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Supramolecular Linear Conglomerates Formed by β-Cyclodextrin Dimers and Sodium Deoxycholate

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The formation of supramolecular structures by the complexation of two bile salts—sodium cholate (NaC) and sodium deoxycholate (NaDC)—with four new head-to-head β-cyclodextrin dimers was studied by NMR techniques. All dimers form 1:2 (dimer:bile salt) stoichiometry complexes with NaC. With NaDC, linear supramolecular conglomerates of an *n*:*n* stoichiometry were obtained for all dimers. ROESY spectra confirmed the presence of electrostatic interactions when a protonated amino group is present in the linking group. The dependence of the pseudo-equilibrium constants with the electrostatic interactions and steric hindrance is discussed.

Keywords: Supramolecular conglomerates; β-Cyclodextrin dimers; Inclusion complexes; Sodium cholate; Sodium deoxycholate

INTRODUCTION

Cyclodextrins are cyclic oligomers built up from 6, 7, or 8 glucopyranose units, linked by α-(l-4)-glycosidic bonds, named α-, β-, and γ-cyclodextrins, respectively. They form inclusion complexes in water with a variety of organic molecules, a property used to increase the bioavailability of poorly soluble drugs [1–7]. Most of these inclusion complexes have a 1:1 stoichiometry [8], although higher stoichiometries are also known. Cyclodextrins also form high order superstructures as polyrotaxanes [9–14], catenanes [15,16] or nanotubes [17–19]. However, the use of cyclodextrins in constructing other types of supramolecular architectures as, for instance, polymer-like conglomerates, is almost unexplored.

To obtain the polymer-like conglomerates, several strategies can be designed. First, a self-complementary monomer or supramolecular synthon can be used. Takahashi *et al.* [20] have obtained a monosubstituted cyclodextrin which forms an intermolecular complex, but daisy-chain polymers similar to those developed by Stoddart *et al.* [21,22] from crown derivatives are not reported. Second, two complementary monomers can be complexed. In this case, one of the monomers has to be a ditopic guest while the other one is a ditopic host (this is the strategy that we have developed in the present paper). Obviously, when at least one of these monomers is a tritopic or polytopic derivative, branched polymer-like supramolecular structures can be formed [23]. Finally, polytopic hosts and polytopic guests can be used to form macromolecular assemblies [24,25].

Over the last 10 years numerous cyclodextrin dimers have been synthesized [26–46], the most common ones being head-to-head and tail-to-tail β-cyclodextrin homodimers. It has been largely proved that the cyclodextrin dimers are excellent ditopic hosts [26–51]. On the other hand, the complexation of ditopic guests by cyclodextrins and derivatives is also well documented in the literature (for a recent survey see Ref. [52]).

When a ditopic guest is complexed by a cyclodextrin dimer, two different situations can be achieved, as schematically represented in Fig. 1. The first situation (Fig. 1a), known as chelate binding,

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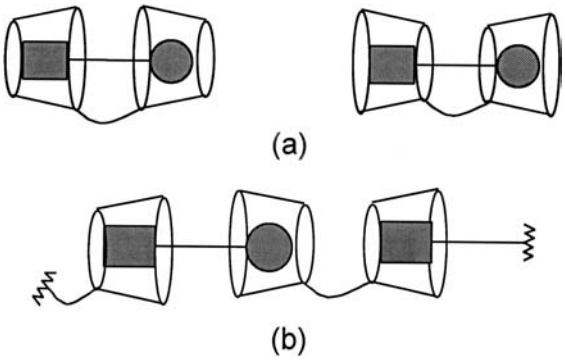

FIGURE 1 Schematic representation of complexes formed by a ditopic guest and a cyclodextrin dimer: (a) 1:1 complexes with chelate effect. (b) Linear supramolecular conglomerates (oligomers or polymers).

