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# Complexation of Sodium Cholate and Sodium Deoxycholate by β-Cyclodextrin and Derivatives†

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The complexation behavior of two bile salts—sodium cholate (NaC) and sodium deoxycholate (NaDC)—with β-cyclodextrin (β-CD), 6-deoxy-6-amino-β-cyclodextrin (β-$CDNH_2$), and dimer I (*N*,*N*′-bis(6-deoxy-β-cyclodextrin)pyromellic acid diamide) was studied by NMR techniques. Complexes formed between β-CD and β-$CDNH_2$ with NaC and NaDC have 1:1 and 2:1 (host:guest) stoichiometries, respectively. Complexes with β-$CDNH_2$ show higher equilibrium constants than those with β-CD because of the electrostatic effect of the protonated amine group. Dimer I showed 1:2 and *n*:*n* stoichiometries with NaC and NaDC, respectively. ROESY spectra stated that bile salts enter first with their 5-C ring forward the inner cavity by the side of the secondary hydroxyl groups of cyclodextrins. In the complexes formed with β-$CDNH_2$, the steroid body of the bile salt enters deeper in the cavity, while the carboxylated side chain is extended toward the protonated amine group at C-6, allowing an electrostatic interaction between both groups. In the case of the 2:1 stoichiometry, the second cyclodextrin complexes ring A of the steroid body.

## Introduction

Bile salts are involved in one of the most important pathways for the metabolism and excretion of cholesterol in mammals and represent an example of the liver capacity to convert lipid-soluble material into excretable water-soluble products.[1] Bile salts have a characteristic steroid structure, with a side chain at C-17, methyl groups at C-10, C-13, and C-20, different numbers of hydroxyl groups at C-3, C-7, and C-12, and a carboxylic acid at C-23, which can or cannot be conjugated with an amino acid.[1] Because of their amphipatic nature, they behave as biosurfactants and are used as drugs in gallstone disease treatments. Among the bile salts, the most often studied are sodium cholate (NaC) and sodium deoxycholate (NaDC)[2,3] (Figure 1a).

Cyclodextrins are cyclic oligomers built up from 6, 7, or 8 glucopyranose units, linked by α-(1−4)-glycosidic linkages, named α, β, and γ-cyclodextrins, respectively (Figure 1b). They form inclusion complexes in water with a variety of organic molecules, a property used to increase the bioavailability of poorly soluble drugs.[4] Several cyclodextrin dimers have been synthesized, and their effect in the inclusion complex formation has been studied. In general, dimers show higher binding constants in comparison with cyclodextrins.[5−10] Dimers can be divided into three groups depending on the side of the cyclodextrin where the linking is carried out: head to head, tail to tail, and head to tail, where head and tail are the primary and secondary hydroxyl sites of cyclodextrin.[9] Several linking bridges have been described: diamine, diether, diester, disulfide, dithioether, imidazolium, and diamide are the most common ones.[11]

The complexation of surfactants by cyclodextrins produces a change in their physicochemical properties, because of the insertion of the hydrophobic chain into the cyclodextrin cavity. A large number of studies have been carried out to study their complexation behavior by a variety of experimental techniques.[12] However, the number of studies on the complexation of biosurfactants, such as bile acids, is really small, and particularly the structure of complexes is still unknown. Yang and Breslow[13] reported that they could not determine the stability constant between β-cyclodextrin (β-CD) and cholic acid by titration calorimetry. Mucci et al.[14,15] reported that cholic acid showed lower stability constants with hydroxypropyl-β-cyclodextrin (HPβCD) compared with those for chenodeoxycholic and ursodeoxycholic acids, because of the hydroxyl group at C-12, which is close to the complexation site. They could not confirm the inclusion of bile acids in HPβCD by differential scanning calorimetry and X-ray

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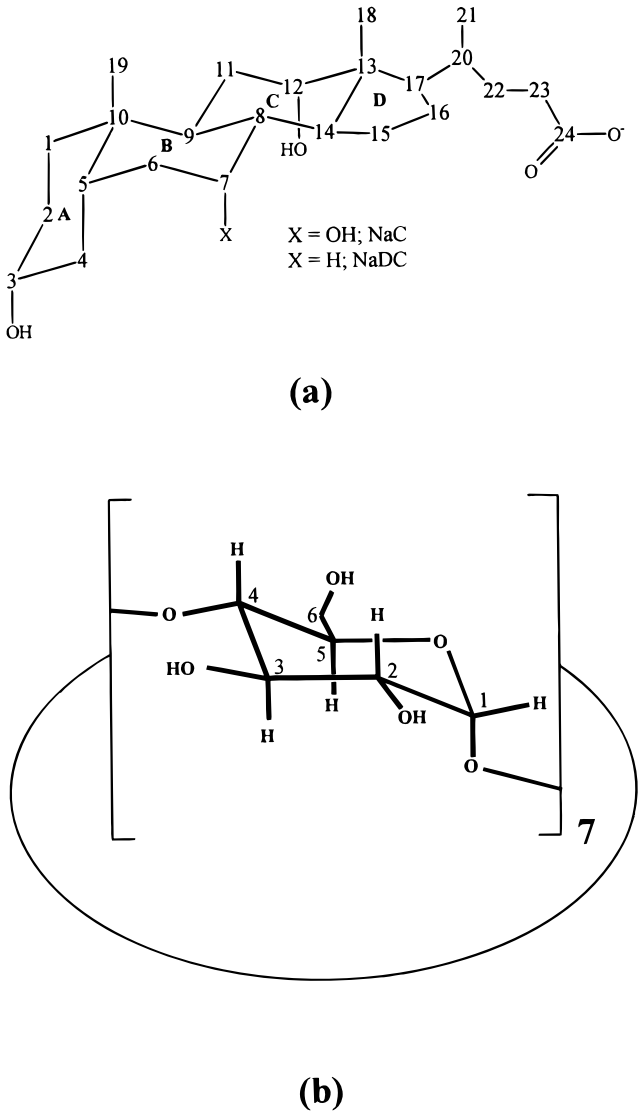


**Figure 1.** Schematic structures of (a) bile salts (NaC and NaDC) and (b) $\beta$-cyclodextrin ($\beta$-CD).

diffractometry. Recently, Hamada et al.[16] have reported that the stability constants obtained between four $\gamma$-cyclodextrin chromophore derivatives with deoxycholic acid were always higher than those obtained with cholic acid. Considering that the physicochemical properties of bile salts are completely different from those of bile acids and that only a few works have been published on their complexation,[17] in this work the complexation of two bile salts (NaC and NaDC) with $\beta$-CD and two derivatives is reported. Attention is paid to the effect of an electrostatic environment and to the formation of supramolecular complexes.

