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Complexation of Bile Salts by Natural Cyclodextrins

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The complexation of three trihydroxy sodium bile salts—cholate, glycocholate, and taurocholate—and their three related sodium dihydroxy bile salts—deoxycholate, glycodeoxycholate, and taurodeoxycholate—by α-, β- and γ-cyclodextrins (α-CD, β-CD and γ-CD), has been studied by using 1D and 2D-NMR techniques. Trihydroxy bile salts form 1:1 complexes with β-CD and γ-CD, while dihydroxy bile salts form 1:2 (bile salt:cyclodextrin) complexes with β-CD and 1:1 complexes with γ-CD. ROESY experiments stated that the side chain, ring D, and part of ring C of the steroid body of the bile salts, are included into the cavities of β-CD and γ-CD, in 1:1 complexes. The A ring of the steroid body is included into the cavity of the second β-CD in the 1:2 complexes.

The only structural difference between related bile salts is the existence or not of a hydroxyl group at C7. The bigger cavity of γ-CD allows this region of bile salts to be located inside the cyclodextrin cavity and therefore γ-CD does not discriminate between both types of bile salts. However, in trihydroxy bile salts, this region clearly remains outside the β-CD cavity. The absence of the C7 hydroxyl group enlarges the hydrophobic region of the steroid body, allowing a favourable interaction with the hydrophobic cavity of a second β-CD. It is concluded that β-CD molecularly recognises bile salts, distinguishing di- from trihydroxy ones, while γ-CD does not.

The steroid body of bile salts is too big to enter into the α-CD cavity and only an interaction between their side chain and α-CD is observed.

Keywords: Bile salts; Natural cyclodextrins; α-, β- and γ-cyclodextrins; 1D and 2D-NMR techniques

INTRODUCTION

Bile salts are naturally occurring molecules segregated into the gallbladder by the liver as main components of bile [1]. They play an important role in the digestive process, acting as biological surfactants interacting with food lipids, allowing their solution and absorption by the body. Common bile salts have two or three hydroxyl groups at the C3, C7 or C12 positions of the characteristic tetracyclic skeleton of steroids. Bile salts also possess a mobile side chain at the C17 position which ends with a carboxylic group that can be conjugated with taurine or glycine (Fig. 1a).

Cyclodextrins (Fig. 1b) are natural cyclic oligomers built up from 6, 7 or 8 glucopyranose units (named α-, β- and γ-CD, respectively) linked by α-(1-4)-glycosidic bonds. They take the shape of a truncated cone with a hydrophobic cavity in which molecules, *guests*, can be trapped forming inclusion complexes. A wide variety of organic molecules, including steroids, can be included into the cyclodextrin cavities [2]. The inclusion processes lead to important modifications of the guest properties, and as a result, cyclodextrins have important and increasing technological applications in biomedicine, pharmacy and engineering [2–4].

The increasing interest [5–28] in studying the cyclodextrin–bile salt systems in aqueous solution is clearly justified by the following reasons: (i) bile salts have a relevant physiological role in life and cyclodextrins are widely used in pharmacy as carriers for oral drug formulations; the *in vivo* interactions between both kinds of molecules could alter the metabolic paths of the bile salts. Studies in this direction have been published relating the effects on bile salts levels in animals fed with

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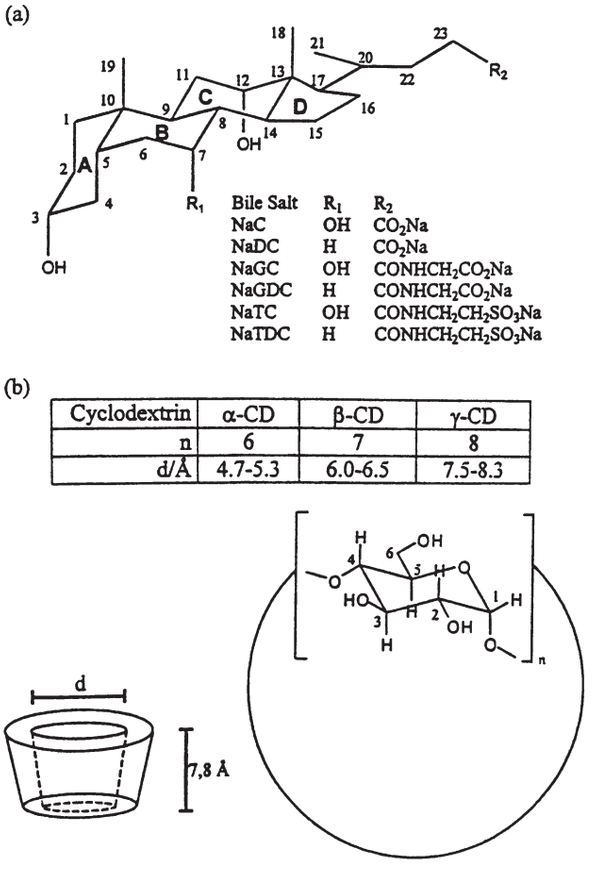

FIGURE 1 Schematic structures of (a) bile salts and (b) cyclodextrins.

a cyclodextrin-enriched diet [7–9] and have promoted the *in vitro* studies. (ii) Cholesterol can be bound to cyclodextrin [29–35] but its low solubility in water prevents the determination of the complex's structure in solution. On the contrary, bile salts and their complexes are highly soluble and therefore can be used as models of the cholesterol–cyclodextrin complex. (iii) Combinations of cyclodextrins/bile salts are now increasingly used in chromatographic and electrophoretic chiral separation procedures [36–40]; the results have shown that the separation enhancements in surfactant–cyclodextrin mixtures are greater than the sum of the effects of each component alone. Therefore, the bile salt–cyclodextrin interaction does appear to be the responsible for such effects. Finally, (iv) for a better understanding of the cyclodextrin inclusion phenomena, systematic studies have to be carried out. Bile salts are very suitable molecules for this purpose since the presence or absence of the hydroxyl groups in the steroid body barely affects the size and shape of the bile salt, but their number, position, and spatial orientation (α or β) have a great influence on their physico-chemical properties. For instance, the polarity significantly changes from sodium cholate

