacetylated compound **9** was upfield shifted to 133 ppm likely through the formation of hydrogen bonds with the numerous close-space free hydroxyls of the CD. Altogether, these results prove undoubtedly the proposed molecular structure.



Scheme 1. Synthesis of water-soluble host **9**. Reagents and conditions: (i) BnBr, Na_2CO_3, EtOH/H_2O, 80 °C, 5 h, 78%; (ii) $LiAlH_4$, THF/Et_2O, rt, 5 h, 86%; (iii) 2,2′-bis-dichlorodiethylether, NaOH, NBu_4HSO_4, 4 °C, 14 h, 61%; (iv) H_2, HCO_2H, $Pd(OH)_2$, MeOH, rt, 14 h, 99%; (v) NaI, Na_2CO_3, ref. MeCN, 4 d then BnBr, 18 h, 43%; (vi) H_2, $Pd(OH)_2$, MeOH/CH_2Cl_2, rt, 14 h, 98%; (vii) CO_2, PPh_3 on resin, rt, 24 h, method A, THF, 24%; method B, DMF, 50.4%; (viii) MeOH/Na catalytic (95%).

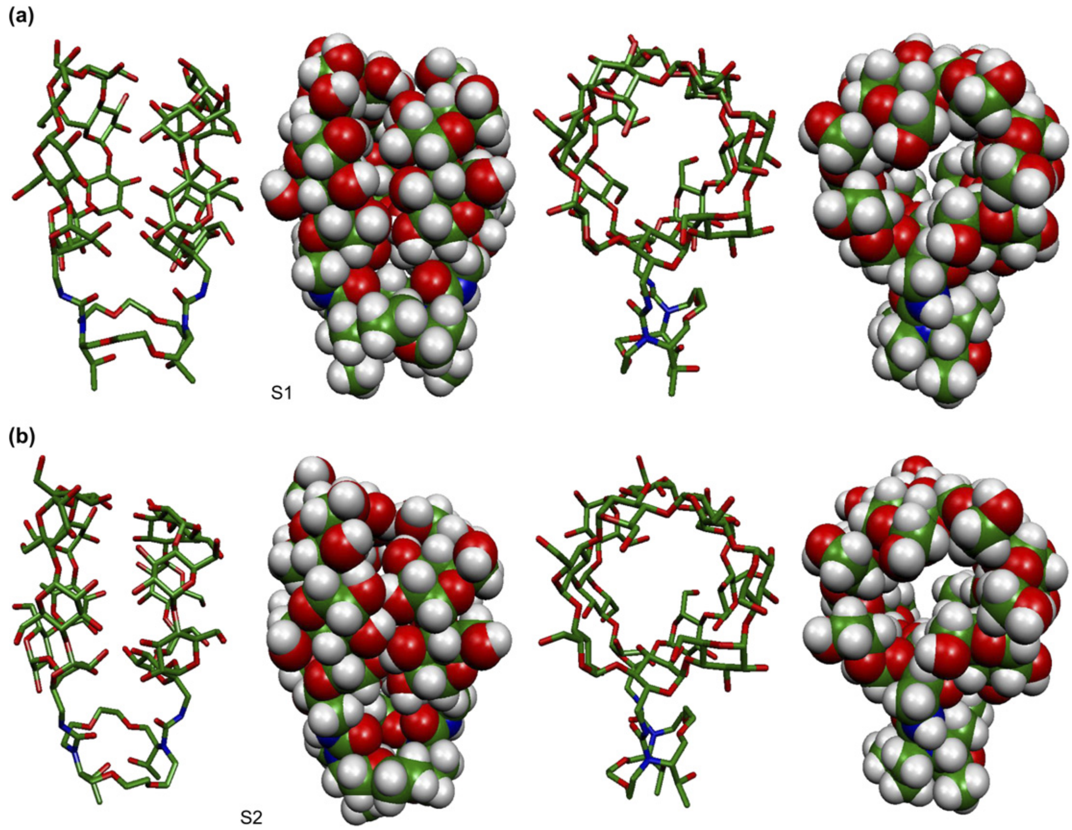


Figure 2. Side and face views of the backbone (without hydrogen atoms) and spacefill (with hydrogen atoms) structures with lowest energy after dynamic simulations: S_1 conformation in vacuum; S_2 conformation in water.

2.2. Conformation of the free bis-CD-aza-crown 9 in water

It is well known that elucidation of the crystal structure is one of the most convincing methods of unequivocally illustrating the geometrical CD derivatives. Unfortunately, isolation of suitable single crystals for X-ray crystallography of this kind of strongly modified CDs is also often a big difficulty too and many attempts to prepare such single crystals from **8** or **9** failed. To elucidate the possible conformations, we performed molecular modelling calculations on the dimer **9**. The converged structures of the bis-CD ligand in both vacuum (S_1) and water medium (S_2) obtained by using dynamic molecular method at the MM3 force field level are depicted in Figure 2.

In both S_1 and S_2 conformations, the two CD wide rims face each other, strongly connected each other through multiple hydrogen bonds involving secondary hydroxyl groups. The closest distances between O-donor and O-acceptor atoms were found equal to 3.05 and 2.88 Å for S_1 and S_2 conformations, respectively. This shows that in water medium, the attraction between the two CDs is more pronounced as could be expected from the known hydrophobicity of the cavities of these fragments.

