Synthesis and Physicochemical, Biological, and Pharmacological Properties of New Bile Acids Amidated with Cyclic Amino Acids†

Aldo Roda,*,‡ Carolina Cerrè,‡ Anna C. Manetta,‡ Gianfranco Cainelli,§ Achille Umani-Ronchi,§ and Mauro Panunzio▽

Dipartimento di Scienze Farmaceutiche, Università di Bologna, Via Belmeloro, 6, 40126 Bologna, Italy, Dipartimento di Chimica "G. Ciamician", Università di Bologna, Via Selmi, 2, 40126 Bologna, Italy, and CSFM-CNR, Via Selmi, 2, 40126 Bologna, Italy

Received November 20, 1995⊗

New analogs of cyclic amino acid-conjugated bile acids were synthesized, and their physicochemical and biological properties were compared with those of natural analogs. Ursodeoxycholic acid was amidated with D-proline, L-proline, 4-hydroxy-L-proline, and 4-methoxy-L-proline. Hyocholic and hyodeoxycholic acids were amidated with L-proline. The physicochemical properties were similar to those of the natural analogs. All of them were highly stable toward enzymatic C-24 amide bond hydrolysis and 7-dehydroxylation. Their transport, metabolism, and effect on biliary lipid secretion were evaluated in bile fistula rat after intravenous infusion. All the analogs were secreted in bile unmodified. The 4-methoxy-L-proline derivative produced the highest secretion rate, much higher than those of all the other natural and synthetic analogs. This was associated with a selective reduction of cholesterol secretion with normal phospholipid secretion and choleresis. When coinfused, all the analogs were able to prevent the hepatotoxicity induced by intravenous taurochenodeoxycholic acid, as revealed by normal choleresis, alkaline phosphatase, and lactate dehydrogenase values in bile. Considering the overall data, 4-methoxy-L-proline, 4-hydroxy-L-proline, and L-proline derivatives of ursodeoxycholic acid were more potent than the natural analogs.

Introduction

Ursodeoxycholic acid (UDCA) has been widely used for cholesterol gallstone dissolution and the treatment of cholestatic liver disease.[1−6] With long term administration, it accumulates in the bile acid (BA) pool, mainly in the form of its hepatic metabolites, glyco- and tauroursodeoxycholic acid (GUDCA and TUDCA), which, in all likelihood, are the pharmacologically active compounds. It has been also demonstrated that the passive intestinal absorption of the UDCA is incomplete and it undergoes 7-dehydroxylation by intestinal microflora to form the toxic lithocholic acid (LCA).[7]

Both UDCA and its conjugates have a poor bioavailability, and total enrichment of the BA pool by chronic oral administration of UDCA does not exceed 40−50% of total BA.[8−10] Thus, there is a need for more potent agents, which has prompted a search for UDCA synthetic analogs that are more stable toward intestinal and hepatic metabolism but, at the same time, have physicochemical and biological properties that facilitate their accumulation in the enterohepatic circulation and are able to displace and replace endogenous lipophilic BA.

Many synthetic UDCA analogs have been developed and studied by us, as well as other investigators, but few of these molecules have shown the characteristics necessary for them to supplant UDCA. Two different strategies have been used in designing new BA synthetic analogs. One is to modify the steroid nucleus by introducing groups able to prevent the major metabolic step of 7-dehydroxylation while optimizing lipophilicity, detergency, polarity, and acidity. These properties were measured and well defined in a previous study relating the physicochemical properties of BA to their structure.[11,12] It was found that the presence of a substituent, such as a methyl group or a fluorine atom in 6-position in the UDCA molecule, sterically or electronically blocked the 7-position, thus hindering 7-dehydroxylase enzymatic attack.[13,14] The same is also true in 7β-methylcholic and 7β-methylchenodeoxycholic acids and 7α-methyl-UDCA.[15−17]

The second strategy tried was to modify the BA side chain by adding a molecule other than glycine or taurine. The primary goal of this structural modification is to hinder hydrolysis of the C-24 amide bond; given that 7-dehydroxylation occurs only to free BA, this latter process is additionally blocked.[18]

Previous papers reported blocking C-24 amide bond hydrolysis by introducing a *N*-methyl group or methyl or cyclopropyl ring in C-23 or C-22,C-23 positions, respectively; the resulting compounds were more stable to bacterial deamidation than the corresponding natural conjugated molecules.[19−22] Other strategies that have been tried successfully include lengthening or shortening the length of the steroid chain, forming C-25 homo-, C-23 nor-, or C-22 bis-nor-amidated BA.[11,12,23] In these cases, as well, the BA were metabolically highly stable. Finally, the amide bond has also been replaced with

† Abbreviations: BA, bile acid; UDCA, ursodeoxycholic acid; GUDCA, glycoursodeoxycholic acid; TUDCA, tauroursodeoxycholic acid; LCA, lithocholic acid; HCA, hyocholic acid; HDCA, hyodeoxycholic acid; TCDCA, taurochenodeoxycholic acid; CMC, critical micellar concentration; CMpH, critical micellar pH; WS, water solubility; r*K*', relative lipophilicity measured by C-18 HPLC; S_{max}, maximal secretion rate; T_{max}, time of the maximal secretion rate; R_s, residual secretion rate; LDH, lactate dehydrogenase; ALP, alkaline phosphatase.

* Author to whom correspondence should be addressed. Tel/Fax: +39-51-343398. E-mail: RODA_A@BIBLIO.CIB.UNIBO.IT.

‡ Dipartimento di Scienze Farmaceutiche.

§ Dipartimento di Chimica "G. Ciamician".

▽ CSFM-CNR.

⊗ Abstract published in *Advance ACS Abstracts,* April 15, 1996.

S0022-2623(95)00850-8 CCC: $12.00

others such an ester or reversed amide bonds.[11,24] All of these BA have been only partially characterized in terms of their physicochemical properties and pharmacological activity.

If lipophilicity and detergent characteristics are modified too much, the BA may not meet all pharmacologic requirements. For example, the homotaurine conjugates of UDCA are too detergent due to the presence of one more carbon atom in the side chain. On the contrary, nor-conjugates are too hydrophilic and behave more like organic anions and not as detergent-like molecules.

In this work we describe the synthesis and physicochemical, biological, and pharmacological properties in a series of new BA amides, in which the C-24 carboxyl has been conjugated with a cyclic amino acid (proline) containing different substituents. UDCA was amidated with L-proline, D-proline, 4-hydroxy-L-proline, and 4-methoxy-L-proline. Hyodeoxycholic acid (HDCA) and hyocholic acid (HCA) were amidated with L-proline.

These amino acids were chosen because they contain a nitrogen atom in a cyclic ring, a structure which should prevent bacterial deamidation by steric hindrance. Positive results had been previously obtained with compounds like *N*-methyltaurine- and sarcosine-amidated BA[11,19] in which a methyl group is bonded to the nitrogen. The increased lipophilicity induced by the proline ring of these new analogs can be in part lowered by the hydroxy or methoxy group in the side chain ring.

