Bile Acids with a Cyclopropyl-Containing Side Chain. 3.[1] Separation, Identification, and Properties of All Four Stereoisomers of 3α,7β-Dihydroxy-22,23-methylene-5β-cholan-24-oic Acid

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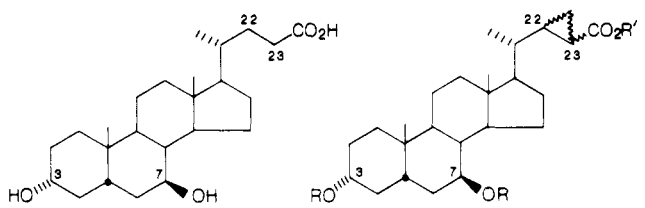
3α,7β-Dihydroxy-22,23-methylene-5β-cholan-24-oic acid (CUDCA) (**2a**), a side-chain cyclopropylog of ursodeoxycholic acid (UDCA) (**1**), was shown to be a mixture of four stereoisomers (CUDCA A–D). The 22*S*,23*S* (**3**), 22*R*,23*R* (**4**), 22*S*,23*R* (**5**), and 22*R*,23*S* (**6**) diastereoisomers have been separated, their respective configurations assigned by ^{13}C NMR spectroscopy, and original synthetic schemes for their preparation elaborated. Moreover, theoretical models of the structure of UDCA and CUDCA A–D were built by using molecular computer graphic techniques. It was shown that the four diastereoisomers (**3–6**) greatly differ in hydrophilicity, in critical micellar concentration (CMC) in water, and exhibit a different interaction with intestinal bacterial enzymes. It was also shown that CUDCA A–C are not conjugated with glycine or taurine in the liver, while CUDCA D is secreted into bile predominantly as taurine and glycine conjugate.

As a part of a program devoted to the synthesis and biological evaluation of bile acids derivatives, we have recently reported the preparation of 3α,7β-dihydroxy-22,23-methylene-5β-cholan-24-oic acid CUDCA (**2a**), a side-chain cyclopropylog of ursodeoxycholic acid (UDCA) (**1**),[2] and of its corresponding taurine conjugate tauro-cyproursodeoxycholic acid (TCUDCA).[3]



1 (UDCA) **2a**: R = H; R' = H (CUDCA) **2b**: R = Ac; R' = Et

CUDCA (**2a**) was obtained in large quantities by the dirhodium(II) tetraacetate catalyzed decomposition of ethyl diazoacetate in the presence of the Δ^{22}-24-norcholene derivative, followed by alkaline hydrolysis of the resulting diacetoxy ester **2b**, and shown to be a mixture of four diastereoisomers, CUDCA A–D (TLC, GLC; Table I) as could have been anticipated by the utilization of the nonstereospecific carbenoidic addition route for the formation of the two new stereogenic centers at C(22) and C(23).

Preliminary physicochemical and biological studies[2,3] performed on CUDCA (**2a**) and TCUDCA as diastereoisomeric mixtures showed that these compounds were endowed with favorable properties, such as increase in biliary secretion of bile acids and phospholipids. It was shown, moreover, that the presence of the cyclopropyl moiety α to the carboxyl group deters metabolic and bacterial degradation to account for the enhanced in vivo activity.

Separation of all four diastereoisomers constituting CUDCA would provide a clue to clarify the side-chain stereochemistry–bioactivity relationship by submitting all of the isomers to careful physicochemical and bioassay studies. In this paper, we describe the isolation of the four stereoisomers, the assignment of their absolute configuration, an alternative stereoselective synthesis of two of them (CUDCA C and D), and the results of preliminary tests of their properties.[4]

Table I. Chromatographic Data of CUDCA Diastereoisomers

CUDCA	TLC, R_f[a]	GLC, t_R,[b,c] min
A	0.45	1.28
B	0.41	1.00
C	0.38	1.30
D	0.35	1.13

[a] Solvent system: $CHCl_3$/MeOH, 9:1, v/v; silica gel plates; T = 20 °C. [b] As ethyl ester, 3,7-diacetoxy derivatives. [c] SP 2250, 1%, 6 ft; T = 265–275 °C; rate 0.33 °C/min; N_2 flow 20 mL/min.

Chemistry. 3α,7β-Dihydroxy-22,23-methylene-5β-cholan-24-oic acid (CUDCA) (**2a**) was prepared by improving an already reported procedure.[2] Thus, oxidative decarboxylation of 3α,7β-diformylursodeoxycholic acid[5] followed by alkaline hydrolysis, acetylation, and dirhodium(II) tetraacetate catalyzed cyclopropanation with ethyl diazoacetate of the Δ^{22} olefin thus obtained afforded diacetyl CUDCA ester **2b** (35% overall yield from **1**). This ester appeared by TLC (petroleum ether–ether, 1:1) as a mixture of two compounds, which were separated by medium-pressure chromatography into a less polar (27%) and a more polar ester fraction (59%). Alkaline hydrolysis of the *less polar* ester fraction yielded a mixture of acids (TLC, R_f 0.45 and 0.41, respectively, in $CHCl_3$–MeOH, 9:1, as a mobile phase), which was subjected to medium-pressure chromatography to give a less polar acid, CUDCA A (61%), and a more polar acid, CUDCA B (30%). Analogously, alkaline hydrolysis of the *more polar* ester fraction followed by medium-pressure chromatography of

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Table II. ^{13}C NMR Chemical Shifts of Side Chain Carbon Atoms

compound	C(16)	C(17)	C(20)	C(21)	C(22)	C(23)	C(24)	C(25)
1, UDCA	28.4	54.9	35.1	18.2	30.9	30.9	174.5	
3, CUDCA A (22*S*,23*S*)	28.9	57.6	34.8	21.3	17.8	30.6	175.8	16.3
4, CUDCA B (22*R*,23*R*)	27.7	57.7	31.8	21.8	30.5	29.9	176.1	12.0
5, CUDCA C (22*S*,23*R*)	28.7	57.2	39.7	20.1	31.1	19.1	177.9	17.5
6, CUDCA D (22*R*,23*S*)	28.5	57.2	39.4	20.0	30.3	23.3	177.3	12.7

[a] Given as δ values.