Figure 3 enlightens how the crown ether can be distorted in the present system.

A higher distortion is found in S_2 conformation, which displays a close atomic distance for both urea and crown

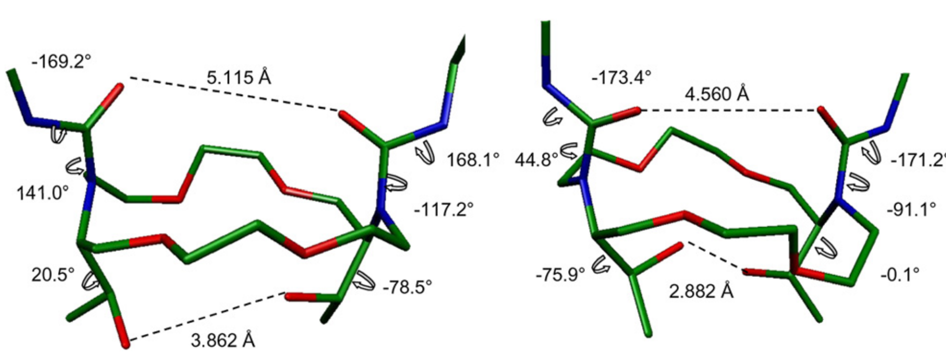


Figure 3. Close view of the crown ether moiety with geometrical features for S_1 and S_2 conformations.

oxomethylene oxygen atoms. These calculations are in good agreement with previous results obtained with the ureido-cyclam tri- and tetra-substituted-β-CD ligand family[12] by molecular-dynamics computations, which have shown a spatial 'bouquet' conformation adopted by these kind of molecules. This essentially arises from strong intramolecular hydrogen bonds between urea functions and also from the spatial distribution of the CDs that face each other with their wide rims strongly connected on the same side of the crown.

2.3. Interaction of 9 with Busulfan as guest

The formulation of molecules with a trend to crystallise is a major problem in pharmacy. Typically, one of the main side effects observed clinically with high dose rate of Busulfan (1,4-butanediol-dimethylsulfonate, a powerful antitumoural agent in leukaemias[13]) is the hepatic veno-occlusive complication due to microcrystallisation in the microvenous system of the liver.[14] In the course of developing a more appropriate formulation based on supramolecular strategy, the possibility of encapsulating the Busulfan molecule into the bis-CD host **9** was investigated. Signs of interaction were first detected by chemical-induced shifts (CIS) of some protons of the guest signals compared to those of the free compound. The signals of both the sulfomethyl and methylene of the butyl chain are slightly shifted downfield (−0.005 ppm) and upfield (+0.014 ppm), respectively as shown in Figure 4.

Stoichiometry of the complex [Busulfan/**9**] could be confirmed by the continuous variation method known as Job plot illustrated in Figure 5.

A value of R=0.5 was reached at the maximum, which strengthens the 1:1 stoichiometry for the complex with an apparent complexation constant (K_a) of ca. 1600 mol^{-1} at 300 K. On the other hand, H_3 and H_5 proton signals located inside the CD cavity of the host remained unchanged. This suggests that the Busulfan is not embedded in CDs hydrophobic cavities but is likely in interaction with the ureas and crown ether part of **9**. This feature is in fair agreement with recent results on the interaction of Busulfan by β-CD[15] and the above results of molecular modelling show that the CD cavities are strongly connected in water by their wide rims so that they probably prohibit a free access to Busulfan. This feature is consistent with some attempts we performed in parallel and that failed to obtain inclusion complexes of **9** in water at 298 K with some hydrophobic guest molecules, for example, bis-nitrophenyl phosphate, which are well known to form currently [1:1] inclusion complexes with bis-CD systems.[16]

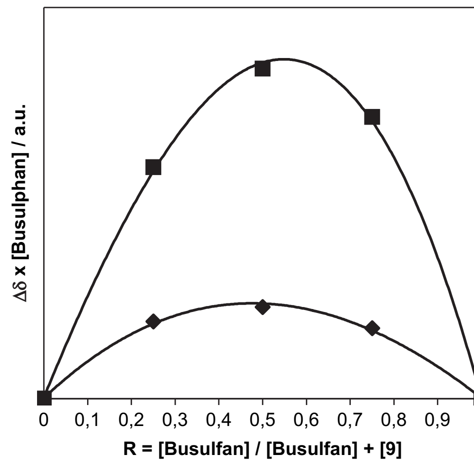


Figure 5. Job plot corresponding to the chemical shift displacement of the sulfomethyl and methylene protons of Busulfan for [Busulfan/**9**] in D_2O at 300 K.

Elsewhere, two-dimensional NMR spectroscopy has recently become a very valuable method for the study of the structures of CD dimers and their complexes in solution[17] since one can conclude on the spatial proximity of two protons if an NOE/ROE cross peak is detected between the relevant proton signals in the 2D NOESY or 2D ROESY spectrum. So it was possible to estimate the local spatial interactions and the orientation of the Busulfan guest molecule inside the bis-CD-aza-crown host using the assigned ROE correlations (Fig. 6).