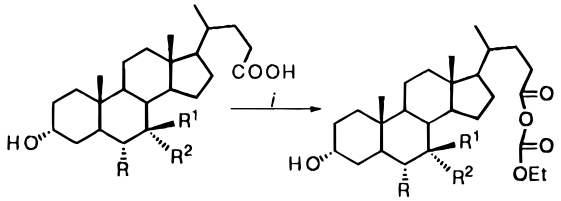
Thus, the rationale for this conjugated BA design may be summarized as follows: (1) to avoid the need of hepatic amidation of the free BA formed by deconjugation in the intestine, which is known to be a rate limiting step in BA biliary secretion,[25,20] and to avoid the formation of indesiderable metabolites that could be formed if a high amount of an unconjugated BA reaches the liver; (2) to increase the biological half-life of the analog by simply amidating the side chain, which also serves to block steroid metabolism (7-dehydroxylation[18]), as well as continuous side chain amidation by the liver and deamidation in the intestine, as the analog cycles through the enterohepatic circulation; and (3) to develop conjugated BA which are still actively absorbed by the intestine, possibly with a higher efficiency in respect to the natural analogs.

Finally with these properties, the selected analogs should be able to inhibit selectively cholesterol secretion or prevent hepatotoxicity in the animal model. Additional studies are required to verify their pharmacological activity on long term administration in vivo in the appropriate animal model to test their efficiency as new cholesterol-dissolving agents or as drugs for cholestatic liver disease therapy.

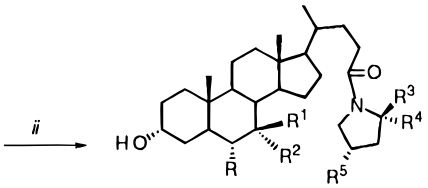
Results and Discussion

Chemistry. The proline derivative-amidated bile acids were obtained in good yield by the mixed anhydride method (see Scheme 1) after chromatographic purification on silica gel with the appropriate eluting solvent following a previously reported procedure.[26] They proved to be >96% pure by C-18 reverse phase HPLC using both an evaporative light-scattering mass detector and a precolumn fluorescence derivatization method.[27,28]

Scheme 1. Synthesis and Structures of the Studied Proline Derivatives of UDCA, HCA, and HDCA[a]



1a, 2a: R=H; R^1=OH; R^2=H;
1b, 2b: R=H; R^1=OH; R^2=H;
1c, 2c: R=H; R^1=OH; R^2=H;
1d, 2d: R=H; R^1=OH; R^2=H;
1e, 2e: R=OH; R^1=H; R^2=OH;
1f, 2f: R=OH; R^1=H; R^2=H;



3a: R=H; R^1=OH; R^2=H; R^3=COOH; R^4=H; R^5=H
3b: R=H; R^1=OH; R^2=H; R^3=H; R^4=COOH; R^5=H
3c: R=H; R^1=OH; R^2=H; R^3=COOH; R^4=H; R^5=OH
3d: R=H; R^1=OH; R^2=H; R^3=COOH; R^4=H; R^5=OMe
3e: R=OH; R^1=H; R^2=OH; R^3=COOH; R^4=H; R^5=H
3f: R=OH; R^1=H; R^2=H; R^3=COOH; R^4=H; R^5=H

[a] (i) Bu_3N, ClCOOEt, dioxane, 10 °C; (ii) proline derivative/NaOH.

Table 1. Physicochemical Properties of the New Analogs and Those of Naturally Occurring Bile Acids[a]

bile acid	WS (μM)	CMC (mM)	pK_a	CMpH	log *P* (calcd)	r*K'* (C-18)
UDCA	8	26	5.0	8.4	5.69	1.00
GUDCA	8	12	3.9	7.1	3.53	0.85
TUDCA	450	13	1−2		3.61	0.82
3a	113	15	3.9	6.0	5.83	1.25
3b	59	7	3.9	6.0	4.41	0.92
3c	250	10	3.1	4.7	3.59	0.60
3d	30	29	3.5	6.5	3.86	0.70
HCA	45	17	5.0	7.5	3.57	1.15
HDCA	15	14	5.0	8.1	4.27	0.89
3e	60	30	3.9	6.6	3.71	0.85
3f	52	25	3.9	6.6	4.41	0.94

[a] WS, water solubility of the protonated species; CMC, critical micellar concentration; pK_a, −log of the acidity constant; CMpH, critical micellar pH; log *P*, calculated octanol/water partition coefficient of the protonated species; r*K'*, relative lipophilicity measured by C-18 HPLC.

Physicochemical Properties. The main physicochemical properties of the studied synthetic BA analogs and those of the corresponding natural BA are reported in Table 1. The critical micellar concentration (CMC) values of all new analogs were comparable with those of the corresponding natural BA, suggesting that the conjugation with these amino acids does not compromise the detergent properties.

Proline conjugates showed CMC values different among the two D and L isomers. The orientation of the carboxy group of the L-proline analog toward the β face of the plane, as obtained by molecular mechanics, could explain its higher CMC. The polar carboxy group interrupts the continuity of the hydrophobic β face, thus reducing the micellar-forming capacity, which is based on back to back hydrophobic interaction.[29,30] The addition of a hydroxy group on the L-proline moiety

lowered the CMC, probably by the hydrogen-bonding capacity of this substituent facilitating micellar packing; the 4-methoxy derivative of the L-proline-UDCA, on the contrary, presented the highest CMC value, probably due to the steric hindrance of the methoxy group.

More detailed studies are required to better define the relationship between side chain structure, conformation, micelle formation, packing, and aggregation number, since the presence of a cyclic amino acid on the side chain profoundly modifies the behavior in respect to the common BA with a linear flexible side chain.

The lipophilicity values (Table 1) followed a more regular and foreseeable trend. Among the L-proline derivatives of UDCA, the lipophilicity order (**3a** > **3d** > **3c**), obtained by C-18 reverse phase HPLC, agreed with the physicochemical properties of added substituents, i.e., L-proline > 4-methoxy-L-proline > 4-hydroxy-L-proline. Among the two analogs amidated with L- and D-proline, the L isomer was the more lipophilic. The lipophilicity of these new amidated analogs is comparable to those of GUDCA and TUDCA, suggesting that lipophilicity-mediated biological properties will be similar.

The reported values have been obtained using the relative capacity factor on C-18 reverse phase HPLC. Despite the fact that this method has been extensively used for many drugs and other bile acids, the values order is affected by mobile phase composition and the BA interaction with the stationary phase which is not only a simple partition phenomenon because absorption also takes place.[31] The log *P* values, calculated by the molecular mechanics, were in agreement with the r*K*' values giving the same order of lipophilicity (Table 1) only among the dihydroxylated analogs (UDCA and HDCA).

The water solubility of the protonated form of the studied analogs (Table 1) was generally slightly higher than those of UDCA and GUDCA, ranging from 30 to 250 μM. The pK_a values were similar to that of GUDCA but lower than that of UDCA, as a result of the withdrawing effect of the amide bond. Accordingly, the presence of a hydroxy or methoxy group on the 4-position of the proline ring further reduced the pK_a values.

As a result of the CMC, pK_a, and solubility values, the critical micellar pH (CMpH) values, i.e., the pH at which a protonated acid is dissolved to form micellar aggregates, were much lower than that of UDCA and also GUDCA, suggesting that these analogs are soluble in the intestinal content (pH 7−8) and only poorly soluble at the acid pH of the gastric juice (2−3); moreover, all of them have values below 7, and this represents an improvement in respect to UDCA, which, having a high CMpH (8.4), is poorly soluble in the intestine and, consequently, poorly absorbed.[10]

The current results, together with previous studies on a large number of natural and synthetic BA,[11,12] suggest that some of these new analogs should have "ideal" physicochemical properties to be further investigated as drugs for cholesterol gallstone dissolution and/or therapy for cholestatic liver disease.

