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Bis-β-cyclodextrinyl- and bis-cellobiosyl-diazacrowns: synthesis and molecular complexation behaviors toward Busulfan anticancer agent and two basic aminoacids

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ABSTRACT

The one-step synthesis of two C_2-symmetric receptors including two β-cyclodextrin cores or two disaccharidyl units connected by urea linkers to a diazacrown ether organizing platform is reported. The X-ray structure of the peracetylated bis-ureidocellobiosyl podand could be determined. These molecular systems, long thought to be potent selective carriers for chiral/achiral organic guests at the supramolecular level, were found to be efficient complexing tools toward the Busulfan anticancer agent but also toward L-arginine and L-lysine basic aminoacids.

1. Introduction

We have recently the opportunity to demonstrate that a novel C_2-symmetrical heterotopic co-receptor[1] based on a chiral diazacrown ether covalently linked to two β-cyclodextrin cavities was able to encapsulate efficiently Busulfan (=1,4-butanediol-dimethylsulfonate), a powerful antitumor drug in leukemias for the first time.[2a,b] Moreover, interesting features were obtained, e.g., a noteworthy one hundred fold enhancement of drug water-solubility[1] and the in vitro and in vivo total lack of toxicity of the receptor.[3] By another way, we came to conclusions on the co-receptor conformational equilibrium and on the structure of the host–guest complex in which the guest was not embedded in the cyclodextrins hydrophobic cavities but lay across the crown ether macrocycle and was connected to the urea functions at each extremity of the crown by hydrogen bonds.[1] On the light of these first results, it appears the next step of our research should be the investigation of new compounds in which the crown ether will be substituted by two disaccharides units and/or in which the chirality of the azacrown part will be removed. This last option was supported by the fact that chirality was likely not implied in the molecular recognition process of an achiral guest.

Thus, we decided to synthesize after the previously reported C_2-symmetrical pseudo-cryptand **8**[1] two types of new ligands, i.e., **4** and **7** as illustrated in Figure 1, and to explore their complexation behavior toward Busulfan and L-arginine and L-lysine basic aminoacids. We expected the new lariats suitable to form host–guest complexes with the former guests, too.

2. Results and discussion

2.1. Synthesis

Bis-cellobiosyl-diazacrown ligand **4** was synthesized over two steps in high yield (99%) from the hepta-*O*-acetyl-azido-β-D-cellobiose **1** and the tetraoxa-diazacyclooctadecane **2** via a tandem Staudinger–aza-Wittig one-pot coupling reaction (alias phosphine imide reaction)[4] and after a quantitative deacetylation step of ligand **3** using Zemplén conditions (Scheme 1).[5] Likewise, a similar synthesis strategy was used for the bis-β-cyclodextrin ligand derivative **6** obtained over one step in a moderate 38% yield from the 6^A-isocyanato-6^A-deoxy-per-*O*-acetylated-β-cyclodextrin **5**[6] as starting material.

The deacetylated final product **7** was obtained by a similar quantitative deacetylation step of **6** using Zemplén conditions. Spectroscopic data of **4** and **7** by IR, NMR, ESIMS, and elemental analysis are in full accordance with the proposed structures. The FTIR spectra of **3**, **4**, **6**, and **7** display absorption bands at 1654, 1637,

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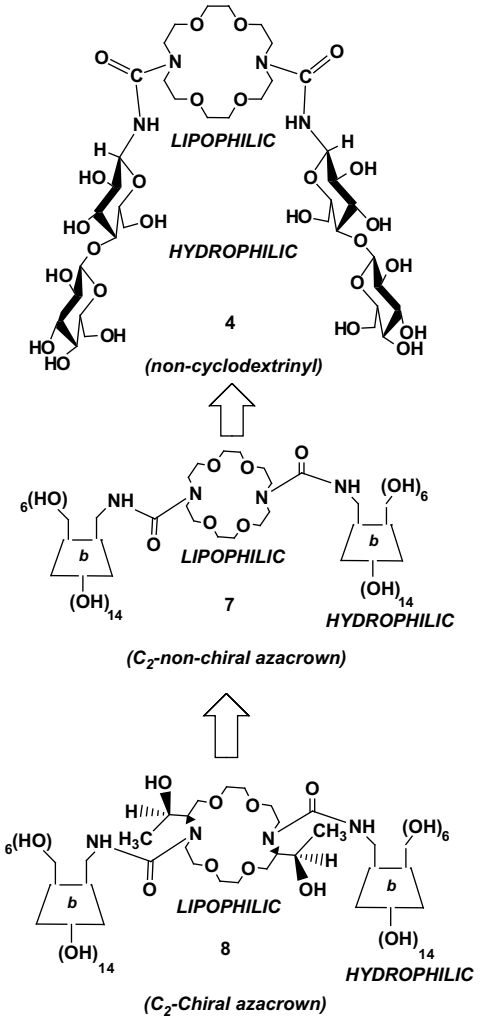


Figure 1. Comparative structure evolution of the three ligands **8**, **7**, and **4**.

1663, and 1635 cm^{-1} characteristic of the urea carbonyl bonds, which were also confirmed by the quaternary carbon corresponding ^{13}C NMR signals at 159.1, 157.7, and 159.7 ppm. This proved that coupling between cyclodextrins or disaccharidyl moieties and azacrown was effective. Furthermore, positive ESI high resolution mass spectra of podands **4**, **6**, and **7** have been recorded and showed the presence of monocharged species $[M+H]^+$ at 997.4160 amu for compound **4**, the dicharged and tricharged species $[M+2Na]^{2+}/2$ at 2153.6602 amu and $[M+3Na]^{3+}/3$ at 1436.1092 amu for compound **6** and the dicharged species $[M]^{2+}/2$ at 1291.4669 amu for compound **7**. Altogether, these data strengthen the proposed structures for **4**, **6**, and **7**.