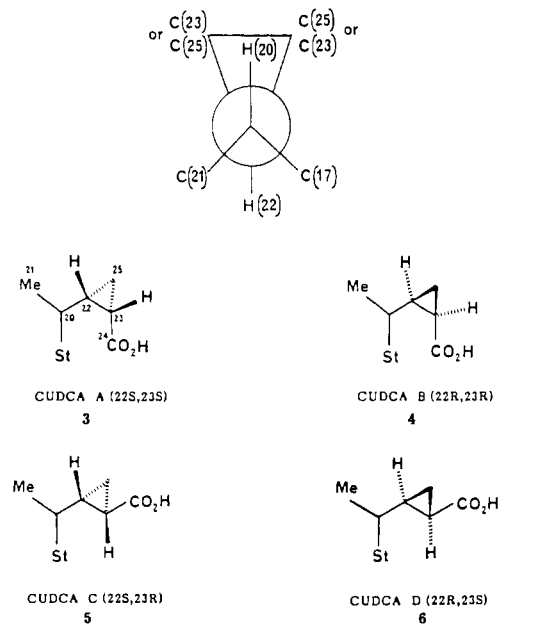
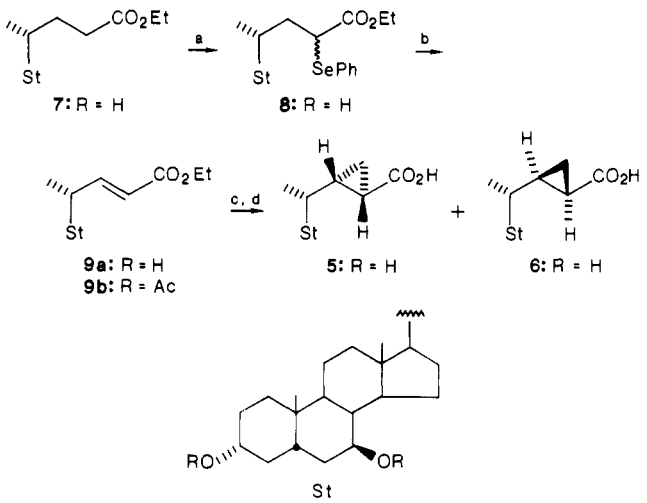


Figure 1. Top: Newmann projection of the preferred C(20)–C(22) rotamer population of CUDCA stereoisomers. Bottom: absolute configurations of CUDCA stereoisomers; in formulas **3–6**, carbons **20–24** lie in the plane of the paper.

the mixture of other two acids (TLC, R_f 0.38 and 0.35, in $CHCl_3$–MeOH, 9:1) yielded a less polar acid, CUDCA C (49%), followed by the more polar CUDCA D (33%).

Stereochemical Assignments. ^{13}C NMR spectroscopy, an excellent tool for the clarification of subtle stereochemical features, has been previously applied in the bile acid field.[6] The ^{13}C NMR of UDCA methyl ester, in particular, was assigned by Lida and co-workers.[7] The above data were of help for the assignment of the chemical shift values to CUDCA A–D isomers. In Table II are reported chemical shifts values of some ring D carbon atoms and those of the side chain of CUDCA A–D, relevant for the assignment of the respective absolute configuration; chemical shifts for the corresponding carbon atoms of UDCA (1) useful for comparison are also reported. Since two isomers must be C(20)–C(24) cis related and two trans related and since the cis isomer must have C(20) shielded by ca. 5 ppm (vis-à-vis the trans isomers) and C(24) by a lower value, compounds A and B are cis substances. In the two trans C(20)–C(24) isomers, the preferred C(20)–C(22) rotamer population can be assumed to be that depicted in Figure 1. Since C(24) is separated spatially from ring D and the C(17) side chain, carbons 16, 17, 20, and 21 show nearly the same δ values in the two compounds.[8]

Scheme I[a]



[a] (a) LDA, THF, PhSeSePh; (b) 1. H_2O_2, CH_2Cl_2, 2. Ac_2O, PPy, Et_3N; (c) CH_2N_2, $Pd(OAc)_2$, Et_2O; (d) NaOH, H_2O/EtOH. The ratio of **5** to **6** was 52:48.

However the γ-effect of C(21) on one of the cyclopropane carbons reveals the site of attachment of the carboxyl group. Thus in C the cyclopropane methine is shielded by ca. 4 ppm (vis-à-vis D) and in D the cyclopropane methylene is shielded by ca. 5 ppm (vis-à-vis C). Inversion of C(23) in either isomer C or D would be expected to shield C(20) by ca. 5 ppm and C(24) by a lesser extent, should the C(20)–C(22) rotamer population not be (or only slightly be) perturbed. This requirement appears to make CUDCA A the C(23) epimer of CUDCA C. The similarity of the cyclopropane methylene shifts of CUDCA A and C, as well as those of CUDCA B and D, appears to make each isomer set a C(23) epimer pair. The four isomers can also be separated into two pairs by inspecting the C-25 methylene of cyclopropanes. CUDCA A and C have downfield methylene shifts (16.3 and 17.5 ppm, respectively) due to δ interaction between C-25 and C-16, while CUDCA B and D methylenes are upfield (12.0 and 12.7 ppm, respectively) due to removal of this interaction. While on the basis of the above arguments CUDCA A–D were identified as 22*S*,23*S* (**3**), 22*R*,23*R* (**4**), 22*S*,23*R* (**5**), and 22*R*,23*S* (**6**) diastereoisomers (Figure 1), respectively, alternative stereospecific synthesis to the *Z* (CUDCA A and B) and *E* (CUDCA C and D) diastereoisomeric couples was undertaken.

Syntheses of CUDCA C and CUDCA D. Our starting material for the synthesis of the thermodinamically more stable *E* isomers CUDCA C and D was UDCA ethyl ester (**7**), prepared from UDCA (**1**), which was subjected to a selenylation–deselenylation procedure,[10] as shown in Scheme I. Ethyl (*E*)-3α,7β-dihydroxy-Δ^{22}-cholenoate (**9a**) was thus obtained via **7** and **8** in 77% overall yield from **1**. The transoid nature of the double bond in **9a** was confirmed by the 15-Hz coupling between the two vinylic