The 2D ROESY spectrum of the [Busulfan/bis-CD-aza-crown] 1:1 complex in D_2O displays on one hand two cross peaks between the methylene protons of the crown and the methylene protons of the Busulfan-butyl chain and on the other hand, between the methyl protons of the Busulfan and the methylene protons of the CD (C-6 connected to the urea N–H atom). These results corroborate the above-observed chemical-induced shifts and indicate that the Busulfan is

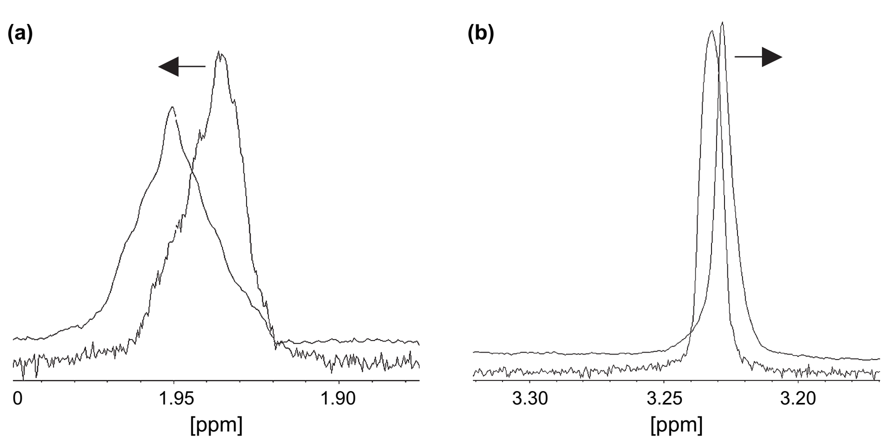


Figure 4. ^{1}H NMR spectrum of [Busulfan/aza-crown-bis-β-CD] [1:1] complex at 400 MHz; (a) view of β-methylene protons of the Busulfan-butyl chain; (b) view of sulfomethyl protons of Busulfan.

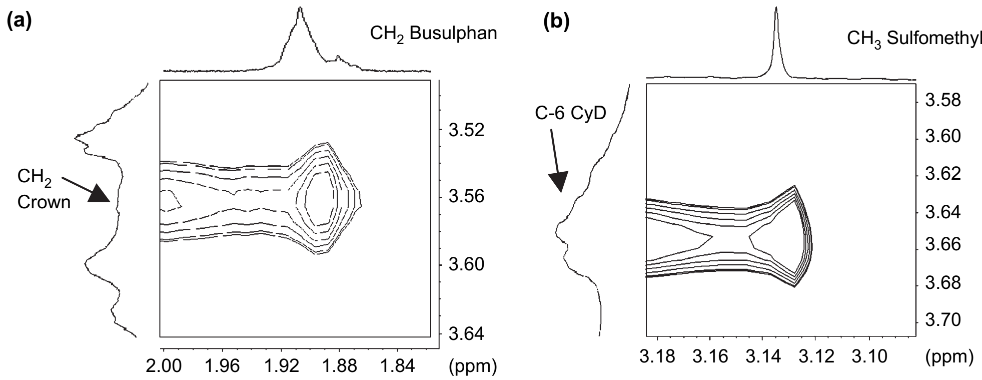


Figure 6. Sectional ^{1}H NMR 2D ROESY spectrum of the [Busulfan/bis-CyD-aza-crown] 1:1 complex in D_2O (8.57×10^{-3} M) at 298 K, mixing time 400 ms. (a) Cross peak between CH_2 β of Busulfan and CH_2 of the crown; (b) cross peak between the CH_3 ester of Busulfan and CH_2 of the CyD C-6 linker.

connected to the ureas N–H atoms at each extremity of the crown, probably by H-bonds with its two ester oxygens, that consequently forced the sulfomethyl protons to be located in close proximity to the CD C-6 methylene atom connected to the urea N–H nitrogens. Thus, concerning the Busulfan central lipophilic butyl chain, it should lie across the crown ether macrocycle in close proximity with the oxoethylene bridges. Considering the dimension of the bis-ureido crown ether existing cavity (which was estimated between 5.1 to 5.4 Å), the distance between the two O_3 ester oxygens (∼5.2 Å), the conformation and the electron density map of the guest,[15] there is a high probability of N–H$\cdots$$O_3$ strong H-bond formation between N–H of ureas and ester oxygen atoms in the [Busulfan/**9**] inclusion complex as illustrated in Figure 7.

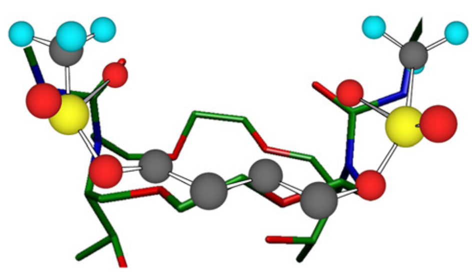


Figure 7. Postulated interactions of Busulfan with the bis-ureido-aza-crown moiety of **9**.

