Mechanisms for Steroid-induced Conformational Enantiomerism of Bilirubin in Protic Solvents

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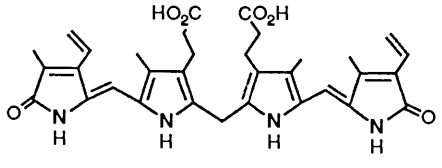
CD spectroscopy has been applied to clarify the mechanism for the conformational enantiomerism of bilirubin (BR) upon complexation with bile salts in water. The BR dianion when bound to the deoxycholate (**1**), cholate (**2**), chenodeoxycholate (**3**), ursodeoxycholate (**4**) or taurodeoxycholate ion (**6**) shows a (+)-to-(−) bisignate CD Cotton effect in water at pH 10.8 (NaOH), indicating that BR bound to these bile salts selectively adopts a (*R*)-helix configuration. Solubilization of BR in bile salt micelles is not necessary to induce the conformational enantiomerism of BR. The CD spectroscopic results on complexation of BR with various bile acids and their derivatives in methanol indicate that the hydrogen-bonding interactions between the hydroxy groups of a steroid and the carboxy groups of BR promote the conformational enantiomerism of BR. The mechanism for the enantioselective complexation in water at pH 10.8 has been deduced from the results obtained in methanol as that hydrogen bonding between the carboxylate ions of BR and the hydroxy groups of bile salt, as well as van der Waals interactions between the host and the guest, dominates the enantioselectivity in the BR–bile salt complexation.

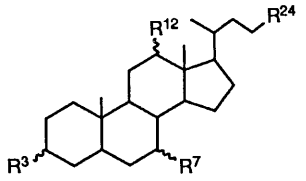
Bile salts are biological detergents which are produced by the metabolism of cholesterol in liver. During metabolism, lipophilic cholesterol is converted to amphiphiles by hydroxylation of a steroid nucleus at the 7- and/or 12-positions, epimerization of a β-hydroxy group at the 3-position to an α-one, and introduction of a carboxy group to the 24-position. From a chemical point of view, the bile salts are very interesting molecules because they have several chiral centres in a steroid nucleus. It can be expected, therefore, that bile salts may be used as hosts in chiral recognition systems. Indeed, it has recently been revealed that bile salt micelles can be utilized as chiral separation agents for capillary zone electrophoresis.[1] Perrin and Wilsey[2] found that sodium deoxycholate, **1**, induces a conformational enantiomerism of (4*Z*,15*Z*)-bilirubin-IX (BR) in a 0.1 mol dm^{-3} phosphate buffer solution at pH 8.0. Since



BR, a yellow-orange bile pigment, is not optically active in solution, no CD Cotton effect is observed. In the presence of **1**, however, negatively and positively signed CD signals have been measured at longer and shorter wavelengths, respectively, on the CD spectrum of BR. Exciton-coupling theory[3] explains this (−)-to-(+) bisignate CD Cotton effect as that the transition dipoles of the two dipyrrinone chromophores of BR preferentially adopt a (*S*)-helix configuration when BR is bound to **1**.[4] The oppositely signed CD Cotton effect has been found at higher pH (8.78).[2] Although Perrin and Wilsey[2] and Reisinger and Lightner[4] have assumed that the conformational enantiomerism of BR induced by **1** is ascribed to an enantioselective binding of BR with the micelle of **1**, no detailed mechanism of this enantiomerism has been presented.