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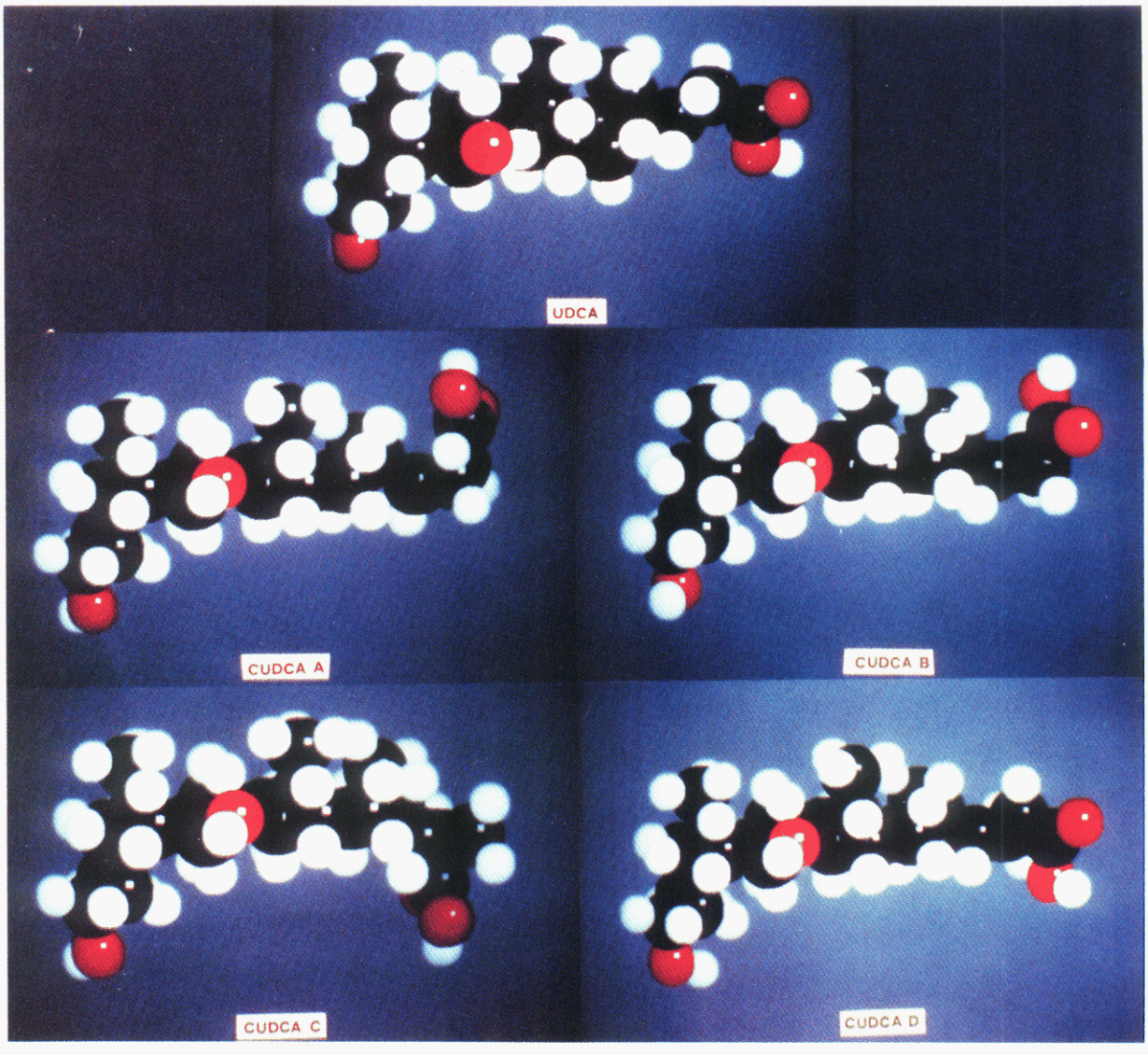
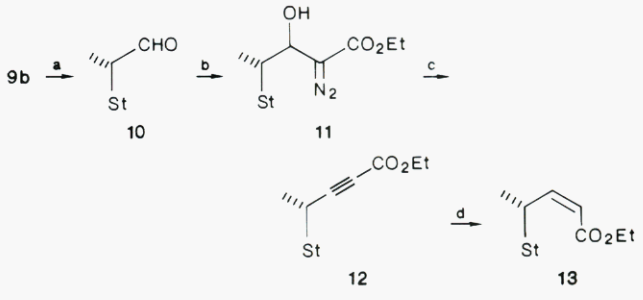


Figure 2. Comparison of the computer-generated, energy-minimized space-filling structures of CUDCA A–D and UDCA.

protons. Cyclopropanation of *E* olefinic ester **9b** with $Pd(OAc)_2/CH_2N_2$ reagent, which adds cis to α,β-unsaturated carbonyl systems[11,12] followed by alkaline hydrolysis, afforded with 77% yield an approximately 1:1 mixture of (22*S*,23*R*)-CUDCA C (**5**) and (22*R*,23*S*)-CUDCA D (**6**).

Attempted Syntheses of CUDCA A and CUDCA B. It was felt that a stereoselective approach analogous to that employed for the synthesis of CUDCA C (**5**) and CUDCA D (**6**) above described, namely the cyclopropanation of a olefin such as **13**, could be applied to the synthesis of *Z* isomers CUDCA A and B. The *E* olefinic ester **9a** was chosen as precursor of the corresponding *Z* olefinic ester **13**, as shown in Scheme II. Acetylation of **9a** followed by ozonolysis in chloroform–methanol at −15 °C of the diacetoxy olefin **9b** thus obtained, followed by zinc–acetic acid workup, afforded the unstable aldehyde **10** (87.7% from **9b**), which was treated with ethyl lithiodiazoacetate in THF at −78 °C to afford the corresponding α-diazo-β-hydroxy ester **11** in 79% yield. Deaminative dehydration of **11** by treatment with $BF_3{\cdot}Et_2O$ in acetonitrile at room temperature for 10 min afforded ethyl 3α,7β-diacetoxy-5β-chol-22-yn-24-oate (**12**) in 30% yield.[13] Hydrogenation

Scheme II[a]



[a] (a) O_3, $CHCl_3$/MeOH; (b) LDA, THF, EDA; (c) $BF_3{\cdot}Et_2O$, CaH_2, CH_3CN; (d) H_2, 2% $Pd/CaCO_3$, AcOEt.

of **12**, over Lindlar catalyst in ethyl acetate, afforded *Z* olefinic ester **13** in 70% yield. The overall yield of **13** from **9b** was 15%. Unfortunately, several attempts to methylenate the C(22)–C(23) double bond of *Z* olefin **13** with palladium acetate–diazomethane, with dimethyloxosulfonium methylide or via Simmons–Smith reaction under a variety of conditions, resulted in the recovery of starting material. While we are developing an alternative synthetic strategy for the synthesis of CUDCA A and B, we think

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Table III. Selected Physicochemical Properties of UDCA and the Four CUDCA Diastereoisomers[a]

	UDCA	CUDCA A	CUDCA B	CUDCA C	CUDCA D
CMC,[b] mM	19	25	20	14	10
K'[c]	0.95	0.21	0.80	1.30	2.05
SWo,[d] M	9	2.5	6	6	5
pK_a	5.08	5.04	5.05	5.02	5.04

[a] See the Experimental Section for conditions. [b] Critical micellar concentration. [c] C-18 HPLC retention factor. [d] Water solubility of the protonated acid.

it appropriate to report our synthetic approach, unprecedented, to a steroidal *Z* olefin such as **13**.