Biological Properties: In Vitro Studies. As shown in Table 2, all the studied analogs presented an extraordinary stability to both cholylglycine hydrolase and intestinal bacteria, when compared to natural BA. The new analogs were not deconjugated by cholylglycine hydrolase enzyme, responsible for the deconjugation of all naturally occurring glycine and taurine amidates and natural BA conjugated with other linear amino acids, like metionine, asparagine, glutamine, alanine, valine, lysine, etc.[11,32]

Table 2. Bile Acid Stability toward C-24 Deamidation (Incubation with cholylglycine hydrolase) and 7-Dehydroxylation (Incubation with human stools)

	half-life (h)	
bile acid	C-24 deamidation	7-dehydroxylation
UDCA		2
GUDCA	4	2
TUDCA	4	2
3a	>72	>72
3b	>72	>72
3c	>72	>72
3d	>72	>72
HCA		4
GHCA	4	6
THCA	4	6
3e	>72	>72
GHDCA	5	
THDCA	6	
3f	>72	>72

The new BA were not 7-dehydroxylated and were stable for at least 3 days of exposure to the intestinal bacteria. The presence of a cyclic ring hindered enzymatic cleavage of the amide bond, and in turn, these conjugates could no longer serve as a substrate for the 7-dehydroxylase enzyme.[18]

The first objective stated was therefore achieved by making these structural modifications that increase the metabolic stability of the BA in the enterohepatic circulation.

In Vivo Studies. Biliary Secretion and Hepatic Metabolism in Rats. The BA analogs were administered iv to bile fistula rats at a dose of 10 μmol/min/kg (1 h infusion). As can be seen in Table 3, they were secreted into the bile with different kinetics and efficiency.

The S_{max} values (range 5.7−11.1 μmol/min/kg) were similar or higher than those of TUDCA and GUDCA (7.5 and 6.1 μmol/min/kg, respectively) suggesting that these BA are taken up and secreted into bile efficiently. Only **3d** presented a S_{max} (11.1 μmol/min/kg) significantly higher ($p < 0.01$) than the natural analogs. Biliary secretion of all the new analogs is not compromised by the structural side chain modifications; on the contrary, this property is enhanced, as a result of ideal lipophilicity, polarity, and structure recognition by hepatocytes. These results point out the role of conjugation in facilitating biliary secretion and improving the hepatic transport of an unconjugated BA; in fact, in order to be secreted into bile, UDCA must be conjugated in the liver with glycine and taurine, and this process is rate-limiting for its secretion into bile.[25] HPLC analysis showed that none of the new analogs were metabolized by liver cells; in fact, they all were secreted into the bile as such.

As far as the effect on biliary lipid secretion is concerned, most of the analogs exhibited parallel stimulation of cholesterol and phospholipid secretion (Table 3). Only **3d** showed a selective and significant ($p < 0.01$) reduction of cholesterol secretion, with a sustained phospholipid secretion; as a result, the secreted bile was

Table 3. Bile Flow and Biliary Lipid Secretion Parameters in Bile Fistula Rates after iv Administration of the Studied Bile Acids[a]

	bile			bile acids			phospholipids			cholesterol		
bile acid infused	S_{max} (μL/min/kg)	R_s (μL/min/kg)	T_{max} (min)	S_{max} (μmol/min/kg)	R_s (μmol/min/kg)	T_{max} (min)	S_{max} (μmol/min/kg)	R_s (μmol/min/kg)	T_{max} (min)	S_{max} (nmol/min/kg)	R_s (nmol/min/kg)	T_{max} (min)
saline	52 ± 12	33 ± 8	60	0.9 ± 0.2	0.5 ± 0.2	60	0.17 ± 0.06	0.07 ± 0.02	60	25 ± 5	7 ± 3	15
UDCA	116 ± 45	47 ± 10	60	4.2 ± 1.2	0.8 ± 0.2	45	0.26 ± 0.13	0.12 ± 0.03	45	55 ± 14	24 ± 10	60
GUDCA	77 ± 11	45 ± 8	45	6.1 ± 3.3	0.8 ± 0.1	45	0.23 ± 0.08	0.10 ± 0.01	45	51 ± 8	18 ± 6	30
TUDCA	72 ± 17	48 ± 11	60	7.5 ± 1.8	0.4 ± 0.1	30	0.20 ± 0.01	0.07 ± 0.02	45	45 ± 10	22 ± 7	45
3a	90 ± 24	50 ± 7	60	6.0 ± 1.8	0.9 ± 0.2	60	0.28 ± 0.07	0.11 ± 0.02	45	55 ± 10	31 ± 11	45
3b	65 ± 6	51 ± 7	60	7.9 ± 0.8	1.6 ± 0.1	60	0.20 ± 0.06	0.09 ± 0.02	60	52 ± 7	28 ± 9	60
3c	97 ± 11	46 ± 7	60	6.3 ± 1.0	0.9 ± 0.2	60	0.25 ± 0.05	0.12 ± 0.01	60	68 ± 15	31 ± 10	45
3d	90 ± 17	44 ± 5	45	11.1 ± 2.1[b]	0.8 ± 0.6	60	0.28 ± 0.03	0.12 ± 0.01	45	28 ± 4[b]	12 ± 5	45
3e	60 ± 2	38 ± 3	60	6.0 ± 0.2	1.8 ± 0.5	60	0.28 ± 0.04	0.26 ± 0.02	90	52 ± 13	23 ± 9	30
3f	67 ± 8	47 ± 3	60	5.7 ± 0.5	1.4 ± 0.2	60	0.34 ± 0.07	0.21 ± 0.04	60	60 ± 9	46 ± 10	30

[a] Bile acids were administered at a dose of 10 μmol/min/kg. S_{max}, maximum secretion rate; T_{max}, time of the maximum secretion rate; R_s, residual secretion rate. Values are expressed as mean ± SD ($n = 6$). [b] Value is significantly different ($p < 0.01$) from the control natural bile acids.

Table 4. Bile Flow, Calcium Output, and Lactate Dehydrogenase and Alkaline Phosphatase Biliary Concentrations in Bile Fistula Rats during iv Coinfusion of TCDCA and the Studied Bile Acid Analogs[a]

bile acid coinfused	bile S_{max} (μL/min/kg)	calcium S_{max} (μmol/min/kg)	$[LDH]_{max}$ (UI/L)	$[ALP]_{max}$ (UI/L)
saline	37 ± 2	0.10 ± 0.02	12100 ± 2500	910 ± 350
UDCA	72 ± 3	0.18 ± 0.05	115 ± 78	217 ± 5
GUDCA	63 ± 26	0.22 ± 0.08	37 ± 9	150 ± 50
TUDCA	48 ± 8	0.30 ± 0.07	37 ± 2	89 ± 32
3a	86 ± 5	0.42 ± 0.11	16 ± 2	32 ± 2
3b	97 ± 16	0.38 ± 0.09	71 ± 12	30 ± 6
3c	90 ± 7	0.37 ± 0.07	10 ± 12	31 ± 19
3d	84 ± 18	0.71 ± 0.11	14 ± 9	30 ± 12
3e	100 ± 12	0.39 ± 0.01	48 ± 4	34 ± 11
3f	83 ± 21	0.42 ± 0.03	95 ± 41	57 ± 13

[a] TCDCA and the studied bile acid were both iv coinfused at a dose of 8 μmol/min/kg. Values are expressed as mean ± SD ($n = 6$).