TABLE I Published equilibrium constant values for the inclusion complexes formed by bile salts (studied in this paper) with natural cyclodextrins. The subscripts indicate the stoichiometry of the complexes

Trihydroxy bile salts				
Cyclodextrin	NaC	NaTC	NaGC	Reference
β-CD K_{11}/M^{-1}	8000			[5]
	2830	2034	2340	[7]
	1100		410	[13]
	2200			[16]
	3400			
	3162	2630	1950	[19]
	1488		336	[20]
γ-CD K_{11}/M^{-1}		3544		[7]
	362		210	[20]
Dihydroxy bile salts				
Cyclodextrin	NaDC	NaTDC	NaGDC	Reference
β-CD K_{11}/M^{-1}	7567	6637	5113	[7]
	61660	34674	18621	[19]
	4545	2632		[28]
β-CD K_{12}/M^{-1}	724	537	457	[19]
β-CD $K_{11} \times K_{12}/M^{-2}$	39000			[5]
γ-CD K_{11}/M^{-1}		16212		[7]
		12195		[28]

(NaC), a trihydroxy bile salt, to sodium deoxycholate (NaDC), a dihydroxy bile salt, with the result that NaC behaves as a monotopic guest molecule while NaDC is a ditopic guest. This difference has been recently used to demonstrate the formation of supramolecular polymer structures [5,6]. Furthermore, since bile salts are longer than the cyclodextrin cavity, in complexes of 1:1 stoichiometry only part of the bile salt can be included inside the cyclodextrin cavity. This phenomenon is illustrative of molecular recognition, a fundamental concept in supramolecular chemistry.

Different experimental techniques have been used to study bile salt–cyclodextrin systems. Among them we can mention UV–Vis spectroscopy [10], fluorescence spectroscopy [11–14], circular dichroism [16], NMR [5,6,15,23], DSC [17,19,22], solubility [21,22,24], FT-IR [17,21,22], surface tension [25,26], speed of sound [16] and X-rays [17,21,22]. However, serious discrepancies related to the complexes stoichiometries, structures, and the values of the equilibrium constants can be deduced from the analysis of the literature. For instance, Tan *et al.* [20] have reported a structure for the cholate-β-CD complex in which the steroid body of the bile salt is completely outside of the cavity of the cyclodextrin, while Ramos Cabrer *et al.* [5] have given evidence that the C (partially) and D (totally) rings, and the side chain of the bile salts are included into the cyclodextrin cavity. Important discrepancies in the complex stoichiometry and the values for the equilibrium constant are also noticeable (see Table I). On the other hand, no systematic studies have been

reported about the influence of the cavity size on the values of the equilibrium constants for the formation of the complexes and on their structure.

The entrance of a guest molecule into the cyclodextrin cavity with the release of water molecules bound inside the cyclodextrin cavity, induces changes in the electronic environment of the nuclei of both the cyclodextrin and the guest, resulting in changes in the chemical shifts of these nuclei. Therefore, 1D and 2D NMR spectroscopies can be used as suitable experimental techniques for the determination of equilibrium constants, stoichiometry, and structure of the complexes [5,6]. ^{13}C-NMR was used instead of ^{1}H-NMR for two reasons: (i) the observed experimental differences in the chemical shifts are higher for carbons than for protons, and (ii) the carbon signals appear well isolated from each other, while, in large molecules, superposition of proton signals is common; therefore the uncertainty in the measurements is reduced.

In this paper we present the results obtained for the complexes formed by three trihydroxy bile salts (NaC, sodium glycocholate NaGC, and sodium taurocholate NaTC) and three related dihydroxy derivatives (NaDC, sodium glycodeoxycholate NaGDC, and sodium taurodeoxycholate NaTDC) and three natural cyclodextrins (α-, β- and γ-CD).