2.2. X-ray crystallography

Crystallization of the peracetylated bis-cellobiosyl podand **3** gave us the opportunity to perform X-ray diffraction analysis and allowed its structural determination (Fig. 2). Single crystals of **3** were obtained by slow diffusion of hexane into a solution of **3** in CH_2Cl_2. Compound **3** crystallizes in the triclinic system (space group P_1) with two disordered H_2O molecules (occupancy 0.5). Regarding the relative conformation of the two disaccharide moieties, they are oriented in opposite directions with respect to the diazacrown macrocycle. The dihedral angles between the mean plane of the diaza-macrocycle (18 atoms) and the mean plane of the disaccharide moieties (13 atoms) are equal to 25.8° and 19.4° for the disaccharide bonded to N1 and the disaccharide bonded to N2, respectively. The dihedral angle between the mean planes of the disaccharides is 40.9°. This conformation allows the formation of intramolecular hydrogen bonds between the nitrogen atoms N3 and N4 of each urea groups and two oxygen atoms (O2 and O3) of the central macrocycle (d_{N3-O2}=2.878 and d_{N4-O3}=2.857 Å). Finally, both urea goups are also involved in intermolecular H-bonds with H_2O solvent molecules (d_{O-O} of ca. 2.7 Å).

3. Binding studies

3.1. Interaction of ligands 4 and 7 with Busulfan

Signs of interaction were firstly detected by chemical-induced shifts (CIS) of protons of the guest signals compared to those of the free compounds in D_2O. The signals of both sulfomethyl and methylene protons of the butyl chain are downfield shifted (−0.28 ppm) and upfield (+0.15 ppm), respectively as shown in Figure 3. The stoichiometries of the complexes were established on the basis of the Job plots continuous variation method as illustrated in Figure 4, values of $R\sim0.5$ being reached at the maximum, which ascertain the 1:1 stoichiometry of these complexes.

Complexation constants (K_a) were determined using Scott's plot method[7] and found to be ca. 2600 M^{-1} and 2700 M^{-1}, respectively, for [Busulfan/**4**] and [Busulfan/**7**] at 300 K. Furthermore, it was possible thanks to 2D ROESY spectroscopy to infer the local spatial interaction and the orientation of the Busulfan guest molecule inside the podand **4** host using the assigned ROE correlations (Fig. 5).

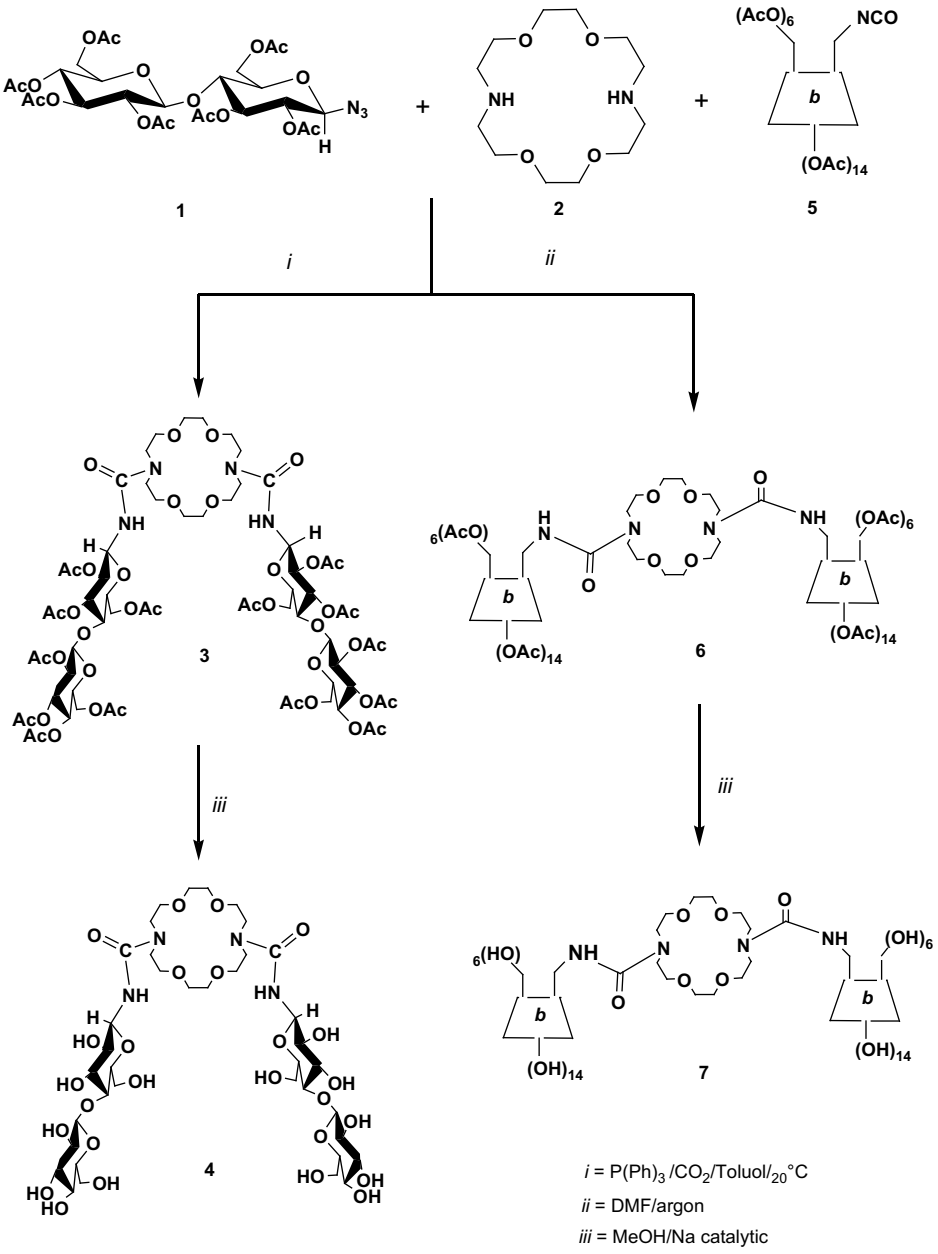
Indeed, the 2D ROESY spectrum of the [Busulfan/**4**] complex in D_2O displays two characteristic cross-peaks, a first one between the sulfomethylene protons and the H-1 anomeric proton of the cellobiose units and a second one between the methylene protons of the Busulfan-butyl chain and the oxo-methylene protons of the crown ether. Interestingly, both anomeric protons are involved at the time scale of the NMR in the complexation of Busulfan; that implies that after deacetylation, the two cellobiose units are now localized on the same side of the crown in water. Altogether, these data corroborate fairly the observed CIS and are in a good agreement with previous obtained results with podand **8**.[1]