Because the inclusion of a guest inside cyclodextrin's cavity produces a change in the electronic environment of their atoms, differences in the chemical shifts of the nuclear magnetic resonance (NMR) spectra of both free and complexed cyclodextrin are expected. For the purpose of this paper, NMR spectroscopy is one of the most useful techniques to study the CD/bile salt complexes because it provides microscopic information on the structure,[4] stoichiometry, and equilibrium constants.

## Experimental Section

**General Procedure.** Commercial bile salts (Sigma-Aldrich), $\beta$-cyclodextrin (kindly supplied by Roquette), and synthesized derivatives were dried in a vacuum oven. Other chemicals were of high quality and used without further purification. Thin-layer chromatography (TLC) was performed on aluminum-backed silica gel plates 60 $F_{254}$ (Merck) eluting ethyl acetate/isopropyl alcohol/water/concentrated $NH_4OH$ (2:3:4:0.3) and visualized with ultraviolet light, 5% $H_2SO_4$ in MeOH, or 0.2% ninhydrin in EtOH sprays followed by charring. Melting points were determined on a Gallenkamp apparatus. Mass spectra were recorded on a HP 1100 LC/Ms spectrometer. $^1$H, $^{13}$C, and DEPT 135 NMR spectra were recorded on a Bruker AC spectrometer at 300 and 75 MHz at 298.1 ($\pm$ 0.1) K. Rotating-frame Overhauser effect spectroscopy (ROESY) experiments were recorded on a Bruker AMX spectrometer at 500 MHz. Conditions for ROESY were as follows: total sample concentration, 10 mM [cyclodextrin (or derivatives) plus bile salts] with a stoichiometric ratio corresponding to the maximum of Job's plot (samples were kept 24 h before measurement for equilibration); 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 $D_2O$.

**Synthesis. 6-Deoxy-6-amino-$\beta$-cyclodextrin ($\beta$-CDNH$_2$).** The monoamine derivative was synthesized by two methods (Scheme 1): (a) the well-known two-step protocol that involves the formation of the azide derivative,[18] which is then reduced with Pd/C[19] (50−55%, from 6-O-tosyl-$\beta$-cyclodextrin[20]); (b) a modification of the method of Fragoso et al.[21] in which 6-*O*-tosyl-$\beta$-cyclodextrin[20] (1 g, 0,77 mmol) was dissolved in a 25% ammonium solution (25 mL) and stirred at 50° C overnight. After evaporation of the solvent under reduced pressure, the resulting white solid was redissolved in water and purified with a Sephadex C-25 cationic column using water and 0.1 M $NH_4HCO_3$ as eluents, to give product in 45−50% yield: $R_f$ 0.2; mp 202−203 °C; $^1$H NMR $\delta$ 4.97 (s, 7, H-1), 3.44−3.89 (m, H-2, H-3, H-4, H-5, H-6), 3.36 (t, 1, H-4′), 3.24 (dd, 1, $CH_2-NH_2$), 2.94 (dd, 1, $CH_2-NH_2$); $^{13}$C and DEPT 135 NMR $\delta$ 104.45 (C-1), 85.82 (C-4′), 83.89 (C-4), 75.71 (C-3), 74.43 (C-2), 74.37 (C-5), 62.93 (C-6, negative signal in DEPT), 42.82 (C-6′, negative signal in DEPT) MS $m/z$ 1156.3 (M + Na). Although the yield obtained by the first method was higher, the second one showed to be faster and cleaner.

***N,N′*-Bis(6-deoxy-$\beta$-cyclodextrin)pyromellic Acid Diamide (Dimer I).** Dimer I was synthesized by the reaction of $\beta$-CDNH$_2$ (0.5 g, 0.44 mmol) with 1,2,4,5-benzenetetracarboxylic dianhydride (0.038 g, 0.176 mmol) in dry dimethylformamide (DMF; 50 mL) (Scheme 1). The reaction was stirred for 48 h at 50 °C. The solvent was removed under reduced pressure at low temperature, and the resulting solid was redissolved in water. Water was added and removed several times until no DMF was observed; finally the product was purified through a Sephadex C-25 column using water and 0.1 M $NH_4HCO_3$ as eluents, obtaining a white solid (0.31 g, 62%): $R_f$ 0.6; mp 185−190 °C (dec); $^1$H NMR $\delta$ 7.85−7.45 (m, 1, Ar−H), 4.95 (s, 7, H-1), 3.47−3.89 (m, 42, H-2, H-3, H-4, H-5, H-6); $^{13}$C and DEPT 135 NMR $\delta$ 176.09 (COOH, signal not present in DEPT), 173.79 (CONH, not present in DEPT), 141.38, 140.51, 139.4, 138.51, 137.44 (substituted aromatic carbons, not present in DEPT), 130.2, 129.79 (CH aromatic ring), 104.54 (C-1), 85.38 (C-4′), 83.75 (C-4), 75.69 (C-5), 74.73 (C-3), 74.38 (C-2), 62.83 (C-6, negative signal in DEPT), 43.14 (C-6′, negative signal in DEPT); MS $m/z$ 2508.5 (M + Na). From this information (multiplet, instead of singlet at the aromatic region) it was concluded that the product was a mixture of 1−3 and 1−4 derivatives, as shown in Scheme 1. When analyzing with more detail the aromatic region, we observe three doublets (7.85, 7.6, and 7.45 ppm) with an integration relation of 1:4:1. Considering that the first and last doublets belong to the 1−3 derivative (asymmetric hydrogens) and the middle one to the 1−4 derivative, we can assume that our product is a mixture of both meta and para isomers in a 1:2 ratio.