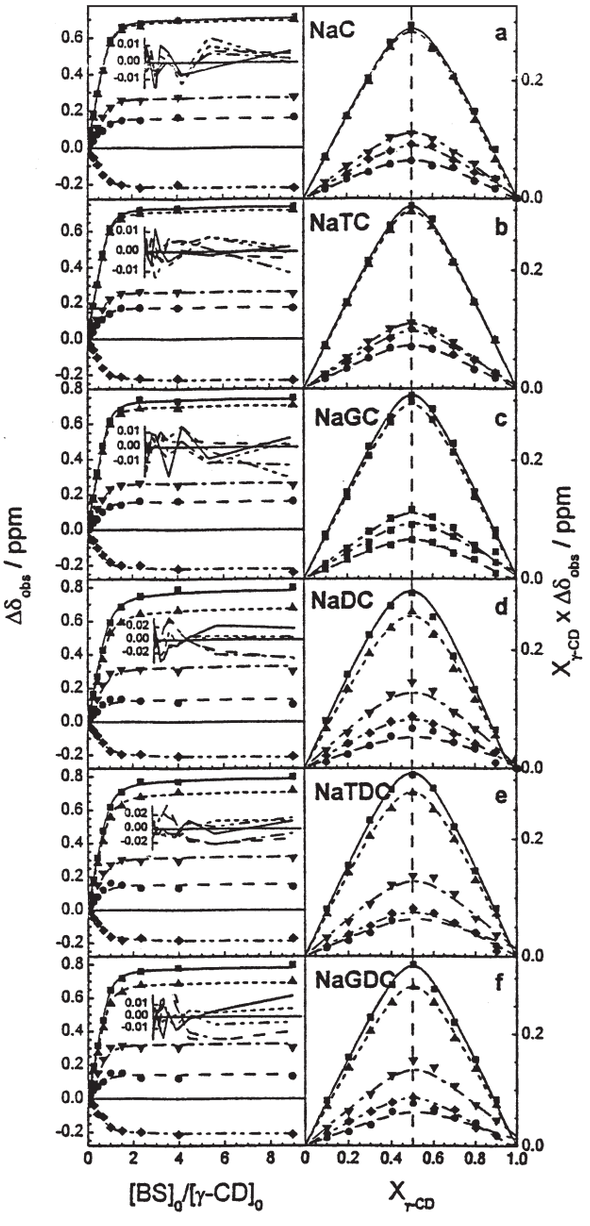

FIGURE 2 Plots of the observed chemical shift displacements, $\Delta\delta_{obs}$, of different cyclodextrin carbons vs. $[\text{Bile Salt}]_o/[\gamma\text{-CD}]_o$ ratio and the corresponding Job's plots. Solid lines are the result of the non linear fitting. The inserted figures correspond to the plots of residuals vs. $[\text{Bile Salt}]_o/[\gamma\text{-CD}]_o$.

EXPERIMENTAL

Commercial bile salts (Sigma–Aldrich) were purified by re-crystallization from appropriate solvents as previously reported [5]. β-CD and γ-CD (kindly supplied by Roquette and Wacker Chemie, respectively), and α-CD (Wacker Chemie) were re-crystallised from hot Milli-Q grade water. All chemicals were dried until constant weight in a vacuum oven at 70°C. Solutions for NMR experiments were prepared in D_2O (99.90%, from SDS). ^{13}C-NMR experiments were carried out at 298.1 (± 0.1 K) in a Bruker AC 300 spectrometer at 75 MHz. 2D-NMR ROESY were carried out in a Bruker AMX 500 spectrometer at 500 MHz. In these experiments samples of 10 mM total concentration (bile salt + cyclodextrin) were used, prepared at the stoichiometric ratio previously determined by ^{13}C studies. A spin-locking time of 250 ms was used in the measurements.

The stoichiometries of the inclusion complexes were deduced by the continuous variation method (Job's plot). Samples (total volume 0.5 ml) were directly prepared in the NMR tubes by mixing variable volumes of two stock solutions (cyclodextrin and bile salt) of the same concentration. Thus, the total concentration (cyclodextrin + bile salt) is constant along a series, while molar fractions of both components vary from 0 to 1 in steps of 0.1.

RESULTS AND DISCUSSION

Inclusion Complex Formation with γ-CD

Figure 2 shows the chemical shift displacements of carbon atoms (1,3-6) of γ-CD, determined from ^{13}C-NMR experiments, vs. the $[\text{Bile Salt}]_o/[\gamma\text{-CD}]_o$ ratio for the six bile salts studied in this work, and the corresponding Job's plots. For the six bile salts, the maxima in the Job's plots clearly appear at

TABLE II Equilibrium constants and maxima chemical shift displacements for the 1:1 complexes formed by di- and trihydroxy bile salts with γ-CD obtained from ^{13}C-NMR measurements. Temperature 25°C

	Trihydroxy bile salts	NaC	NaGC	NaTC
$K_{11}/10^3 M^{-1}$		3.2 ± 0.4	4.5 ± 0.7	4.7 ± 0.4
$\Delta\delta_{max}$/ppm	C1	0.733 ± 0.007	0.756 ± 0.008	0.752 ± 0.005
	C3	0.167 ± 0.006	0.169 ± 0.009	0.179 ± 0.005
	C4	0.728 ± 0.008	0.721 ± 0.010	0.730 ± 0.006
	C5	0.282 ± 0.007	0.267 ± 0.008	0.272 ± 0.005
	C6	−0.222 ± 0.007	−0.216 ± 0.008	−0.232 ± 0.005
Dihydroxy bile salts		NaDC	NaGDC	NaTDC
$K_{11}/10^3 M^{-1}$		2.0 ± 0.4	4.1 ± 0.9	2.6 ± 0.5
$\Delta\delta_{max}$/ppm	C1	0.860 ± 0.010	0.808 ± 0.013	0.831 ± 0.014
	C3	0.133 ± 0.014	0.136 ± 0.012	0.148 ± 0.018
	C4	0.733 ± 0.025	0.713 ± 0.018	0.747 ± 0.032
	C5	0.344 ± 0.015	0.317 ± 0.018	0.323 ± 0.019
	C6	−0.224 ± 0.015	−0.219 ± 0.012	−0.196 ± 0.018

$X_{\gamma-CD} = 0.5$ indicating that all the bile salts form complexes with a 1:1 stoichiometry with γ-CD.

Under fast exchange conditions [41], in a mixture of free and 1:1 complexed cyclodextrin, the observed chemical shift displacement of each carbon nucleus of cyclodextrin is expressed as

$$\delta_{obs} = f_{CD}\delta_{CD} + f_{C_{11}}\delta_{C_{11}} \quad (1)$$

where δ_{CD}, $\delta_{C_{11}}$, f_{CD} and $f_{C_{11}}$ represent the chemical shift of a given nucleus of free and complexed cyclodextrin, respectively, and f fractions are given by $f_{CD} = [CD]/[CD]_o$ and $f_{C_{11}} = [C_{11}]/[CD]_o$. As usual, terms in brackets are free, complexed and initial cyclodextrin molar concentrations. From a mass balance for cyclodextrin and rearrangement of $\Delta\delta_{obs} = \delta_{obs} - \delta_{CD}$, and $\Delta\delta_{max} = \delta_{C_{11}} - \delta_{CD}$, Eq. (2) is obtained:

$$\Delta\delta_{obs} = \Delta\delta_{max} f_{C_{11}} \quad (2)$$

The 1:1 complex formation between the host and the guest is represented by Eq. (3)

$$BS + CD \rightleftharpoons C_{11} \quad (3)$$

and the equilibrium constant of this equilibrium is given by

$$K_{11} = \frac{[C_{11}]}{[BS][CD]} \quad (4)$$

From the mass balances of cyclodextrin and bile salt,

$$[BS]_o = [BS] + [C_{11}] \quad (5)$$

$$[CD]_o = [CD] + [C_{11}] \quad (6)$$

and Eq. (4), Eq. (7) may be deduced.