Beside the BR–**1** system, the conformational enantiomerism of BR has been known to occur when this guest molecule is bound to chiral hosts such as human serum albumin,[5] cyclodextrins,[6] acyclic oligosaccharides,[7] cinchona alkaloids,[8] nucleosides[9] and (*S*)-(+)-2-aminobutane.[10] We have demonstrated that hydrogen bonding between the carboxylate ions of BR and the hydroxy groups of cyclic and acyclic oligosaccharides plays an essential role in the complexation of BR having a (*S*)-helix configuration with the saccharides.[6c,7] Hydrogen-bond formation is also possible in the BR–**1** system. We then carried out a CD spectroscopic study on the interactions of BR with various bile salts and their derivatives (Table 1) in water at pH 10.8 as well as in methanol. Since no

Table 1 Host steroids used in this study



Compound	R^3	R^7	R^{12}	R^{24}
1	α-OH	H	α-OH	CO_2Na
2	α-OH	α-OH	α-OH	CO_2Na
3	α-OH	α-OH	H	CO_2Na
4	α-OH	β-OH	H	CO_2Na
5	α-OH	H	H	CO_2Na
6	α-OH	H	α-OH	$CONHC_2H_4SO_3Na$
7	α-OH	H	α-OH	CO_2H
8	α-OH	H	α-OH	CO_2CH_3
9	α-OH	H	α-OH	CH_2OH
10	α-OH	H	α-OH	CH_2I
11	α-OH	α-OH	α-OH	CO_2H
12	α-OH	α-OH	α-OH	CO_2CH_3
13	α-OH	α-OH	H	CO_2H
14	α-OH	α-OH	H	CO_2CH_3
15	α-OH	α-OH	H	CH_2OH
16	α-OH	β-OH	H	CO_2H
17	α-OH	β-OH	H	CO_2CH_3
18	α-OH	β-OH	H	CH_2OH
19	α-OH	H	H	CO_2H
20	α-OH	H	H	CO_2CH_3
21	α-OH	H	H	CH_2OH
22	β-OH	H	α-OH	CO_2CH_3
23	H	H	H	CO_2H

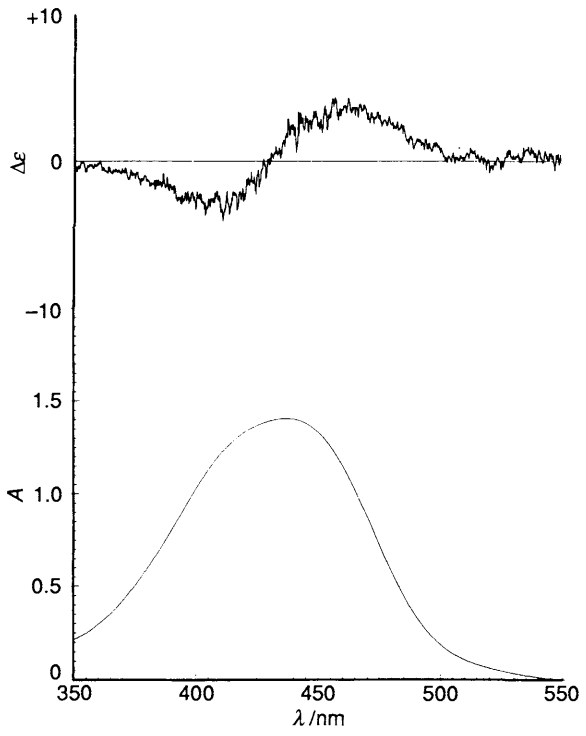


Fig. 1 Absorption (lower) and CD spectra (upper) of BR (2.5×10^{-5} mol dm^{-3}) in aqueous **1** (1.2×10^{-2} mol dm^{-3}) solution at pH 10.8 and 20 °C