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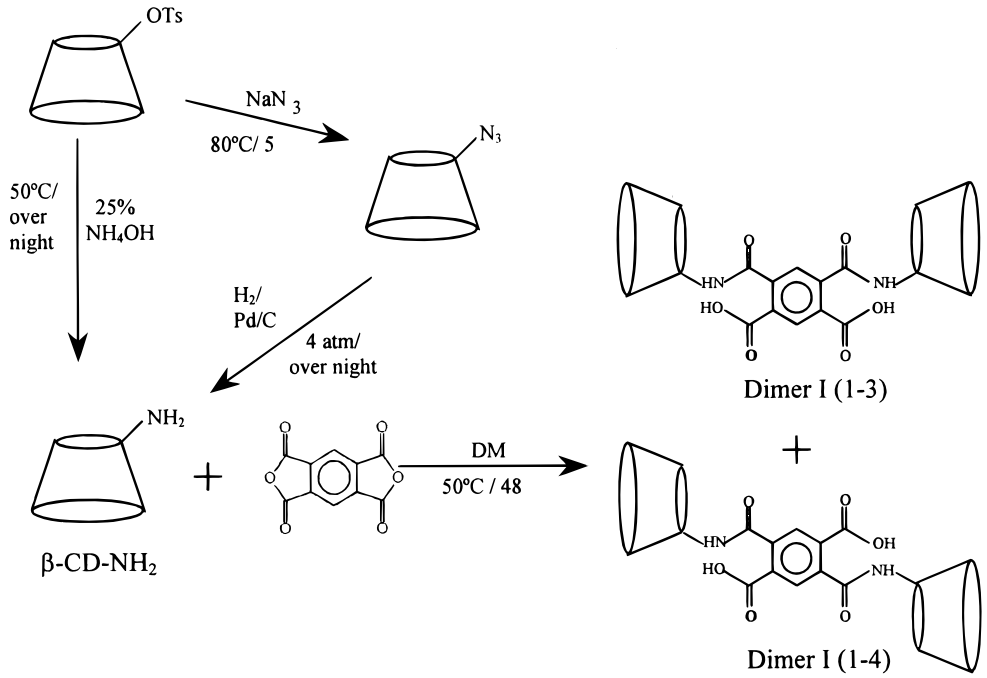
**Scheme 1**




**Complexes Formation.** The stoichiometries of the inclusion complexes formed were provided using the continuous variation technique (Job's plot)[22] based on the difference observed in the chemical shifts of different carbons of cyclodextrin (or derivatives) in the presence of increasing amounts of the bile salt. The plot of $\Delta\delta_{obs}x_{HOST}$ against the mole fraction of the host or guest shows a maximum at $x_{CD} = n/(n + m)$ or $x_{BS} = m/(n + m)$, respectively, where $n$ and $m$ represent the stoichiometric ratios of cyclodextrin and bile salts according to eq 1. For each system, 10 solutions with different cyclodextrin (or derivative) and bile salt molar ratios were prepared, where the total molar concentration was kept constant at 10 mM (concentration below the two bile salts critical micelle concentration (cmc) values[3]). Samples were left overnight for equilibration before measurement.

**Theoretical Background.** The $n$:$m$ complex formation ($C_{nm}$) between a host (cyclodextrins, CD) and guest (bile salts, BS) is represented by eq 1:

$$mBS + nCD \rightleftarrows C_{nm} \quad (1)$$

The stability constant of this equilibrium is given by

$$K_{nm} = \frac{[C_{nm}]}{[BS]^m[CD]^n} \quad (2)$$

Under fast exchange conditions,[23] the observed $^{13}C$ chemical shift for a CD atom is expressed as

$$\delta_{obs} = f_{CD}\delta_{CD} + nf_{C_{nm}}\delta_{C_{nm}} \quad (3)$$

where $\delta_{CD}$, $\delta_{C_{nm}}$, $f_{CD}$, and $f_{C_{nm}}$ represent the chemical shift of a given nucleus of free and complexed CD and their molar fractions, respectively. From a mass balance for CD and rearrangement of $\Delta\delta_{obs} = \delta_{obs} - \delta_{CD}$ and $\Delta\delta_{Cm} = \delta_{C_{nm}} - \delta_{CD}$, eq 4 is obtained:

$$\Delta\delta_{obs} = nf_{C_{nm}}\Delta\delta_{C_m} \quad (4)$$

Therefore, the chemical shift displacement of a specific nucleus of CD is expressed as

$$\Delta\delta_{obs} = \frac{n\Delta\delta_{C_m}}{[CD]_0}[C_{nm}] \quad (5)$$

From the mass balances of cyclodextrin and bile salt

$$[BS]_0 = [BS] + m[C_{nm}] \quad (6)$$

$$[CD]_0 = [CD] + n[C_{nm}] \quad (7)$$

and eq 2, the concentration of the complex in solution may be deduced. This concentration is given by eq 8 for a 1:1 complex and by eq 9 for a 2:1 complex:

$$[C_{11}]^2 - ([BS]_0 + [CD]_0 + 1/K_{comp})[C_{11}] + ([BS]_0[CD]_0) = 0 \quad (8)$$

$$-4[C_{21}]^3 + 4([CD]_0 + [BS]_0)[C_{21}]^2 - (4[BS]_0[CD]_0 + [CD]_0{}^2 + 1/K_{comp})[C_{21}] + [BS]_0[CD]_0{}^2 = 0 \quad (9)$$

When using proper initial concentrations of bile salt and cyclodextrin, the cubic term in eq 9 is negligible. Finally, when either eq 8 or eq 9 is combined with eq 5, an expression for the chemical shift displacement of cyclodextrin as a function of the concentrations of bile salt and cyclodextrin added is obtained.