Molecular Structures. Figure 2 shows Corey–Pauling–Koltun (CPK) space-filling models of UDCA and CUDCA A–D generated from a modified SMILES[14] description of the connectivity and chirality of the compounds using the program CONCORD.[15] The geometry of the five structures was then optimized by using the AM1 Hamiltonian contained in the molecular orbital package MOPAC.[16,17] A point of particular interest in these energetically favored conformers is that the introduction of the cyclopropyl moiety in the side chain of UDCA induces a different orientation of the carboxylate moiety relative to the rest of the molecule; thus, the cis compounds **3** and **4** have the carboxylate group projecting out from the β face of the molecule, as shown. The carboxylate group of the trans compounds **5** and **6**, on the other hand, is oriented on the α face. Among the four diastereoisomers A–D, CUDCA D is the one that superimposes more closely to UDCA.

Physicochemical Properties. The physicochemical properties of the four diastereoisomers CUDCA A–D are reported in Table III. First notice that the CMC of these cyclopropyl analogues is CUDCA A > CUDCA B > CUDCA C > CUDCA D, and therefore, the detergency properties of these four diastereoisomers, i.e., the tendency to self-aggregate to form micelles, widely differ. It is well known that natural BA self-aggregate via hydrophobic back-to-back interactions to form dimers, tetramers, and, by increasing the concentration, larger micelles with not well established aggregation number. The high CMC values observed for the cis isomers CUDCA A and CUDCA B can be explained with the position of the carboxylate group relative to the rest of the molecule in the minimal energy conformation (see Figure 2 and the discussion above), which reduces the continuity of the hydrophobic area, destabilizing at the same time with its polarity the hydrophobic bond between two initially formed monomers. On the contrary, trans isomers CUDCA C and CUDCA D exhibit CMC values lower than the cis isomers as well as UDCA. The position of the carboxylate group, in this case, favors an energy-stable hydrophobic interaction between two monomers. The lower critical micellar concentration (CMC) value of CUDCA C and CUDCA D with respect to UDCA can be explained by an increased continuity of the hydrophobic area due to the presence of one more carbon atom in the side chain, in agreement with that observed in homologous series of amphypatic molecules with detergent properties.

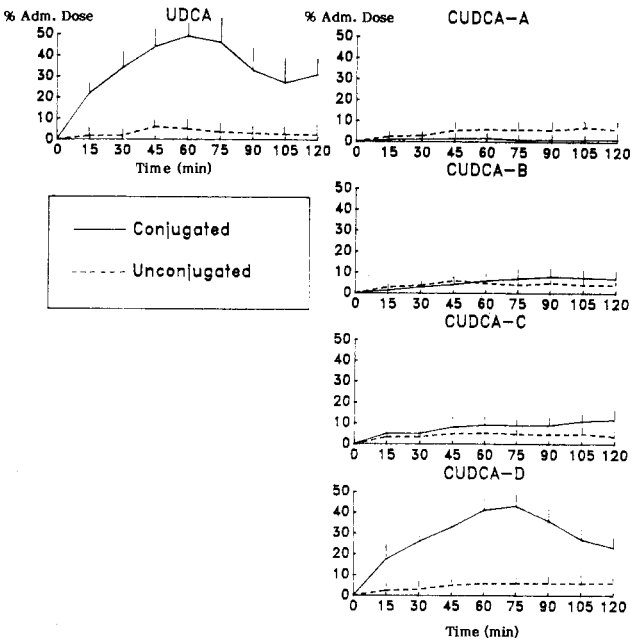

Figure 3. Kinetics of appearance in bile of free and conjugated bile acids after intravenous administration (6 mol/min per kg over 1 h) of UDCA and the four CUDCA isomers. The results are expressed as mean values ± SD of five experiments.

The four diastereoisomers CUDCA A–D greatly differ also in their hydrophobic–hydrophylic balance as evidentiated by their "retention factor"[18,19] value K' (Table III) with an order of hydrophylicity D > C > B > A, inversely related to the CMC values discussed above. Other physicochemical properties such as the water solubility of the protonated form (SWo) and the pK_a values are not affected by the presence and the stereochemistry of the cyclopropane ring and are similar to those of natural UDCA.

Evaluation of Hepatic Amidation in Vivo. The kinetics of appearance in bile of ursodeoxycholic acid (UDCA, **1**) and the four CUDCA isomers (**3**–**6**), expressed as percentage of administered dose vs time over a 1-h period and 1 h after the infusion was stopped, are reported in Figure 3. UDCA, used as a control, is rapidly and efficiently conjugated with taurine and glycine and excreted into bile in the amidated form in more than 90%, and in this form is present in bile after 1 h of infusion with a maximum concentration value of 48 ± 7%. Once the infusion is stopped, the level of conjugated UDCA drops, reaching a steady state value of approximately 30%. Cis isomers CUDCA A (**3**) and B (**4**), on the contrary, are conjugated with taurine and glycine for less than 5% and are poorly excreted into bile as unconjugated acids. The trans isomer CUDCA C (**5**) is slightly conjugated (8–12%) while CUDCA D (**6**) behaves analogously to UDCA, being conjugated for approximately 45%. A large amount is excreted as taurine or glycine conjugate, and the maximum secretion value (42 ± 3%) is reached within 60 min. The kinetics of CUDCA D is quite similar to that of UDCA; in this case, a steady state value of 22 ± 3% is reached after 2 h. The above results clearly indicate that side-chain structural parameters are important for substrate recognition by the bile acids hepatic conjugating systems.[20,21]

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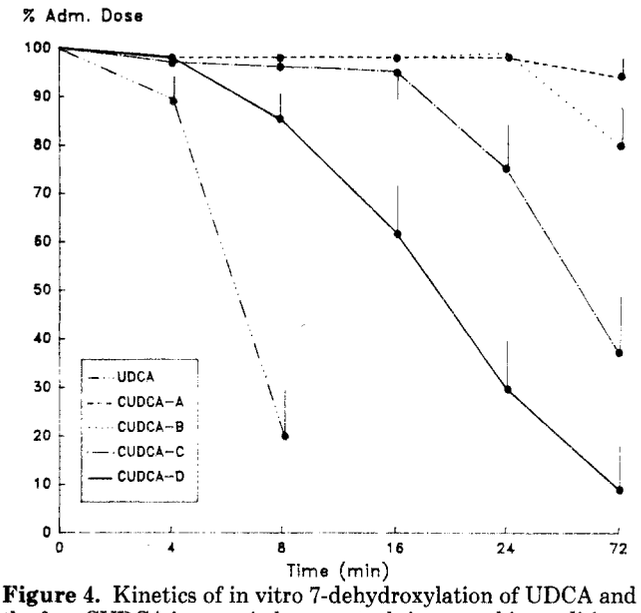

Figure 4. Kinetics of in vitro 7-dehydroxylation of UDCA and the four CUDCA isomers in human stools in anaerobic conditions. The results are expressed as the percentage of unmetabolized compound recovered in stools and are the mean values of five sets of experiments ± SD.

