J Incl Phenom Macrocycl Chem (2009) 63:309–317
DOI 10.1007/s10847-008-9524-3

ORIGINAL ARTICLE

Solubilization of cholesterol in aqueous solution by two β-cyclodextrin dimers and a negatively charged β-cyclodextrin derivative

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Received: 27 June 2008 / Accepted: 1 December 2008 / Published online: 12 December 2008

Abstract Two βCD dimers (linked by succinic acid, **2**, or ethylenediaminetetraacetic acid, EDTA, **3**, bridges) and a negatively charged monomer derivative of βCD, **1**, have been synthesized and their ability to solubilize cholesterol in aqueous solution was studied. The three compounds exhibit a great capacity in solubilizing cholesterol as, for instance, concentrations up to 6 mM of cholesterol were measured in the presence of 25 mM of **3**. The phase-solubility diagrams of the two dimers exhibit A_L type profiles while the monomer **1** follows an A_P isotherm. The cholesterol/dimer complexes have 1:1 stoicheiometries while monomer **1** forms two complexes with molar ratios of 1:1 and 1:2 (cholesterol/**1**). The equilibrium constants are $K_{1:1} = (5.9 \pm 0.3) \times 10^4\ M^{-1}$ and $K_{1:1} = (8.8 \pm 0.2) \times 10^4\ M^{-1}$ for **2** and **3**, respectively, and $K_{1:1} = 73 \pm 19\ M^{-1}$ and $K_{1:2} = 204 \pm 65\ M^{-1}$ for **1**. The comparison of $K_{1:1}$(**3**) with the product $K_{1:1} \times K_{1:2}$ (**1**) reveals that a chelate effect in binding the cholesterol by **3** exists. The structure of the cholesterol/**3** complex was studied by ROESY experiments and by molecular dynamics simulations.

Keywords Cholesterol · Phase-solubility diagrams · Inclusion complex · Chelate effect · Cyclodextrin dimers

Introduction

Cyclodextrins are cyclic oligomers of α-D-glucopyranose units linked by α-(1-4)-glucosidic bonds [1]. As a consequence of the $^4C_1$ conformation of the glucopyranose units, all secondary hydroxyl groups are situated on one of the two edges of the ring, and all primary ones are placed on the other edge. Natural cyclodextrins (αCD, βCD, and γCD, having 6, 7 or 8 glucopyranose units, respectively) have a rigid structure due to the formation of intramolecular hydrogen bonds between the O2–H and O3–H hydroxyl groups of adjacent glucose units. They are toroidal or truncated cone shaped, the larger diameter corresponding to the secondary rim, and contain a relatively hydrophobic cavity [1]. The interior of the cavity is not a smooth cone but has constrictions in the neighbourhoods of the H3 and H5 atoms [2] and all of the O4 atoms are virtually coplanar [1]. Because of this structure, they can form inclusion complexes in water with a large variety of organic molecules [3], a property used to increase the bioavailability of poorly soluble drugs [4, 5]. The complexation of cholesterol (Fig. 1) by natural cyclodextrins and derivatives has been widely studied [5–14].

There are several reasons which justify the interest on cholesterol/cyclodextrin interactions all over the years. Among them, we can mention: (i) Cholesterol is a lipid found in the cell membranes of all tissues. It plays a central role in many biochemical processes, such as the composition of cell membranes (regulating membrane fluidity). Since cyclodextrins can be used for drug formulations [4, 5, 15], including some steroids [16–19], it is suspected that cholesterol/cyclodextrin interactions can lead to undesirable side effects. In fact, it is well known that cyclodextrins can remove cholesterol from membranes [6, 20] and erythrocytes [21], and cholesterol depletion by βCD disrupts caveolae structure [22]. The effects of cyclodextrins on membranes differ from those of detergents that first incorporate themselves into membranes and then extract membrane components since cyclodextrins do not incorporate into membranes [6]. (ii) The cholesterol/

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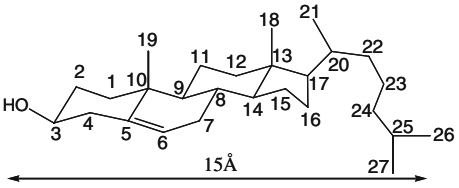