3.2. Interaction of ligands 4, 7, and 8 with L-lysine and L-arginine

As previously observed for Busulfan[1] as guest and on the ground of structural and electronic similarities with basic aminoacids, formation of host–guest complexes between hosts **4**, **7**, **8** and L-arginine and L-lysine was investigated (see Fig. 6). As indicated above, interactions were first detected by CIS of guest proton signals compared to those of the free guest in the same solvent. For example, signals of both β and ω methylene protons of L-lysine were upfield shifted (+0.5 ppm) as enlighten in Figure 7. The Job plots continuous variation method was also applied and shows that values of $R\sim0.5$ were reached at the maximum with the three ligands **4**, **7**, and **8** (Fig. 8) indicating 1:1 stoichiometries in all the complexes. Complexation constants (K_a) of ca. 4000 M^{-1} were estimated for [L-arginine/**8**], 5500 M^{-1} for [L-lysine/**8**], and 6000 M^{-1} for [L-arginine/**7**] and 4500 M^{-1} for the [L-lysine/**7**].

Positive ESI high resolution mass spectra of the complexes formed by ligand **4** showed the presence of monocharged species at 1171.5311 amu and at 1143.5250 amu corresponding to the 1:1 [L-arginine/**4**] and [L-lysine/**4**] complexes, which strengthen results enlighten in Figure 7.

Unfortunately, detection of Busulfan complexes by ESI mass spectrometry failed. This feature may be analyzed in terms of lowest K_a values observed for the Busulfan with the three ligands **4**, **7**, and **8** (e.g., 1600 M^{-1} for **8**).[1] On the ground of the results



Scheme 1.

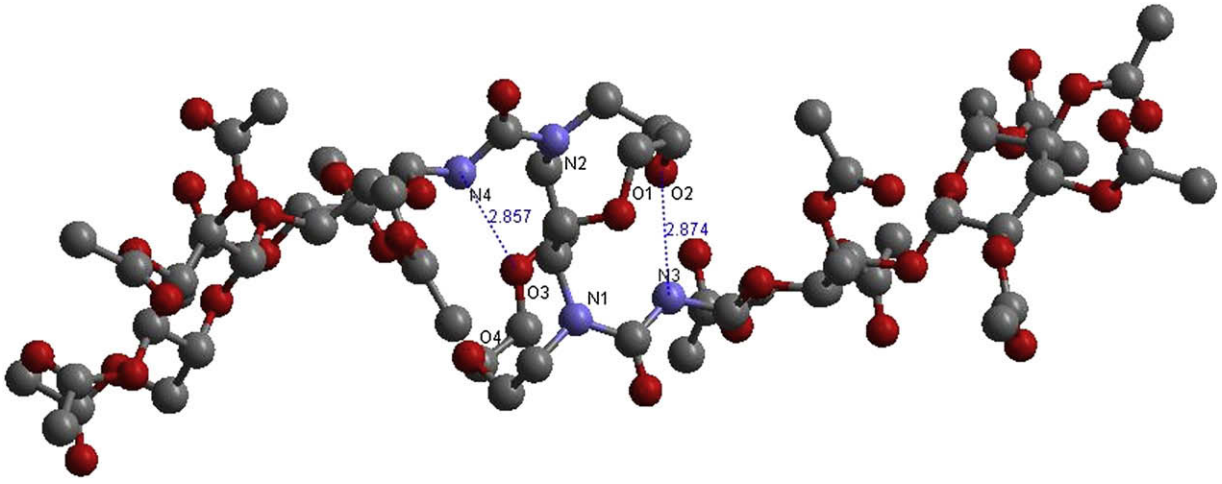


Figure 2. X-ray structure of peracetylated ligand **3**. Hydrogen atoms are omitted for clarity.

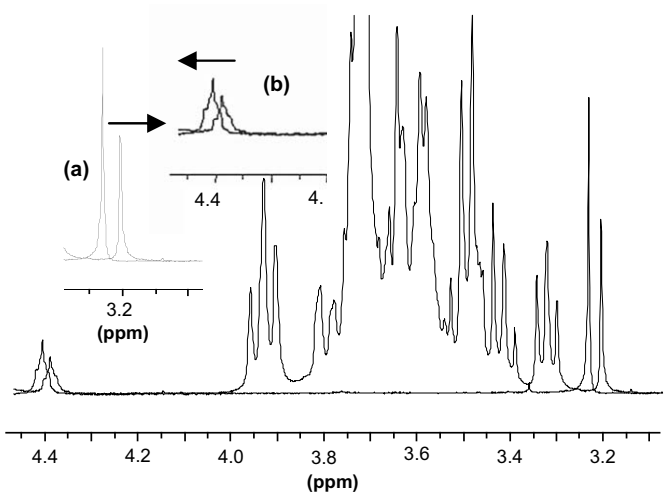


Figure 3. ^{1}H NMR spectrum of [Busulfan/**4**] [1:1] complex and free Busulfan at 400 MHz. Zoom views of sulfomethyl (a) and β-methylene (b) protons of the Busulfan-butyl chain.