For the 1:1 complex this expression is represented by eq 10:

$$\Delta\delta_{obs} = \frac{\Delta\delta_{C_m}}{2[CD_0]}\left\{[BS_0] + [CD_0] + \frac{1}{K_{comp}} - \left(\left([BS_0] + [CD_0] + \frac{1}{K_{comp}}\right)^2 - 4([BS_0][CD_0])\right)^{1/2}\right\} \quad (10)$$

That for the 2:1 complex is represented by eq 11:

$$\Delta\delta_{obs} = \Delta\delta_C\left\{4[BS_0][CD_0] + [CD_0]^2 + \frac{1}{K_{comp}} - \left(\left(4[BS_0][CD_0] + [CD_0]^2 + \frac{1}{K_{comp}}\right)^2 - 16[BS_0][CD_0]^2([CD_0] + [BS_0])\right)^{1/2}\right\}/4[CD_0]([CD_0] + [BS_0]) \quad (11)$$

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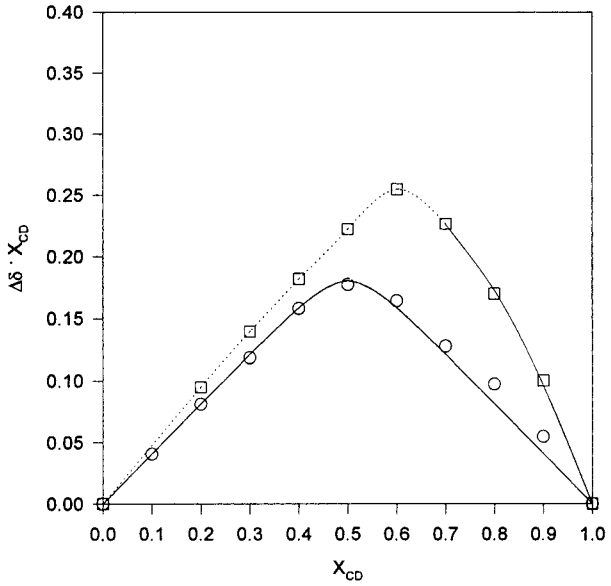


**Figure 2.** Job's plot corresponding to the chemical shift displacement of Carbon 1 of $\beta$-CD for the 1:1 $\beta$-CD/NaC (○) and 2:1 $\beta$-CD/NaDC (□) complexes. Solid lines are the result of the nonlinear fitting.

Experimental data ($\Delta\delta_{obs}$) are fitted to the corresponding expression by using a nonlinear least-squares computer program to obtain the stability constant ($K_{comp}$) and the chemical shift of the complex ($\delta_{C_m}$) as fitting parameters.[23] For the 2:1 complexes, only those values for $x_{CD} \geq 0.67$ were used to estimate the stability constant (solid line in Figure 2).

## Results and Discussion

**Complexation of NaC and NaDC by $\beta$-CD.** Although chemical shift of any atom of both host (cyclodextrin) and guest (bile salt) can be used to study the complexation process, in this work the displacements of cyclodextrin atoms are studied. Because $^1$H nucleus chemical shifts are small and overlap between them, while $^{13}$C signals are clear singlets with higher chemical shift displacements, $^{13}$C experiments have been carried out. To determine the stoichiometry and stability constants, the chemical shift displacement of carbons 1 and 4 were analyzed, because these atoms are more influenced than carbons 2, 3, 5, and 6 (in fact, these signals remain practically constant).

Figure 2 shows the resulting continuous variation plots for the complexes formed between $\beta$-CD with NaC and NaDC. Obviously, the plots show a 1:1 and 2:1 (host:guest) stoichiometry for NaC and NaDC, respectively (maxima at $x_{CD} = 0.5$ and $x_{CD} = 0.67$). These results are in agreement with those obtained by Tan and Lindebaum,[17] who studied the complexation of bile salts by $\beta$-CD with flow microcalorimetry and NMR techniques.

Because in our experiments all of the 2:1 systems present a maximum in Job's plot at exactly 0.67, it can be considered that the concentration of the 1:1 complex present in solution is negligible, as described by Werner.[24] This fact means that the equilibrium may be considered as a global complexation process (in which two cyclodextrins complex one bile salt in a single step) for which only one equilibrium constant is required. The deduced stability constants and $\delta_{C_m}$ values for both complexes are shown in Table 1.

The agreement between the experimental and calculated $^{13}$C chemical shift displacement of the three carbons showed very good fits for both 1:1 and 2:1 systems. Table 1 shows that $K_{21}$ values are higher than $K_{11}$ values, following the same pattern as previous results obtained for both bile salts (and acids) with different cyclodextrins.[14−16] Hamada et al.[16] have reported that the stability constants obtained between four $\gamma$-cyclodextrin chromophore derivatives with deoxycholic acid were always higher than the ones obtained with cholic acid. Similarly, Mucci et al.[14,15] reported that cholic acid showed lower stability constants with HP$\beta$CD than chenodeoxycholic and ursodeoxycholic acids, because of the hydroxyl group at C-12, which is close to the complexation site.