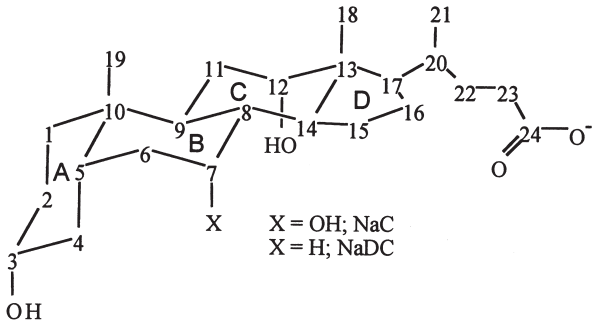

FIGURE 2 Structures of sodium cholate (NaC) and sodium deoxycholate (NaDC).

arises when the ditopic guest is complexed simultaneously by both cyclodextrins of the same dimer forming a 1:1 complex. When a cooperative effect is present, a higher stability constant than that expected for the complexation by isolated cyclodextrins, is observed. The chelate effect or cooperative binding has been largely studied by Breslow *et al.* [27,47–50] and others [29,32,33,35,37–39,43–45,51]. The chelate effect of cyclodextrin dimers can be exploited in different chemical and biological applications [30,31,34,38,41,42,47,53,54]. The second situation (Fig. 1b), much less explored in the literature, arises when a ditopic guest is complexed by two cyclodextrins belonging to two dimers, resulting in the formation of high order supramolecular entities (oligomer or polymer-like) with an *n*:*n* stoichiometry [26,40].

Bile salts are natural surfactants [55–60] which play an important role in the digestive process, interacting with food lipids, allowing their solubilization and absorption by the body. Common bile salts have two or three hydroxyl groups at C3, C7 or C12 positions of the characteristic tetracyclic skeleton of steroids, and a mobile side chain at the C17 position which ends with a carboxyl group (Fig. 2).

As a result of an increasing interest [23,35,40,46,61–79], the main features of complexing bile salts by cyclodextrin monomers have been well established. For instance, trihydroxy bile salts (as sodium cholate, NaC) behave as monotopic guests, while dihydroxy bile salts (as sodium deoxycholate, NaDC) behave as ditopic guests when they are complexed by β-cyclodextrin [23,40]. When complexing ditopic bile salts with cyclodextrin dimers both situations of Fig. 1 have been found. High order supramolecular structures have been obtained when a head-to-head β-cyclodextrin dimer was used [40], while tail-to-tail dimers form 1:1 complexes [35]. It seems that the key differentiating factor is the type of dimer used during complexation.

Therefore, the first aim of this study is to view the different possibilities for head-to-head cyclodextrin dimers. The second aim of this study is to evaluate the effect of the bridge-linking group (amine vs. amide) and the conformation of the spacer on the formation of the supramolecular conglomerates.

MATERIALS AND METHODS

Commercial bile salts (Sigma–Aldrich) and β-cyclodextrin (kindly supplied by Roquette) were purified as previously reported [40,72] and dried in a vacuum oven. DMF was dried over CaH_2 and distilled under reduced pressure before use. Other chemicals were used without further purification. Thin-layer chromatography (TLC) was performed on aluminium-backed silica gel plates $60F_{254}$ (Merck) eluting with ethyl acetate: isopropyl alcohol: water: 25% NH_4OH (2:3:4:0.3) and developed with ultraviolet light, 5% H_2SO_4 in MeOH, or 0.2% ninhydrin in EtOH sprays followed by charring. L-SIMS + Mass spectra were determined on a Micromass Autospec Spectrometer. ^1H, ^{13}C and DEPT 135 NMR spectra were recorded on a Brucker spectrometer at 300 and 75 MHz at 298.1 (± 0.1) K. ROESY spectra were recorded on a Brucker spectrometer at 500 MHz. Conditions for ROESY were as follows: total sample concentration, 10 mM (dimer plus bile salt) with a stoichiometric ratio corresponding to the maximum of Job's plot (samples were kept 24 h for equilibration before measurement); relaxation delay, 0 s; mixing time, 300 ms; spectral width, 10 ppm with 1024 complex points in *f*2; 128 *t*1 values and 8 scans per *t*1 value. All NMR experiments were carried out in D_2O.

The procedure for the determination of the equilibrium constants can be found elsewhere [85,86]. Experimental data were fitted to the appropriate equations by using a nonlinear least-squares computer program to obtain K_s and $\Delta\delta_{max}$ as fitting parameters.