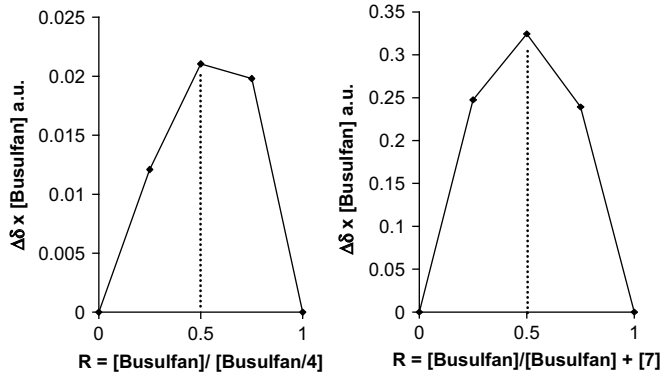


Figure 4. Job plots corresponding to the chemical shifts displacements of the sulfomethylene protons of Busulfan for [Busulfan/**4**] and [Busulfan/**7**] in D_2O at 300 K.

acquired initially with Busulfan, one can suggest that similar complexation mode should be involved between Busulfan, L-lysine or L-arginine and podands **4**, **7**, and **8**. According to the hydrophilic status of aminoacids, it was also admitted that aminoacids could not be embedded into CyDs hydrophobic cavities of the bis-CyD podand **7** or **8**.

In addition, H_3 and H_5 proton signals located inside the CyD cavity of the host **7** remained unchanged upon complexation as observed earlier with ligand **8** and Busulfan,[1] just as no CIS could be observed in the cellobiose moiety in ligand **4**. Consequently, the aminoacids guests are very likely in close interaction with the two ureas and the crown ether parts of **4**, **7**, and **8**. Nevertheless, an interesting and rather surprising result was observed with the [Busulfan/**7**] complex (Fig. 9), which clearly exhibits a downfield CIS of H_3 proton signal (−0.1 ppm) of the CyD internal cavity.

Contrary to the previously depicted [Busulfan/**8**] complex[1] where no CIS on H_3 and H_5-CyD protons occurred, a different complexation mode of the lipophilic Busulfan guest by host **7** could be inferred. One explanation may be found regarding the conformational status difference of the chiral crown ether part in ligand **8** compared to achiral crown ether **7**. Previous results of molecular modeling calculations on **8**[1] in water predicted a strong distortion of the crown ether macrocycle induced by the formation of a short H-bond between secondary alcohols of the chiral moiety then inducing an open channel at one extremity of the crown. So that, a little rod as the Busulfan guest should find an easier way to enter the bis-ureido diazacrown pocket. The same situation cannot exist with the achiral crown ether of the ligand **7**, so that the best access for the Busulfan guest is probably the narrow rims of the CyD cores. These results are in good agreement with the above observed CIS and sustain the hypothesis that either L-lysine or L-arginine are connected to the ureas N–H atoms at both extremities of the crown. Thus, the central lipophilic methylene atoms should be forced to lie across the crown ether macrocycle in a close vicinity of the oxoethylene bridges. Considering the dimension of the bis-ureido crown ether cavity (ca. 7.2 Å), the average distances between polar groups of L-arginine and L-lysine, and previous observations made on the [Busulfan/**8**] complex,[1] it does exist a high probability of N–H···O_3 and N–H···NH=C–NH_2 or H_2N···H–N–CO–NH multiple H-bond formation between polar SO_3Me, NH_2, NH_2–C=NH, and NH_2–CH–CO_2H groups and N–H ureas at the ends of the crown.

Furthermore, the Busulfan or L-lysine inclusion mode was also supported by the IR spectra of the complexes in which the characteristic C=O urea frequency is up-shifted from 1635 cm^{-1} to 1625 cm^{-1} for **4**. Lastly, complexation experiments were also run with D-lysine and D-arginine enantiomers in order to check a possible discrimination by the chiral podand **8**. Unfortunately, we were not able to specify any significant differences between L and D series

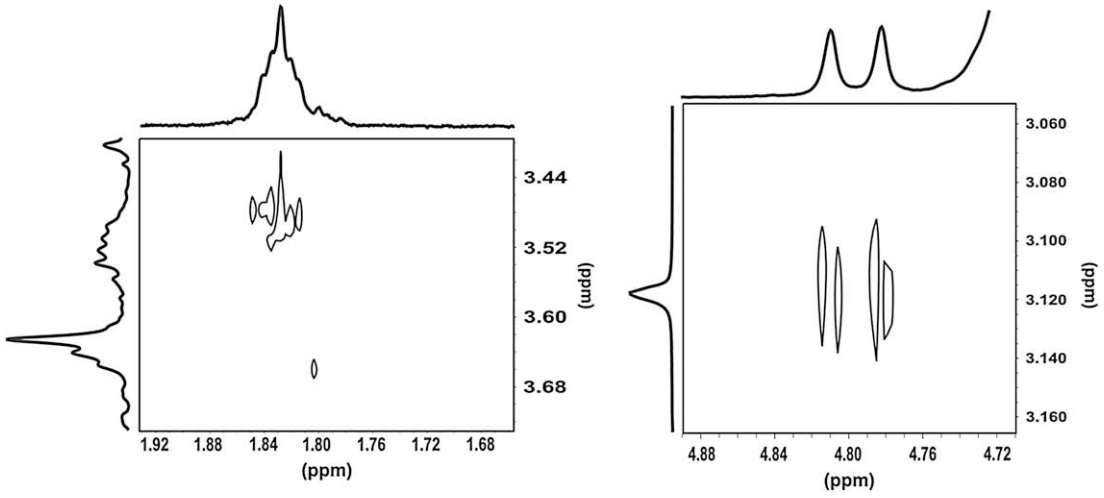


Figure 5. Sectional ^{1}H NMR 2D ROESY spectrum of the [Busulfan/**4**] 1:1 complex in D_2O (6.66×10^{-3} M) at 298 K, mixing time 400 ms. (a) Cross-peak between CH_2 β of Busulfan and CH_2 of the crown; (b) Cross-peaks between the CH_3 ester of Busulfan and anomeric H-1 of cellobiose unit.