The analogy between the conformation of natural UDCA side chain and that of the side chain of the partially rigid analogue CUDCA D (**6**) as already evidentiated by computer graphic (see above), seems to be confirmed by the similarity in substrate specificity and kinetics exhibited by these two substances. CUDCA A (**3**), B (**4**), and to a less extent CUDCA C (**5**), on the contrary, behave differently from UDCA, due probably to a poor substrate specificity toward one, or both, of the enzymes involved in the conjugation process, i.e., bile acid CoA ligase and bile acid CoA glycine/taurine-*N*-acyl-transferase.

Evaluation of Bacterial 7-Dehydroxylase. The kinetics of the biotransformation of the four diastereoisomers CUDCA A–D and that of UDCA expressed as percent of amount of unmetabolized bile acid vs time are reported in Figure 4. As can be seen, also in this case the stereochemistry of the side chain plays a relevant role in modulating the substrate specificity. Cis isomers CUDCA A and B and trans isomer CUDCA C are poor substrates for the 7-dehydroxylase enzyme, and the kinetics of 7-dehydroxylation are, accordingly, highly delayed. Trans isomer CUDCA D, although more metabolized than the others CUDCA isomers, still exhibits a half-life 4 times higher than that of UDCA.

Conclusions

The introduction of a cyclopropyl moiety at level of the C(22)–C(23) bond of UDCA imposes rigidity to the side chain of the resulting four diastereoisomers (**3–6**), thus inducing a different spatial disposition of the C(24) carboxylate group. By studying the properties of these diastereoisomers, we have clearly shown that the conformation of the bile acid side chain plays an important, hitherto unrecognized, role on physicochemical parameters directly related to detergency such as CMC and hydrophobic/hydrophylic ratio, as well as on biological properties of these amphipatic molecules. Of particular interest, in this connection, is the finding that the spatial disposition of the polar group at the side-chain terminus is essential for substrate recognition by amidating enzymes in liver and by intestinal bacterial 7-dehydroxylases. Finally, from the above data, CUDCA D emerges as a particularly interesting molecule, with the following features: (a) efficient conjugation by the liver and, consequently, quantitative recovery into bile and active absorption in the ileal portion of the intestine (ileum) and (b) increased bioavailability of the drug due to a reduced biotransformation in the intestine and consequent decrease in the formation of the potentially toxic monohydroxy derivative. Additional studies carried out in order to assess the potential therapeutic value of CUDCA D as a possible substitute for UDCA are in course and will be reported in due time.

Experimental Section

General Methods. Melting points were determined on a Kofler micro hot-stage apparatus and are uncorrected. Specific rotations were recorded on a Roussel Jouan digital 71 polarimeter. IR spectra were determined with a Perkin-Elmer 1320 spectrometer. Analytical gas chromatography (GLC) was performed on a Hewlett-Packard 5830 A chromatograph equipped with a flame-ionization detector and a 6 ft × 2 mm i.d. 1% SP 2250 on 100/100 Chromosorb W column. The oven temperature was 265–275 °C (5 °C/min increase) with nitrogen as the carrier gas at a flow rate of 20 mL/min. ^{1}H NMR spectra were taken on a Varian EM 390 spectrometer. ^{13}C NMR spectra were taken on a Nicolet NT-200, wide-bore, broad-band spectrometer, operating with an Oxford magnet at 50.31 MHz in the Fourier transform mode. The carbon shifts are in parts per million downfield from TMS; δ(TMS) = δ(pyridine-d_5) + 123.5 ppm. The asterisks shifts may be interchanged. All reactions involving organometallic reagents were performed in a dry apparatus under argon. Ether and THF were distilled from $LiAlH_4$ immediately prior to use. Diisopropylamine was distilled from calcium hydride and stored over 4A molecular sieves. Column chromatography was performed on Merck silica gel (0.063–0.200 mm). Flash chromatography was performed on Merck silica gel (0.040–0.063 mm). Medium-pressure chromatography was performed on Merck LiChroprep Si 60 (0.040–0.063 mm, lobar columns).

Carbethoxycarbenoid Addition to Δ^{22} Olefin. Cyclopropanation of the Δ^{22} olefin (2.0 g, 4.65 mmol) with ethyl diazoacetate (1.60 g, 14.03 mmol) in the presence of dirhodium(II) tetraacetate as catalyst was carried out as previously described[2] to afford **2b** as a mixture of the four diastereoisomeric esters of CUDCA A–D (GLC; see Table I). A first separation of the diastereoisomeric mixture was realized by medium-pressure chromatography of the crude reaction mixture (2.4 g). Four fractions, I–IV, in a ratio 1:3.8:1:8.3 were collected and identified as follows.

Fraction I. Elution with petroleum ether–ether, 4:1, afforded 0.158 g (7.9%) of unreacted olefin.

Fraction II. Elution with petroleum ether–ether, 4:1, afforded 0.642 g (26.7%) of a mixture, mp 130–205 °C, constituted (GLC) by esters of CUDCA A and B in a ratio 73:27.

Fraction III. Elution with the same solvents afforded 0.155 g (6.5%) of a mixture of the four diastereoisomeric CUDCA A–D esters (TLC, GLC).

Fraction IV. Elution with petroleum ether–ether, 7:3, afforded 1.405 g (58.5%) of a mixture, mp 60–65 °C, constituted (GLC) by esters of CUDCA C and D in a ratio 52:48.

Hydrolysis of Fraction II. A solution of 10 N NaOH (10 mL) was added to a warm solution of fraction II (0.642 g, 1.24 mmol) in EtOH (15 mL). The mixture was heated at reflux for 4 h, cooled, poured onto iced water (50 mL), acidified with 2 N hydrochloric acid, and extracted with ethyl acetate (3 × 15 mL). The combined organic phases were washed with water (2 × 10 mL), dried (Na_2SO_4), and concentrated in vacuo. The residue (0.524 g) was further dried (0.5 Torr, 100 °C, 4 h) and subjected to medium-pressure chromatography. Elution with chloroform–methanol, 97.5:2.5 afforded 0.304 g (60.5%) of CUDCA A, identified as (22*S*,23*S*)-3α,7β-dihydroxy-22,23-methylene-5β-cholan-24-oic acid (**3**): mp 248–251 °C; $[\alpha]^{25}_D$ +56.9° (*c* 1.1, EtOH); ν_{max} (Nujol) 3380 (OH), 1700 cm^{-1} (CO); NMR (CD_3OD + $CDCl_3$) δ 0.67 (3 H, s, 18-Me), 0.93 (3 H, s, 19-Me), 1.00 (3 H, d, 21-Me), 3.33–3.70 (2 H, m, C*H*OH), 4.53 (3 H, s, OH and CO_2H). Anal. ($C_{25}H_{40}O_4$) C, H, O.