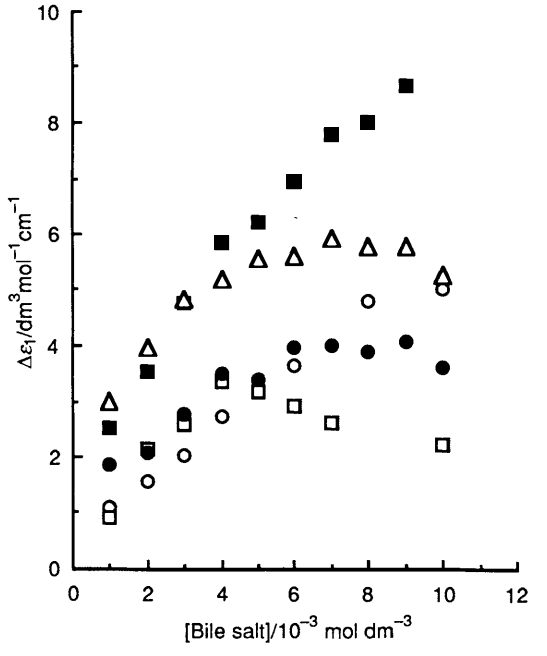


Fig. 2 Changes in $\Delta\varepsilon_1$ observed for BR (1.25×10^{-5} mol dm^{-3}) in aqueous **1** (○), **2** (●), **3** (□), **4** (■) and **6** (△) solutions upon addition of the bile salts

direct evidence for hydrogen-bond formation in solution was obtained because of experimental difficulties, we tried to prove it chemically using various steroids as hosts with or without hydrogen-bonding ability. The results suggest that hydrogen bonding between the carboxylate ions of BR and the α-hydroxy groups of **1** promotes the enantioselective complexation in water at pH 10.8.

Table 2 Critical micelle concentrations (cmc) of bile salts, and CD spectroscopic data obtained for BR–bile salt systems in water[a]

Compound	cmc/mol dm^{-3}	$\Delta\varepsilon_1$ (λ_{max}/nm)	$\Delta\varepsilon_2$ (λ_{max}/nm)
1	5×10^{-3} [b]	+4.3 (463)	−3.3 (411)
2	1.4×10^{-2} [c]	+4.3 (460)	−5.3 (406)
3	6×10^{-3} [d]	+4.7 (460)	−4.5 (406)
4	1.5×10^{-2} [e]	+5.0 (454)	−5.5 (400)
6	4×10^{-3} [f]	+5.3 (460)	−4.0 (410)

[a] The CD spectra were measured for BR (2.5×10^{-5} mol dm^{-3}) in water containing bile salts (1.2×10^{-2} mol dm^{-3}) at pH 10.8 and at 20 °C. The CD intensities are shown by the molar circular dichroism ($\Delta\varepsilon$/dm^3 mol^{-1} cm^{-1}). [b] Ref. 11. [c] A. Helenius and K. Simons, *Biochim. Biophys. Acta*, 1975, **415**, 29. [d] The cmc of **3** was determined by measuring the turbidities of the dispersions of steroid **14** in water at pH 10 as a function of the concentration of **3** (see ref. 11). [e] The cmc of **4** was evaluated by measuring the fluorescence spectra of pyrene as a function of the concentration of **4** (see: K. Kalyanasundaram and J. K. Thomas, *J. Am. Chem. Soc.*, 1977, **99**, 2039). [f] J. P. Kratohvil, W. P. Hsu and D. I. Kwok, *Langmuir*, 1986, **2**, 256.