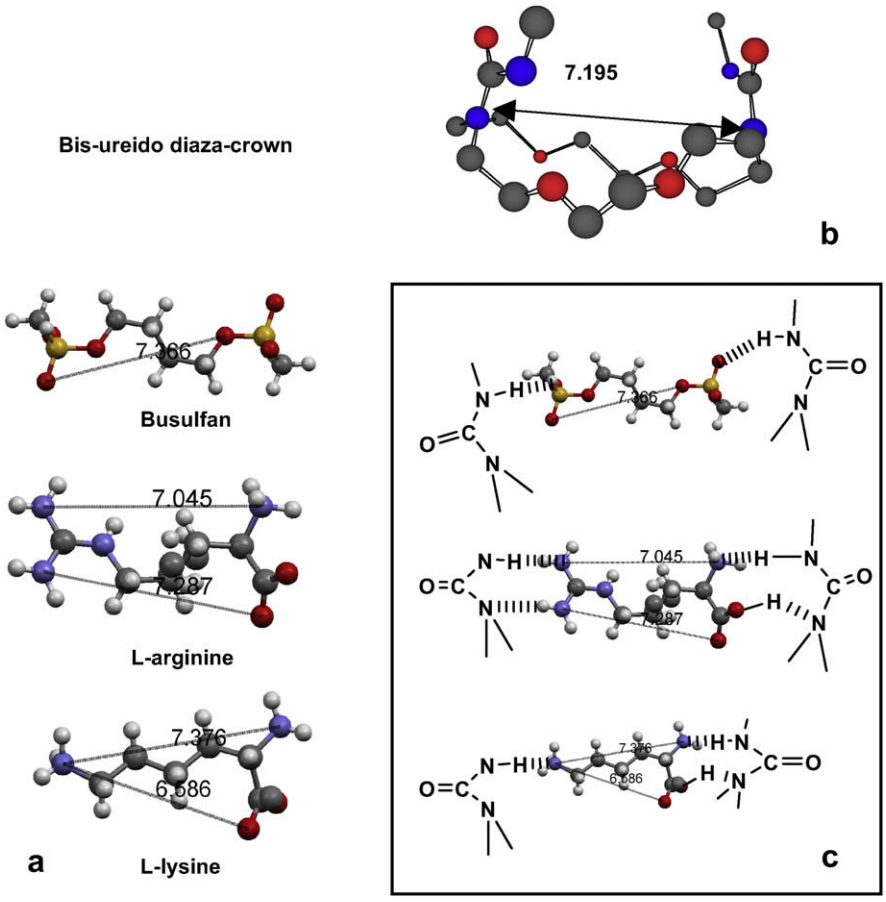


Figure 6. (a) Comparison of X-ray structures of L-lysine,[9] L-arginine,[9] and Busulfan[10] guests. Selected distances between polar centers are given in Å; (b) close view of the crown ether moiety structure with lowest energy obtained after dynamic simulation in vacuo; (c) graphical representation of possible H-bond interactions for Busulfan, L-lysine, and L-arginine with ureas of the azacrown moiety.

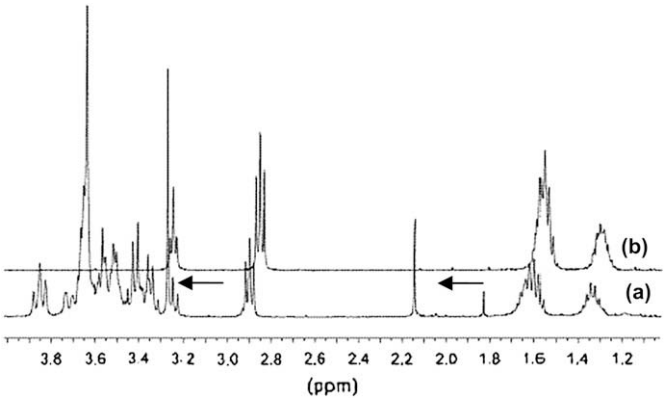


Figure 7. ^{1}H NMR spectrum of (a) [L-lysine/**4**] [1:1] complex and (b) free L-lysine at 400 MHz in D_2O.

in our hands. This may suggest that the secondary hydroxyls on chiral centers of the crown **8** are not placed in a correct spatial position to induce enantiomers discrimination. Interestingly, looking at the solubilities of the Busulfan complexes with podands **4** and **7** in water, we observed they were maintained at the same level (ca. 10 g L^{-1}) as early measured with podand **8**.[1]

4. Conclusions

Two water soluble new podands have been synthesized in high yields by the tandem Staudinger–aza-Wittig one-pot coupling reaction. The X-ray structure of the peracetylated bis-cellobiosyl compound **3** was resolved showing a particular conformation of the cellobiosyl moieties in the crystal. Unfortunately, valuable crystals of the deacetylated podand **4** could not be obtained so far and precluded for instance to analyze the conformation of the cellobiosyl part related to the bis-ureido azacrown macrocycle. Concerning the binding studies, it was established experimentally that the new hosts interact efficiently either with Busulfan or basic aminoacids as L-arginine or L-lysine to form [1:1] supramolecular species. The present 1D- and 2D-NMR results clearly established that a similar mode of complexation is involved for both studied aminoacids as guests.

In summary, some general features could be stressed: (i) the investigated aminoacid guests are not embedded in the CyDs cavities but anchored across the azacrown macrocycle to the urea functions at the ends of the crown ether by H-bonds with the polar groups of the guests; (ii) higher association constants were found for Busulfan with the ligand **4**, this effect might be related to the lower rigidity of the architecture of **4** allowing an easier access to the 'boat shaped' bis-ureido crown ether receptacle compared to those of **8** in which the lower face of the azacrown is kept closed by an intramolecular H-bond between the two secondary hydroxyls of the chiral centers;[1] (iii) we have demonstrated the ability of the above receptors to operate an efficient molecular recognition toward linear guest molecules having two polar groups separated by an adapted length of lipophilic alkyl spacer; (iv) an unexpected but fine tuning complexation mode for Busulfan was revealed between ligands **8** and **7**; (v) interestingly, the new synthesized molecular receptors were also able to enhance the water-solubility (to ca. 10 g L^{-1}) of the slightly