To study the structure of the complexes formed, ROESY experiments were carried out. ROESY is a two-dimensional technique based on the nuclear Overhauser effect (NOE), in which cross-peaks may be observed between protons if the corresponding internuclear distance is smaller than 3−4 Å.[25] This technique has been previously used to study the complexation of steroids[26] and other compounds[25] with cyclodextrins. Mucci et al.[26] studied the complexation of ursodeoxycholic acid with $\beta$-cyclodextrin by ROESY, finding that the aliphatic side chain of the bile acid enters the cyclodextrin cavity by the side of the secondary rim. The complete assignment of the NaC and NaDC protons was made following the results reported by Campedron et al.[2] and Barnes and Geckle.[27] Only cross-peak interactions with H-3, H-5, and H-6 of cyclodextrins

**Table 1. Stability Constants ($K_{nm}$) and $^{13}$C NMR Chemical Shift Displacements ($\Delta\delta_{C_m}$, ppm) for the Complexes Formed between $\beta$-CD and Derivatives with Sodium Cholate (NaC) and Sodium Deoxycholate (NaDC)**

| | | NaC ($K_{11}$/10$^3$ M$^{-1}$) | NaDC ($K_{21}$/10$^3$ M$^{-2}$) |
|---|---|---|---|
| | | $\beta$-CD Complexes | |
| Carbon 1 | $K_{nm}$ | 8.3 ± 4.8 | 39.0 ± 0.4 |
| | $\Delta\delta_{C_m}$ | 0.414 ± 0.009 | 0.62 ± 0.01 |
| Carbon 4 | $K_{nm}$ | 7.7 ± 1.2 | 39.0 ± 0.6 |
| | $\Delta\delta_{C_m}$ | 0.574 ± 0.004 | 0.83 ± 0.02 |
| | | $\beta$-CDNH$_2$ Complexes | |
| Carbon 1 | $K_{nm}$ | 11.5 ± 12.5 | 49.1 ± 0.1 |
| | $\Delta\delta_{C_m}$ | 0.39 ± 0.02 | 0.54 ± 0.02 |
| Carbon 4 | $K_{nm}$ | 10.5 ± 8.7 | 49.10 ± 0.04 |
| | $\Delta\delta_{C_m}$ | 0.55 ± 0.02 | 0.76 ± 0.01 |

**Table 2. ROESY Intermolecular Cross-Peaks Observed between Sodium Cholate (NaC) and Cyclodextrin ($\beta$-CD, $\beta$-CDNH$_2$, and Dimer I) Protons (× Weak; ××, Medium; ×××, Strong Interactions)**

| | $\beta$-CD | | | $\beta$-CD-NH$_2$ | | | dimer | | |
|---|---|---|---|---|---|---|---|---|---|
| NaC | H-3 | H-5 | H-6 | H-3 | H-5 | H-6 | H-3 | H-5 | H-6 |
| P-23 | ××× | | | ××× | | × | ××× | | |
| P-22 | ××× | | | ×× | × | × | ××× | | |
| P-21 | ××× | ×× | | ××× | ×× | × | ××× | ×× | |
| P-20 | ×× | | | ×× | | | ×× | | |
| P-19 | | | | | | | × | | |
| P-18 | ×× | | | ××× | | | ××× | | |
| P-17 | ××× | | | × | ×× | | ××× | | |
| P-16 | ××× | | | ×× | ×× | | ××× | | |
| P-15 | ××× | | | × | ×× | | ××× | | |
| P-14 | ×× | | | | | | ×× | | |
| P-12 | ×× | | | × | | | ×× | | |
| P-11 | | | | × | | | ×× | | |
| P-9 | | | | ×× | | | ×× | | |
| P-8 | | | | × | | | ×× | | |
| P-7 | | | | ××× | | | | | |

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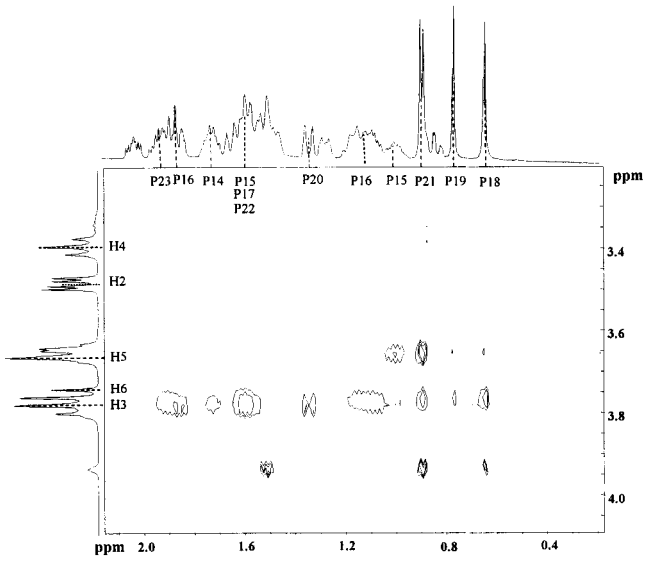