Results and Discussion

BR–Bile Salt Complexes in Water.—The absorption and CD spectra of BR in an aqueous solution of **1** (1.2×10^{-2} mol dm^{-3}) at pH 10.8 (NaOH) are shown in Fig. 1. A (+)-to-(−) bisignate CD Cotton effect was observed, indicating that BR bound to **1** preferentially adopts a (*R*)-helix configuration. Since the critical micelle concentration (cmc) of **1** in water at pH 10 (NaOH) is 5×10^{-3} mol dm^{-3},[11] micelles of **1** should be formed under these conditions. Micelle formation, however, is not necessary for the conformational enantiomerism of BR as exhibited in Fig. 2. The CD intensity at 460 nm increases continuously with concentration increasing of **1** below the cmc and tends to saturate at [**1**] > 8×10^{-3} mol dm^{-3}. Similar phenomena were observed for **2**, **3**, **4** and **6** (see Table 2 and Fig. 2). The pK_a value of **1** has been reported to be 5.17 at concentrations below the cmc and 6.35 at concentrations well above the cmc.[12] Therefore, the carboxy group of **1** should be dissociated in water at pH 10.8. Meanwhile, the pK_a values for dissociation of the two propionic acid residues of BR have not been determined accurately.[13] Both carboxy groups of BR, however, are ionized at pH 10.8.[14] The complexation of BR with a bile salt under the present conditions, therefore, should occur to minimize an electrostatic repulsion between the anionic groups of BR and of the bile salts. This means that the $-CO_2^-$ group at the 24-position of the bile salt is not a chiral-recognition site. Each of the bile salts **1**–**4** or **6** has at least two hydroxy groups. If the enantioselective complexation of BR with the bile salts is promoted by hydrogen-bonding interactions, the hydroxy groups attached to the steroid nucleus should be the binding sites. According to a 'three-point attachment model' for chiral recognition,[15] a guest molecule should interact with a chiral host at at least three points. In the cases of the steroids **1**–**4** and **6**, two hydrogen bonds and a steric hindrance may contribute to chiral recognition. In order to confirm whether hydrogen bonds are formed at two points, we should use steroids such as **5** having only one hydroxy group at the 3-position and the salt of **23**, without a hydroxy group, as the referential hosts. However, **5** as well as **23** does not dissolve satisfactorily even in aqueous alkaline solution. Therefore we studied complex formation in methanol where a great variety of the host–guest pairs can be examined.

BR–Steroid Complexes in Organic Solvents.—We used methanol as a protic organic solvent whose properties are somewhat similar to those of water. In methanol, the carboxy groups of both bile acids and BR may not be ionized and bile acids do not form micelles. Therefore, the carboxy groups of the

Table 3 CD spectroscopic data obtained for the BR–steroid systems in methanol

Compound	$\Delta\varepsilon_1$ (λ_{ext}/nm)	$\Delta\varepsilon_2$ (λ_{ext}/nm)
7	+4.8 (466)	−3.5 (415)
8	+1.8 (465)	−1.9 (416)
9	+1.4 (470)	−1.2 (416)
10	+2.4 (459)	−1.3 (413)
11	+1.3 (462)	−0.8 (407)
12	+1.3 (473)	−1.3 (413)
13	+1.3 (467)	−1.3 (415)
14	+1.8 (470)	−1.5 (417)
15	+2.0 (462)	−1.5 (417)
16	no CD signal	
17	no CD signal	
18	no CD signal	
19	+3.4 (466)	−3.1 (425)
20	+2.8 (461)	−2.3 (414)
21	no CD signal	
22	no CD signal	
23	no CD signal	

[a] The CD spectroscopic data were collected using methanolic solutions of BR (2.5×10^{-5} mol dm^{-3}) in the presence of steroids (1.2×10^{-2} mol dm^{-3}) at 20 °C.

host and the guest may also act as hydrogen-bonding sites in methanol. The CD spectroscopic data are summarized in Table 3. As shown in Fig. 3, the guest steroids can be classified into two categories: steroids which induce bisignate CD Cotton effect (category A) and steroids whose mixtures of BR do not show any CD signal (category B). In Fig. 3 (category A), the steroids are arranged in the order of magnitude of the molar circular dichroism ($\Delta\varepsilon$) observed upon complexation with BR.

Effect of hydroxy group at C-3 *position of bile acids.* Relatively intense CD signals were observed for the complex of BR and **19** whereas no CD signal was detected in the BR–**23** system. The steroid **19** has an α-hydroxy group at the 3-position and a carboxy group at the 24-position as hydrogen-bonding sites, while **23** has only one hydrogen-bonding site at the 24-position. Since solubilization of BR in micelles can be excluded from the mechanism for interaction between BR and bile acid in methanol, van der Waals, dipole–dipole and/or hydrogen-bonding interactions should be taken into consideration for the complexation. If the van der Waals and/or dipole–dipole interactions participate meaningfully in the enantioselective complexation, the steroid **23** as well as other steroids which belong to category B, especially **21** (an analogue of **19**), should induce a bisignate CD Cotton effect. The difference between **19** and **23**, therefore, strongly suggests that at least two-point hydrogen bonding between BR and steroid is needed to promote the conformational enantiomerism of BR. We assume that one of the carboxy groups of BR interacts with the carboxy group at the 24-position of **19** and another carboxy group of BR is bound to the α-hydroxy group at the 3-position of the steroid through hydrogen-bonding interactions. The hydroxymethyl group of **21** may show much weaker hydrogen-bonding ability compared with the carboxy group of **19**. The steroid **20** is the methyl ester of **19** and shows a bisignate CD Cotton effect. The $-CO_2CH_3$ group seems to act as a proton acceptor in the hydrogen-bonding interaction.