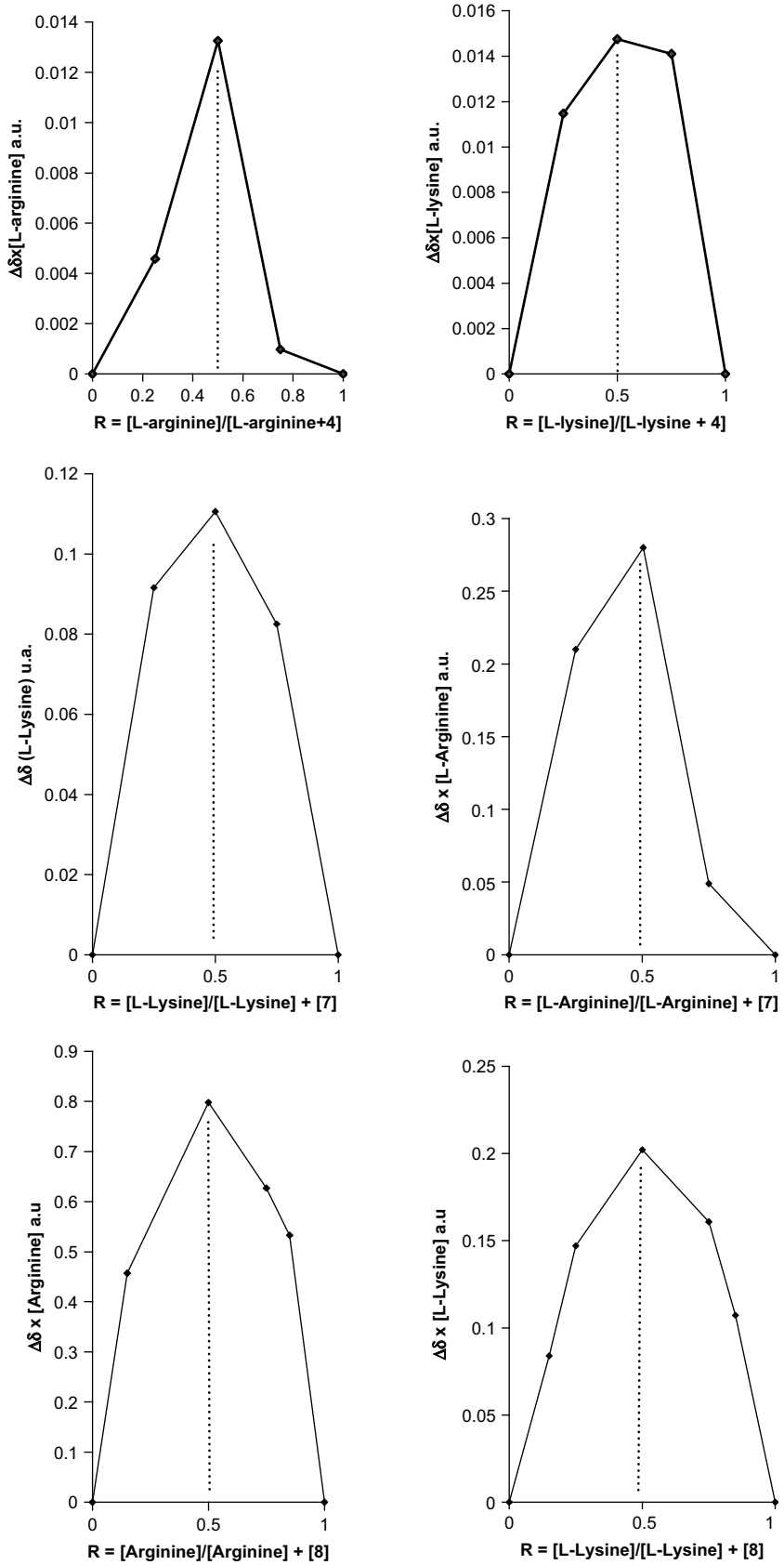


Figure 8. Job plots corresponding to the chemical shifts displacements of the H_4 protons of L-arginine and H_5 of L-lysine for [L-arginine/**4**], [L-lysine/**4**], [L-arginine/**7**], [L-lysine/**7**], and [L-arginine/**8**], [L-lysine/**8**] in D_2O at 300 K.

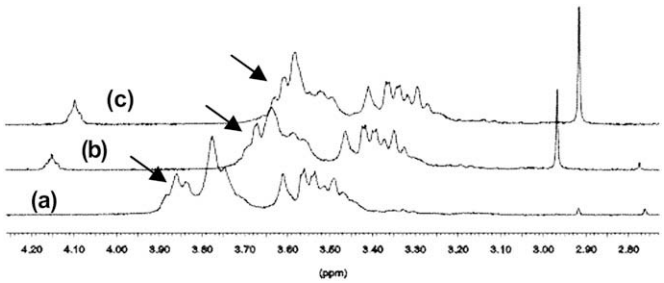


Figure 9. ^{1}H NMR stack plots of: (a) free podand **7** (CyD+crown) zoom part; (b) podand **7**+0.2 equiv of Busulfan; (c) podand **7**+0.6 equiv of Busulfan. Shifts of H_3 protons are marked by arrows.

soluble lipophilic Busulfan drug by complexation. Considering the azacrown alcohol chiral centers of podand **8**, they do not participate nor induce any measurable effect on chiral discrimination between L- and D-aminoacids series. Investigations on the stability of different complexes of Busulfan in biological media along their anti-neoplastic activity are presently engaged. The new molecular devices introduced here should also contribute to the future development of oligosaccharidyl based nano-materials.