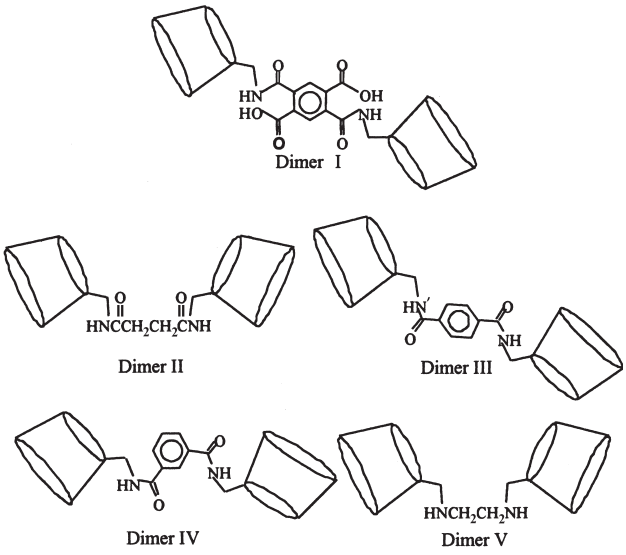

FIGURE 3 Structures of β-cyclodextrin head-to-head dimers. Dimer I has been previously reported [26,40].

Synthesis

6-O-tosyl-β-cyclodextrin (β-CDTs) [87], 6-deoxy-6-amino-β-cyclodextrin (β-$CDNH_2$) [40], 6-deoxy-6-(aminoethyl)amino-β-cyclodextrin (β-CDen) [72] were synthesized according to the procedures described in the cited literature.

Synthesis of Dimer II (*N,N′*-bis(6-deoxy-β-cyclodextrin)succinamide) was carried out by two methods, the *m*-nitrophenyl procedure [80] and an acyl chloride procedure [23] (also used for the synthesis of the other dimers). Succinoyl chloride (0.034 g, 0.22 mmol) was added to a cold solution (0°C) of β-$CDNH_2$ (0.5 g, 0.44 mmol) and triethylamine (75 μl, 0.54 mmol) in dry DMF (20 ml). The mixture was stirred at 0°C for 1 h and at r.t. overnight. The solvent was partially removed under reduced pressure, and precipitated in acetone ($3-4x$). Finally the product was purified through Sephadex C-25 and A-25 columns, using water and 0.2 M NH_4HCO_3 as eluent, to obtain a white solid in a 58% yield. R_f 0.6; ^{1}H NMR δ 4.95 (s, 14H, H1), 3.46–3.88 (m, 80H, H2, H3, H4, H5, H6), 3.22–3.33 (m, 4H, H6′), 2.37–2.5 (m, 4H, CH_2CH_2); ^{13}C and DEPT 135 δ 177.35 (CONH), 104.48 (C-1), 85.72 (C-4′), 83.77 (C-4), 75.70 (C-5), 74.70 (C-3), 74.43 (C-2), 72.82 (C-5′), 62.96 (C-6), 42.79 (C-6′), 33.61 (CH_2CH_2); L-SIMS + m/z 2348.91 (M^+).

Dimer III (*N,N′*-bis(6-deoxy-β-cyclodextrin)terephthalamide) was synthesized by reaction of β-$CDNH_2$ (0.5 g, 0.44 mmol) with terephthaloyl chloride (0.0406 g, 0.2 mmol), according to the former method. A 60% yield was obtained. R_f 0.6; ^{1}H NMR δ 7.86 (s, 2H, aromatic H), 5.05 (m, br, 14H, H1), 3.56–3.97 (m, 80H, H2, H3, H4, H5, H6), 3.27 (d, J = 11.37 Hz, 4H, H6′); ^{13}C and DEPT 135 δ 172.10 (CONH), 139.19 (substituted aromatic carbons), 130.16 (CH aromatic ring), 104.48 (C-1), 86.28 (C-4′), 83.71 (C-4), 75.69 (C-5), 74.67 (C-3), 74.41 (C-2), 72.74 (C-5′), 62.91 (C-6), 43.74 (C-6′); L-SIMS + m/z 2398.05 (M^+).

Dimer IV (*N,N′*-bis(6-deoxy-β-cyclodextrin)isoterephthalamide) was synthesized by reaction of β-$CDNH_2$ (0.5 g, 0.44 mmol) with isoterephthaloyl chloride (0.406 g, 0.2 mmol), in a 53% yield. R_f 0.58; ^{1}H NMR δ 8.08 (s, 1H, aromatic H), 7.87 (d, J =