Effect of hydroxy group at C-12 *position of deoxycholic acid derivatives.* The steroid **7**, which shows the strongest bisignate Cotton effect amongst the steroids employed, has three hydrogen-bonding sites: two α-hydroxy groups at the 3- and 12-positions and a carboxy group at the 24-position. Although the hydrogen-bonding ability at the 24-position of **10**, whose 24-substituent is the $-CH_2I$ group, should be much weaker than that of **7**, the steroid **10** induces fairly intense bisignate CD signals. This means that hydrogen-bond formation at the α-hydroxy groups at the 3- and 12-positions of deoxycholic acid derivatives also promotes the conformational enantiomerism of BR. This is very important to elucidate the mechanism for the conformational enantiomerism in water at pH 10.8 where all carboxy groups of BR and bile acid are ionized.

Effect of hydroxy group at C-7 *position of chenodeoxycholic acid derivatives.* Each steroid **13**, **14** or **15** has two α-hydroxy groups at the 3- and 7-positions. The complexes of these steroids and BR also exhibit the (+)-to-(−) bisignate CD Cotton effect. The CD intensity of BR decreases in the order of **15** > **14** > **13**, which may correspond to the order of hydrogen-bonding ability at the 24-position of these steroids. In the case of **15**, the hydrogen bonds may be formed mainly at the hydroxy groups attached to the steroid nucleus. Meanwhile, the carboxy group at the 24-position of **13** can interact with BR more strongly than the hydroxymethyl group of **15**. The point is that hydrogen-bond formation between the α-hydroxy groups at the 3- and 7-positions of a steroid and the carboxy groups of BR can yield a complex of BR, having a (*R*)-helix configuration, and steroid. This aspect can be applied to explain the results obtained for the BR–**3** system in water.

Effects of β-hydroxy groups of steroids. The steroids **16**, **17**, **18** or **22** have a β-hydroxy group at the 3- or 7-position. None of these steroids exhibit CD signals. These data are in conflict with the CD spectroscopic data obtained for **4** in water at pH 10.8 where the (+)-to-(−) bisignate CD Cotton effect was clearly observed (see Table 2). A plausible explanation for this conflict appears in the following section.

Mechanism for Conformational Enantiomerism in Water.— The results obtained in methanol reveal that the hydrogen-bonding interactions between BR and steroid play an essential role in the conformational enantiomerism of BR and that the pairs of α-hydroxy groups at the 3- and 12-positions and at the 3- and 7-positions can act as the two-point hydrogen-bonding sites. In methanol, the $-CO_2H$ and $-CO_2CH_3$ groups at the 24-position of the steroids also act as the binding sites. However, all carboxy groups of the bile salt and BR are ionized in water at pH 10.8 leading to an electrostatic repulsion between the carboxylate ions of the host and the guest. The conformational enantiomerism of BR induced by **1** in water, therefore, seems to be achieved through hydrogen bonding between the hydroxy groups at the 3- and 12-positions of **1** and the carboxylate ions of BR. Although the NH groups of the pyrrole rings of BR may also interact with **1** through hydrogen bonding, a Corey–Pauling–Koltun (CPK) molecular model indicates that the interactions between the NH groups of BR and the hydroxy groups of **1** are spatially implausible. The CPK molecular model also suggests that the carboxylate ions of both conformational enantiomers of BR can be bound to the α-hydroxy groups of **1**. Probably a small difference in the stability between the complexes of (*R*)- and (*S*)-helix BRs dominates the enantioselectivity. The difference in the complex stabilities may be ascribed to the difference in the strength of intermolecular interactions such as van der Waals, dipole–dipole and/or hydrophobic interactions. These interactions are much stronger in water than in organic solvents. Recently, Smithrud and Diederich[16] and Kano *et al.*[17] demonstrated that the van der Waals interactions between hydrophobic host and guest are strongest in water. In order to maximize van der Waals contact, the BR molecule tends to face a plane of the chiral steroid nucleus of **1**. The plausible structures of the hydrogen-bonded complexes of **1** and (*R*)- and (*S*)-helix BRs, which are speculated from a consideration of the CPK molecular models, are shown in Fig. 4. In the case of (*R*)-helix BR, a dipyrrinone moiety of BR can spatially face the α-plane of the steroid **1**. Meanwhile, overlapping of the dipyrrinone moieties of (*S*)-helix BR with the steroid plane is much smaller than that for the (*R*)-helix BR–**1**