Lastly, the Busulfan inclusion mode was also unambiguously supported by the IR spectrum of the complex in which the characteristic C=O urea frequency is up-shifted from 1685 to 1642 cm^{-1}. Interestingly, the solubility of the complex in water was estimated to be at least 10 g L^{-1}. This is a fairly good solubility to an almost water insoluble drug, which is at the origin of its major side effects.[14]

3. Conclusions

A water-soluble C_2-symmetric receptor including two β-CDs connected by urea linkers to a chiral diaza-crown ether organising platform has been synthesised in eight steps and characterised. Its inclusion properties towards Busulphan have been experimentally evaluated. Molecular modelling simulations, either in vacuum or in water as solvent, gave a convergent set of unexpected conformations in which the CD cavities are tightly connected together by their wide rims above the crown ether moiety to form a pseudo-cryptand molecular system. It was established experimentally that the new host interact efficiently with the Busulfan antitumoural agent thus strongly enhancing its water solubility. The 1D and 2D NMR results clearly established that the lipophilic guest is not embedded in the hydrophobic CD cavities, but connected across the aza-crown macrocycle to the urea functions at each extremity of the crown ether, likely by hydrogen bonds with the sulfomethyl oxygen atoms. In light of these first investigations, a deeper study on the stability of the inclusion complex of Busulfan in biological media along its anti-neoplasic activity is now under way. The new molecular devices introduced here should contribute to the future development of CD-based nano-biomaterials.

4. Experimental

4.1. General comments

All new compounds gave satisfactory spectroscopic data. ^{1}H and ^{13}C NMR spectra were recorded on Bruker DRX-400 and AC 250 spectrometers, FTIR spectra on a Perkin–Elmer 1600 and a Bruker Vector22 spectrometers. Mps were determined on a Büchi apparatus in capillary tubes and are uncorrected. Optical rotations were measured on a Perkin–Elmer 141 automatic polarimeter in a 1 dm cell at rt. In all experiments, DMF was dried over $CaSO_4$, filtered off, distilled over CaH_2 and flushed with argon to eliminate dimethylamine. Electrospray mass spectra in the positive ion mode were obtained on a Q-TOF *Ultima Global* hybrid quadrupole/time-of-flight instrument (Waters-Micromass, Manchester, UK) equipped with a pneumatically assisted electrospray (Z-spray) ion source and an additional sprayer (Lock Spray) for the reference compound. The source and desolvation temperatures were kept at 80 and 150 °C, respectively. Nitrogen was used as the drying and nebulising gas at flow rates of 350 and 50 L/h, respectively. The capillary voltage was 3 kV, the cone voltage 100 V and the RF lens1 energy was optimised for each sample (30–150 V). Lock mass correction, using appropriate cluster ions of an orthophosphoric acid solution (0.1% in 50:50 acetonitrile/water) or of a sodium iodide solution (2 μg/μL in 50/50 propan-2-ol/water+0.05 μg/μL caesium iodide), was applied for accurate mass measurements. The mass range was typically 50–4050 Da and spectra were recorded at 4 s/scan in the profile mode at a resolution of 10^4 (FWMH). Data acquisition and processing were performed with MassLynx 4.0 software. Busulphan was purchased from Sigma–Aldrich (Schnelldorf, Germany) and compounds **7** and $\mathbf{7}^{bis}$ were synthesised according to the literature.[18]

4.2. Synthesis of host 9

4.2.1. (2*S*,3*R*)-2-(*N*,*N*-Dibenzyl)amino-3-hydroxybenzyl-butanoate 1. Benzyl bromide (66 mL, 0.55 mol, 3.3 equiv) was dropped over 2 h on a mechanically stirred dispersion of L-threonine (98%, 20.2 g, 166 mmol) and 38.75 g of dry Na_2CO_3 (195 mmol, ~2.2 equiv) in 75% aq EtOH (200 mL) below 25 °C. The resulting mixture was then refluxed for 5 h (the formation of **1** being monitored by SiO_2 TLC: R_f=0.43; AcOEt/cyclohexane, 1:4), cooled to rt, concentrated under reduced pressure and partitioned between CH_2Cl_2 and H_2O (2×200 mL). The aqueous phase was extracted with CH_2Cl_2 (2×100 mL). The combined organic phases were washed with satd aq $NaHCO_3$ (50 mL), H_2O (2×50 mL), dried over $MgSO_4$ and finally concentrated under vacuum to afford the crude ester **1** as a pale yellow syrup used for the next step without further purification. Yield ~67 g (172 mmol, 78%). An analytical sample was isolated by LC (SiO_2, AcOEt/hexane, 1:9) as a colourless gum: $[\alpha]_D^{20}$ −149 (*c* 2.5, $CHCl_3$). MS (70 eV, EI^+) calcd for $C_{25}H_{27}NO_3$ (389): found *m*/*z* (%) 344 (32) $[M-C_2H_5O]^{\bullet+}$. IR (film): 1730 cm^{-1}. ^1H NMR (250 MHz, $CDCl_3$, 25 °C): δ (ppm) 7.15–7.55 (15H, m, arom.); 5.28 (1H, d, *J*=12.4 Hz, CO_2C*H*HPh); 5.22 (1H, d, CO_2CH*H*Ph); 4.07 (1H, m, H-β); 4.00 (2H, d, *J*=13.2 Hz, NC*H*HPh); 3.50 (1H, s, OH); 3.39 (2H, d, NCH*H*Ph); 3.11 (1H, d, *J*=9.5 Hz, H-α); 1.09 (3H, d, *J*=5.8 Hz, CH_3). ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ (ppm) 139.5 (*C* arom.); 135.7 (*C* arom.); 129.1, 128.7, 128.6, 128.5, 127.4 (*C*H arom.); 67.2 (*C*-β); 66.3 ($PhCH_2O$); 63.2 (*C*-α); 54.8 ($PhCH_2N$); 19.2 (CH_3). Elemental analysis calcd (%) for $C_{25}H_{27}NO_3$ (389.49): C 77.09, H 6.99, N 3.60; found: C 76.91, H 6.81, N 3.67.