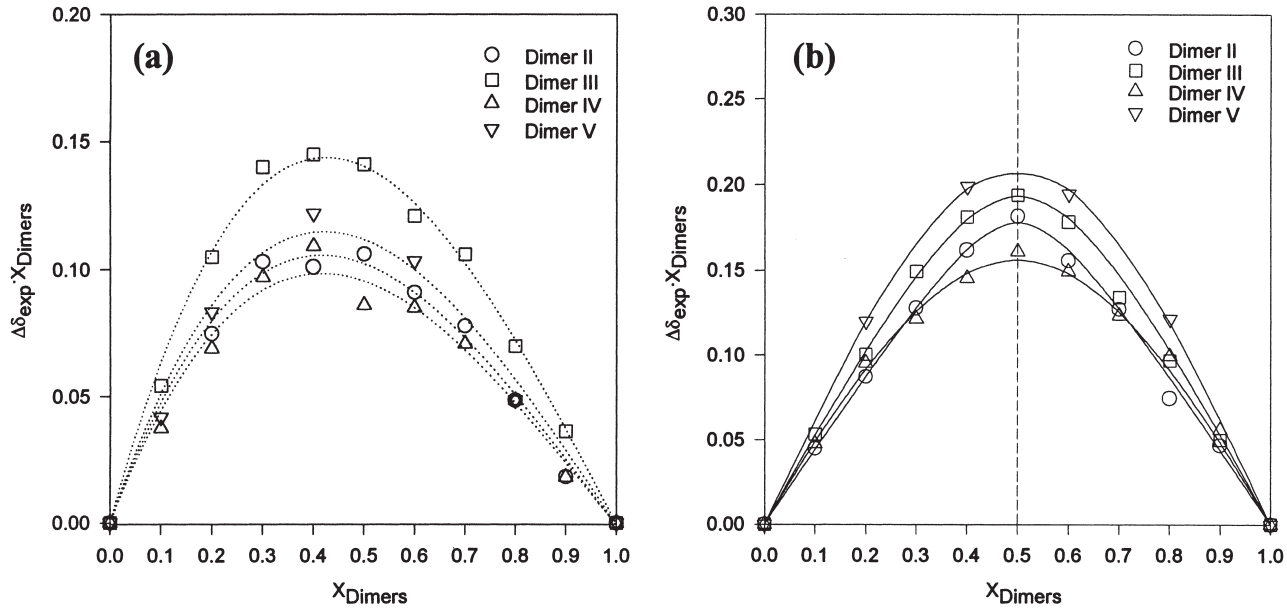

FIGURE 4 Job's Plot corresponding to the chemical shift displacement of carbon 1 of β-cyclodextrin dimers with NaC (a) and NaDC (b). Total concentration: [Dimer] + [Bile salt] = 10 mmol. Dot lines in (a) are polynomial fits of experimental data and are used as guides for the eye. Solid lines in (b) are the result of a nonlinear fit of the data to a 1:1 system with parameters given in Table II. Results for Dimer I have been published elsewhere [26,40].

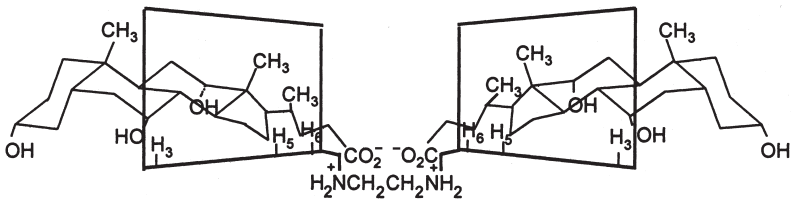

FIGURE 5 Schematic representation of the 1:2 Dimer V/NaC complex deduced from ROESY intra and intermolecular cross-peaks.

7.58 Hz, 2H, aromatic H), 7.55 (d, $J = 7.58$ Hz, 1H, aromatic H), 4.95 (m, br, 14H, H1), 3.35–3.86 (m, 80H, H2,H3, H4, H5, H6), 3.26 (d, $J = 10.95$ Hz, 4H, H6′); ^{13}C and DEPT 135 δ 172.23 (CONH), 136.63 (substituted aromatic carbons), 133.27, 132.00, 128.49 (CH aromatic ring), 104.49 (C-1), 86.26 (C-4′), 83.70 (C-4), 75.70 (C-5), 74.69 (C-3), 74.42 (C-2), 72.85 (C-5′), 62.92 (C-6), 43.75 (C-6′); L-SIMS + m/z 2398.05 (M^+).