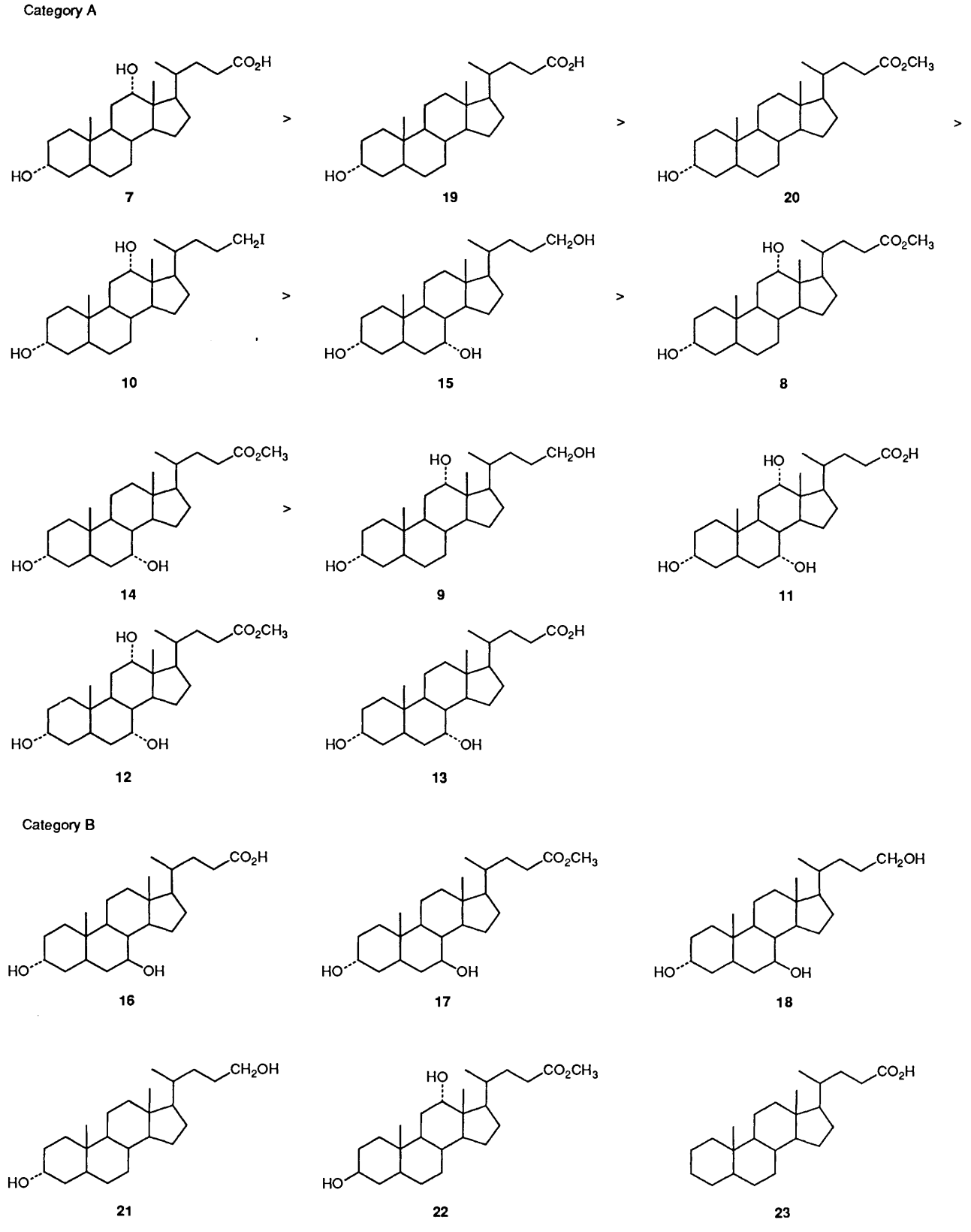


Fig. 3 Structures of the steroids which induce the conformational enantiomerism of BR (category A) and do not provide any CD signal of BR (category B). In category A, the steroids are arranged in the order of magnitude of the CD signal observed upon complexation with BR.

complex. These models clearly indicate that the stability of the (*R*)-helix **BR–1** complex is greater than that of the (*S*)-helix **BR–1** complex because of the difference in the van der Waals stabilization energy. Weaker CD intensities in methanol may also be interpreted in terms of the van der Waals interactions in methanol being much weaker than those in water.