$$[C_{11}]^2 - \left([BS]_o + [CD]_o + K_{11}^{-1}\right)[C_{11}] + [BS]_o[CD]_o = 0 \quad (7)$$

Finally, from Eqs. (2) and (7), Eq. (8) is deduced,

$$\Delta\delta_{obs} = \frac{\Delta\delta_{max}}{2[CD]_o}\left\{K_{11}^{-1} + [BS]_o + [CD]_o - \sqrt{\left(K_{11}^{-1} + [BS]_o + [CD]_o\right)^2 - 4[BS]_o[CD]_o}\right\} + \Delta\delta_o \quad (8)$$

which relates the experimental $\Delta\delta_{obs}$ with initial bile salt and cyclodextrin concentrations. These equations are fitted to experimental data by using a non linear least-squares computer program to obtain K_{11}, $\Delta\delta_{max}$ and $\Delta\delta_o$ (for each carbon atom) as fitting parameters. $\Delta\delta_o$ is a fitting parameter to account for the experimental error at zero bile salt concentration. In a previous paper [5] these equations were used to obtain K_{11} for the complex NaC:β-CD, but only experimental data from two carbon atoms (1 and 4) were used and independent fittings of the two set of data were performed. In this paper, a global non linear least squares fitting of all the data is carried out, i.e. the same K_{11} but different $\Delta\delta_{max}$ (for each carbon atom) fitting parameters are used for optimising the data of all cyclodextrin carbon atoms simultaneously. This global data fitting reduces the standard deviations of the parameters leading to the best values for the fitting parameters which can be obtained from this array of experimental data.

Solid lines in Fig. 2 are the result of the non linear fitting, the optimised parameters being summarised in Table II. Inserts in the figures are the residuals of the fitting indicating the quality of the fits. It is evident that the equilibrium formation constants are very similar, i.e. γ-CD does not discriminate between the six bile salts studied here. The obtained K_{11} values are lower [7,28] or higher [20] than those published by other authors (see Table I).

It is known that cyclodextrins form inclusion complexes with surfactant monomers, reducing

the micelle concentration [42]. As bile salts form aggregates above a critical concentration [43–47], *cmc*, it is necessary to guarantee that no micelles are formed in the experiments. This means that the total concentration of bile salt must be selected in such a way that the concentration of free bile salt is always lower than *cmc*, i.e. [BS] < *cmc*. Otherwise, (i) Eq. (5) is no longer valid as the concentration of formed aggregates should be considered in the mass balance, (ii) possible interferences in the measurements from the formation of micelles could appear, and, (iii) the cyclodextrin could take part in the formation of the micelles.

Working at $[CD]_o < [BS]_o$, accepting that (i) a 1:1 complex bile salt:cyclodextrin stoichiometry is formed, and (ii) the maximum concentration of free bile salt is equal to *cmc* [48,49], the maximum concentration of initial bile salt, which can be used in the experiments, is given by [50]

$$[BS]_{o,max} = cmc\left(1 + \frac{K_{11}[CD]_o}{1 + K_{11}cmc}\right) \qquad (9)$$

This equation justifies the observed increment of the apparent critical micelle concentration, *cmc**, upon the addition of cyclodextrins to surfactant solutions [51–53] by substituting $[BS]_{o,max} = cmc^*$. Equation (9) is reduced to

$$[BS]_{o,max} = cmc + [CD]_o \qquad (10)$$

for high K_{11} values, which is equivalent to accepting that all the cyclodextrin added is in the complexed form. Equation (10) yields a good estimation for the maximum bile salt concentration which can be used.

The range of published values for *cmc* for each bile salt is rather wide [43,47], but 5–7 mM for dihydroxy derivatives and 10–15 mM for trihydroxy ones, can be considered as good values. Once K_{11} is obtained, Eq. (8) is used to confirm that $[BS]_o < [BS]_{o,max}$, or equivalently [BS] < *cmc*. This limitation has not always been considered by previous authors [16] to fit $\Delta\delta_{obs}$ data.

Very similar values for $\Delta\delta_{max}$ are observed for each carbon atom for the six bile salts (Table II). C3 and C6 carbon atoms of the cyclodextrins are located at the two opposite hydroxyl rims, while C1 and C4 (which participate in the formation of glycosidic bonds) can be considered to be located in the middle of the truncated cone. Therefore, all bile salts induce practically the same change in the electronic environment of each carbon atom along the whole γ-CD cavity. The existence or not of a hydroxy group at C7 and the conjugation of glycine or taurine with the carboxylate group of the side chain in bile salts fundamentally modifies their polarity as evidenced by their *cmc* values and aggregation behaviour [43]. The conclusions are (i) the polarity of bile salts does not have any influence on their complexation behaviour with γ-CD and (ii) all complexes have almost identical structures since the arrangement of each bile salt inside the cyclodextrin cavity must be the same, a consequence of their almost identical geometry.