Fig. 1 Structure of cholesterol

cyclodextrin interaction can modify the cholesterol metabolism. For instance, experiments with rabbits show that a single intravenous administration of hydroxypropyl-βCD temporarily decreased the level of total cholesterol in serum and repeated administration of this cyclodextrin led to a gradual increase in total cholesterol in circulation and eventually to a slight relief of atherosclerotic lesions in the thoracic aorta [23]. On the other hand, the cholesterol-βCD complex has a limited aqueous solubility and its crystallization in renal tissue might explain the toxicity [24] of parenterally administered βCD. The absence of such crystallization might explain the lower nephrotoxicity of hydroxypropyl-βCD after intravenous administration [7]. (iii) Cyclodextrins can be used to obtain low cholesterol foods even at industrial scale [25–32]. (iv) Standardization of cholesterol assays often involve the use of solutions of this steroid containing organic solvents. Karuppiah et al. [33] have suggested the use of the cholesterol/hydroxylpropyl-βCD complex as a primary cholesterol standard, offering an excellent alternative to the utilization of organic solvents or detergents.

Many cyclodextrin oligomers have been synthesized during recent years [34–36]. The complexation of a ditopic guest with cyclodextrin oligomers (particularly dimers) can conduct to either a chelate effect [37, 38] or to the formation of polymers [39]. Although some dimers have been used to complex steroids, mainly bile salts [40–45], as far as we know only Breslow and Zhang [8] have studied the enhancement of cholesterol solubility in water by a βCD dimer (in which the two cyclodextrin residues are linked by a thioether bridge). If a chelate effect is present, the equilibrium constant for the formation of a guest/cyclodextrin complex by a dimer will be higher than that for the complexation of the same guest by two independent cyclodextrins. In this context, it is important to remark that the cholesterol length is almost twice the high of the cyclodextrin cone [1], allowing a complexation by two cyclodextrin units, as demonstrated for some bile salts [40, 46, 47]. If the dimer has a better solubility than the cyclodextrin monomer, this will offer an excellent chance of enhancing the cholesterol solubility in water and of forming a more stable inclusion complex.

With these precedents in mind we have synthesized the three βCD derivatives shown in Fig. 2 and explored their ability of solubilizing cholesterol in aqueous solution. When a ditopic guest (as cholesterol) is complexed by a ditopic host (as **2** and **3**) a chelate binding [38] can arise when the ditopic guest is complexed simultaneously by both cyclodextrin residues of the same dimer, forming a 1:1 complex. [48, 49]. Otherwise, polymers with an *n*:*n* stoichiometry can be formed [39]. To check whether a chelate effect exists when cholesterol is complexed by **2** or **3**, the associated equilibrium constant has to be compared with the equilibrium constant determined for suitable monomers. Since **2** is neutral, β-cyclodextrin is an appropriate reference compound. However, since **3** is double negatively charged, the reference monomer should be single charged in order to guarantee that a hypothetical 1:2 (cholesterol/refence monomer) complex is double charged. For this reason, the anionic cyclodextrin derivative **1** has been synthesized and the cholesterol/**1** interaction studied.