[16,17] In the cases of ursodeoxycholic acid (**16**) and its derivatives (**17**, **18**) in methanol, the complexation may not occur because of very weak van der Waals interactions in the organic solvent. In water, however, a dipyrrinone moiety of BR can face the steroid nucleus of ursodeoxycholate (**4**) to cause van der Waals interactions between the host and the guest. The hydrogen-

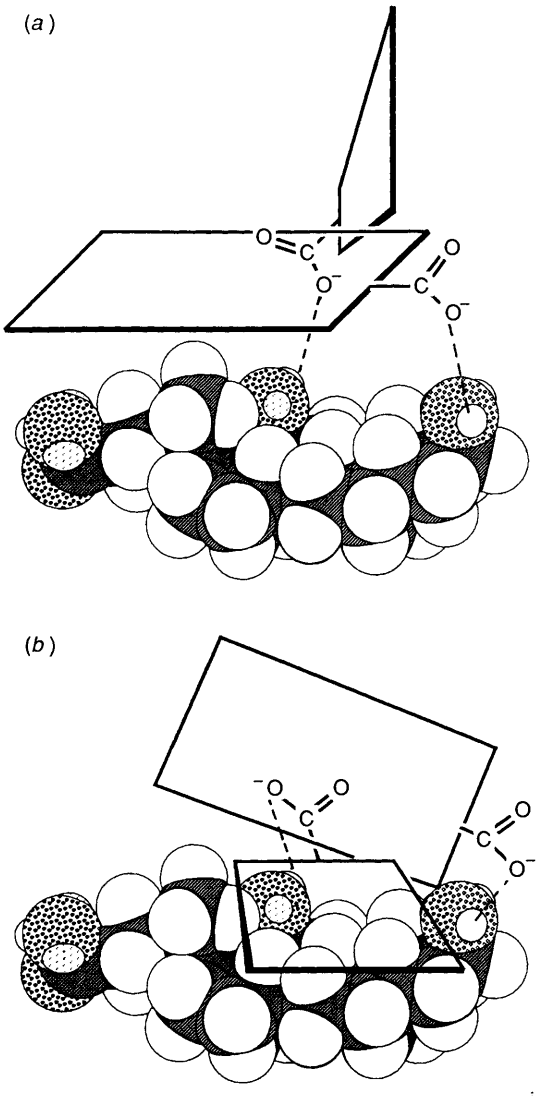


Fig. 4 Plausible structures of (*a*) the (*R*)-helix BR–**1** and (*b*) (*S*)-helix BR **1** complexes, which are speculated from the CPK molecular models. The dotted lines denote the hydrogen bonds.

bonding interactions seem to cooperate with the van der Waals interactions leading to the bile salt-induced conformational enantiomerism of BR in water.