4.2.2. (2*R*,3*R*)-2-*N*,*N*-Dibenzylamino-1,3-butane-diol 2. To a mechanically stirred suspension of 95% $LiAlH_4$ (7.8 g, ~195 mmol, 1.15 equiv) in THF (450 mL) under Ar and cooled below 4 °C was carefully dropped a solution of crude ester **1** (66.2 g, 170 mmol) in abs ether (150 mL) over 2 h. The mixture was then stirred for 5 h at rt and refluxed for one more hour. After completion of the reaction (checked by SiO_2 TLC: ethyl acetate/*n*-hexane; 1:1, R_f~0.29), the mixture was cooled to 0 °C, quenched by slow addition of ethyl acetate (50 mL), satd aq Na_2SO_4 (35 mL) and stirred overnight at rt in open air. The suspension was filtered through a sintered glass and the remaining salts thoroughly washed with a CH_2Cl_2/ethanol mixture (1:1; 250 mL). The filtrates were concentrated under reduced pressure and the residue dissolved in CH_2Cl_2 (400 mL), washed with H_2O (3×50 mL), dried over $MgSO_4$, concentrated under reduced pressure and finally stored at 4 °C in the dark. The resulting solids were isolated by filtration and crystallised twice from ethyl acetate/hexanes to yield pure alcohol **2** (ca. 32 g, 66% over two steps) as white crystals (an extra 20% crop could be obtained from the mother liquors by preparative chromatography on SiO_2 with ethyl acetate/hexanes; 1:2): mp=90–91 °C; $[\alpha]_D^{20}$ −55 (*c* 1, $CHCl_3$). MS (70 eV, EI^+): *m*/*z* 286.1 $[M+H]^+$. ^1H NMR (250 MHz, $CDCl_3/\varepsilon D_2O$): δ (ppm) 7.20–7.42 (m, 10H, arom.); 3.99 (2H, d, *J*=13.2 Hz, NC*H*HPh); 3.85 (1H, m, H-β); 3.80 (2H, d, *J*=13.2 and 5.8 Hz, CH_2OD); 3.72 (2H, d, NCH*H*Ph); 2.62 (1H, d, *J*=8.8 Hz, H-α); 1.15 (3H, d, *J*=5.8 Hz, CH_3). ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ (ppm) 139.5 (*C* arom.); 129.4, 128.7, 127.5 (*C*H arom.); 65.5 (*C*-β); 64.8 (*C*-α); 59.1 ($PhCH_2$); 54.7 (CH_2–OH); 20.4 (CH_3). Elemental analysis calcd (%) for $C_{18}H_{23}NO_2$ (285.39): C 75.76, H 8.12, N 4.91; found: C 75.83, H 8.12, N 4.99.

4.2.3. (2*R*,3*R*)-1-[(2-Chloroethoxy)-2-ethyl]-2-*N*,*N*-dibenzylamino-butan-3-ol 3. To a mechanically stirred dispersion of diol **2** (57.1 g, ~200 mmol) and 98% $N(butyl)_4HSO_4$ (69.3 g, 1 equiv), 2,2′-bis-dichloro-diethylether (650 mL) in 2-L Morton flask was slowly added chilled 50% M/v NaOH (0.7 L) over 15 min. The two-phase system was vigorously stirred below 6 °C for 14 h, the reaction being monitored by SiO_2 TLC: ethyl acetate/*n*-hexane/toluene; 1:1:1, R_f~0.52. The resulting emulsion was partitioned between H_2O (0.7 L) and CH_2Cl_2 (0.6 L). The isolated aqueous phase was extracted with CH_2Cl_2 (3×100 mL) and the combined organic phases washed with H_2O (3×50 mL), dried over $MgSO_4$, concentrated under reduced pressure, the excess of reagent being removed by distillation under vacuo around 50 °C (~85% recovery) under an efficient fume board. The residue was purified by HPLC (SiO_2, ethyl acetate/*n*-hexane; 1:4) to yield the pure ether **3** (47.8 g, 61%) as a colourless gum: $[\alpha]_D^{20}$ −73.0 (*c* 3, $CHCl_3$); MS (70 eV, EI^+) calcd for $C_{22}H_{30}ClNO_3$ (391.19): found *m*/*z* 392.0 $[M+H]^+$, 346.0 $[M-C_2H_5O]^{\bullet+}$; HRMS (70 eV, EI^+) calcd for $C_{22}H_{30}ClNO_3$ (391.1914): found *m*/*z* 392.1976 $[M+H]^+$. ^1H NMR (250 MHz, $CDCl_3$, 25 °C): δ (ppm) 7.37–7.20 (10H, m, arom.); 4.10 (1H, br s, OH); 3.96 (2H, d, *J*=13.1 Hz, 2×C*H*H–N); 3.85–3.58 (13H, m, H-β, $ClCH_2$, 4×OCH_2, 2×CH*H*–N); 2.62 (1H, m, H-α); 1.11 (3H, d, *J*=5.8 Hz, CH_3). ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ (ppm) 139.3 (*C* arom.); 129.2, 128.5, 127.2 (*C*H arom.); 71.4, 70.8, 70.7 (3×OCH_2), 68.1 (N–CH–CH_2–O); 64.0 (*C*-β); 63.7 (*C*-α); 54.5 ($PhCH_2$); 42.9 (CH_2Cl); 19.7 (CH_3). Elemental analysis calcd (%) for $C_{22}H_{30}ClNO_3$ (391.94): C 67.42, H 7.72, N 3.55; found: C 67.29, H 7.68, N 3.65.