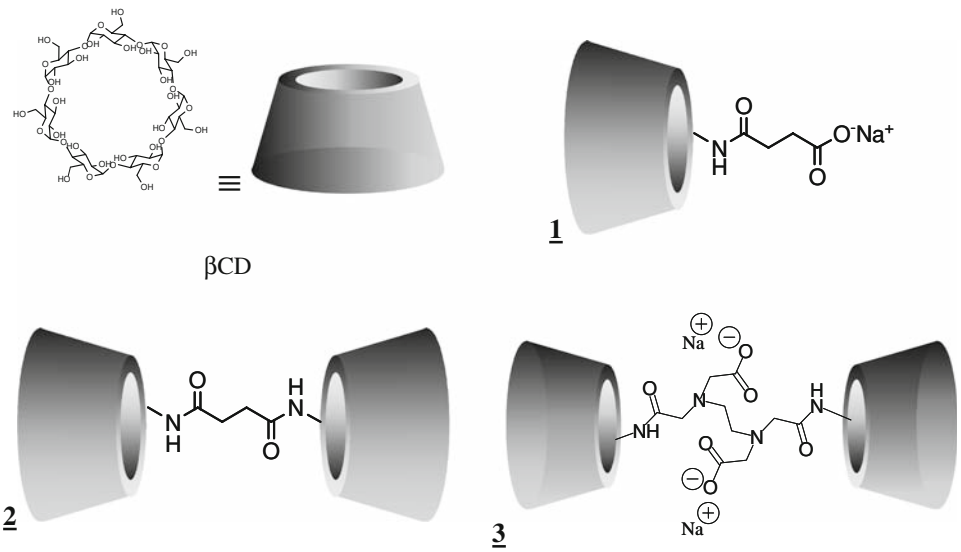


Fig. 2 βCD derivatives studied in this paper

Experimental

Solutions containing various concentrations of **1**–**3** in water were stirred with an excess of solid cholesterol for 24 h at 25.0 ± 0.2 °C. Then the solution was filtered through a membrane filter (0.22 μm) and cholesterol in the filtrate was determined by an enzymatic method (SPINREACT kit). Stirring for 72 h did not show any statistical difference with results after 24 h indicating that one day is enough to reach the thermodynamic equilibrium. Measurements were carried out in a bicarbonate/carbonate buffer (50 mM) at pH = 9.75. This high pH was chosen to prevent the existence of any protonated species in the solution, in particular those derived from the protonation of the nitrogen atoms of the bridge since pK_a values in the ranges 3.5–4.4 and 6.7–7.3 have been published for the dissociation of the tertiary amino groups of EDTA diamides [50].

Synthesis of the compounds

Synthesis of **1**: 1.2 g of 6-NH_2-βCD (1.03 mmol) and 0.5 ml de triethylamine were solved in 5 mL of dry DMF. The mixture was added over a solution of 0.12 g of succinic anhydride (1.2 mmol) and stirred at 50 °C for one day under argon atmosphere. H_2O (~5 mL) was then added and the resultant mixture stirred 1 h at room temperature. After evaporation, the residue was taken up in H_2O (~5 mL) and purify by column (Sephadex C-25) chromatography, eluted with water. Yield 79%. NMR. ^{1}H NMR (D_2O, 300 MHz, δ/ppm): 4.99–4.96 (m, 7H–C(1)), 3.89–3.38 (m, 42H, cyclodextrin), 2.51–2.45 (m, 4H, CH_2-succ)); ^{13}C NMR; (D_2O, 300 MHz, δ/ppm): 181.05 (COOH); 177.5 (CONH); 102.6 C(1); 83.6, 82.1 C(4), C(40); 73.9, 73.6, 72.9, 72.8, 72.7 (C(2), C(3), C(5), C(20), C(30)); 70.7 (C(50)); 62.2 (C(6)); 42.8 C(60); 30.8 (succ-CH2COOH); 29.8 (succ-CH_2CONH). MALDI-TOF: Calculated = 1233.40 g mol^{-1}; observed = 1256.40 ($[M + Na]^+$).

Synthesis of **2**: 0.12 g of succinic acid (1.02 mmoles) and 0.33 g of 1-hydoxybenzotriazole, HOBT, (2.4 mmoles) were dissolved in 5 ml of dry DMF. Then 0.38 mL (2.4 mmoles) of N,N′-Diisopropylcarbodiimide, DIC, were added. The mixture was stirred for 30 min and then 3 g (2.6 mmoles) of 6-NH_2-β-CD were added. The reaction was stirred at room temperature for 24 hours under argon atmosphere. Then 0.33 g of HOBT, 0.38 mL of DIC and 1 g of 6-NH_2-β-CD were added. The reaction is stirred for 48 h at room temperature. The mixture is added on 50 mL of water, concentrated in vaccuo and purified in a Sephadex-C25 column with water as eluent. Yield 82%. NMR. ^{1}H NMR (D_2O, 300 MHz, δ/ppm): 4.99–4.96 (m, 14H-C(1)), 3.89–3.38 (m, 84H, cyclodextrin), 2.50–2.37 (m, 4H, CH_2-succ)); ^{13}C NMR; (D_2O, 300 MHz, δ/ppm): Main peaks 177.3 (CONH); 42.7 C(6'); 33.5 (succ-CH_2COOH); MALDI-TOF: Calculated = 2348.78 g mol^{-1}; observed 2371.78 ($[M + Na]^+$).