**Figure 3.** Partial ROESY NMR spectrum of the 1:1 $\beta$-CD/NaC complex (intermolecular region).

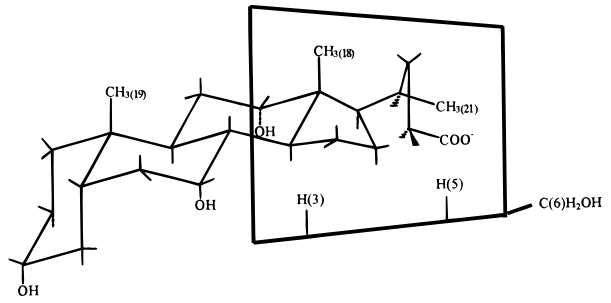


**Figure 4.** Schematic representation of the 1:1 $\beta$-CD/NaC complex, deduced from ROESY cross-peak interactions.

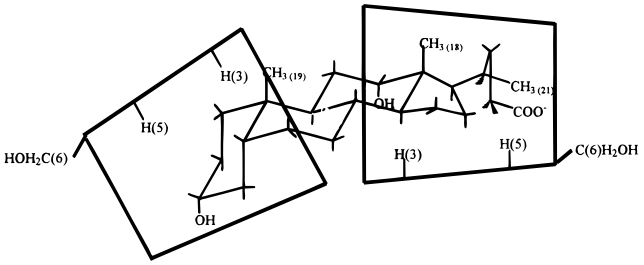


**Figure 5.** Schematic representation of the 2:1 $\beta$-CD/NaDC complex, deduced from ROESY cross-peak interactions.

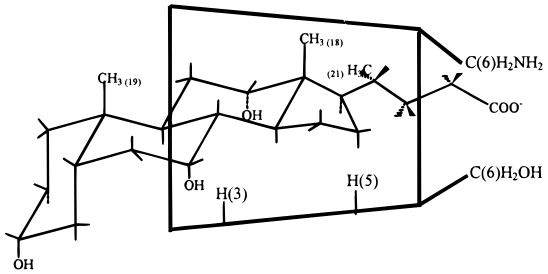


**Figure 6.** Schematic representation of the 1:1 $\beta$-CD$NH_2$/NaC complex, deduced from ROESY cross-peak interactions.

were considered to analyze the results, because H-2 and H-4 are not facing the inner cavity and H-1 is affected by $D_2O$ (Figure 1b).

Table 2 summarizes the cross-peak interactions between $\beta$-CD and NaC protons (Figure 3). The following cross-peak interactions are observed **(**the notation used is *n*-H

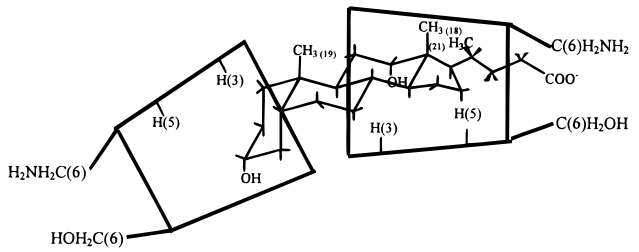


**Figure 7.** Schematic representation of the 2:1 $\beta$-CD$NH_2$/NaDC complex, deduced from ROESY cross-peak interactions.

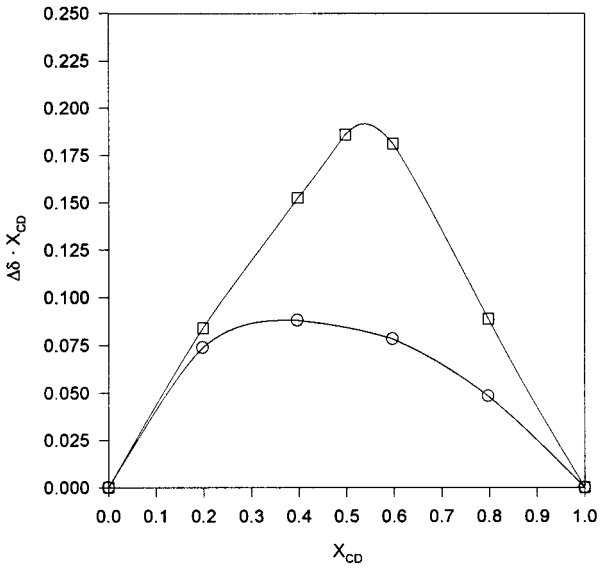


**Figure 8.** Job's plot corresponding to the chemical shift displacement of Carbon 1 of $\beta$-CD for the 1:1 (*n*:*n*) dimer I/NaC (○) and 1:2 dimer I/NaDC (□) complexes. Solid lines are the result of a nonlinear fit of the data.

for $\beta$-CD protons and P-*n* for bile salts protons, where n is the carbon number indicated in Figure 1**)**. 3-H interacted with P-12, P-14 to P-18, and P-20 to P-23; 5-H showed interaction with P-21 of NaC. Significant interactions were found between the side chain and the steroid body (P-23 with P-16 (××); P-22 with P-16 (×); and P-20 with P-17 (××)).

From the following statements, (a) 3-H is interacting with P-18 but not with P-19 of NaC, (b) 3-H is interacting with protons from rings C (P-12, P-14) and D (P-14 to P-17) and the side chain (P-20 to P-23), (c) 5-H presents cross-peak interactions with protons from the side chain (P-21), and (d) the interactions between the side-chain protons, it may be confirmed that in the 1:1 complex between $\beta$-CD and NaC the steroid body enters forward into the inner cavity of $\beta$-CD by the side of the secondary hydroxyl groups, with the side chain folded toward the steroid body (Figure 4), i.e., rings D and C are totally and partially included, respectively. These results are in agreement with those obtained by Mucci et al.[26] for ursodeoxycholic acid.

For the $\beta$-CD/NaDC complex the observed interactions (see Table 3) were 3-H with P-1 to P-6 and P-12 to P-23 and 5-H with P-16, P-17, and P-20 to P-22. The side chain also interacts with the steroid body (P-23 with P-16 (××); P-22 with P-16 (×) and P-17 (××); and P-20 with P-17 (××)).