These conclusions are confirmed by the ROESY spectra of the six bile salts with γ-CD, since all of them showed to be almost identical. Table III summarises the interactions between different protons existing in bile salt-γ-CD systems. The observed interactions suggest a structure for the complex in which the rings C and D are completely inside the cyclodextrin cavity (as an example the structure for the NaDC–γ-CD complex is shown in Fig. 3). The comparison of these structures with the NaC–β-CD previously published [5] indicates a deeper penetration of the bile salt guest inside the γ-CD. It must be noticed that (i) the substituents at C7 position are now included into the γ-CD cavity while they remain outside it in the case of the β-CD [5] (see also below); (ii) there are no interactions between the protons of the A ring with any protons of the γ-CD, confirming the 1:1 stoichiometry; and (iii) the H6 protons of γ-CD only interact with protons of the side chain (P23 and P21) of the bile salt. The conclusion is that all bile salts enter the γ-CD cavity by the side of the secondary hydroxyl rim.

Inclusion Complex Formation with β-CD

Figure 4 shows the chemical shift displacements of β-CD carbon atoms vs. $[\text{Bile Salt}]_o/[\beta\text{-CD}]_o$ ratio for the six bile salts studied in this work, and the corresponding Job's plots. These plots indicate that the three trihydroxy bile salts (NaC, NaGC and NaTC) form 1:1 stoichiometric complexes (maxima at $X_{\beta-CD} = 0.5$), while the three dihydroxy bile salts (NaDC, NaGDC, NaTDC) exhibit curves with maxima at $X_{\beta-CD} \approx 0.67$ indicating the formation of 1:2 (bile salt:β-CD) complexes. Therefore β-CD clearly distinguishes between tri- and dihydroxy bile salts, which was not the case with γ-CD. It should be noticed that only Tan and Lindenbaum [1],

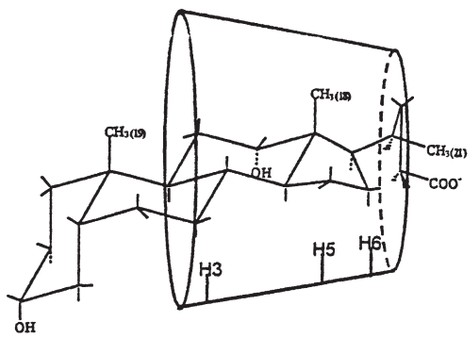

FIGURE 3 Schematic representation of the 1:1 γ-CD/NaDC complex, deduced from ROESY experiments.

TABLE III ROESY intramolecular cross-peaks observed between bile salt protons (named P) and γ-CD protons (named H). s: strong; m: medium; w: weak

		NaC			NaGC			NaTC			NaDC			NaGDC			NaTDC		
Location	Proton	H3	H5	H6	H3	H5	H6	H3	H5	H6	H3	H5	H6	H3	H5	H6	H3	H5	H6
Side chain	P23										m	w	w						
	P22	m			m			w			m	w		s			m		
	P21	m	w	w	m	w	w	m	m		m	w	w	s	m	w	m	m	w
	P20	m			m			m			m			m			m		
A/B rings	P19	m			w			w			w								
C/D rings	P18	m			m			s			m			m			s		
D ring	P17	m			w			m	w		w			m			m		
	P16	m	w		m	w		m	w		m	m		w	m		w	m	
	P15	m			m			s			w			m			m		
C/D rings	P14																m		
C ring	P12	m			w						m			m					
	P11	w			w			m			w			m			m		
B ring	P7	m			w			w			m			m			m		
Amino acid chain	$-CH_2-$glycine					w	w												

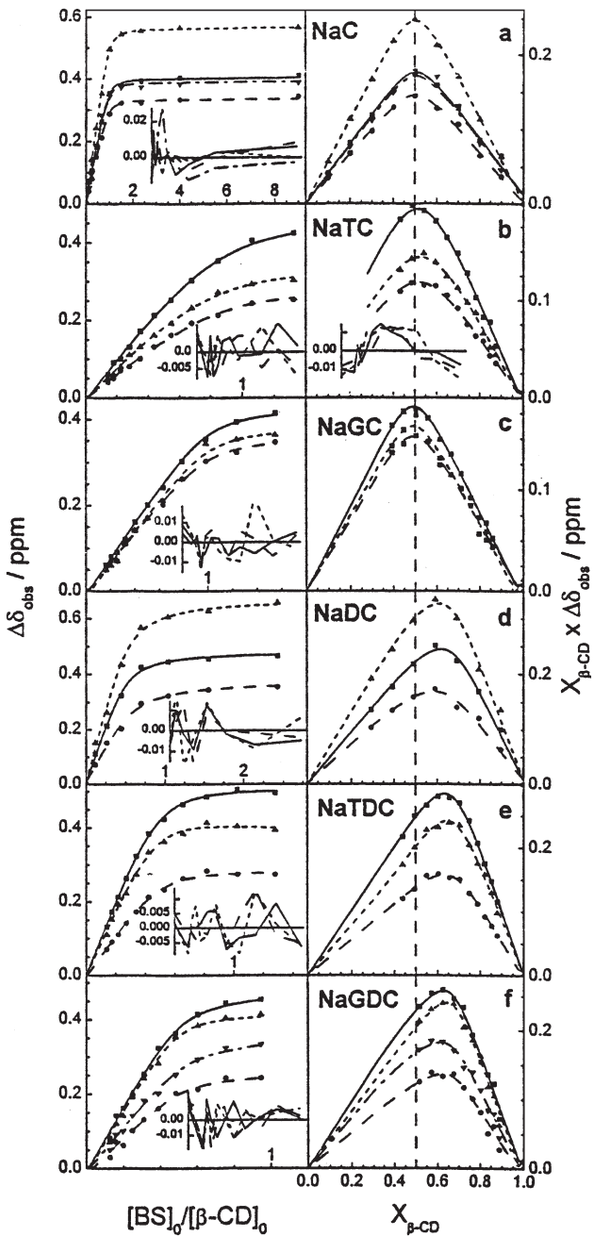

FIGURE 4 Plots of the observed chemical shift displacements, $\Delta\delta_{obs}$, of different β-CD carbons vs. $[\text{Bile Salt}]_o/[\beta\text{-CD}]_o$ ratio and the corresponding Job's plots. Solid lines are the result of the non linear fitting. The inserted figures correspond to the plots of residuals vs. $[\text{Bile Salt}]_o/[\beta\text{-CD}]_o$.