Synthesis of **3** (it has been synthesized previously by Yan et al. [51]): 6-NH_2-β-CD (1.2 g, 1.06 mmol) and EDTA dianhydride (0.12 g, 0.47 mmol) were dissolved in dry DMF (10 mL). Then 0.3 mL of triethylamine was added and the mixture stirred at 50 °C for one day under argon atmosphere. H_2O (~5 mL) was then added and the resultant mixture stirred 1 h at room temperature. After evaporation, the residue was taken up in H_2O (~5 mL) and purified is a Sephadex C-25 column with water as eluent. Yield 85%. R_f = 0.17 (Ethyl acetate:2-propanol:water:ammonia/2:3:4:0.3). NMR. ^{1}H NMR (D_2O, 500 MHz, δ/ppm): 5.13–5.04 (bs, 12H–C(1), 2H–C(1′)); 4.04–3.76 (m, 12H–C(3), 12H–C(5), 24H–C(6), 2H–C(3′), 2H–C(6′), 2H–C(5′), 4CH_2COO, 4CH_2CON); 3.72–3.64 (m, 12H–C(2), 2H–C(2′)); 3.63–3.50 (m, 12H–C(4), 2H–C(4′); 3.44 (t, J = 9.2, 2H–C(6′)); 3.22 (s, CH_2CH_2). ^{13}C NMR; (D_2O, 500 MHz, δ/ppm): 181.8 (COO); 177.6 (CON); 104.6 (C(1), C(1′)); 85.8 C(4′); 83.9 C(4); 75.9 C(2); 75.6 C(2′); 74.8 C(3); 74.7 C(3′); 74.6 C(5): 72.6 C(5′); 63.1 C(6); 61.1 (CH_2COO); 60.9 (CH_2CON); 55.5 (CH_2CH_2); 42.6 C(6′). MALDI-TOF: Calculated = 2524.21 g mol^{-1}; observed = 2544.82 ($[M + Na]^+$).

Results and discussion

Figures 3–5 show phase-solubility profiles of the increased solubility of cholesterol in water at various concentrations of the cyclodextrin derivatives. Maxima concentrations used were 2.49 mM (**2**), 20.1 mM (**1**) and 25 mM (**3**). The two derivatives carrying negative charges (**1** and **3**) are much more soluble than βCD in aqueous solution. In fact,

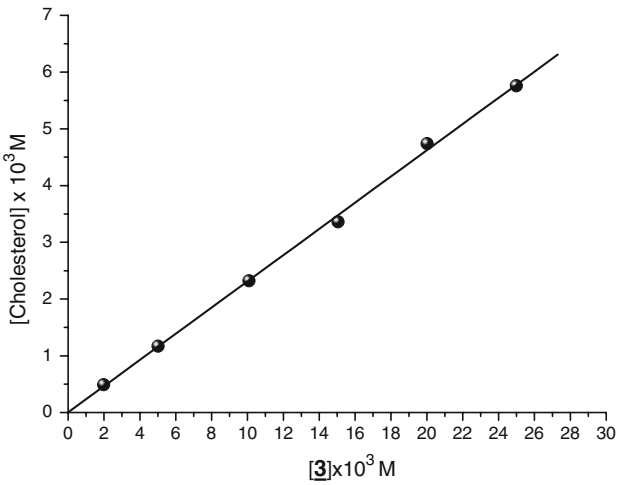


Fig. 3 Phase-solubility diagrams of cholesterol with **3** in bicarbonate/carbonate (50 mM) buffer at pH 9.75. Temperature was kept constant at 25.0 ± 0.2 °C

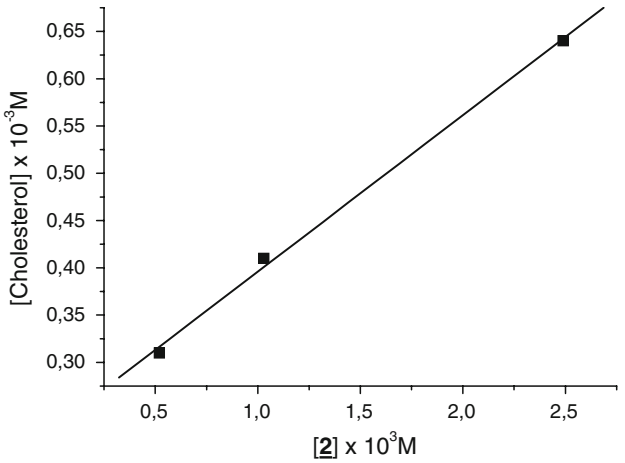


Fig. 4 Phase-solubility diagrams of cholesterol with **2** in bicarbonate/carbonate (50 mM) buffer at pH 9.75. Temperature was kept constant at 25.0 ± 0.2 °C

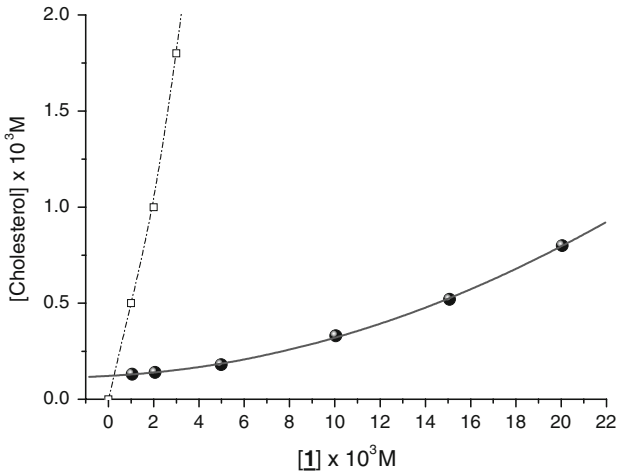


Fig. 5 Phase-solubility diagram of cholesterol with **1** in bicarbonate/carbonate (50 mM) buffer at pH 9.75 (●). Temperature was kept constant at 25 ± 0.2 °C. For comparative purposes the solubility curve for DOMβCD (taken from [12]) is also shown (□)