Because of the hydrogen at the C-7 position of the steroid body of NaDC, its hydrophobic region is larger than that in NaC, allowing the formation of a stable inclusion complex with two cyclodextrins. This can be confirmed by

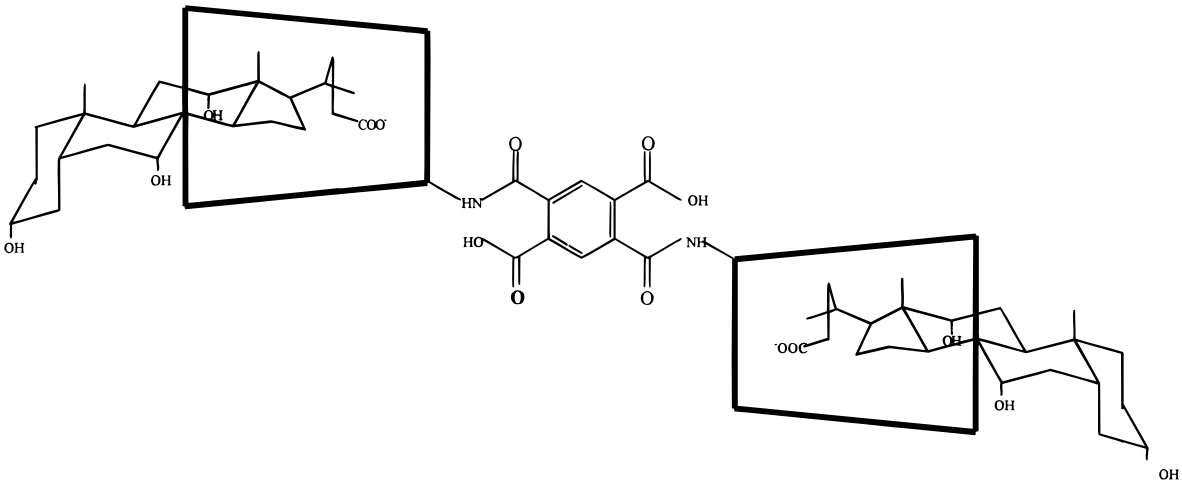

**Figure 9.** Schematic representation of the 1:2 dimer I/NaC complex, deduced from ROESY cross-peak interactions. Both isomers 1−3 and 1−4 can be present.

**Table 3. ROESY Intermolecular Cross-Peaks Observed between Sodium Deoxycholate (NaDC) and Cyclodextrin (*β*-CD, *β*-CDNH$_2$, and Dimer I) Protons (×, Weak; ××, Medium; ××× Strong Interactions)**

| | *β*-CD | | *β*-CD-NH$_2$ | | dimer I | |
|---|---|---|---|---|---|---|
| NaDC | H-3 | H-5 | H-3 | H-5 | H-3 | H-5 |
| P-23 | × | | × | | × | |
| P-22 | ×× | × | ××× | ×× | ×× | |
| P-21 | ××× | ×× | ××× | ××× | ××× | |
| P-20 | ××× | ×× | ××× | ×× | ×× | |
| P-19 | ×× | | | | | |
| P-18 | ××× | | ××× | | ××× | |
| P-17 | ××× | × | ×× | ×× | ×× | |
| P-16 | ××× | × | ×× | | ×× | |
| P-15 | ×× | | ×× | | ×× | |
| P-14 | × | | × | | ×× | |
| P-12 | × | | × | | | |
| P-11 | | | × | | | |
| P-9 | | | | | | |
| P-8 | | | × | | | |
| P-7 | | | × | | | |
| P-6 | × | | | | | |
| P-5 | × | | × | | × | |
| P-4 | × | | × | | ×× | |
| P-3 | ××× | × | ××× | | ××× | |
| P-2 | ××× | | × | | ×× | |
| P-1 | ×× | | × | | ×× | |

the interaction of 3-H with protons of the four rings of the bile salt (see above) and the interactions between 5-H with rings A (P-3) and D (P-16 and P-17) and the side chain (P-20 to P-22). These interactions confirm the 2:1 stoichiometry for the *β*-CD/NaDC complex. The interaction between the side chain and the steroid body also suggests that the side chain is folded toward the steroid body.

From this information, it is deduced that (a) the steroid body enters forward into the inner cavity of one cyclodextrin by the side of the secondary hydroxyl groups, with the side chain folded toward the steroid body, and (b) the second *β*-CD complexes rings A (totally) and B (partially) by the second cyclodextrin (Figure 5).

**Complexation of NaC and NaDC by *β*-CDNH$_2$.** As previously, Job's plots for carbons 1 and 4 of *β*-CDNH$_2$ showed maxima at 0.5 for NaC and 0.67 for NaDC; i.e., the complexes formed have the same stoichiometries as those with *β*-CD.

Table 1 summarizes the values of the equilibrium constants and $\delta_{C_m}$. In comparison to former results, the stability constants are now higher, in agreement with results obtained by other authors.[28,29] These higher values may be due to the electrostatic interactions between the protonated amino group and the negative carboxylate group of bile salts. This is confirmed by the ROESY results.

Table 2 resumes the interactions between *β*-CDNH$_2$ and NaC protons. The main interactions are 3-H of *β*-CDNH$_2$ with P-7 to P-12, P-15 to P-18, and P-20 to P-23; 5-H with P-15 to P-17, P-21 and P-22; and 6-H with P-21 to P-23.

The main differences between these results and those for *β*-CD are the interactions of the side chain with 5-H (P-22) and 6-H (P-21 to P-23) of *β*-CDNH$_2$. No intramolecular interactions between the side chain and the steroid body are observed. These facts indicate that the side chain is unfolded, with the negative carboxylate group moving toward the positive protonated amino group. Furthermore, the interactions of 3-H with P-7 to P12 indicate that the side-chain elongation produces a deeper penetration of the steroid body in the inner cavity of the *β*-CDNH$_2$ (Figure 6). As a consequence, the complexation constant should be higher, in agreement with the experimental results.

In the case of the *β*-CDNH$_2$/NaDC complex (Table 3), the main cross-peak interactions were 3-H with P-1 to P-5, P-7, P-8, P-11 to P-18, and P-20 to P-23 and 5-H with P-17 and P-20 to P-22.

From this information, it is deduced that NaDC enters the first *β*-CDNH$_2$ with the side chain unfolded because the influence of the amine group, as in the case of NaC. However, it penetrates less than NaC, as can be deduced from the absence of interactions with 6-H. This deeper inclusion in the first *β*-CDNH$_2$ causes the second *β*-CDNH$_2$ to complex the steroid body in a lower extension, because only ring A is, in fact, included in the *β*-CDNH$_2$ cavity (Figure 7).