Elution with the same solvents afforded 3 mg (0.6%) of a mixture of CUDCA A and B.

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Elution with the same solvents afforded 0.153 g (30.4%) of CUDCA B, identified as (22*R*,23*R*)-3α,7β-dihydroxy-22,23-methylene-5β-cholan-24-oic acid (**4**): mp 252–255 °C; $[\alpha]^{25}_D$ +7.5° (*c* 2.7, EtOH); ν_{max} (Nujol) 3380 (OH), 1700 cm^{-1} (CO); NMR (CD_3OD + $CDCl_3$) δ 0.70 (3 H, s, 18-Me), 0.97 (3 H, s, 19-Me), 1.07 (3 H, d, 21-Me), 3.30–3.67 (2 H, m, C*H*OH), 4.80 (3 H, s, OH and CO_2H). Anal. ($C_{25}H_{40}O_4$) C, H, O.

Hydrolysis of Fraction IV. A solution of 10 N NaOH (10 mL) was added to a warm solution of fraction IV (0.695 g, 1.35 mmol) in EtOH (15 mL). The mixture was heated at reflux for 4 h, cooled, poured onto ice water (50 mL), acidified with 2 N hydrochloric acid, and extracted with ethyl acetate (3 × 20 mL). The combined organic phases were washed with water (2 × 20 mL), dried (Na_2SO_4), and concentrated in vacuo. The residue (0.5 g) was further dried (0.5 Torr, 100 °C, 4 h) and subjected to medium-pressure chromatography. Elution with chloroform–methanol, 98:2, afforded 0.264 g (48.5%) of CUDCA C, identified as (22*S*,23*R*)-3α,7β-dihydroxy-22,23-methylene-5β-cholan-24-oic acid (**5**): mp 133–136 °C; $[\alpha]^{25}_D$ +14.5° (*c* 1.3, EtOH); ν_{max} (Nujol) 3380 (OH), 1700 cm^{-1} (CO); NMR (CD_3OD + $CDCl_3$) δ 0.67 (3 H, s, 18-Me), 0.97 (3 H, s, 19-Me), 3.17–3.73 (2 H, m, C*H*OH), 4.63 (3 H, s, OH and COOH). Anal. ($C_{25}H_{40}O_4$) C, H, O.

Elution with the same solvents afforded a mixture of CUDCA C and D (50 mg, 9.2%).

Elution with the same solvents, finally, gave 0.180 g (33.0%) of CUDCA D, identified as (22*R*,23*S*)-3α,7β-dihyroxy-22,23-methylene-5β-cholan-24-oic acid (**6**): mp 168–170 °C; $[\alpha]^{25}_D$ +59.9° (*c* 1.1, EtOH); ν_{max} (Nujol) 3380 (OH), 1700 cm^{-1} (CO); NMR (CD_3OD + $CDCl_3$) δ 0.67 (3 H, s, 18 Me), 0.97 (3 H, s, 19 Me), 1.00 (3 H, d, 21-Me), 3.37–3.82 (2 H, m, C*H*OH), 4.37 (3 H, s, OH and CO_2H). Anal. ($C_{25}H_{40}O_4$) C, H, O.

Ethyl 3α,7β-Dihydroxy-5β-cholan-24-oate (7). A solution of UDCA (1) (1.0 g, 2.55 mmol) and toluene-4-sulfonic acid (0.1 g) in EtOH (20 mL) was kept with stirring at room temperature for 24 h. The solution was concentrated under vacuo, ethyl acetate (50 mL) added, and the resulting solution washed with sodium hydrogen carbonate solution (2 × 10 mL) and then with water (2 × 10 mL), dried ($MgSO_4$), and evaporated to give pure ethyl ester **7** (1.0 g, 93%): mp 64–67 °C; ν_{max} ($CHCl_3$) 3610, 3450 (OH), 1725 cm^{-1} (CO); NMR ($CDCl_3$) δ 0.67 (3 H, s, 18-Me), 0.93 (3 H, s, 19-Me), 1.33 (3 H, t, *J* = 7 Hz, OCH_2CH_3), 3.33–3.73 (2 H, m, C*H*OH), 4.10 (2 H, q, *J* = 7 Hz, OCH_2CH_3). Anal. ($C_{26}H_{44}O_4$) C, H, O.

Ethyl 3α,7β-Dihydroxy-23-(phenylseleno)-5β-cholan-24-oate (8). A solution of ester **7** (12 g, 28.5 mmol) in THF (80 mL) was added dropwise to a solution of lithium diisopropylamide [from addition of *n*-butyllithium in hexane (95 mL of a 1.35 M solution) to a solution of diisopropylamine (14.3 g) in tetrahydrofuran (150 mL)] kept under nitrogen at −78 °C. After the mixture was stirred for 15 min, a solution of diphenyldiselenide (17.8 g, 57 mmol) in THF (70 mL) was added dropwise to the solution of the enolate, and the resulting solution was kept with stirring at −78 °C for 2 h; the reaction mixture was allowed to warm to room temperature, poured onto a 10% NH_4Cl solution (500 mL), and then extracted with ethyl acetate (5 × 50 mL). The combined organic phases were washed with 1.6 N hydrochloric acid (3 × 30 mL) and with saturated aqueous sodium hydrogen carbonate solution (3 × 30 mL). The organic phase was dried ($MgSO_4$) and concentrated in vacuo. Chromatography of the oily residue (30 g) on silica gel column and elution with chloroform–methanol, 99:1, gave 13.86 g (84%) of the 23-phenylseleno derivative (**8**): mp 63–65 °C; ν_{max} ($CHCl_3$) 3610, 3450 (OH), 1725 cm^{-1} (CO); mixture of isomers NMR ($CDCl_3$) δ 0.63 and 0.70 (3 H, ss, 18 Me), 0.97 (6 H, s, 19 and 21-Me), 1.16 (3 H, t, *J* = 7 Hz, OCH_2CH_3), 3.33–3.88 (2 H, m, C*H*OH), 4.05 (2 H, dq, *J* = 7 Hz, OCH_2CH_3), 7.10–7.64 (5 H, br m, aromatic H's). Anal. ($C_{26}H_{48}O_4Se$) C, H.