Dimer V (*N*,*N*′-bis(6-deoxy-β-cyclodextrin)ethylendiamine) was synthesized as follows: β-CDTs (0.55 g, 0.425 mmol) and NaI (0.006 g, 0.04 mmol) were added to a solution of β-CDen (0.25 g, 212 mmol) in dry DMF (20 ml), and the reaction was stirred at 40°C for 48 h. The solvent was partially removed under reduced pressure, precipitated in acetone (3–4x), and finally purified through Sephadex C-25, obtaining a white solid in a 80% yield. R_f 0.2; ^{1}H NMR δ 4.92 (m, br, 14H, H1), 3.44–3.86 (m, 80H, H2, H3, H4, H5, H6), 3.26 (m, 4H, H6′), 2.75 (m, 4H, CH_2CH_2); ^{13}C and DEPT 135 δ 104.49 (C-1), 86.15 (C-4′), 83.74 (C-4), 75.70 (C-5), 74.68 (C-3), 74.44 (C-2), 72.42 (C-5′), 62.89 (C-6), 51.49 (C-6′), 50.27 (CH_2CH_2); L-SIMS + m/z 2294.01 (M^+).

RESULTS AND DISCUSSION

Three amide-linked (Dimer II, III and IV) and one amine-linked (Dimer V) dimers were synthesized (see Fig. 3). The Dimer II was synthesized by the methods proposed by Easton *et al.* [80] and Alvarez-Parrilla *et al.* [23]. The second method involves fewer steps and shows to give higher yields of products than the former method. This method was also adopted for synthesizing all other amide dimers. The amine Dimer V was synthesized by the reaction of β-CDTs with β-CDen (see "Materials and methods" section) in the presence of NaI, by a modification of the method proposed by May *et al.* [81] for the synthesis of polyamine cyclodextrin monomers.

The stoichiometry of the complexes formed between β-cyclodextrin dimers and bile salts (NaC and NaDC) was determined by the continuous variation technique (Job's method), by measuring the chemical shift displacement of carbon 1 in cyclodextrins for different concentrations of hosts and guests. Figure 4 shows the obtained results for all dimer:NaC and dimer:NaDC systems. The maxima in Fig. 4a for all the four dimers synthesized ($x_{\text{Dimer}} = 0.3–0.4$) indicate that NaC forms 1:2 (Dimer: NaC) complexes. This is in agreement with previously reported results for Dimer I [40]. This indicates that NaC behaves as a monotopic guest and that the complex structure does not correspond to those shown in Fig. 1 (see below). For NaDC the maxima at $x_{CD} = 0.5$ (Fig. 4b) indicate an *n*:*n* stoichiometry for the complexes. This behavior of NaDC as a guest clearly differs from that observed for NaC. However, de Jong *et al.* [35] observed 1:1 stoichiometries for both bile salts when they are complexed by tail-to-tail dimers, and suggest a cooperative binding of both cyclodextrin units in these dimers as shown in Fig. 1a.

It has been demonstrated that trihydroxy bile salts form 1:1 complexes with β-cyclodextrin, and that they enter into the β-cyclodextrin cavity by its secondary hydroxyl rim. This fact is mainly supported by the high number of interactions of H3 with hydrogens of the C and D rings, and the absence of similar interactions with H6 [40,82]. The absence of interactions between cyclodextrin protons and P1–6 of the A and B steroidal rings is also noticeable (the notation Pn is used for the bile salts protons, where *n* is the carbon number indicated in Fig. 2). However, dihydroxy bile salts form inclusion complexes with β-cyclodextrin with a 1:2 stoichiometry. In this case, the ROESY spectra clearly show interactions of P1-6 with H3 [82]. That is to say, trihydroxy and dihydroxy bile salts are monotopic and ditopic guests, respectively, when they are complexed by β-cyclodextrin monomers.

The different behavior of NaC and NaDC in their complexation with head-to-head dimers can be explained as follows: (i) NaC behaves as a monotopic guest and each β-cyclodextrin unit of the dimer complexes one bile salt molecule. This results in a 1:2 stoichiometry complex (see Fig. 5). (ii) NaDC behaves as a ditopic guest entering each β-cyclodextrin unit of a dimer by its secondary hydroxyl rim and leaving free its second guest position which is complexed by a second β-cyclodextrin unit belonging to a different dimer molecule. This results in the formation of supramolecular conglomerates of *n*:*n* stoichiometry as shown in Fig. 1b.