the mentioned maxima concentrations for these two derivatives are far below their maxima concentration in water. That is important in order to extract large amounts of cholesterol in industrial or biological applications. However, the neutral derivative **2** is much less soluble than βCD and its maximum solubility in water is around 5 mM.

Higuchi and Connors [52, 53]have analyzed the phase-solubility diagrams and classified them according to the shape of the plot of solubilized compound versus solubilizer agent. The analysis of these diagrams allows to determine the value of the stability constant and also to give insight into the stoichiometry of the complex. Following their classification, **2** and **3** dimers exhibit A_L type diagrams since cholesterol solubility linearly increases with dimer concentration. This behavior is similar to the one observed by Breslow and Zhang [8] for the thioether βCD dimer **4** (see Table 1). However, monomer **1** follows an A_P isotherm and the cholesterol solubility deviates upward from linearity. These isotherms have been observed by Frijlink et al. [7], Nishijo et al. [12], and Loftsson et al. [11, 14] for several derivatives of βCD with different degrees of substitution. βCD gives a B_s profile [7, 10] due to that the cholesterol-βCD complex has a limited aqueous solubility. This is not the case of complexes exhibiting A_L or A_P type diagrams. For instance, the cholesterol-**3** complex reaches a concentration of 6×10^{-3} M at [**3**] = 0.025 M (maximum studied concentration). The difference between the observed diagrams is a first indication that the stoichiometry of the inclusion complex cholesterol:**1** is different than those of the two dimers. The linearity of the plots in Figs. 3 and 4 indicates the formation of complexes with a 1:1 (cholesterol:dimer) stoichiometry while the A_L diagram (Fig. 5) suggests that higher stoichiometries are involved. If the complex has a 1:1 stoichiometry, the constant $K_{1:1}$ for the equilibrium

$$CD + Ch \rightleftarrows Ch - CD$$

is given by Eq. (1),

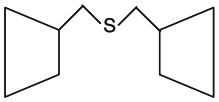
$$K_{1:1} = \frac{[Ch - CD]}{[Ch][CD]} \tag{1}$$

(*CD*: cyclodextrin; *Ch*: cholesterol; *Ch-CD*: inclusion complex). For an A_L diagram, $K_{1:1}$ can be calculated by Eq. (2) [52]

$$K_{1:1} = \frac{\text{slope}}{g_0(1 - \text{slope})} \tag{2}$$

where *slope* refers to the slope of the A_L diagram and g_o is the solubility of cholesterol in water. g_o is often obtained as the intercept of the [cholesterol] versus [solubilizer] plot. This method can be appropriate when the solubilized cholesterol is not too far from its own solubility in water [8]. Loftsson et al. [54] have pointed out that generally the intercept values derived from phase-solubility analysis are only good estimates for g_o in cases where the solubility of the drug is ≥1 mM. In other cases, its use can lead to spurious results. Results in Figs 3 and 4 show that the enhancement of cholesterol solubility by the two cyclodextrin derivatives is around 10^3 times greater than that reported for cholesterol in water, even accepting the highest published values, ranging from 1×10^{-6} M to 25×10^{-6} M [8, 55, 56]. Matsuoka et al. [57] and Cabral et al. [58] have published results in the range 2.6–3.3×10^{-8} M. Furthermore, intercepts in Figs. 3 and 4 are not statistically different from zero, and they should not be used to calculate $K_{1:1}$. Slopes of Figs. 3 and 4 have been used together with the value for g_o measured by Nishijo et al. [12] (equal to 3.4×10^{-6} M at 298.15 K) to estimate

Table 1 Equilibrium constant values for the formation of cholesterol/cyclodextrin dimer complexes obtained from phase-solubility diagrams of Figs. 3 and 4