(*E*)-Ethyl 3α,7β-Dihydroxy-5β-chol-22-en-24-oate (9a). A solution of 36% hydrogen peroxide (1.5 mL) is added dropwise to a solution of ester **8** (1.2 g, 2.09 mmol) in dichloromethane (30 mL) kept with stirring at room temperature. After 15 min, the reaction mixture is washed with water (2 × 10 mL), filtered by passing over silica gel–Celite (5 g, 1/1, w/w), dried ($MgSO_4$), and concentrated in vacuo. Flash chromatography of the residue over silica gel and elution with $CHCl_3$–MeOH, 99:1, afforded *E* olefin **9a** (0.86 g, 99%): mp 78–81 °C; ν_{max} 3600, 3420 (OH), 1705 (CO), 1650 cm^{-1} (C=C); NMR ($CDCl_3$) δ 0.72 (3 H, s, 18-Me), 0.97 (3 H, s, 19-Me), 1.27 (3 H, t, *J* = 7 Hz, OCH_2CH_3), 2.40 (2 H, m, OH) 3.53 (2 H, m, C*H*OH), 4.13 (2 H, q, *J* = 7 Hz, OCH_2CH_3), 5.67 (1 H, d, *J* = 16 Hz, 23-CH), 6.73 (1 H, dd, J_{20-22} = 9.5 Hz and J_{22-23} = 16 Hz, 22-CH). Anal. ($C_{26}H_{42}O_4$) C, H, O.

(*E*)-Ethyl 3α,7β-Diacetoxy-5β-chol-22-en-24-oate (9b). 4-Pyrrolidinopyridine (30 mg, 0.2 mmol) is added to a solution of olefin **9a** (1.2 g, 2.87 mmol) in CH_2Cl_2 (10 mL) containing Ac_2O (0.85 mL, 8.3 mmol) and Et_3N (1.19 mL, 8.6 mmol). The resulting solution is kept with stirring at room temperature for 1 h. Dichloromethane (10 mL) is then added, and the resulting solution washed with 3 N hydrochloric acid (3 × 10 mL) and with saturated sodium hydrogen carbonate solution (3 × 10 mL), dried ($MgSO_4$), and concentrated in vacuo to give diacetoxy derivative **9b** (1.38 g, 96%): mp 56–59 °C; ν_{max} 1715 (CO), 1650 cm^{-1} (C=C); NMR ($CDCl_3$) δ 0.70 (3 H, s, 18-Me), 1.00 (3 H, s, 19-Me), 1.28 (3 H, t, *J* = 7 Hz, OCH_2CH_3), 1.97 (3 H, s, 7-$OCOCH_3$), 2.03 (3 H, s, 3-$OCOCH_3$), 4.13 (2 H, q, *J* = 7 Hz, OCH_2CH_3), 4.40–4.93 (2 H, m, C*H*OAc), 5.67 (1 H, d, *J* = 16 Hz, 23-CH), 6.73 (1 H, dd, J_{20-22} = 9.5 Hz and J_{22-23} = 16 Hz, 22-CH). Anal. ($C_{30}H_{46}O_6$) C, H, O.

(*E*)-Ethyl 3α,7β-Diacetoxy-22,23-methylene-5β-cholan-24-oate. Diazomethane was generated slowly by adding dropwise *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide (1.72 g) in ether (18 mL) to a mixture of potassium hydroxide (0.48 g) in water (1.0 mL) and carbitol (3.0 mL), which was being warmed at 70 °C. The ethereal diazomethane (5.5 mmol) distillate was delivered directly into a reaction flask containing ester **9b** (0.8 g, 1.59 mmol) and palladium diacetate (5.0 mg) in benzene (50 mL) at 0 °C. After the addition, the mixture was stirred for 24 h until gradually attaining room temperature, after which the mixture was filtered over Celite and evaporated in vacuo. Flash chromatography of the residue (0.8 g) and elution with petroleum ether–ether, 3:2, afforded a mixture of CUDCA C and CUDCA D ethyl esters (0.7 g, 85%).

3α,7β-Diacetoxy-5β-bisnorcholan-22-al (10). Ozone was bubbled at −15 °C through a solution of ethyl 3α,7β-diacetoxy-5β-chol-22-en-24-oate (**9b**) (4.0 g, 7.97 mmol) in chloroform–methanol, 1:1, (50 mL) until a light blue color appeared (1 h). Zinc dust (18 g) and 5 N acetic acid (90 mL) were added to the cold solution, and the mixture was stirred for 2 h at room temperature. The zinc dust was filtered off and washed with chloroform (3 × 15 mL), and the aqueous layer was separated and extracted with chloroform (3 × 40 mL). The combined organic phases were washed with saturated solutions of aqueous sodium hydrogen carbonate and sodium chloride, dried (Na_2SO_4), and evaporated under reduced pressure. Flash chromatography of the residue (3.5 g) and elution with petroleum ether–ether, 4:1, gave aldehyde **10** (3.02 g, 87.7%): mp 62–64 °C; ν_{max} (Nujol) 1740 cm^{-1} (CO); NMR ($CDCl_3$) δ 0.73 (3 H, s, 18-Me), 1.0 (3 H, s, 19-Me), 1.97 (3 H, s, 7-OCOMe), 2.00 (3 H, s, 3-OCOMe), 4.40–5.00 (2 H, br m, 3 and 7-C*H*OAc), 9.50 (1 H, d, *J* = 3 Hz, CHO). Anal. ($C_{26}H_{40}O_5$) C, H, O.

Ethyl 3α,7β-Diacetoxy-22-hydroxy-23-diazo-5β-cholan-24-oate (11). A cold (−78 °C) solution of lithium diisopropylamide [from addition of *n*-butyllithium in hexane (1.74 mL of a 1.49 M solution) to a solution of diisopropylamine (0.42 mL, 3.01 mmol) in anhydrous THF (20 mL)] was added during 20 min to a stirred solution of the aldehyde **10** (1 g, 2.31 mmol) and ethyl diazoacetate (0.27 mL, 2.55 mmol) at −78 °C. After the mixture had been stirred at −78 °C for 15 min, a cold solution of acetic acid (0.18 g, 3.0 mmol) in ether (5 mL) was added, and the reaction mixture was allowed to warm to room temperature. Water (200 mL) was added, the organic layer was separated, and the aqueous layer was extracted with ether (2 × 50 mL). The combined organic phases were washed with saturated aqueous sodium hydrogen carbonate, dried ($MgSO_4$), and evaporated under reduced pressure. Chromatography of the residue (1.2 g) on Al_2O_3 (neutral, activity IV) and elution with benzene followed by benzene–ethyl acetate, 9:1, afforded α-diazo-β-hydroxy ester **11** (1.0 g, 79%): ν_{max} (Nujol) 3470 (OH), 2100 (C=N_2), and 1740 cm^{-1} (CO); NMR ($CDCl_3$) δ 0.70 (3 H, s, 18-Me), 0.97 (3 H, s, 19-Me), 1.24 (3 H, t, *J* = 7 Hz OCH_2CH_3), 1.93 (3 H, s, 7-$OCOCH_3$), 1.97 (3 H, s, 3-$OCOCH_3$), 3.68 (1 H, m, 22-OH), 4.10 (2 H, q, *J* = 7 Hz, OCH_2CH_3), 4.30–4.90 (3 H, br m, 3- and 7-C*H*OAc, 22-C*H*OH). Anal. ($C_{30}H_{46}O_7N_2$) C, H, O.