TABLE I ROESY intermolecular cross-peaks observed between protons (Pn) of the bile salts sodium cholate (NaC) and sodium deoxycholate (NaDC) with protons (Hn) of the Dimers II–V (w, weak; m, medium; s, strong interactions)

		NaC			NaDC						
		Dimer V			Dimer II	Dimer III	Dimer IV		Dimer V		
Location	Hydrogen	H3	H5	H6	H3	H3	H3	H5	H3	H5	H6
Side Chain	P23	s							s		
	P22	s		w	m	m	m		s		w
	P21	s	w	w	s	s	s	w	s	w	w
	P20	s			m	m	m	w	m		
C/D ring	P18	s			s	s	s		s		
D ring	P17	s	w		m	m	m	w	s		
	P16	s	w		m	m	m		m		
	P15	s			m	m	m	w	m		
C/D ring	P14	m			w				m		
C ring	P12	m							w		
	P11	m									
B/C ring	P9	w									
	P8	m									
B ring	P7	w									
	P6				w	w	w				
A ring	P5				w	w	w		w		
	P4				m	w	w		w		
	P3				m	m	m		m		
	P2				m	m	m		m		
	P1				m	m	m		m		

To confirm the previous hypothesis, ROESY experiments were carried out for all systems. The assignment of NaDC protons was made following Campedron *et al.* [83] and Barnes and Geckel [84]. As an example, Table I summarizes the intermolecular cross-peaks between cyclodextrin protons of Dimer V and NaC protons. It must be noticed that H3 experiences interactions with protons of the B, C and D rings of the steroid nucleus. No interactions have been observed with the protons of the A steroid ring. Interactions have also been observed with protons P20–23 of the side chain. Furthermore, H5 shows interactions with protons of the D ring while H5 and H6 only show cross-peaks with protons of the side chain. It is also noticeable that H5 and H6 do not interact with A–C rings. All this information suggests the structure shown in Fig. 5 in which each cyclodextrin unit of the dimer is complexing one NaC molecule. The steroid body enters into the cavity through the secondary hydroxy1 rim of the cyclodextrin, i.e. by the tail of the dimer, as suggested by the interactions with H3 and the absence of similar interactions with H5 and H6. The interactions of H6 with P21 and H3 with P7 (B ring) can be accepted as a measurement of the depth of the inclusion of the guest in the host. The distances H3–H6 (approximately 4.9 Å) and P7–P21 (approximately 7.3 Å) are compatible with such an interpretation.

Vázquez Tato *et al.* [40,72] have suggested the unfolding of the side chain of the bile salts towards the protonated amine group of cyclodextrin derivatives. The observed interactions between the protons of the side chain and the steroid body when NaC is complexed by Dimer V (results not shown) are also in agreement with that interpretation. The comparison of present results (interactions between H3 and B ring protons) with those obtained for Dimer I [40], suggests a deeper inclusion of the bile salt into the cavity of the cyclodextrin due to the electrostatic interactions between the carboxylate anion of NaC and the protonated amine group of Dimer V.

Table I also summarizes the observed interactions between Dimers II–V with NaDC. The main features deduced from this table are: (i) there are no interactions between H6 and protons of the steroid body; (ii) H3 interacts with protons of A, C and D rings, as well as with the P20–22 protons of the side chain; and (iii) B ring of the steroid body does not experience any interaction. Therefore, it can be deduced that NaDC is included in the β-cyclodextrin cavity by both sides of the steroid body, behaving as a ditopic guest [23,26,40,82]. Previously mentioned interactions and the absence of H6-steroid body interactions unequivocally rule out the chelate structure of Fig. 1a (in which the ditopic guest is complexed by the two cyclodextrins of one dimer molecule) since dimers are linked at the primary hydroxyl rim. If this were the case, the NaDC should enter into the cyclodextrin cavity by the primary hydroxyl rim and interactions between H6 and the steroid body should be observed. The only possible explanation for the observed interactions of both opposite sides of NaDC with the β-cyclodextrin cavity and the absence of interactions of the B steroid ring is that they are complexed by two β-cyclodextrin units belonging to different dimer molecules. Since the observed stoichiometry is 1:1

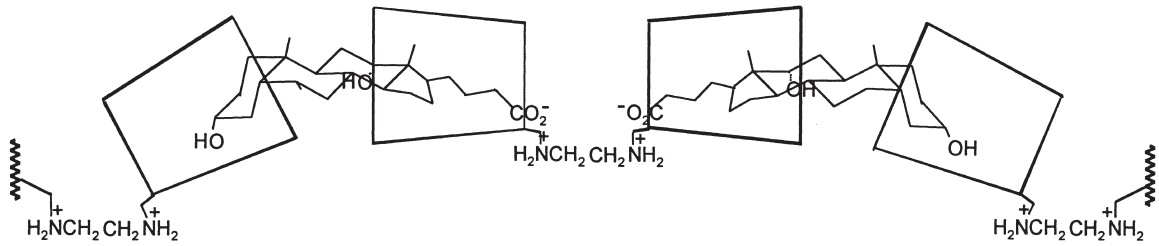

FIGURE 6 Schematic representation of the supramolecular polymers obtained by complexing NaDC with Dimer V.