4.2.4. (2*R*,3*R*)-1-[(2-Chloroethoxy)-2-ethyl]-2-aminobutan-3-ol 4. Ar was bubbled through a solution of tertiary amine **3** (16.5 g, ~42 mmol) and HCO_2H (1.6 mL, 1 equiv) in abs MeOH (100 mL) for 10 min at rt to remove O_2. Moistened Pearlman's catalyst (20% $Pd(OH)_2$/C, 435 mg, ca. 0.02 equiv) was added to the suspension, which was immediately saturated with H_2 (~1 atm) and magnetically stirred for 14 h. Na_2CO_3 (4.45 g, 1 equiv) was added to the suspension, which was stirred for one more hour. The resulting mixture was filtered through a Celite pad, the reactor and pad exhaustively rinsed with a mixture of CH_2Cl_2/EtOH (1:1; 200 mL), the filtrates concentrated under reduced pressure to yield the primary amine **4** (8.7 g, 98%) as a pale yellow syrup: R_f=0.31 (SiO_2 TLC, CH_2Cl_2/MeOH, 95:5); $[\alpha]_D^{20}$−0.2 (*c* 4, $CHCl_3$); HRMS (70 eV, EI^+) calcd for $C_8H_{18}ClNO_3$ (211.0975): found *m*/*z* 212.1038 $[M+H]^+$. ^1H NMR (250 MHz, $CDCl_3$, 25 °C): δ (ppm) 3.74–3.46 (10H, m, 4×OCH_2, CH_2Cl); 3.38 (1H, m, H-β); 2.68 (1H, m, H-α); 2.3 (3H, br s, NH_2, OH); 1.14 (3H, d, *J*=6.6 Hz, CH_3). ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ (ppm) 74.2, 71.3, 70.5, 70.45 (4×OCH_2); 67.8 (*C*-β); 56.3 (*C*-α); 42.8 (CH_2Cl); 20.1 (CH_3).

4.2.5. (2*R*,11*R*)-1,10-Diaza-1,10-*N*,*N*′-benzyl-2,11-bis-[(2′*R*)-2′-hydroxyethyl]-4,7,13,16-tetraoxa-cyclooctadecane 5. Ar was bubbled through a solution of primary amine **4** (8.5 g, ~40 mmol) in abs MeCN (200 mL) for 10 min at rt to remove O_2. Anhydrous Na_2CO_3 (5.51 g, 1.3 equiv) and NaI (6.0 g, 1 equiv) were slowly added to the solution and the resulting suspension magnetically stirred and heated to gentle reflux for 20 h under Ar. The mixture was allowed to cool to rt and benzyl bromide (1.23 mL, 1.5 equiv) was dropped over 5 min on the mixture, which was refluxed again for 10 h. After complete cooling, the reaction mixture was concentrated under reduced pressure and the residue partitioned between H_2O and CH_2Cl_2 (2×250 mL). The isolated aqueous phase was extracted with CH_2Cl_2 (3×75 mL) and the combined organic phases washed with satd NH_4Cl, H_2O twice (50 mL), dried over $MgSO_4$, concentrated under reduced pressure and the residue purified by LC (SiO_2, ethyl acetate/*n*-hexane/HN(*iso*-Pr)$_2$; 800:199:1) to yield pure crown ether **5** (4.58 g, 43%) as a pale yellow gum: R_f= 0.29 (SiO_2 TLC, ethyl acetate); $[\alpha]_D^{20}$ −71.6 (*c* 1.2, $CHCl_3$). HRMS (70 eV, EI^+) calcd for $C_{30}H_{46}N_2O_6$ (530.3356): found *m/z* 530.3353 $[M]^{\bullet+}$, 485.2 (100%) $[M-C_2H_5O]^{\bullet+}$. ^1H NMR (400 MHz, $CDCl_3$, 25 °C): δ (ppm) 7.35–7.20 (10H, m, H_{arom}); 4.20 (2H, sl, OH); 3.93 (2H, d, *J*=13.3 Hz, 2×PhC*H*H); 3.70–3.45 (18H, m, 2×PhCH*H*, 7×OC*H*$_2$, 2×H-β); 3.40 (2H, m, *J*=6.2 and 8.0 Hz); 3.03 (4H, m, *J*=14.5 Hz, 2×OC*H*$_2$N); 2.62 (2H, 2×H-α); 1.14 (6H, d, *J*=6.1 Hz, 2×C*H*$_3$). ^{13}C NMR (100 MHz, 25 °C, $CDCl_3$): δ (ppm) 139.6 (*C* arom.); 129.0, 128.4, 127.2 (*C*H arom.); 70.8, 70.5, 68.4 (O*C*H$_2$); 67.3 (*C*-β); 63.6 (*C*-α); 56.1 (Ph*C*H$_2$); 50.4 (N–*C*H$_2$ aliph.); 19.7 (*C*H$_3$).