In general, hydrogen-bond formation in water hardly occurs because of strong hydration of both host and guest molecules. However, the situation is somewhat different in an amphiphilic host–guest system. Although acetic acid scarcely dimerizes in water through hydrogen bonding between the carboxy groups, the dimerization constant of the carboxylic acid is known to increase with increasing alkyl-chain length of the acid.[18] Recently we found the β-cyclodextrin-induced conformational enantiomerism of BR, where hydrogen bonds are formed between the secondary hydroxy groups of β-cyclodextrin and the carboxylate ions of BR.[6c] When the hydrophobicity of the cyclodextrin cavity increases upon inclusion of a hydrophobic coexisting guest, the BR–β-cyclodextrin hydrogen-bonded complex becomes more stable.[6c] Since both BR and bile salt are amphiphiles, the hydrophobic backbones of this host and guest may prepare the microscopic environments which are favourable for forming hydrogen bonds.

Perrin and Wilsey[2] measured the (−)-to-(+) bisignate CD Cotton effect for BR in 0.1 mol dm^{-3} phosphate buffer at pH 8.0 in the presence of **1** (7.24×10^{-2} mol dm^{-3}). The same CD pattern has been observed in the 0.1 mol dm^{-3} Tris buffer at pH 8.02.[4] We could reproduce these results. Bile salts forms micelles at concentrations above the cmcs and their micelle structures are drastically altered in the presence of inorganic salts.[19] We found the (−)-to-(+) bisignate CD Cotton effect ($\Delta\varepsilon = -6.2$ and $+4.7$ dm^3 mol^{-1} cm^{-1} at 463 and 411 nm, respectively) for the mixture of BR (1.2×10^{-5} mol dm^{-3}) and **1** (0.01 mol dm^{-3}) in 0.1 mol dm^{-3} phosphate buffer at pH 8.0. However, the signs are opposite in water at pH 8.0 (NaOH) in the absence of inorganic salt ($\Delta\varepsilon = +2.6$ and -3.3 dm^3 mol^{-1} cm^{-1} at 468 and 410 nm, respectively). These results indicate that the preferred configuration of BR depends upon the structure of the micelle of **1**. At the present stage, the mechanism for the micelle-induced conformational enantiomerism of BR is out of our area of interest.

Experimental

BR (Sigma) was dissolved in chloroform and washed with aqueous $NaHCO_3$. Chloroform was evaporated under reduced pressure and the yellow-orange residue was washed with a small amount of methanol. The steroids used in this study are listed in Table 1. The bile acids, **7**, **11**, **13**, **19** (Nacalai), **16** (Sigma) and **23** (Aldrich) and the bile salt **6** (Nacalai) were purchased and used without further purification. The sodium salts of the bile acids, **1**–**5**, were prepared according to the procedures described previously.[11] The steroids **8**, **9**, **12**, **14**, **15**, **17**, **20**, **21** and **22** were the same as those used in the previous work.[11] Compound **10** was synthesized according to the procedures described in the literature,[20] yield 70%, m.p. 67–69 °C. Compound **18** was prepared by a method described in the literature,[20] yield 73%, m.p. 110–112 °C.

The UV–VIS absorption and CD spectra were measured using a Shimadzu UV-2100 spectrophotometer and a JASCO J-500 spectropolarimeter with a data processor, respectively, at 20 °C. For preparing the BR solutions, the solvents used were previously bubbled with nitrogen gas for 20 min.

Acknowledgements

This work was supported by a Grant-in-Aid for Science Research on Priority Areas, 'Multiplex Organic Systems' (No. 01649006).

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Paper 2/02956A
Received 5*th June* 1992
Accepted 21*st July* 1992