Ramos Cabrer *et al.* [5] and Cooper *et al.* [28], have reported the formation of 1:2 stoichiometric complexes.

For the 1:1 complexes, Eq. (8) is fitted to experimental data to obtain K_{11} and $\Delta\delta_{max}$ (for each carbon atom) in a global fit. The values of the fitting parameters are given in Table IV. Values for NaC were recalculated from previously published data [5]. Lines in Fig. 4a–c are the result of the global fitting. As previously, inserts in the figures are the residuals of the fitting.

TABLE IV Equilibrium constants and maxima chemical shift displacements for the complexes formed by di- and trihydroxy bile salts with β-CD obtained form ^{13}C-NMR measurements. The subscripts indicate the stoichiometry of complexes. Temperature 25°C

		NaC	NaGC	NaTC
Trihydroxy bile salts				
$K_{11}/10^3\,M^{-1}$		7.5 ± 1.7	3.6 ± 1.6	1.8 ± 0.9
$\Delta\delta_{max}$/ppm	C1	0.397 ± 0.008	0.425 ± 0.008	0.469 ± 0.015
	C3	0.339 ± 0.008		
	C4	0.572 ± 0.008	0.355 ± 0.008	0.281 ± 0.015
	C5	0.386 ± 0.008	0.390 ± 0.008	0.339 ± 0.015
Dihydroxy bile salts		NaDC	NaGDC	NaTDC
$K_{11}/10^3\,M^{-1}$		11 ± 11	3.6	3.0 ± 2.1
$\Delta\delta_{max,\,C_{11}}$/ppm	C1	0.469 ± 0.012	0.556 ± 0.036	0.498 ± 0.009
	C3	0.359 ± 0.012	0.323 ± 0.036	0.284 ± 0.009
	C4	0.658 ± 0.012	0.479 ± 0.036	0.379 ± 0.009
	C5		0.461 ± 0.036	
	C6		−0.273 ± 0.036	
$K_{12}/10^3\,M^{-1}$		0.7 ± 0.7	1.2 ± 0.7	0.5 ± 0.2
$\Delta\delta_{max,\,C_{12}}$/ppm	C1	0.464 ± 0.025	0.426 ± 0.016	0.57 ± 0.13
	C3	0.282 ± 0.025	0.230 ± 0.016	0.32 ± 0.13
	C4	0.591 ± 0.025	0.409 ± 0.016	0.50 ± 0.13
	C5		0.286 ± 0.016	
	C6		−0.023 ± 0.016	

The formation of a 1:2 complex from the addition of a second cyclodextrin to the 1:1 complex,

$$C_{11} + CD \rightleftharpoons C_{12} \tag{11}$$

leads to the equilibrium constant, K_{12},

$$K_{12} = \frac{[C_{12}]}{[C_{11}][CD]} \tag{12}$$

Modifying the mass balances (Eqs. (5) and (6)) by taking into account the concentration of the 1:2 complex, Eq. (13) can be deduced

$$K_{11}K_{12}[CD]^3 + K_{11}\{2K_{12}[BS]_o - K_{12}[CD]_o + 1\}[CD]^2 + \{K_{12}[BS]_o - K_{11}[CD]_o + 1\}[CD] - [CD]_o = 0 \tag{13}$$

the concentrations of the 1:1 and 1:2 complexes are given by Eqs. (14) and (15)

$$C_{11} = \frac{K_{11}[BS]_o[CD]}{1 + K_{11}[CD] + K_{11}K_{12}[CD]^2} \tag{14}$$

$$C_{12} = \frac{K_{11}K_{12}[BS]_o[CD]^2}{1 + K_{11}[CD] + K_{11}K_{12}[CD]^2} \tag{15}$$

For these systems, Eq. (1) is transformed to

$$\delta_{obs} = f_{CD}\delta_{CD} + f_{C_{11}}\delta_{C_{11}} + 2f_{C_{12}}\delta_{C_{12}} \tag{16}$$

where $\delta_{C_{12}}$ represents the chemical shift of a given nucleus of complexed cyclodextrin in the 1:2 complex, and $f_{C_{12}}$ is given by $f_{C_{12}} = [C12]/[CD]_o$. Equation (16) is easily transformed to

$$\Delta\delta_{obs} = f_{C_{11}}\Delta\delta_{max,C_{11}} + 2f_{C_{12}}\Delta\delta_{max,C_{12}} \tag{17}$$

where $\Delta\delta_{max,C_{11}} = \delta_{C_{11}} - \delta_{CD}$ and $\Delta\delta_{max,C_{12}} = \delta_{C_{12}} - \delta_{CD}$.

As previously, Eqs. (4) and (11)–(17) can be used to fit the experimental values of $\Delta\delta_{obs}$ for each of the carbon atoms of the β-CD. The obtained values for equilibrium constants and $\Delta\delta_{max}$ for both complexes are given in Table IV. Values for NaDC were recalculated from previously published data [5].

As in the case of γ-CD, the equilibrium constants K_{11} are similar for the six bile salts studied, independently of the stoichiometry of the complex, the highest values corresponding to NaC and NaDC. The K_{11} and $\Delta\delta_{max}$ values observed for the six bile salts, suggest that the structure of the 1:1 complexes must be very similar. Exception is made by the $\Delta\delta_{max}$ values for C4 of β-CD when complexing NaC and NaDC, suggesting a specific interaction between these two bile salts and β-CD which could explain the higher equilibrium constants observed for these two bile salts. K_{12} ranges from 0.5×10^3 to $1.2 \times 10^3\,M^{-1}$ suggesting again that the complexation by a second β-CD is very similar for the three dihydroxy bile salts.