Solubilizer	Slope intercept	*CE*	$K_{1:1}/M^{-1}$
2	$(1.65 \pm 0.08) \times 10^{-1}$ $(2 \pm 1) \times 10^{-5}$ M	0.20 ± 0.01	$(5.9 \pm 0.3) \times 10^{4}$
3	$(2.31 \pm 0.04) \times 10^{-1}$ $(3 \pm 6) \times 10^{-6}$ M	$(3.00 \pm 0.07) \times 10^{-1}$	$(8.8 \pm 0.2) \times 10^{4}$
Thioether βCD dimer[8]	~0.77	~3.3	$3.3–5.5 \times 10^{6}$
 **4**			

The value determined for the complexation with dimer **4** by Breslow and Zhang [8] is also given

$K_{1:1}$. This allows a close comparison of present results with published values by Nishijo et al. [12, 13] for the complexation of cholesterol by DOMβCD and TOMβCD. Furthermore that value is also close to the value published by Breslow and Zhang [8] (who used two different methods for the determination of cholesterol) allowing a direct comparison of the equilibrium constant values corresponding to the dimer studied here and the one published by these authors. Table 1 shows the resulting values. The value obtained by Breslow and Zhang [8] for **4** is also given. It can be noticed that the last value is one order of magnitude greater than those for **2** and **3**, which are very close to each other. This fact suggests that the bridge has, if any, a very low influence on the ability of the two dimers in complexing cholesterol. The higher value obtained by Breslow and Zhang [8] can be understood on the basis that the shorter bridge of **4** (in comparison to **2** and **3**) would facilitate a simultaneous deeper inclusion of the cholesterol in the two cyclodextrin residues of the dimer.

To prevent the influence of g_o on the calculation of $K_{1:1}$ and to facilitate comparative analysis, it can be more convenient to use the complexation efficiency (Eq. 3) [5, 54], *CE*, which is less sensitive to errors related to the estimation of g_o. Values are given in Table 1.

$$CE = \frac{[Ch - CD]}{[CD]} = g_0 K_{1:1} = \frac{\text{slope}}{(1 - \text{slope})} \quad (3)$$

We can observe that *CE* for **4** is 10 times more efficient. However it must be noticed that the ranges of concentration of the cyclodextrin dimers are very different since the maximum concentration used by Breslow and Zhang ($\sim 30 \times 10^{-6}$ M) is almost 1,000 times lower than those used for present studies with dimers **2** and **3** (see Figs. 2 and 3). Since we do not know the maximum solubility of **4**, but attending to the lower solubility limit of dimer **2** (neutral) compared to dimer **3** (a di-anion), it is possible that the different range of studied concentration arise from solubility limits of the solubilizers.

Figure 6 shows part of the ROESY intermolecular cross-peaks between cholesterol protons (named P) and dimer **3** protons (named H) in deuterium oxide at 25 °C. The assignment of cholesterol protons was based on Ravichandran and Divagar work [59]. Interactions between cholesterol protons, located in far sites of the molecule, with protons of the cyclodextrin residues are evident. For instance, the methyl P19 protons (located at the A and B rings junction) and P21-27 (belonging to the side chain) interact with cyclodextrin protons H3, H5 and H6, suggesting that cholesterol is being simultaneously complexed by two cyclodextrin residues. No interactions between protons of the bridge and cholesterol have been observed.

The structure of the colesterol/**3** complex was minimized by molecular dynamics simulations (MM2). Figure 7 shows the final geometry of the complex. Some slight differences on this final structure can be observed depending on the starting geometry, but they can be considered negligible for the purposes of these calculations. First it must be noticed that cholesterol is located inside of the two cyclodextrin cavities. This is in agreement with ROESY experiments as interactions of cholesterol protons located at far distances to each other (longer than the height of the cyclodextrin residue) with cyclodextrin protons have been observed. This complexation is facilitated by the almost linear structure of cholesterol due to the *cis* junction between its A and B rings. Second the primary rim sides of the two cyclodextrin residues are in a face to face orientation. This is in agreement with calculations on the inclusion complex formation of cholesterol with dimer **4** performed by Choi et al. [60]. Third, the two glucosidic planes are forming an angle of 14.7° meaning that the two cyclodextrin residues are clamping the cholesterol molecule, the extreme of jaws (cyclodextrin residues) being as