Table 5. Maximum Biliary Secretion Rates of TCDCA and the Studied Bile Acid Analogs during iv Coinfusion in Bile Fistula Rats[a]

bile acid coinfused	S_{max} (μmol/min/kg)	
	TCDCA	studied BA
saline	1.10 ± 0.12	
UDCA	1.56 ± 0.35	1.72 ± 0.40
GUDCA	3.13 ± 0.86	3.33 ± 0.69
TUDCA	5.72 ± 1.26	8.08 ± 1.81
3a	5.43 ± 0.95	3.22 ± 0.76
3b	3.64 ± 1.06	2.07 ± 0.59
3c	4.98 ± 1.31	3.27 ± 0.63
3d	6.96 ± 1.42	4.67 ± 1.08
3e	5.07 ± 1.51	3.41 ± 0.90
3f	3.88 ± 0.84	3.02 ± 0.75

[a] TCDCA and the studied bile acid were both iv coinfused at a dose of 8 mmol/min/kg. Values are expressed as mean ± SD ($n = 6$).

enriched with the administered BA, phospholipid, and, to a less extent, cholesterol. This selective acute effect in reducing the cholesterol secretion suggests a potential use of this analog as a cholesterol-dissolving agent, since it is able to produce a bile undersaturated in cholesterol.

The stimulation of bile flow induced by the infusion of the new analogs was similar to that obtained during infusion of the natural analogs. During and after iv infusion of all the studied analogs, the biochemical liver function tests were normal, suggesting a lack of toxicity of these BA.

Acute Coinfusion Model of Liver Protection: Prevention of the Hepatotoxicity Induced by Taurochenodeoxycholic Acid. The activity of the new analogs as drugs for the treatment of cholestatic liver diseases has been evaluated in bile fistula rats. It is well known[33−36] that taurochenodeoxycholic acid (TCDCA) administered to rats at 4−8 μmol/min/kg doses is highly hepatotoxic, as revealed by cell necrosis, release in bile of cellular enzymes such as alkaline phosphatase (ALP) and lactate dehydrogenase (LDH), block of biliary calcium secretion, and cholestasis. The simultaneous infusion of UDCA or its conjugates is able to reduce or prevent liver injury.

We carried out experiments of iv coinfusion of TCDCA and the new BA analogs at the same dose of 8 μmol/min/kg. UDCA, GUDCA, and TUDCA were used as controls. In Table 4 the bile flow, calcium output, and ALP and LDH biliary concentrations are reported. The effects of UDCA and its conjugates were comparable to those reported in the literature. All of the new analogs were able to prevent the TCDCA hepatotoxic effects, as revealed by increased bile flow and calcium secretion and reduced ALP and LDH leaking.

Bile flow reached values similar to those of healthy animals receiving only saline solution; all the new analogs were more choleretic than the natural ones. Calcium secretion increased in respect to both saline control and the natural BA. The more potent effect was given by **3d** ($p < 0.01$). LDH and ALP activity in bile were significantly reduced with respect to saline control; all the new analogs were even more effective than the natural ones as far as ALP is concerned; **3a**,**c**,**d** were the more effective in reducing LDH leaking.

The maximum biliary secretion rates (S_{max}) of TCDCA and the studied analogs, as determined by HPLC analysis of bile acid composition, are reported in Table 5. When only saline solution was coinfused with TCDCA, the mean maximum secretion rate of TCDCA was 1.10 ± 0.12 μmol/min/kg, and this was increased by the coinfusion of UDCA, GUDCA, TUDCA, and the new analogs. The most potent effect was due to **3d**; its efficient secretion rate was associated with a concomitant increase in the maximum secretion rate of TCDCA (6.9 ± 0.56 μmol/min/kg), indicating its efficient displacement, thus reducing the hepatic residence time of TCDCA and the adverse effects of this BA on hepatocytes, as shown by the normalization of the studied biochemical parameters.

Further selection of these analogs as clinical candidates for hepatoprotection activity cannot be done only on the basis of these results alone that, more or less, demonstrate that all of them are able to prevent hepatotoxicity induced by TCDCA. To be suitable for

clinical use, the new analogs described here must fulfill additional criteria, such as efficient intestinal absorption, transport, and accumulation in bile when chronically administered to suitable animals such as hamster.

Experimental Section

Chemistry. Melting points (uncorrected) were determined on a Buchi 530 apparatus. Optical rotations were measured on a Perkin-Elmer Model 141 polarimeter with a 1 cm microcell. Proton and carbon NMR spectra were obtained on a Varian Gemini-200 apparatus and are measured in δ units. Flash chromatography was performed on silica gel 200−400 mesh (Merck). log *P* values were calculated using HyperChem software (registered trademark of Hypercube, Inc., Waterloo, Ontario, Canada). All solvents were purified by distillation. UDCA, TUDCA, HDCA, and HCA were purchased from Sigma (St. Louis, MO); GUDCA was from Calbiochem (La Jolla, CA).

General Procedure for the Preparation of Bile Acid Amides.[26] To a well-stirred and cooled at 10 °C solution of bile acid (50 mmol) and tri-*N*-butylamine (55 mmol) in anhydrous dioxane (300 mL) was added ethyl chloroformate (55 mmol) dropwise. Stirring was continued for 30 min; then a solution of the amino acid (60 mmol) in 10% NaOH (p/v) (25 mL) was added dropwise at the same temperature and the mixture left for 4 h. The reaction mixture was allowed to warm to room temperature and poured onto 400 g of ice. The pH was adjusted to 1 by 12 N HCl. The white precipitate was filtered and dissolved in 2.5 M NaOH (20 mL). The pH was adjusted to pH 5.4 with 0.1 M HCl. A white precipitate occurred. This precipitate was removed and the mother liquor acidified with 1 M HCl to pH 1.2. The new precipitate formed was essentially constituted by the pure target **3**. Alternatively the product was purified by flash chromatography and recrystallized from diethyl ether.

***N*-[(3α,5*β*,7*β*)-3,7-Dihydroxy-24-oxocholan-24-yl]-L-proline (3a).** To a suspension of UDCA (**1a**) (19.6 g, 50 mmol) in anhydrous 1,4-dioxane (300 mL) was added tributylamine (13.2 mL, 55 mmol) at 10 °C. This temperature was kept with stirring while a solution of ethyl chloroformate (5.3 mL, 55 mmol) in dioxane (10 mL) was added during 15 min. After the solution was stirred for an additional 30 min, a solution of L-proline (6.9 g, 60 mmol) in 10% NaOH (p/v) (25 mL) was added at the same temperature, and the resulting solution was stirred for 4 h while the temperature was allowed to reach room temperature spontaneously. Ice (400 g) was added, and the mixture was stirred and acidified (pH 2, concentrated HCl). A white precipitate occurred. After stirring for a further 30 min, the precipitate was collected and dissolved in $NaOH_{aq}$ (20 mL, 2.5 N). The pH was adjusted to 5.4 (1 N HCl). The solution was allowed to stand for 15 min and filtered, and the aqueous layers were acidified at pH 1.5 with concentrated HCl at 0 °C. The new collected precipitate (20.7 g) showed to be pure target **3a**: mp 133−137 °C; $[\alpha]^{20} = -23.7°$ (*c* 0.88, $CHCl_3$); IR (Nujol) 3360, 1719, 1623 cm^{-1}; ^{1}H-NMR ($CDCl_3$) 5.0 (broad, 2H), 4.57 (t, 1H), 3.6 (m, 2H), 3.0 (m, 2H), 2.4−0.8 (complex pattern, 28H), 1.0 (d, 3H), 0.94 (s, 3H), 0.67 (s, 3H); ^{13}C-NMR ($CDCl_3$) 176.3, 175.0, 72.4, 72.2, 59.3, 57.4, 56.8, 49.5, 49.3, 48.7, 48.4, 45.0, 44.7, 44.3, 41.8, 41.0, 38.9, 36.3, 35.4, 31.3, 29.9, 28.2, 24.2, 22.6, 19.4, 12.9. Anal. ($C_{29}H_{47}NO_5$) C, H, N.