Ethyl 3α,7β-Diacetoxy-5β-chol-22-yn-24-oate (12). A solution of α-diazo-β-hydroxy ester **11** (0.5 g, 0.92 mmol) in acetonitrile (5 mL) was added dropwise to a stirring mixture of boron trifluoride etherate (0.12 mL, 0.98 mmol) and calcium hydride (0.25 g) in acetonitrile (5 mL) at room temperature. After 10 min, the mixture was filtered, the residue was washed with ether (50 mL), and the combined organic phases were washed with brine, dried ($MgSO_4$), and evaporated. Chromatography of the residue (0.4 g) on Florisil and elution with petroleum ether–ether, 4:1, afforded pure **12** (0.14 g, 30%): ν_{max} ($CHCl_3$) 2240 (C≡C), 1720 cm^{-1} (CO); NMR ($CDCl_3$) δ 0.77 (3 H, s, 18-Me), 1.00 (3 H, s, 19-Me), 1.27 (3 H, t, J = 7 Hz, OCH_2CH_3), 1.93 (3 H, s, 7-$OCOCH_3$), 2.00 (3 H, s, 3-$OCOCH_3$), 2.40–2.70 (1 H, m, 20-CH), 4.17 (2 H, q, J = 7 Hz, OCH_2CH_3), 4.40–4.80 (2 H, m, 3- and 7-CHOAc). Anal. ($C_{30}H_{44}O_6$) C, H, O.

(*Z*)-Ethyl 3α,7β-Diacetoxy-5β-chol-22-en-24-oate (13). A solution of the acetylenic ester **12** (0.5 g, 1.0 mmol) in ethyl acetate (30 mL) was hydrogenated at normal pressure and room temperature over Lindlar catalyst (40 mg). After 30 min, the black suspension was filtered by passing over Celite, and the solvent was evaporated. Chromatography of the residue (0.5 g) on silica gel and elution with petroleum ether–ether, 85:15, gave pure *Z* olefin **13** (0.36 g, 70%): mp 105–113 °C; ν_{max} ($CHCl_3$) 1715 (CO), 1640 cm^{-1} (C=C); NMR ($CDCl_3$) δ 0.77 (3 H, s, 18-Me), 1.00 (3 H, s, n 19-Me), 1.27 (3 H, t, J = 7 Hz, OCH_2CH_3), 1.93 (3 H, s, 7-$OCOCH_3$), 2.00 (3 H, s, 3-$OCOCH_3$), 3.40–3.80 (1 H, m, 20-CH), 4.12 (2 H, q, J = 7 Hz, OCH_2CH_3), 4.40 and 4.70 (2 H, m, 3- and 7-CHOAc), 5.52 (1 H, d, J = 10 Hz, 23-CH), 5.87 (1 H, dd, J_{22-20} = 10 Hz, J_{22-23} = 10 Hz, 22-CH). Anal. ($C_{30}H_{46}O_6$) C, H, O.

Physicochemical Properties. Critical micellar concentration (CMC) values were measured in water by the dye solubilization method[22] and, in particular, with azulene (Aldrich Chemical Co., Milwaukee, WI) and Orange OT (gift from Dr. K. Mysels, Chemistry Department, University of California at San Diego, La Jolla, CA) as water insoluble dyes. The water solubility of the protonated form were measured on saturated solutions a pH 3 as previously reported[23] after filtration on a Millipore (0.22 μm).

Acidity constants were determined by potentiometric measurements in solutions of aqueous methanol at different mole fractions. The pK_a values, estimated in water by means of previously assessed correlations from the pK_a values in mixed solvents, are in close agreement with each other.[24] All measurements were carried out at 25 ± 0.01 °C. Hydrophobic–hydrophylic properties of bile salts were determined by reverse-phase high-performance liquid chromatography.[19] HPLC was performed with a Water Inc. (Milford, MA) liquid chromatograph. A C-18 reverse-phase column 5 μ pore size and 10 cm in length was used. The analysis was carried out under isocratic conditions. As a mobile phase a mixture of 2-propanol/H_2O, 8/17 v/v, pH 7 was used. A retention factor (K') was calculated from the relative mobilities of the separated bile acids.[18]

Molecular Modeling. Theoretical models of the structure of the UDCA and the four stereoisomers of CUDCA were built with a Digital Equipment Corp. VAX 11/750 computer system, a raster scan high-resolution color graphic terminal Lundy 5688, a Summagraphics Corp. Bit Pad One digitizer (used for real-time interactive input of commands and data).

Biological Properties. Sprague–Dawley male rats (300–330 g) were used. The rats were anesthetized with ethyl carbamate, and the bile was collected. The bile acids were administered intravenously as sodium salts through the femoral vein at a dose of 6 mol/kg per min per animal. Bile samples were collected at 15-min intervals for a 1-h period. The biliary bile acids composition were determined by TLC–enzymatic combined methods as previously reported.[2]

The substrate specificity for 7-dehydroxylase was measured in human stools in anaerobic conditions as previously described.[3] Data were expressed by the time required to metabolize half of the initial CUDCA to the 3α-monohydroxy derivative (cyprolithocholic acid). In particular, the recovered chemical forms were quantified in the bile samples, i.e., the glycine and taurine conjugates, after separation on TLC with a solvent system composed by propionic acid/isoamyl acetate/water/1-propanol, 3:4:2:1, v/v/v/v. Once visualized by exposure to iodine vapor, the bands corresponding to unconjugated and glycine and taurine conjugates were eluted with methanol, and bile acids concentration was measured using a specific 3-hydroxysteroide dehydrogenase enzyme.[25] The percentage of amidation was calculated by the ratio between the sum of glycine and taurine conjugates and total BA recovered into bile.

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