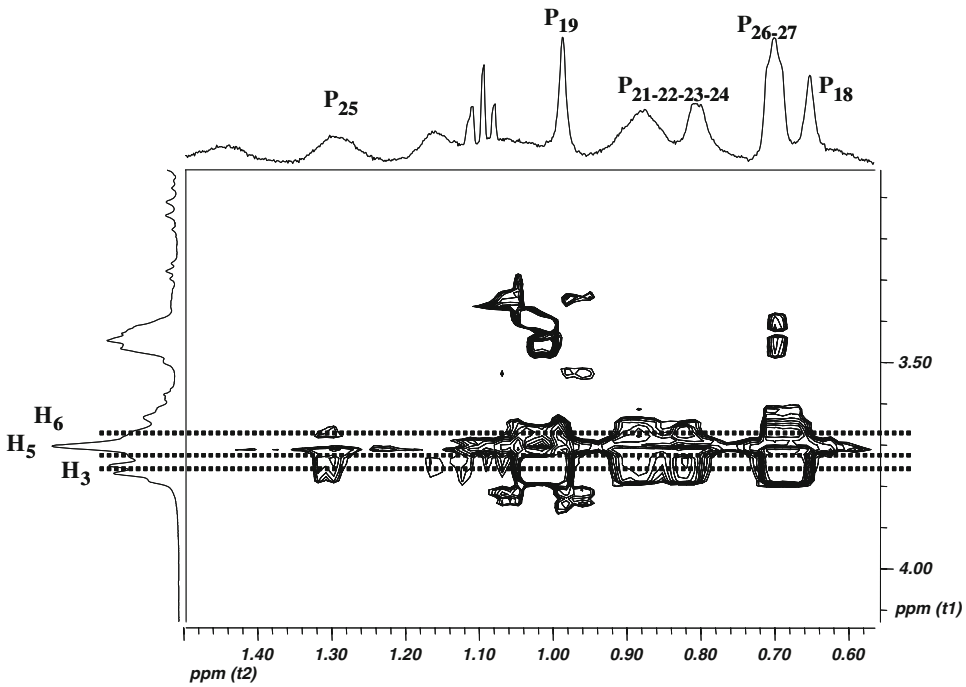


Fig. 6 ROESY spectrum of the cholesterol/**3** system. [**3**] = 10 mM and [cholesterol] = 2 mM in deuterium oxide

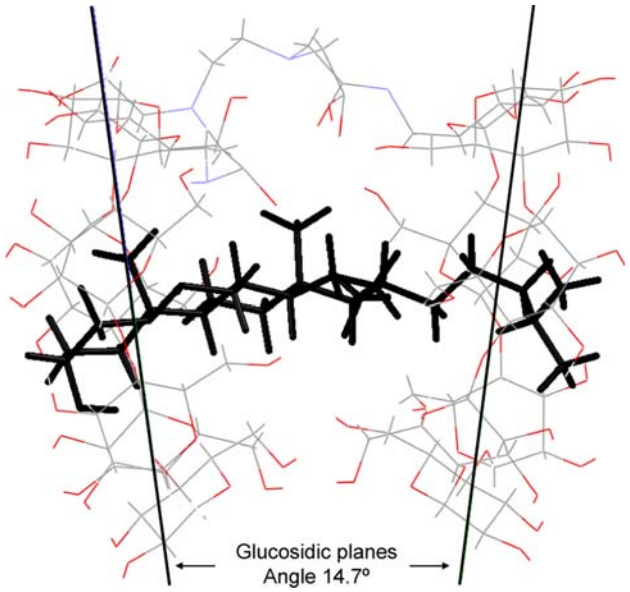


Fig. 7 Structure of cholesterol/**3** complex obtained by molecular dynamics simulations. Cholesterol in black colour

close to each other that they can form hydrogen bonds between the $-CH_2OH$ hydroxyl groups of opposite glucose units. This probably assures a tightly holding of cholesterol inside the complex. Fourth, the carbon skeleton of the bridge between the cyclodextrin units is rather far from cholesterol, justifying the absence of ROESY interactions between protons of the bridge and cholesterol.

For an A_P isotherm, and accepting that two complexes with stoichiometries 1:1 (Eq. 1) and 1:2 (Eq. 4) are formed, the equilibrium constants can be calculated as follows. The 1:2 complex is formed by a second binding of a cyclodextrin to the complex of equilibrium (1), i.e.,

$$Ch - CD + CD \rightleftarrows Ch - CD_2$$

$$K_{1:2} = \frac{[Ch - CD_2]}{[CD][Ch - CD]} \tag{4}$$

The mass balance equations for cholesterol and cyclodextrin are (Eqs. 5 and 6)

$$[Ch]_t = g_o + [Ch - CD] + [Ch - CD_2] \tag{5}$$

$$[CD]_t = [CD] + [Ch - CD] + 2[Ch - CD_2] \tag{6}$$

where $[Ch]_t$ and $[CD]_t$ are cholesterol and cyclodextrin total concentrations, respectively. The combination of previous equations gives Eqs. 7 and 8

$$[Ch]_t = g_o + K_{1:1}g_o[CD] + K_{1:1}K_{1:2}g_o[CD]^2 \tag{7}$$

$$[CD] = \frac{-(K_{1:1}g_o + 1) + \sqrt{(K_{1:1}g_o + 1)^2 + 8K_{1:1}K_{1:2}g_o[CD]_t}}{4K_{1:1}K_{1:2}g_o} \tag{8}$$