***N*-[(3α,5*β*,7*β*)-3,7-Dihydroxy-24-oxocholan-24-yl]-D-proline (3b).** The protocol was analogous to that of **3a** starting from UDCA (10 mmol, 3.92 g) in 60 mL of dioxane, tributylamine (11 mmol, 2.64 mL), ethyl chloroformate (11 mmol, 1.05 mL), and D-proline (12 mmol, 1.38 g) dissolved in 10 mL of 10% NaOH. The target was purified by flash chromatography (THF/MeOH, 9:1, v/v) and recrystallized from ether, obtained 2.50 g of **3b**: mp 140−144 °C; $[\alpha]^{20} = +76.5°$ (*c* 0.098, CH_3OH); IR (Nujol) 3360, 1719, 1625 cm^{-1}; ^{1}H-NMR ($CDCl_3$) 4.4 (broad, 2H), 4.35 (t, 1H), 3.6 (m, 4H), 3.0 (m, 2H), 2.4−0.8 (complex pattern, 28H), 1.0 (d, 3H), 0.94 (s, 3H), 0.67 (s, 3H); ^{13}C-NMR ($CDCl_3$) 175.1, 174.0, 72.0, 71.8, 60.3, 57.5, 56.5, 49.8, 48.1, 44.7, 44.4, 44.0, 42.4, 40.6, 36.1, 35.2, 31.9, 29.6, 27.9., 23.9, 22.4, 19.1, 12.7. Anal. ($C_{29}H_{47}NO_5$) C, H, N.

***N*-[(3α,5*β*,7*β*)-3,7-Dihydroxy-24-oxocholan-24-yl]-4-*trans*-hydroxy-L-proline (3c).** This compound was obtained from UDCA (19.6 g, 50 mmol) and *trans*-4-hydroxy-L-proline (7.86 g, 60mmol) following the procedure described above. The residue was subjected to flash chromatography. Elution with THF/acetic acid, 98:2 (v/v), afforded the target (22.8 g): mp 98−100 °C; $[\alpha]^{20} = +16.2°$ (*c* 0.28, CH_3OH); IR (Nujol) 3360, 1719, 1625 cm^{-1}; ^{1}H-NMR ($CDCl_3$) 5.0 (broad, 2H), 4.57 (dd, 1H), 3.6 (m, 4H), 3.0 (m, 2H), 2.4−0.8 (complex pattern, 28H), 1.0 (d, 3H), 0.94 (s, 3H), 0.67 (s, 3H); ^{13}C-NMR ($CDCl_3$) 176.3, 174.5, 72.5, 72.3, 60.5, 57.9, 56.9, 49.9, 49.3, 48.8, 48.5, 49.9, 44.4, 41.9, 41.1, 39.0, 36.4, 35.5, 32.7, 31.4, 30.8, 30.1, 22.1, 22.8, 19.0, 13.0. Anal. ($C_{29}H_{47}NO_6$) C, H, N.

***N*-[(3α,5*β*,7*β*)-3,7-Dihydroxy-24-oxocholan-24-yl]-4-*trans*-methoxy-L-proline (3d).** This compound was prepared according the procedure described for compound **3a** starting from UDCA (3.52 g, 9 mmol) and L-methoxyproline[37] (1.72 g, 12 mmol), obtained 2.32 g of **3d**: mp 118−124 °C; $[\alpha]^{20} = +17.3°$ (*c* 0.104, CH_3OH); IR (Nujol) 3360, 1719, 1625 cm^{-1}; ^{1}H-NMR ($CDCl_3$) 4.47 (t, 1H), 4.0 (broad, 2H), 3.68 (m, 1H), 3.5 (s, 3H), 2.4−0.8 (complex pattern, 30H), 0.9 (d, 3H), 0.85 (s, 3H), 0.67 (s, 3H); ^{13}C-NMR ($CDCl_3$) 176.3, 174.5, 78.6, 70.9, 65.7, 57.6, 56.9, 54.8, 49.6, 49.3, 48.7, 48.5, 43.5, 43.3, 42.3, 40.0, 39.0, 36.8, 35.3, 33.9, 31.4 30.5, 29.8, 26.7, 23.2, 21.0, 18.4, 14.9, 12.0. Anal. ($C_{30}H_{49}NO_6$) C, H, N.

***N*-[(3α,5*β*,6α,7*β*)-3,6,7-Trihydroxy-24-oxocholan-24-yl]-L-proline (3e).** This compound was obtained from 20.4 g (50 mmol) of **1e** (HCA) following the procedure described for compound **3a.** The crude reaction mixture was purified by flash chromatography using as eluent ethyl acetate/methanol/acetic acid, 8.5:1:0.5, yielding **3e** (8 g): mp 218−222 °C; $[\alpha]^{20} = -18.1°$ (*c* 0.38, CH_3OH); IR (Nujol) 3360, 1715, 1630 cm^{-1}; ^{1}H-NMR ($CDCl_3$) 5.1 (broad, 1H), 4.6 (t, 1H), 3.72 (m, 6H), 3.0 (m, 2H), 2.5−0.9 (complex pattern, 27H), 1.03 (d, 3H), 0.95 (s, 3H), 0.69 (s, 3H); ^{13}C-NMR ($CDCl_3$) 176.3, 175.5, 70.9, 67.9, 64.9, 59.1, 56.8, 54.9, 49.5, 49.3, 48.7, 43.8, 43.4, 42.4, 40.1, 39.1, 35.9, 34.8, 31.4, 30.5, 29.8, 28.7, 26.6, 24.2, 21.2, 18.5, 15.0, 12.3. Anal. ($C_{29}H_{47}NO_6$) C, H, N.

***N*-[(3α,5*β*,6α,)-3,6-Dihydroxy-24-oxocholan-24-yl]-L-proline (3f).** This compound was obtained as above described from **1f** (HDCA) (19.6 g, 50 mmol). The crude reaction mixture was purified by flash chromatography using as eluent ethyl acetate/methanol/acetic acid, 8.5:1:0.5 yielding **3f** (13 g): mp 203−209 °C; $[\alpha]^{20} = -13.9°$ (*c* 0.49, CH_3OH); IR (Nujol) 3360, 1719, 1625 cm^{-1}; ^{1}H-NMR ($CDCl_3$) 4.1 (broad, 2H), 3.8 (m, 2H), 3.5 (m, 2H), 3.3 (t, 1H), 2.4−0.8 (complex pattern, 29H), 1.03 (d, 3H), 0.95 (d, 3H), 0.85 (s, 3H), 0.60 (s, 3H); ^{13}C-NMR ($CDCl_3$) 176.4, 174.5, 70.8, 67.7, 65.9, 59.2, 55.7, 54.8, 49.4, 49.3, 48.6, 43.9, 43.2, 42.1, 40.1, 36.1, 35.9, 34.7, 31.2, 30.4, 29.8, 29.5, 28.6, 26.5, 24.0, 21.0, 18.4, 14.8, 11.9. Anal. ($C_{29}H_{47}NO_5$) C, H, N.