5. Experimental

5.1. General comments

All the new compounds gave satisfactory spectroscopic data. ^{1}H and ^{13}C NMR spectra were recorded on Bruker DRX-400 spectrometer, FTIR spectra on a Bruker Vector22 spectrometer. ESI-HRMS spectra in the positive ion mode were obtained on a Bruker microTOF-Q98. Busulfan was purchased from Sigma–Aldrich (Schnelldorf, Germany) and compounds **1** and **5** were synthesized according to the literature.[6]

5.2. Synthesis of hosts 3–7

*5.2.1. 1,10-N,N′-Bis-(2,3,4,6,2′,3′,4′,6′-hexa-O-acetyl-β-D-ureidocellobiosyl)-4,7,13,16-tetraoxa-1,10-diazacyclooctadecane **3***

A solution (0.699 g, 1.0 mmol) of 2,3,6,2′,3′,4′,6′-hepta-*O*-acetyl-azido-β-D-cellobiose and triphenylphosphane (3.3 mmol) in anhydrous toluene (15 mL) was stirred for 30 min at rt then 4,7,13,16-tetraoxa-1,10-diazacyclooctadecane (0.130 g, 0.5 mmol) was added to the mixture, which was stirred for 24 h under CO_2 bubbling. After evaporation of the solvent, the residue was chromatographed on silicagel column (eluent AcOEt/MeOH 8:1) to give 99% (0.784 g, 0.495 mmol) of a pure white powder. Anal. Found: C, 49.68; H, 6.05; N, 3.47. $C_{66}H_{96}N_4O_{40}$ requires C, 50.00; H, 6.10; N, 3.53. IR: ν_{max} 3478, 1741, and 1663 cm^{-1}. ^{1}H NMR (700 MHz, $CDCl_3$): 6.47 [2H, d, 2 N*H*, *J*=9.1], 5.26 [2H, t, 2H$_{3'}$, *J*=9.1–9.8], 5.14 [2H, t, 2H$_3$, *J*=9.1–9.8], 5.09 [2H, t, 2H$_1$, *J*=9.1–7.7], 5.09 [2H, t, 2H$_{4'}$, *J*=9.1–9.8], 4.93 [2H, t, 2H$_{2'}$, *J*=7.7–9.1], 4.85 [2H, t, 2H$_2$, *J*=7.7–9.1], 4.53 [2H, d, 2H$_{1'}$, *J*=7.7], 4.39 [2H, dd, 2H$_{6b}$, *J*=4.9–12.6], 4.05 [2H, d, 2H$_{6a}$, *J*=12.6], 3.79 [2H, t, 2H$_{4'}$, *J*=9.1–9.8 Hz], 3.60–3.75 [28H, m, 2H$_5$, 2H$_{5'}$, C*H*$_2$], 2.00–2.14 (42H, s, C*H*$_3$). ^{13}C NMR (176 MHz, $CDCl_3$): 170.3–169.0 [*C*O–CH$_3$], 157.7 [N*C*ONH], 100.7 [C_1 cellobiosyl], 80.4 [C_1 cellobiosyl], 76.4 [C_4 cellobiosyl], 74.1 [$C_{5'}$ cellobiosyl], 72.9 [C_3 cellobiosyl], 73.9 [$C_{3'}$ cellobiosyl], 71.9 [C_5 cellobiosyl], 71.6 [*C*2′ cellobiosyl], 70.8 [C_2 cellobiosyl], 71.0–70.1 [*C*H$_2$ crown], 67.9 [$C_{4'}$ cellobiosyl], 62.0 [$C_{6'}$ cellobiosyl], 61.6 [C_6 cellobiosyl], 20.9–20.5 (*C*H$_3$).

*5.2.2. 1,10-N,N′-Bis-(β-D-ureidocellobiosyl)-4,7,13,16-tetraoxa-1,10-diazacyclooctadecane **4***

A solution of **3** (0.784 g, 0.495 mmol) in anhydrous MeOH (15 mL) was chilled in an ice bath at 0 °C and a 1 M MeONa solution was added dropwise. The mixture was stirred for 1 h under Ar at 0 °C, then 1 h at rt. Small amounts of IRN77® ion exchange resin were added until neutralization at pH=7.0. The resulting suspension was filtered off, the filtrate was evaporated to dryness, the solid product was dissolved into distilled water (20 mL) and finally lyophilized to give 99% (0.776 g; 0.778 mmol) of a pure white amorphous powder. Anal. Found: C, 43.21; H, 6.87; N, 5.18. $C_{38}H_{68}N_4O_{26}\cdot 3H_2O$ requires C, 43.43; H, 7.10; N, 5.33. IR: ν_{max} 3384, 1635, and 1030. ^{1}H NMR (400 MHz, D_2O): 4.91 [2H, d, H$_1$, *J*=9.3], 4.53 [2H, d, H$_{1'}$, *J*=8.0], 3.94 [4H, t, H$_3$, H$_{3'}$, *J*=9.8], 3.82–3.35 [14H, m, H$_2$, H$_4$, H$_5$, H$_6$, H$_{4'}$, H$_{5'}$, H$_{6'}$, and 12H C*H*$_2$], 3.34 [2H, t, H$_{2'}$, *J*=8.0–9.1]. ^{13}C NMR (175 MHz, D_2O): 159.7 [NH–*C*O–N], 102.9 [C_1 cellobiosyl], 81.9 [C_1 cellobiosyl], 78.8 [C_4 cellobiosyl], 76.5, 76.4, 75.9, 75.6, 73.5, 71.9, 70.4 [C_2, C_3, C_5, $C_{2'}$, $C_{3'}$, $C_{4'}$, $C_{5'}$ cellobiosyl], 69.8 [*C*H$_2$ crown], 61.0, 60.5 [C_6, $C_{6'}$], 48.9 [*C*H$_2$ crown]. HR-ESIMS *m*/*z*: [$C_{38}H_{69}N_4O_{26}$]; 997.4160 [M+H]$^+$.

*5.2.3. 1,10-N,N′-Bis-{[hexakis-(2,3,6-tri-O-acetyl)]2,3-di-O-acetyl-cyclomaltoheptaosyl-6A-deoxy-6A-ureido}-4,7,13,16-tetraoxa-1,10-diazacyclooctadecane **6***