(instead of the 1:2 observed for NaC), the only conclusion in agreement with all these evidences is that the formation of linear conglomerates has taken place. Figure 6 shows the proposed structure of these conglomerates (oligomers or polymers) for Dimer III (see also Fig. 1b).

The degree of inclusion of NaDC into the cyclodextrin cavity, and consequently the complex stability, can be explained by electrostatic interactions[¶] and steric hindrance. This is confirmed by the values of the pseudo-equilibrium constants determined from ^{13}C NMR chemical shift displacements of the carbon 1 of the cyclodextrin. The pseudo-equilibrium constants, K_s, and the maximum chemical shift displacements, $\Delta\delta_{max}$, for *n*:*n* Dimer: NaDC systems are shown in Table II.

Dimer II (with an aliphatic chain as linker) has the highest pseudo-equilibrium constant value, while Dimer IV (with a meta-substituted aromatic ring as linker) has the lowest one. Dimers I [40] and III (both with para-substituted aromatic rings as linkers) have similar pseudo-equilibrium constants. The lowest K_s value observed for Dimer IV may be due to the steric hindrance produced by the proximity of both cyclodextrin units in the dimer. This is supported by the fact that the obtained yield in the synthesis of Dimer IV is lower ($\approx$10% less) than that for Dimer III. Furthermore, the synthesis of the dimer with an ortho-substituted aromatic linker was not possible due to the large steric hindrance arising from the proximity of the two bulk cyclodextrins. However the flexible linker of Dimer II allows the reduction of the steric hindrance during the formation of the supramolecular structure. Consequently the pseudo-equilibrium constant value is higher.

The difference between the pseudo-equilibrium constant values for Dimers II and V is quite interesting. Both dimers have a flexible (amide and amine, respectively) chain as the linker. The protonated amine group in Dimer V and the negative carboxylated side chain of NaDC interact electrostatically, as commented on above. This favorable interaction promotes a deeper inclusion of the steroid body into the cavity of the first cyclodextrin but a less profound inclusion of the second complexing site into the cavity of a second cyclodextrin, reducing the stability of the complex. The smaller pseudo-equilibrium constant of this supramolecular structure suggests that the stabilization effect is weaker than the destabilization one.

From these results, it is possible to conclude that the stability of the linear supramolecular structures depends on the inclusion degree of the two sites of NaDC inside the cavity of each cyclodextrin. The deeper is the inclusion of the first site, the lower will be that of the second one. The corresponding effects on the global complex equilibrium are opposite to each other. Therefore, the overall change in the observed pseudo-equilibrium constant depends on their relative importance. The degree of inclusion is controlled by steric hindrances and electrostatic interactions. Present results indicate that to form supramolecular conglomerates structures, an aliphatic neutral bridge between the two cyclodextrins is a better choice than a rigid ring or a charged linker when the host is a charged ditopic guest like NaDC.

TABLE II Pseudo-equilibrium constants, K_s and ^{13}C NMR maxima chemical shift displacements $\Delta\delta_{max}$ of C-1 of cyclodextrin dimers, for the *n*:*n* complexes formed between sodium deoxycholate with Dimers IβV

Host	$K_s/10^3\,M^{-1}$	$\Delta\delta_{max}$/ppm
Dimer I	2.0 ± 0.3	0.43 ± 0.01
Dimer II	3.2 ± 0.8	0.46 ± 0.01
Dimer III	1.2 ± 0.3	0.58 ± 0.02
Dimer IV	0.4 ± 0.1	0.66 ± 0.06
Dimer V	0.6 ± 0.1	0.80 ± 0.03

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[¶]As in the case of NaC, the observed interactions between the side chain and the steroid body suggest that the side chain is unfolded toward the amine group in protonated Dimer V, as it was observed with amino derivatives of β-cyclodextrin [40,72].

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