Physicochemical Properties: Critical Micellar Concentration. Critical micellar concentration values in water were obtained by surface tension measurements, using a maximum bubble-pressure method, as previously reported.[38]

Water Solubility. The water solubility (WS) of the protonated form was measured on saturated solutions at pH 1, as previously reported.[39]

Acidity. Acidity constants were determined by potentiometric measurements in solutions of aqueous methanol of different mole fractions at 25.0 °C. The pK_a values were estimated in water by means of previously established correlations at pK_a values in mixed solvents.[40]

Lipophilicity. The relative lipophilicity of bile salts was measured by C-18 reverse phase HPLC.[31] The analyses were carried out under isocratic conditions (0.9 mL/min), using aqueous methanol 65% (v/v) containing 2 mM ammonium acetate at pH 7 as the mobile phase, in order to insure complete ionization of all BA. A retention factor (K') was calculated from the relative mobilities of the separated bile acids using the formula: $K' = (t_v - t_o)/t_o$, where t_o is the retention time of the solvent and t_v is the retention time of bile salts. Data are expressed as the relative retention factor, rK' (relative to UDCA).

Critical Micellar pH. The estimation of the pH−solubility relationship of each bile acid and its salt was carried out by aqueous acidometric titration of the bile salt; 20 mL of 50 mM solution of each bile salt studied was titrated with 0.1 M HCl. The CMpH is the pH at which the precipitation of the

protonated form is first visualized by turbidimetry at 500 nm. This value was confirmed by back-titration with 0.1 M NaOH until the suspension became transparent.

Biological Properties. In Vitro Studies: Stability to Cholylglycine Hydrolase. Hydrolysis of the C-24 amide bond was studied in a solution consisting of acetate buffer (0.3 M, pH 5.6, 200 μL), EDTA (0.2 M, 40 μL), mercaptoethanol (0.2%, 40 μL), enzyme (cholylglycine hydrolase from *Clostridium perfringens*, 50 units/mL, 50 μL), and the conjugated bile acid (10 mM, 10 μL). After incubation at different times (0.5, 1, 2, 8, 16, 24, 48, and 72 h) at 37 °C, bile acids were isolated and analyzed using the procedure described below. GUDCA, TUDCA, glycohyocholic acid (GHCA), taurohycholic acid (THCA), glycohyodeoxycholic acid (GHDCA), and taurohyodeoxycholic acid (THDCA) were used as controls.

Stability to Intestinal Bacteria. Homogenized fresh human stools (500 mg) were transferred into sterile vials to which 5 mL of sterilized chopped meat-glucose medium (Scott Lab., Fiskville, RI) was added. BA were then added at a final concentration of 0.05 mM. UDCA, UCA, and their glyco and tauro conjugates were used as controls. Vials were incubated at 37 °C; then, at 0, 4, 8, 16, 20, 24, and 72 h after the addition of the BA, the reaction was stopped with 150 μL of 30% KOH. The samples were centrifuged at 3500 rpm for 10 min; from the supernatant the BA were isolated by C-18 solid-phase extraction and analyzed by TLC (solvent I) and HPLC (solvent B) (see Analytical Methods).

In Vivo Study: Biliary Secretion and Hepatic Metabolism in Rats. The study was performed in male Sprague−Dawley rats (300−330 g). The animals were anesthetized with ethyl carbamate, and the bile duct was cannulated with polyethylene-10 tubing (Clay Adams, Becton Dickinson, Parippany, NJ).

After 1 h of base-line steady state, the BA were administered as sodium salts through the femoral vein at a dose of 10 μmol/min/kg for 1 h; bile was collected for 3 h at 15 min intervals. The controls received UDCA, GUDCA, TUDCA, and saline solution. Each group consisted of six animals.

The bile flow was measured gravimetrically taking the density of bile as 1; the concentrations of BA, cholesterol, and phospholipids in the samples were determined by enzymatic methods.[41−43] BA composition was evaluated by the HPLC method, as described in Analytical Methods, using solvent A. Biliary lipid secretion, calculated from the volume of secreted bile and the biliary lipid concentration, is expressed as μmol/min/kg. The following parameters were calculated by plotting biliary secretion vs time: the mean values ± SD of the maximal secretion rate and relative time (S_{max} and T_{max}) and the residual secretion rate (R_s) (the rate value at the third hour of the experiment).

Acute Coinfusion Model of Liver Protection: Prevention of the Hepatotoxicity Induced by Taurochenodeoxycholic Acid. The sodium salts of TCDCA and the studied analog were coinfused at the same dose of 8 μmol/min/kg for 1 h into the jugular vein of bile fistula rats (Sprague−Dowley, weight range 200−250 g). Bile was collected at 30 min intervals for 3 h. The control rats received saline solution, UDCA, GUDCA, or TUDCA.

Total BA concentration was determined as previously reported;[41] BA composition was evaluated by HPLC (solvent A, see Analytical Methods); calcium concentration, alkaline phosphatase, and lactate dehydrogenase activities were determined in bile by conventional enzymatic methods (Merck, Darmstadt, Germany).

Statistical Analysis. Statistical analysis was carried out using analysis of variance to show differences among the various groups studied. In addition, the Student's *t*-test was used to identify and isolate the agents producing significant results. All values are expressed as the mean ± SD; calculations were performed using the procedure performance analysis of variance of the Statistical Analysis System (SAS Institute Inc., Cary, NC).

Analytical Methods. The qualitative and quantitative compositions of BA in bile or other matrices such as stool were determined by different combined chromatography techniques. The biological fluids underwent a preliminary cleanup procedure by conventional C-18 reverse phase extraction (C-18 Bond Elut; Analytichem International, Harbor City, CA).[13]

Thin-layer chromatography (TLC), utilizing silica gel 0.25 μm thickness plates (Merck, Darmstat, Germany), was employed as the first screening test. The solvent system used for the separation of conjugated BA was composed of propionic acid/isoamyl acetate/water/*N*-propanol (3:4:1:2, v/v/v/v; solvent I), and that of the unconjugated BA was acetic acid/carbon tetrachloride/isopropyl ether/isoamyl acetate/water/*N*-propanol/benzene (1:4:6:8:2:2, v/v/v/v/v/v; solvent II). Separated BA were revealed with 5% phosphomolybdic acid ethanol solution.

The BA were also evaluated by HPLC[27] with a Waters 600E multisolvent delivery system equipped with an autosampler injector (Waters 717; Waters, Milford, MA). The apparatus was connected to an evaporative light-scattering detector, ELSD II (Varex Corp., Burtonsville, MD), and the signal recorded with a Waters 746 data module. A Nova-Pak C-18 Waters steel column (3.9 mm × 300 mm), particle size 4 μm, was used; column temperature was kept at 37 ± 0.2 °C by a Waters TCM thermostat. For the separation of glycine- and taurine-amidated BA, a mobile phase, composed of aqueous methanol 65% (v/v) containing 15 mM ammonium acetate, with an apparent pH of 5.4 ± 0.1 (solvent A) was used under isocratic conditions (0.9 mL/min); unconjugated BA were separated with aqueous methanol 75% (v/v) containing 15 mM ammonium acetate, also with an apparent pH of 5.4 ± 0.1 and a flow rate of 0.9 mL/min (solvent B).

Conclusions

The physicochemical properties of the new studied analogs in term of CMC and lipophilicity are similar to those of the parent compounds and, in part, explain their behavior in aqueous solutions and their biological and pharmacological properties.