The similarity of the structure of the formed complexes was confirmed by ROESY experiments. The intermolecular cross-peaks observed between β-CD and bile salts are given in Table V. Only interactions with H3, H5 and H6 of β-CD are considered, as H2 and H4 are not facing towards the cavity inside and H1 signal is affected by the solvent signal. The most noticeable fact is the absence of interactions between cyclodextrin protons and P1–6 protons of A and B rings of the trihydroxy bile salts, while they are present in dihydroxy bile salt complexes. However, all bile salts present interactions of C and D rings protons and the side chain protons with β-CD protons, mainly with H3.

TABLE V ROESY intermolecular cross-peaks observed between bile salt protons (named P) and β-CD protons (named H). s: strong; m: medium; w: weak

		NaC			NaGC			NaTC			NaDC			NaGDC			NaTDC		
Location	Proton	H3	H5	H6	H3	H5	H6	H3	H5	H6	H3	H5	H6	H3	H5	H6	H3	H5	H6
Side chain	P23	s			m	w	w	m	w	w	w			w	w	w	w	w	w
	P22	s			m	w		m	w		m	w		m	w	w	m	w	w
	P21	s	m	w	s	m	w	m	w	w	s	m		m	w		m	m	
	P20	m			m			m			s	m		s	w		m	w	
A/B rings	P19										m			m			m		
C/D rings	P18	m			m			s			s			s			m		
D ring	P17	s			s			m			s	w		m	w		m	m	
	P16	s	s		s	s		m	w		s	w		m	w		s	w	
	P15	s			m			m			m			m			m		
C/D rings	P14	m			m			s			w			s			m		
C ring	P12	m			w			w			w			w			w		
B ring	P6										w			m			w		
A ring	P5										w			m			m		
	P4										w			w			w		
	P3										s	w		s	m		m		
	P2										s			m			w		
	P1										m			m			m		
Amino acid chain	CH_2 (glycine)				w	w	w							w	w	w			
	CH_2CH_2 (taurine)								w	w								w	w

Therefore, ROESY experiments support the stoichiometry observed for each type of bile salts, suggesting that C and D rings, and the side chain of the bile salt, enter into the cavity of a *first* β-CD, while A and B rings interacts with a *second* β-CD, only in the 1:2 complexes. The only difference between related bile salts (i.e. NaC–NaDC, and so on) is the existence or not of a hydroxyl group at C7. The bigger cavity of γ-CD allows this region of the bile salt to be located inside the cyclodextrin cavity (notice the interactions of P7 with H3 in Table III), and therefore γ-CD does not discriminate between both types of bile salts. However, this region clearly remains outside the β-CD cavity, as no interactions with P7 are observed, allowing the complexation of this region by a second cyclodextrin molecule when its hydrophobicity is large enough to favourably interact with the hydrophobic cavity of β-CD. This is supported by the interactions of P19 with H3 due to the presence of the second β-CD molecule as they are only observed for dihydroxy bile salts.

On the other hand, from (i) the high number of interactions of H3 with protons of the C and D rings, as well as with protons of the A and B rings for dihydroxy bile salts, (ii) the absence of these interactions with H6, and (iii) the existence of some weak interactions of the side chain protons (mainly P23) with H6, it may be concluded that bile salts enter in the cavity of β-CD by the side of the secondary hydroxyl groups. Furthermore, the following facts: (i) for all bile salts, there are intramolecular interactions (results not shown) of P23 proton, placed in the side chain, with P16 and P17, placed in the D ring, and (ii) the four conjugated bile salts present interactions between the protons of the amino acid chain ($-CH_2-$ in glycine and $-CH_2-CH_2-$ in taurine) and H6 of β-CD (Table V), suggest that the side chain (common to the six bile salts) acquires a folded conformation toward the steroid body, but the amino acid chain is unfolded outward the β-CD cavity.

All previous facts allow the structure of the complexes to be proposed. As examples, Fig. 5 shows the proposed structures for the conjugated amino acids with β-CD. The similarity of the structures for each pair di/trihydroxy bile salts (cross-peaks are basically the same for each pair) justifies the close agreement between the K_{11} values obtained for both stoichiometries, supporting the values corresponding to the dihydroxy bile salts affected by a high standard deviation as a consequence of the strong correlation between the fit parameters. This could be reduced by having experimental points at higher $[\text{Bile Salt}]_o/[\beta\text{-CD}]_o$ ratio allowing a better estimation of the fit parameters. However the range of suitable experimental conditions is limited by the formation of bile salt aggregates above *cmc*, the low solubility of β-CD and the minimum concentration