4.2.6. (2*R*,11*R*)-2,11-Bis-[(2′*R*)-2′-hydroxyethyl]-4,7,13,16-tetraoxa-1,10-diaza-cyclooctadecane 6. After Ar was bubbled for **5** min through a solution of *N*,*N*′-dibenzyl-1,10-diaza-crown ether **5** (1.064 g, 2 mmol) and 80 μL of formic acid (1 equiv) in abs MeOH at rt to remove O_2, 20% $Pd(OH)_2$ on charcoal (200 mg) was added and the suspension immediately stirred under 1 atm of H_2 for 14 h. The catalyst was removed by filtration through basic alumina using MeOH/CH_2Cl_2 (1:1) for washings, and volatiles were evaporated under reduced pressure to yield the crude aza-crown **6** (680 mg, 98% yield), which was used without further purification for the next step. Colourless wax: mp<50 °C; $[\alpha]_D^{20}$ −42.0 (*c* 0.65, $CHCl_3$). HRMS (70 eV, EI^+) calcd for $C_{16}H_{34}N_2O_6$ (350.2417): found *m/z* 351.2479 $[M+H]^+$. ^1H NMR (250 MHz, $CDCl_3$, 25 °C): δ (ppm) 3.50–3.70 (20H, m); 3.42 (2H, dd, *J*=4.4 and 9.5 Hz); 3.05 (2H, m, *J*=5.8 Hz); 2.58 (2H, m, *J*=11.7 Hz); 2.34 (2H, m); 1.18 (6H, d, *J*=5.8 Hz, 2×C*H*$_3$). ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ (ppm) 71.1, 70.7, 70.0, 68.6 (4×O*C*H$_2$); 65.8 (*C*-β); 64.2 (*C*-α); 47.0 (*C*H$_2$N); 19.8 (*C*H$_3$).

4.2.7. (2*R*,11*R*,19*R*,21*R*)-2,11-Bis-(2-hydroxyethyl)-1,10-*N*,*N*′-bis-{[hexakis-(2,3,6-tri-*O*-acetyl)]-2,3-di-*O*-acetyl-cyclomaltoheptaosyl-6^A-deoxy-6^A-ureido}-4,7,13,16-tetraoxa-1,10-diaza-cyclooctadecane 8. *Method A*: Polystyrene bound triphenylphosphine resin (2.7 g), 6^A-azido-6^A-deoxy-peracetyl-β-CD **7** (0.562 g, 0.156 mmol) and **6** (0.0547 g, 0.156 mmol) in freshly distilled DMF (40 mL; previously flushed 20 min by argon) were placed into a solid-phase peptide synthesis vessel at rt. The mixture was then stirred for 24 h under continuous CO_2 bubbling. After filtration, the polymer was washed by DMF (3×30 mL) and the solution was concentrated to dryness. The crude product was filtered and washed with ether, then purified on a Chromatotron® (silica gel, CH_2Cl_2/MeOH; 98:2). A white amorphous powder (0.038 mmol, 0.101 g, 24%) was isolated.

Method B: 6^A-isocyanato-6^A-deoxy-peracetyl-β-cyclodextrin **7**^{bis} (0.779 g, 0.389 mmol, 2.1 equiv) into anhyd DMF (40 mL) was added to a DMF (10 mL) solution of diaza-crown ether **6** (0.065 g, 0.186 mmol). The mixture was stirred at rt for 24 h under argon. The mixture was then evaporated to dryness and the residue treated by MeOH (2 mL). The final product was precipitated from the methanolic solution by ether, filtered on a sintered glass and dried in a dessicator over anhydrous KOH. A white amorphous powder (0.408 g, 0.153 mmol, 50.4%) was obtained: $[\alpha]_D^{18}$ +93.5 (*c* 0.1, MeOH). FTIR (KBr): ν=1751 cm^{-1} (C=O acetate), ν=1675 cm^{-1} (C=O urea). ^1H NMR (400 MHz, $CDCl_3$, 25 °C): δ (ppm) 5.42–5.23 (m, 14H, H-3 CD^{a,b}); 5.20–5.05 (m, 14H, H-1 CD^{a,b}); 4.92–4.73 (m, 14H, H-2 CD^{a,b}); 4.68–4.50 (m, 14H, H-5 CD^{a,b}); 4.40–3.80 (m, 32H, H-6^a, crown); 3.88–3.44 (m, 34H, H-^{6b}, crown); 2.20–1.98 (m, 120H, C*H*$_3$ acetates); 1.25–1.17 ppm (m, 6H, CH_3 crown). ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ (ppm) 171.0–170.0 (multiple s, *C*=O acetates); 161.0 (*C*=O, urea); 97.0 (C-1); 76.0 (C-4); 72.0, 71.0, 70.0 (C-2, C-3, C-5); 63.0 (C-6); 41.0 (*C*H, *C*H$_2$, crown); 23.0 (*C*H$_3$, crown); 21.0 (*C*H$_3$, acetates). MS-MALDI (α-cyano matrix) *m/z*: 3998.19 $[M-9CH_3CO]^+$, 2067.26 $[M-5CH_3CO+5H^+]^{2+}$.