A solution of 1,4,10,13-tetraoxa-7,16-diazacyclooctadecane **2**, (0.330 g, 1.3 mmol) in anhydrous DMF was added under Ar onto a solution of 6^A-isocyanato-6^A-deoxy-peracetylated-β-cyclodextrin **5**[6] (0.500 g, 0.249 mmol, 2.1 equiv) in freshly distilled DMF (100 mL) previously flushed for 20 min by Ar. After one night at rt, the mixture was evaporated to dryness and the residue treated by MeOH (2 mL). The crude product was obtained by precipitation by ether from the methanolic solution, filtered, and chromatographed on a silicagel column (eluent CH_2Cl_2/MeOH 98:2) to give 2.05 g (2.80 mmol, 38%) of a pure white powder. Anal. Found: C, 50.08; H, 5.74; N, 1.33. $C_{178}H_{244}N_4O_{114}$ requires C, 50.14; H, 5.77; N, 1.31. IR: ν_{max} 1748, 1654. ^{1}H NMR (400 MHz, $CDCl_3$): 5.40–5.20 [m, 14H, H$_3^{AB}$], 5.20–5.05 [m, 14H, H$_1^{AB}$], 4.90–4.72 [m, 14H, H$_2^{AB}$], 4.65–4.49 [m, 7H, H$_6^A$], 4.40–4.20 [m, 7H, H$_6^A$], 4.20–4.10 [m, 14H, H$_5^{AB}$], 3.85–3.60 [m, 14H, H$_4^{AB}$], 3.60–3.40 [m, 8H, H$_6^B$, C*H*$_2$, crown]. ^{13}C NMR (100 MHz, $CDCl_3$): 170.8, 170.4, 169.4 [*C*OCH$_3$], 159.1 [NH*C*ONH], 96.8 [C_1], 76.6 [C_4], 71.5, 71.1, 69.6 [C_2, C_3, C_5], 62.6 [C_6], 50.4 [$C_{6'}$], 40.9 [*C*H$_2$, crown]. HR-ESIMS *m*/*z*: [$C_{178}H_{244}N_4Na_2O_{114}$: 4307.3204]; 2153.6602 [M+2Na]$^{2+}$/2, 1436.1092 [M+2Na]$^{3+}$/3.

*5.2.4. 1,10-N,N′-Bis-[cyclomaltoheptaosyl-6^A-deoxy-6^A-ureido]-4,7,13,16-tetraoxa-1,10-diazacyclooctadecane **7***

The peracetylated bis-β-CD-crown ether **6** (2.0 g, 0.47 mmol) was dissolved in anhydrous MeOH (33 mL). The solution was chilled in an ice bath at 0 °C and a 1 M MeONa solution (32.9 mmol) was added dropwise. The mixture was stirred for 1 h under Ar at 0 °C, then for 1 h at rt. Small amounts of IRN77® ion exchange resin were added until neutralization at pH=7.0. The resulting suspension was filtered off, the filtrate was evaporated to dryness, the solid product was dissolved in distilled water (15 mL), and finally lyophilized to give 1.78 g (98%, 0.69 mmol) of a pure white amorphous powder. Anal. Found: C, 42.04; H, 5.79; N, 1.76. $C_{98}H_{164}N_4O_{74}\cdot 12H_2O$ requires C, 42.06; H, 6.77; N, 2.00. IR: ν_{max} 3385 (OH) and 1637 (C=O urea) cm^{-1}. ^{1}H NMR (400 MHz, D_2O): 5.08 [m, 14H, H$_1$], 3.90–3.84 [br t, 14H, H$_3^{AB}$], 3.87 [s, 16H, *O*–C*H*$_2$ crown], 3.79–3.70 [complex m, 38H, H$_6^A$, H$_5^{AB}$], 3.63–3.42 [complex m, 32H, H$_6^B$, H$_4^{AB}$, H$_2^{AB}$], 3.35–3.10 [m, 8H, *N*–C*H*$_2$ crown]. ^{13}C NMR (100 MHz, D_2O): 102.3 [C_1], 81.3 [C_4], 73.5 [C_2], 72.5 [C_3], 72.2 [C_5], 70.6 [C_6^A], 60.6 [C_6^B], 49.1 [*C*H$_2$ crown], 41.7 [*C*H$_2$ crown]. HR-ESIMS *m*/*z*: [$C_{98}H_{164}N_4O_{74}$: 2582.9338]; 1291.4669 [M]$^{2+}$/2.

5.3. Preparation of [Busulfan/4] and [Busulfan/7] complexes

A solution of Busulfan (4.8 mg, 0.019 mmol) in DMSO (0.5 mL) was added under Ar to a solution of **4** or **7** (0.018 mmol) in H_2O (25 mL) at rt. The mixture was stirred again for 18 h and then lyophilized to yield quantitatively ivory amorphous powder.

5.4. Preparation of [aminoacid/4 or 7 or 8] complexes

A solution of the L-aminoacid (1×10^{-5} mol, 1 equiv) in 1 mL distilled water was added to a solution of compound **4**, **7** or **8** (1×10^{-5} mol, 1 equiv) in 4 mL distilled H_2O. The mixture was stirred for 18 h at room temperature then lyophilized to yield a white amorphous powder.

5.5. Crystallography

Data were collected at 173(2) K on a Bruker X8APEX diffractometer equipped with a APEX-I CCD area-detector, an Oxford Cryosystem liquid N2 low temperature device and a molybdenum microfocus sealed tube generator with mirror-monochromated Mo Kα radiation (λ=0.71073), operated at 50 kV/600 μA. The space group determination, structure solution and refinement were made using SHELXTL[8] software (2008.4 version). The hydrogen atoms were introduced at calculated positions and not refined (riding model). The position of the H atoms on disordered water molecules was not determined. The absolute configuration has been determined considering the absolute configuration of known stereocenters. Crystal data for **3**: $C_{66}H_{96}N_4O_{40}\cdot H_2O$, M=1603.48, triclinic, space group $P1$, a=10.3352(5) Å, b=10.9255(6) Å, c=19.8389(10) Å, α=76.233(2)°, β=76.428(2)°, γ=88.015(3)°, V=2114.51(19) Å^3, T=173(2) K, Z=1, D_c=1.259 g cm^{-3}, μ=0.106 mm^{-1}, 26,519 collected reflections, 15,650 independent (R_{int}=0.0341), GooF=1.339, R_1=0.1150, wR_2=0.2488 for $I>2\sigma(I)$ and R_1=0.2047, wR_2=0.2768 for all data. Crystallographic data (excluding structure factors) for the structure in this paper have been deposited with the Cambridge Crystallographic Data Center as supplementary publication under no. CCDC 708518. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44 (0)1223 336033 or e-mail: deposit@ccdc.cam.ac.uk).

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