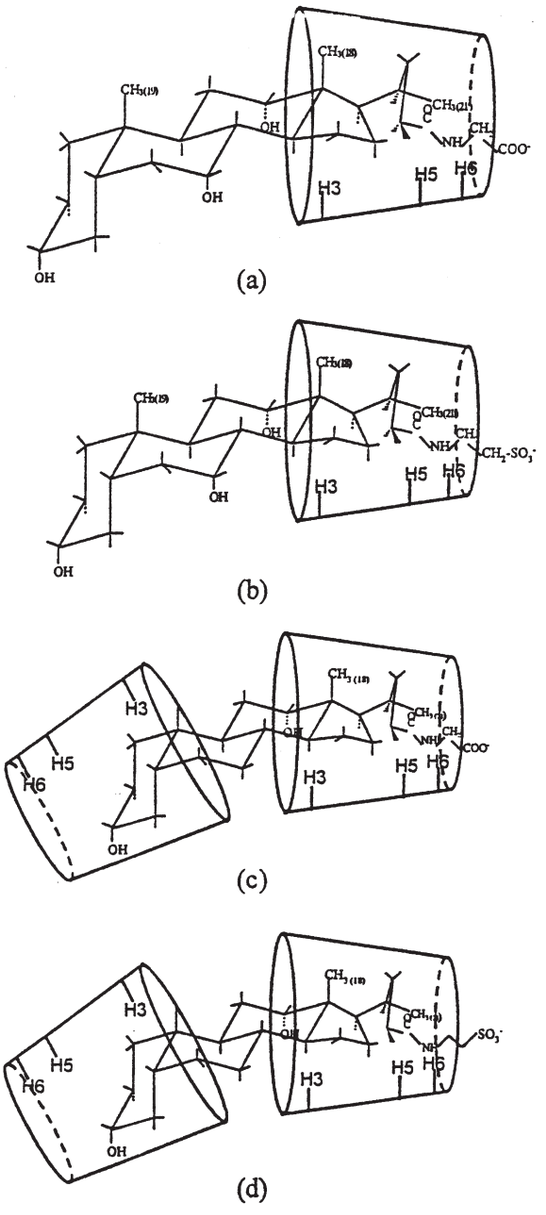

FIGURE 5 Schematic representation of 1:1 and 1:2 Bile Salt/β-CD complexes, deduced from ROESY experiments. Bile salts (a) NaGC, (b) NaTC, (c) NaGDC, and (d) NaTDC.

of the reactants required for NMR experiments. Alternatively, the K_{11} could be fixed for 1:2 complex to that value corresponding to the 1:1 trihydroxy bile salt complex. When it is done so, good fittings with lower standard deviations and similar values to those of the free fittings are observed. For example, for NaDC when K_{11} is fixed to $7.5 \times 10^3\ M^{-1}$, a value of $(1.7 \pm 0.6) \times 10^3\ M^{-1}$ is obtained for K_{12}. The proposed structures are in agreement with the observed similarity between K_{12} values, corresponding to the inclusion of the A ring (this part of the molecule is identical for the three dihydroxy derivatives) of bile salts into β-CD.

TABLE VI ROESY intermolecular and intramolecular cross-peaks observed between NaC and NaDC protons (named P) and α-CD protons (named H). s: strong; m: medium; w: weak

		NaC						NaDC					
		α-CD	Side chain		D ring		C ring	α-CD	Side chain		D ring		C ring
Location	Proton	H6	P21	P18	P17	P16	P12	H6	P21	P18	P17	P16	P12
Side chain	P23	s	s					m	m				
	P22	s			m	m		m			m	w	
	P21						m						s
	P20			s						s			

A careful analysis of the insert in Job's plot of Fig. 4b shows that the residuals present some periodicity which could indicate that some 1:2 complex could be formed, as the maximum seems to be slightly shifted from $X_{\beta-CD}$ 0.5. When the experimental data were analysed by considering the formation of 1:1 and 1:2 complexes, no convergence is achieved unless some optimising parameters were considered constants. For instance, when $\Delta\delta_{max}$ for each carbon atom were fixed to the values of NaTDC, values of $(1.8 \pm 0.3) \times 10^3\,M^{-1}$ and $14 \pm 2\,M^{-1}$ were obtained for K_{11} and K_{12}, respectively. This indicates that, if formed, the amount of 1:2 complex is very low and therefore the 1:1 stoichiometry model is essentially correct.

Inclusion Complex Formation with α-CD

The ^{13}C-NMR titration curves (not shown) observed for α-CD in the presence of increasing amounts of NaC and NaDC show that only small chemical shift displacements are observed as $\Delta\delta_{obs}$ is always lower than 0.05 ppm for all α-CD carbon atoms, indicating a very weak interaction between bile salts and α-CD. The lower diameter of α-CD compared to γ- and β-CD and the proposed structures for the complexes with the other two cyclodextrins, suggest that the steroid nucleus is too big to enter into the cavity of α-CD and that only the side chain of bile salts could enter into it. Inter- and intramolecular interactions deduced from ROESY spectra presented in Table VI, confirm this hypothesis as only interactions between H6 and P22 and P23 (protons of the side chain) are observed for bile salts. Furthermore, the interactions of P22 with P16 and P17, P12 with methyl protons P21, and the absence of interactions between P23 and protons of D ring of the steroid nucleus, suggest that the side chain is unfolded and partially included into the α-CD cavity as shown in Fig. 6. The interaction of the side chain with α-CD is in agreement with the observed complexation of aliphatic chain compounds, as for instance, aliphatic alcohols, [54–56] by α-CD.

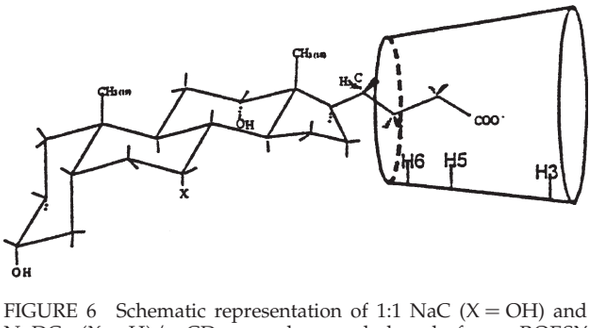

FIGURE 6 Schematic representation of 1:1 NaC (X = OH) and NaDC (X = H)/α-CD complexes, deduced from ROESY experiments.

CONCLUSION

The main conclusion derived from the present study is that β-CD molecularly recognises bile salts, distinguishing di- from trihydroxy ones, while γ-CD does not. This is a consequence of the absence of the hydroxyl group at C7 in the dihydroxy derivatives since it enlarges the hydrophobic region of the steroid body, allowing a favourable interaction of this region with the hydrophobic cavity of the second β-CD. However, the bigger cavity of γ-CD allows this region of the bile salt to be located inside the cyclodextrin cavity and therefore γ-CD does not discriminate between both types of bile salts.

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