The two equilibrium constants are calculated by a least-square iterative process. Table 2 shows the calculated values together with published values for other systems taken from the literature. βCD and HPβCD form 1:1 complexes with cholesterol but DOMβCD and TOMβCD, as monomer **1**, also form 1:2 complexes meaning that cholesterol is a ditopic guest. This is possible since the

Table 2 Equilibrium constants for different cholesterol/cyclodextrin complexes

Host	$K_{1:1}/M^{-1}$	$K_{1:2}/M^{-1}$	Reference
1	73 ± 19	204 ± 65	This paper
βCD	(a) 1.9×10^3		[61]
	1.57×10^3		[10]
	1.7×10^4		[7]
HPβCD hydroxypropyl-βCD	(b) 1.8×10^4		[61]
	1.9×10^4		[7]
DOMβCD heptakis (2,6-di-*O*-methyl)-βCD	(a) $5.5 \cdot 10^5$		[61]
	1.1×10^2	5.68×10^4	[12]
TOMβCD Heptakis (2,3,6-tri-*O*-methyl)-βCD	0.7×10^2	7.55×10^4	[13]

(a) Theoretical value

(b) Stability constant determined from the initial slope of the B_s diagram

cholesterol length (15 Å) is almost twice the heigh of the cyclodextrin cone (7–8 Å) [1], allowing the complexation by two cyclodextrin units. This also justifies the formation of 1:1 complexes with dimers **2** and **3** commented on above.

The calculated value for $K_{1:1}$ is close to published values by Nishijo et al. [12, 13] for the complexation of cholesterol by DOMβCD and TOMβCD. Since $K_{1:2} > K_{1:1}$, the 1:2 complex is formed more easily than that with the 1:1 stoichiometry, in agreement with Nishijo et al. However $K_{1:2}$ is ~300 times lower than those for methyl βCD derivatives. This is graphically illustrated in Fig. 5, where the solubility curve for DOMβCD deviates upward from linearity much faster than the curve for **1**. Nishijo et al. [12, 13] have measured the change in enthalpy and entropy accompanying the sequential cholesterol complexation by two cyclodextrin units. Based on their results, Nishijo et al. [13] concluded that hydrophobic interactions are the driving force for 1:2 complex formation and that $K_{1:2} > K_{1:1}$ is mainly due to an entropy effect. Similar comments also apply to the cholesterol/DOMβCD system [12], although the contribution of entropy terms for standard Gibbs free energy changes is larger in the former case. This difference may arise from the lower number of methyl groups in DOMβCD than in TOMβCD. This could explain the lower value of $K_{1:2}$ observed for **1** since its side chain with amide and carboxylate groups is much less hydrophobic than the methyl groups in both DOMβCD and TOMβCD.

When a ditopic guest (as cholesterol) is complexed by a ditopic host (as cyclodextrin dimers) a chelate binding can arise when the ditopic guest is complexed simultaneously by both cyclodextrin residues of the same dimer, forming a 1:1 complex. In this case a higher stability constant than that for the complexation by isolated cyclodextrins would be expected. This chelate effect has been mainly studied by Breslow et al. [38, 48, 49] Otherwise, polymers with an *n*:*n* stoichiometry can be formed [39]. To check whether a chelate effect exist when cholesterol is complexed by **3**, the associated equilibrium constant $K_{1:1} = 8.8 \times 10^4\ M^{-1}$ has to be compared with the product of the two equilibrium constants determined for the monomer **1**. This is a feasible comparison since the 1:2 (cholesterol/**1**) and the 1:1 (cholesterol/**3**) complexes are both double charged. From values in Table 2 it results $K_{1:1} \times K_{1:2} = (1.5 \pm 0.9) \times 10^4\ M^{-2}$ which is 6 times lower than $K_{1:1}$ for the formation of the cholesterol/**3** complex. Therefore it may be concluded that a chelate effect in binding the cholesterol by **3** exists.

Acknowledgment The authors thank the Ministerio de Educación y Ciencia of Spain (MEC) (Project MAT2004-04606) and Xunta de Galicia (PGIDIT05PXIC26201PN) for financial support. A. Antelo and M. Alvarez also thank MEC for research scholarships.

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