The conjugation of UDCA, HDCA, and HCA with cyclic amino acids prevents the enzymatic hydrolysis of the C-24 amide bond by cholylglycine hydrolase even after a long incubation time. The new analogs are also highly resistant to bacterial 7-dehydroxylase.

The structural modifications do not compromise the hepatic uptake and the transport; on the contrary, some of them, like **3d**, present a maximum transport higher than that of natural UDCA conjugates. **3d** is able to selectively inhibit the cholesterol secretion, and this is associated with the highest secretion rate of phospholipids and BA. A bile undersaturated in cholesterol and enriched in phospholipids is therefore produced, and this strongly encourages the use of this compound for cholesterol gallstone dissolution.

All of them are able to prevent the hepatotoxicity induced in rat by TCDCA iv infusion, and this suggests a potential use as drugs for the treatment of cholestatic liver deseases.

Considering the overall set of data, L-proline, 4-hydroxy-L-proline, and 4-methoxy-L-proline derivatives of UDCA seem the more potent analogs; again, **3d**, combining its high metabolic stability and efficient hepatic transport with a selective inhibition of cholesterol secretion and with normalization of the biochemical parameters during coinfusion with TCDCA, is the more promising analog.

The data, obtained on this series of new cyclic analogs, are extremely useful in a rationale approach to new bile acid drug design and in better understanding the key points in BA metabolism and mechanisms of action.

Acknowledgment. We gratefully acknowledge Alfa Wasserman S.p.A. (Bologna, Italy) for supporting this work and Dr. Egidio Marchi for helpful suggestions and discussion.

References

(1) Makino, J.; Shinizaki, K.; Nakagawa, K.; Yoshino, K. Dissolution of Cholesterol Gallstones by Long-Term Administration of Ursodeoxycholic Acid. *Jpn. J. Gastroenterol.* **1975**, *72*, 690−702.

(2) Bacharach, W. H.; Hofmann, A. F. Ursodeoxycholic Acid in the Treatment of Cholesterol Cholelithiasis. *Dig. Dis. Sci.* **1982**, *27*, 737−761.

(3) Roda, E.; Bazzoli, F.; Morselli, A. M.; Mazzella, G.; Roda, A.; Sama, C.; Festi, D.; Aldini, R.; Barbara L. Ursodeoxycholic Acid Versus Chenodeoxycholic Acid as Cholesterol Gallstone Dissolving Agents: a Comparative Randomized Study. *Hepatology* **1982**, *2*, 804−810.

(4) Hofmann, A. F.; Popper, H. Ursodeoxycholic Acid for Primary Biliary Cirrosis. *Lancet* **1987**, *2*, 398−399.

(5) Leuschner, U.; Fisher, H.; Kurtz, W.; Guldutuna, S.; Hubner, K.; Hellstern, A.; Gatzen, M. Ursodeoxycholic Acid in Primary Biliary Cirrosis: Results of a Controlled Double-Blind Trial. *Gastroenterology* **1989**, *96*, 1268−1274.

(6) Poupon, R. E.; Balkau, B.; Eschwege, E.; Poupon, R. Ursodeoxycholic Acid Primary Biliary Cirrosis Study. A Multicenter, Controlled Trial of Ursodiol for the Treatment of Primary Biliary Cirrosis. *N. Engl. J. Med.* **1991**, *324*, 1548−1554.

(7) Palmer, R. H. Toxic Effects of Lithocholate on the Liver and Biliary Tree. In *The Hepatobiliary Sistem. Fundamental and Pathological Mechanisms*; Taylor, W., Ed.; Plenum Press: New York, 1976; pp 227−240.

(8) Parquet, M.; Metman, E. H.; Raizman, A.; Rambaud, J. C.; Berthaux, N.; Infante, R. Bioavailability, Gastrointestinal Transit, Solubilization and Faecal Excretion of Ursodeoxycholic Acid in Man. *Eur. J. Clin. Invest.* **1985**, *15*, 171−178.

(9) Walker, S.; Rudolph, G.; Raedsch, R.; Stiehl, A. Intestinal Absorption of Ursodeoxycholic Acid in Patients with Extrahepatic Biliary Obstruction and Bile Drainage. *Gastroenterology* **1992**, *102*, 810−815.

(10) Roda, A.; Roda, E.; Marchi, E.; Simoni, P.; Cerrè, C.; Pistillo, A.; Polimeni, C. Efficient Intestinal Absorption of an Enteric-Coated Sodium Ursodeoxycholate Formulation. *Pharm. Res.* **1994**, *11*, 642−647.

(11) Roda, A.; Grigolo, B.; Pellicciari, R.; Natalini, B. Structure-Activity Relationship Studies on Natural and Synthetic Bile Acid Analogs. *Dig. Dis. Sci.* **1989**, *34*, 1−12.

(12) Roda, A.; Grigolo, B.; Roda, E.; Simoni, P.; Pellicciari, R.; Natalini, B.; Fini, A.; Morselli Labate, A. M. Quantitative Relationship Between Bile Acid Structure and Biliary Lipid Secretion in Rat. *J. Pharm. Sci.* **1988**, *77*, 596−605.

(13) Roda, A.; Pellicciari, R.; Cerrè, C.; Polimeni, C.; Sadeghpour, B.; Marinozzi, M.; Cantelli Forti, G.; Sapigni, E. New 6-Substituted Bile Acids: Physico-Chemical and Biological Properties of 6α-Methyl Ursodeoxycholic Acid and 6α-Methyl-7-epicholic Acid. *J. Lipid Res.* **1994**, *35*, 2268−2279.

(14) Roda, A.; Pellicciari, R.; Polimeni, C.; Cerrè, C.; Cantelli Forti, G.; Sadeghpour, B.; Sapigni, E.; Gioacchini, A. M.; Natalini, B. Metabolism, Pharmacokinetics, and Activity of a New 6-Fluoro Analogue of Ursodeoxycholic Acids in Rats and Hamsters. *Gastroenterology* **1995**, *108*, 1204−1214.

(15) Une, M.; Singhal, A. K.; McSherry, C. K.; May-Donath, P.; Mosbach, E. H. Metabolism of 3α,7α-dihydroxy-7β-methyl-5β-cholanoic Acid and 3α,7β-dihydroxy-7α-methyl-5β-cholanoic Acid in Hamster. *Biochim. Biophys. Acta* **1985**, *833*, 196−202.

(16) Kuroki, S.; Mosbach, E. H.; Cohen, B. I.; Stenger, R. J.; McSherry, C. K. 7-Methyl Bile Acids: 7β-Methyl-Cholic Acid Inhibits Bacterial 7-Dehydroxylation of Cholic Acid and Chenodeoxycholic Acid in the Hamster. *J. Lipid Res.* **1987**, *28*, 856−863.

(17) Kuroki, S.; Mosbach, E. H.; Cohen, B. I.; McSherry, C. K. Metabolism of Bile Acid Analogues 7-Methyl-cholic Acid and 7-Methyl-ursocholic Acid. *Gastroenterology* **1987**, *82*, 876−884.

(18) Batta, A. K.; Salen, G.; Arora, R.; Shefer, S.; Batta, M.; Person, A. Side Chain Conjugation Prevents Bacterial 7-Dehydroxylation of Bile Acids. *J. Biol. Chem.* **1990**, *265*, 10925−10928.

(19) Lilienau, J.; Schteingart, C. D.; Hofmann, A. F. Physicochemical and Physiological Properties of Cholyl-sarcosine: a Potential Replacement Detergent for Bile Acid Deficiency States in the Small Intestine. *J. Clin. Invest.* **1992**, *89*, 420−431.