**Complexation of NaC and NaDC by Dimer I.** Keeping in mind the complexation behavior of cyclodextrins with NaC and NaDC, a head to head dimer with a semirigid linker was synthesized in order to obtain supramolecular complexes. Considering that amides are more resistant to hydrolysis than esters,[6] dimer I (*N*,*N*′-bis(6-deoxy-*β*-cyclodextrin)-pyromellic acid diamide) was synthesized as a mixture of 1−3 and 1−4 derivatives in a 1:2 relation (which does not affect the supramolecular

(28) Anand, P. S.; Ramos Cabrer, P.; Alvarez-Parrilla, E.; Meijide, F.; Vázquez Tato, J. Proceeding of the 1st International Conference on Supramolecular Science and Technology, Zakopane, Poland, Sept 27−Oct 3, 1998.

(29) May, B. L.; Kean, S. D.; Easton, C. J.; Lincoln, S. F. *J. Chem. Soc., Perkin Trans. 1* **1997**, 3157.

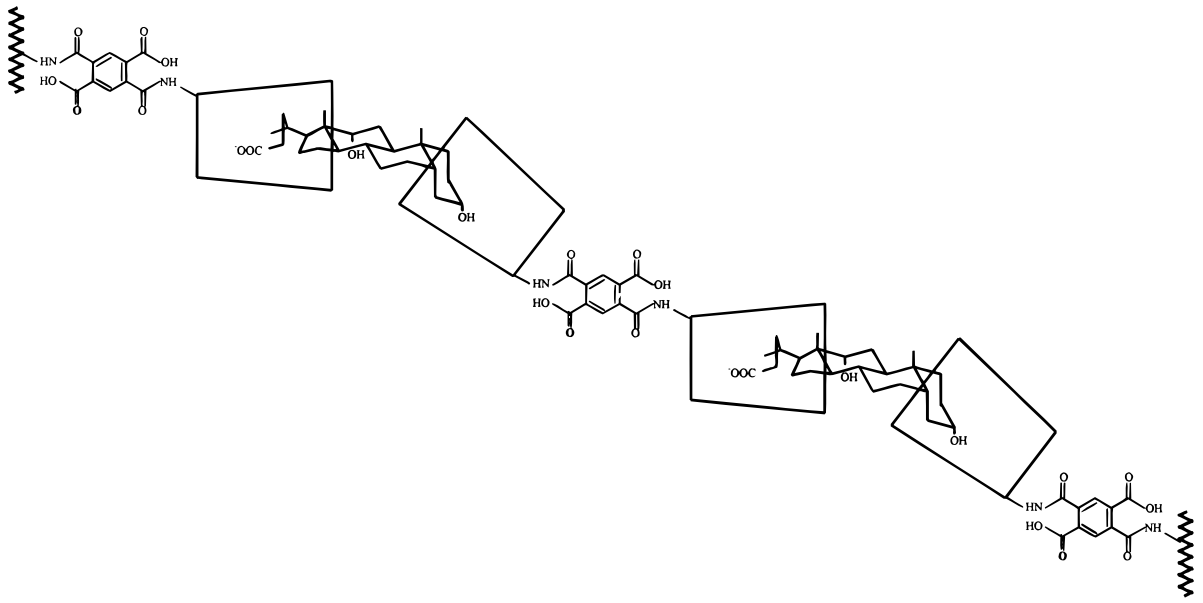

**Figure 10.** Schematic representation of the 1:1 (*n*:*n*) dimer I/NaDC complex, deduced from ROESY cross-peak interactions. Both isomers 1−3 and 1−4 can be present in the supramolecular structure.

interpretation) by reaction of $\beta$-CDNH$_2$ with 1,2,4,5-benzenetetracarboxylic dianhydride (see above).

Figure 8 shows the continuous variation plots for the complexes formed between this host and NaC and NaDC. The maxima $x_{CD}$ values at 0.33 and 0.5 show the formation of 1:2 and 1:1 complexes, respectively.

The observed ROESY cross-peak interactions in dimer I/NaC (Table 2) were 3-H with P-8, P-9, P-11, P-12, and P-14 to P-23 and 5-H with P-21. Interactions between the side chain and the steroid body are also observed (P-23 with P-16 (××) and P-20 (×); P-22 with P-16 (××) and P-17 (×); and P-20 with P-17 (××)). These interactions show that the complexation of NaC by each cyclodextrin unit in dimer I is similar to the one observed for the $\beta$-CD/NaC system (see above). Therefore, the side chain is folded toward ring D, which is entering the cyclodextrin cavity by the secondary rim. Figure 9 represents the 1:2 complex structure in which each cyclodextrin cavity of the dimer complexes one NaC molecule.

Table 3 summarizes the interactions for the complex dimer I/NaDC: 3-H interacted with P-1 to P-5, P-14 to P-18, and P-20 to P-23. Therefore, interactions between the side chain and the steroid body were similar to those of the $\beta$-CD/NaC complex. This means that the bile salt is complexed by two cyclodextrin units.

To understand the complexation behavior of this system, it is necessary to take into account the following facts: (a) the stoichiometry of dimer I/NaC is 1:2, i.e., both cyclodextrin units in the dimer behave independently from each other, allowing the formation of a 1:2 complex; (b) the stoichiometry observed for the dimer I/NaDC system is 1:1, which is equivalent to *n*:*n*; (c) NaDC presents two hydrophobic sites, both of which can be complexed by one cyclodextrin unit; (d) the ROESY experiments clearly suggest that both hydrophobic sites are complexed. As a consequence, a supramolecular complex has to be formed, as shown in Figure 10, where the higher the number of units in the complex, the closer to the n:n stoichiometry. However, its size and structure (which could be a cyclic one) cannot be deduced from the present experiments.

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