4.2.8. (2*R*,11*R*,19*R*,21*R*)-2,11-Bis-(2-hydroxyethyl)-1,10-*N*,*N*′-bis-[cyclomaltoheptaosyl-6^A-deoxy-6^A-ureido]-4,7,13,16-tetraoxa-1,10-diaza-cyclooctadecane 9. The peracetylated bis-β-CD-crown ether **7** (0.049 mmol, 0.214 g) was dissolved in anhyd MeOH (50 mL). The solution was chilled in an ice bath at 0 °C and a 1 M MeONa solution (3.5 mL) was added drop by drop. The mixture was stirred 1 h under argon at 0 °C and then 1 h at rt. Small amounts of IRN77® ion exchange resin were added until neutralisation at pH=7.0. The resulting suspension was filtered off, the filtrate was evaporated to dryness, the solid product was dissolved into distilled water and finally lyophilised to yield **8** as an orange amorphous powder (0.125 g, 0.053 mmol, 95%): $[\alpha]_D^{18}$ +88 (*c* 0.05, H_2O). FTIR (KBr): ν=1685 cm^{-1} (C=O urea). ^1H NMR (400 MHz, D_2O, 25 °C): δ (ppm) 5.09 (m, 14H, H-1 CD^{ab}); 4.08–3.93 (m, 14H, H-3 CD^{ab}); 3.94–3.78 (*complex* m, 36H, H-6 CD^a; H-5 CD^{ab}); 3.77–3.72 (m, 14H, C*H*$_2$ crown); 3.71–3.51 (*complex* m, 42H, H-2 CD^{ab}, H-4 CD^{ab}, H-6 CD^b, C*H*$_2$ crown); 3.42–3.25 (m, 2H, CHβ crown); 1.20 (dd, 6H, C*H*$_3$ crown). ^{13}C NMR (100 MHz, DMSO-d_6, 25 °C): δ (ppm) 133.0 (C=O urea); 129.0 (C-1); 74.0 (C-4); 72.0 (C-2); 70.0 (C-3); 67.0 (C-5); 63.0 (C-6); 61.0 (*C*–H crown), 52.0–46.0 (*C*H$_2$ crown); 24.0 (*C*H$_3$ crown); Elemental analysis calcd (%) for $C_{102}H_{172}N_4O_{76}\cdot 7H_2O$ (2796.58): C 43.81, H 6.70, N 2.00; found: C 43.75, H 6.81, N 1.96.

4.3. Preparation of [Busulfan/9] inclusion complex

A solution of Busulfan (0.0048 g, 0.019 mmol) in DMSO (0.5 mL) was added under argon to a solution of **9** (0.047 g, 0.018 mmol) in H_2O (25 mL) at rt. The mixture was stirred 18 h more and then lyophilised to yield a yellow amorphous powder: $[\alpha]_D^{20}$ +83 (*c* 0.1, H_2O); FTIR (KBr): ν=1642 cm^{-1} (C=O urea). ^{1}H NMR (400 MHz, D_2O, 25 °C): δ (ppm) 5.09 (m, 14H, H-1 CD^{a,b}); 4.42 (m, 4H, CH_2 *Busulfan*); 4.08–3.93 (m, 14H, H-3 CD^{a,b}); 3.94–3.78 (m, 36H, H-6 CD^a, H-5 CD^{a,b}); 3.77–3.72 (m, 14H, CH_2 crown); 3.71–3.52 (m, 40H, H-2 CD^{a,b}, H-4 CD^{a,b}, H-6 CD^b, CH_2 crown); 3.49–3.39 (m, 2H, C$H\beta$ crown); 3.23 (s, 6H, CH_3 *Busulfan*); 1.95 (m, 4H, C$H_2\beta$ *Busulfan*); 1.29–1.10 (m, 6H, CH_3 crown).

4.4. Molecular modelling calculations

A coarse skeleton of the ether crown was initially prepared using ChemDraw and Chem3D software packages[19] and the structure of the native β-CD (native β-CD) was taken from the Cambridge Structural Database by means of ConQuest 1.8.[20] In order to build up the final ligand, two CDs were attached through the urea groups to the ether crown after removing one of their primary hydroxyl groups. The molecular modelling calculations were carried out on a Pentium-4 personal computer using the TINKER programme and its implemented MM3 force field.[21] The energy minimisation MINIMIZE option of the TINKER programme was first used to find a starting geometrical state of the system. The respective conformational minima were sought without constraints for the ligand in vacuum (structure S_1) and in water solvent (structure S_2). Structure S_1 was minimised to a final RMS gradient equal to 0.0948 kJ Å^{-1} mol^{-1} by a modified version of the algorithm of Jorge Nocedal based on a quasi-Newton method (596 cycles).[22] No cut-off option was used in this case. The structure S_2 was obtained with the same method (final RMS gradient equal to 0.0950 kJ Å^{-1} mol^{-1} (1475 cycles)) considering a cubic box of 25×25×25 Å^3 containing 383 water molecules. In this case, the periodic boundary conditions were imposed with a cut-off radius of 9 Å. A stochastic annealing procedure (chosen temperature 1000 K, time step 0.02 ps, 95 snapshots) applied to conformation S_2 gives very similar results showing the robustness of the MINIMIZE method implemented in the TINKER programme.

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