(20) Roda, A.; Aldini, R.; Grigolo, B.; Simoni, P.; Roda, E.; Pellicciari, R.; Lenzi, P.; Natalini, B. 23-Methyl-3α,7β-dihydroxy-5β-cholan-24-oic Acid: Dose-Response Study of Biliary Secretion in Rat. *Hepatology* **1988**, *8*, 1571−1576.

(21) Pellicciari, R.; Natalini, B.; Cecchetti, S.; Porter, B.; Roda, A.; Grigolo, B.; Balducci, R. Bile Acids with a Cyclopropyl-Containing Side Chain. 3. Separation, Identification and Properties of all the Four Stereoisomers of 3α,7β-dihydroxy-22,23-methylene-5β-cholan-24-oic Acid (CUDCA). *J. Med. Chem.* **1988**, *31*, 730−736.

(22) Roda, A.; Grigolo, B.; Aldini, R.; Simoni, P.; Pellicciari, R.; Natalini, B.; Balducci, R. Bile Acids with a Cyclopropyl-Containing Side Chain. IV. Physicochemical and Biological Properties of the Four Diastereoisomers of 3α,7β-dihydroxy-22,23-methylene-5β-cholan-24-oic Acid. *J. Lipid Res.* **1987**, *28*, 1384−1397.

(23) Yoon, Y. B.; Hagey, L. R.; Hofmann, A. F.; Gurantz, D.; Michelotti, E. L.; Steinbach, J. H. Effect of Side-Chain Shortening on the Physiologic Properties of Bile Acids: Hepatic Transport and Effect on Biliary Secretion of 23-nor-ursodeoxycholate in Rodents. *Gastroenterology* **1986**, *90*, 837−852.

(24) Coleman, J. P.; Kirby, L. C.; Klein, R. A. Synthesis and Characterization of Novel Analogs of Conjugated Bile Acids Containing Reversed Amide Bonds. *J. Lipid Res.* **1995**, *36*, 901−910.

(25) Zouboulis-Vafiadis, I.; Dumont, M.; Erlinger, S. Conjugation is Rate Limiting in Hepatic Transport of Ursodeoxycholate in the Rat. *Am. J. Physiol.* **1982**, *243*, G208−G213.

(26) Bellini, A. M.; Quaglio, M. P.; Guarneri, M.; Cavezzini, G. Antimicrobial Activity of Cholane Compounds. *Eur. J. Med. Chem.-Chim. Ther.* **1983**, *18*, 191−195.

(27) Roda, A.; Cerrè, C.; Simoni, P.; Polimeni, C.; Vaccari, C.; Pistillo, A. Determination of Free and Amidated Bile Acids by High-Performance Liquid Chromatography with Evaporative Light Scattering Mass Detection. *J. Lipid Res.* **1992**, *33*, 1393−1402.

(28) Roda, A.; Gatti, R.; Cavrini, V.; Cerrè, C. HPLC Study of the Impurities Present in Different Ursodeoxycholic Acid Preparations: Comparative Evaluation of Four Detectors. *J. Pharm. Biomed. Anal.* **1993**, *11*, 751−760.

(29) Roda, A.; Hofmann, A. F.; Mysels, K. J. The Influence of Bile Salt Structure on Self Association in Aqueous Solutions. *J. Biol. Chem.* **1983**, *258*, 6362−6370.

(30) Balducci, R.; Roda, A.; Pearlman, R. S. A Theoretical Model for the Critical Micelle Concentration of Bile Salts. *J. Sol. Chem.* **1989**, *18*, 355−367.

(31) Roda, A.; Minutello, A.; Angellotti, M. A.; Fini, A. Bile Acid Structure-Activity Relationship: Evaluation of Bile Acid Lipophilicity Using 1-Octanol/Water Partition Coefficient and Reverse Phase HPLC. *J. Lipid Res.* **1990**, *31*, 1433−1443.

(32) Huijghebaert, S. M.; Hofmann, A. F. Influence of the Amino Acid Moiety on Deconjugation of Bile Acid Amidates by Colylglycine Hydrolase or Human Fecal Cultures. *J. Lipid Res.* **1986**, *27*, 742−752.

(33) Heuman, D. M.; Mills, A. S.; McCall, J.; Hylemon, P. B.; Pandak, W. M.; Vlahcevic, Z. R. Conjugates of Ursodeoxycholate Protect Against Cholestasis and Hepatocellular Necrosis Caused by More Hydrophobic Bile Salts: In Vivo Studies in the Rat. *Gastroenterology* **1991**, *100*, 203−211.

(34) Kitani, K.; Ohta, M.; Kanai, S. Tauroursodeoxycholate Prevents Biliary Protein Excretion Induced by Other Bile Salts in the Rat. *Am. J. Physiol.* **1985**, *248*, G407−G417.

(35) Schmucker, D. L.; Ohta, M.; Kanai, S.; Sato, Y.; Kitani, K. Hepatic Injury Induced by Bile Salts: Correlation Between Biochemical and Morphological Events. *Hepatology* **1990**, *12*, 1216−1221.

(36) Galle, P. R.; Theilmann, L.; Raedsch, R.; Otto, G.; Stiehl, A. Ursodeoxycholate Reduces Hepatotoxicity of Bile Salts in Primary Human Hepatocites. *Hepatology* **1990**, *12*, 486−491.

(37) Krapcho, J.; Turk, C.; Cushman, D. W.; Powell, J. R.; DeForrest, J. M.; Spitzmiller, E. R.; Karanewsky, D. S.; Duggan, M.; Ronvak, G.; Schwartz, J.; Natarajan, S.; Godfrey, J. D.; Ryono, D. E.; Neubeck, R.; Atwal, K. S.; Petrillo, E. W., Jr. Angiotensin-Converting Enzyme Inhibitors. *J. Med. Chem.* **1988,** *31*, 1160−1169.

(38) Roda, A.; Cerrè, C.; Fini, A.; Sipahi, A. M.; Baraldini, M. Experimental Evaluation of a Model for Predicting Micellar Composition and Concentration of Monomeric Species in Bile Salt Binary Mixtures. *J. Pharm. Sci.* **1995**, *84*, 593−598.

(39) Fini, A.; Roda, A.; Fugazza, R.; Grigolo, B. Chemical Properties of Bile Acids: III, Bile Acids Structure and their Solubility in Water. *J. Sol. Chem.* **1985**, *8*, 595−603.

(40) Fini, A.; Roda, A. Chemical Properties of Bile Acids: Part II: pKa Values in Water and Aqueous Methanol of Some Hydroxy Bile Acids. *Eur. J. Med. Chem.* **1982**, *5*, 467−470.

(41) Fausa, O. Quantitative Determination of Serum Bile Acids Using a Purified 3α-Hydroxysteroid Dehydrogenase. *Scand. J. Gastroenterol.* **1975**, *10*, 747−751.

(42) Roda, A.; Geminiani, S.; Rossi, R.; Festi, D.; Roda, E.; Barbara, L. Enzymatic Determination of Phospholipids in Bile. *Lab. J. Res. Med.* **1982**, *2*, 119−122.

(43) Roda, A.; Festi, D.; Sama, C.; Mazzella, G.; Aldini, R.; Roda, E.; Barbara, L. Enzymatic Determination of Cholesterol in Bile. *Clin. Chim. Acta* **1975**, *64*, 337−341.

JM9508503