Synthesis and characterization of novel analogs of conjugated bile acids containing reversed amide bonds

James P. Coleman,[1,*] Lyndon C. Kirby,[*] and Robert A. Klein[†]

Department of Microbiology and Immunology,[*] School of Medicine, and Department of Chemistry,[†] East Carolina University, Greenville, NC 27858

Abstract New analogs of amino acid-conjugated bile acids were synthesized in which the amide bond was reversed from its normal configuration. These structural isomers of the β-alanyl conjugates of cholic acid and ursodeoxycholic acid were synthesized by reaction of succinic anhydride with the 24-nor-23-amine derivatives of cholic acid and ursodeoxycholic acid. The chemical and physical properties of these reverse amide conjugated bile acid analogs were compared with those of the normal glycine and β-alanine conjugates. The reverse amide analogs comigrated with their isomeric β-alanine conjugates during thin-layer chromatography using a variety of solvent systems. However, the isomeric pairs could be resolved by reversed-phase high performance liquid chromatography, with the reverse amides having greater retention times compared to the β-alanine conjugates. Critical micelle concentrations, solubility of undissociated forms, and acid dissociation constants were similar for the isomeric pairs. Significant differences in melting points were observed, however. While the isomeric pairs showed no significant differences in sensitivity to base hydrolysis, the reverse amides were not hydrolyzed by the cholylglycine hydrolase from *Clostridium perfringens,* even after long incubation periods.—**Coleman, J. P., L. C. Kirby, and R. A. Klein.** Synthesis and characterization of novel analogs of conjugated bile acids containing reversed amide bonds. *J. Lipid Res.* 1995. **36:** 901–910.

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Cholic acid and chenodeoxycholic acid are cholesterol-derived compounds synthesized by the liver and secreted as conjugates of either glycine or taurine into the gall bladder where they are stored. When required for digestion, the conjugated bile acids (CBAs) are released into the duodenum by contraction of the gall bladder. The CBAs are recycled mainly via a highly efficient mechanism involving active transport by epithelial cells located in the terminal ileum (1, 2). In competition with these transport sites for CBA substrate are bacteria normally found in the digestive tract which are capable of hydrolyzing the CBAs to the corresponding free bile acids (FBAs) and amino acids. There are two important consequences of this hydrolysis. First, it contributes to a reduction in the half-life of bile acids within the body, as FBAs are actively transported less efficiently than CBAs (3–6). Second, the FBA products can be further metabolized to "secondary" bile acids such as deoxycholic acid and lithocholic acid. These 7-dehydroxylated derivatives of the parent bile acids are more hydrophobic and may exhibit a variety of toxic effects, presumably due to their interaction with cell membranes (7–10).

Bile acid deconjugation is an important consideration during the treatment of cholesterol gallstones and other enterohepatic disorders with ursodeoxycholic acid (UDCA). UDCA can be 7-dehydroxylated to lithocholic acid, which is extremely hydrophobic and potentially toxic. If the conjugated form is the active compound then the usefulness of this compound could probably be extended by maintaining UDCA or a suitable analog in the conjugated state for longer periods of time. This increased half-life would allow the drug to be used at lower dosage levels. In addition, the formation of toxic metabolites would also be reduced as a result of the decrease in formation of free bile acids, the preferred substrates for bacterial-mediated 7-dehydroxylation (11–13).

Two approaches have been taken in the design of compounds ultimately resistant to 7-dehydroxylation. First, modifications in the A or B ring can be made to prevent the formation of the 3-oxo-4-ene configuration (which renders the 7-hydroxy group labile) or to sterically hinder

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Abbreviations: CA, cholic acid; GCA, glycocholic acid; GUDCA, glycoursodeoxycholic acid; ACA, cholyl-β-alanine; AUDCA, ursodeoxycholyl-β-alanine; SNCN, succinylnorcholanylamide; SNUDCN, succinylnorursodeoxycholanylamide; NCN, norcholanylamine; NUDCN, norursodeoxycholanylamine; CBA, conjugated bile acid; FBA, free bile acid; UDCA, ursodeoxycholic acid; RP-HPLC, reversed-phase high performance liquid chromatography; CMC, critical micelle concentration; TLC, thin-layer chromatography; PNMR, proton nuclear magnetic resonance.

[1]To whom correspondence should be addressed.

the 7-hydroxy group (14, 15). Second, modifications of the side chain of conjugated bile acids (or analogs) can be made to prevent the amide bond hydrolysis which generates the free C_{24}-carboxyl group. Several groups have reported on investigations into the structural factors influencing the stability of conjugated bile acids to bacterial hydrolysis (16, 17) and on the synthesis of new conjugates or analogs of bile acids designed to be resistant to bacterial hydrolysis (18–22).

All of the analogs tested have been based on structures chemically related to natural bile acids and most were composed of the bile acid ring joined to the amino group of the side chain via an amide bond. One exception was the ester analog synthesized by Roda et al. (22). The fact that this compound was recognized by ileal and hepatic transport sites indicates that there is some tolerance by these transport sites for "abnormal" linkages, provided that the terminal functional group remain unchanged. Another exception was the sulfonated side chain analogs developed by Kihara et al. (18), which lacked an internal amide linkage.

A report (23) on the synthesis of nor-bile acids (C-23 bile acids) through nornitrile intermediates suggested to us the use of nornitriles for other purposes. In particular, the reduction of the nitrile moiety to the amine would yield a compound identical to the original bile acid except for the presence of an amino group at carbon 23 instead of a carboxyl group (**Fig. 1**). Such "bile amines" could be linked via amide bonds to short dicarboxylic acids to produce novel compounds differing from natural CBAs only in the orientation of the amide bond. These "reverse conjugates" would be expected to have gross physical and chemical properties very similar to those of the corresponding natural bile acid conjugates. In view of the stereospecific nature of enzymatic reactions, we predicted that the reversal of the amide bond in these CBA analogs would yield compounds which, while being structurally and chemically similar to the normal CBA, would differ in the stability of their amide bond to enzymatic hydrolysis. This prediction has been tested and confirmed, as described below.

EXPERIMENTAL

Materials

Ursodeoxycholic acid (3α,7β-dihydroxy-5β-cholan-24-oic acid) was obtained from Aldrich Chemical Company and was used without further purification. Cholic acid (3α,7α,12α-trihydroxy-5β-cholan-24-oic acid) was obtained from Sigma Chemical Company and was recrystallized from ethanol–water until the purity was greater than 99% as judged by thin-layer chromatography. *Clostridium perfringens* cholylglycine hydrolase, carboxypeptidase A, carboxypeptidase B, and pancreatin were obtained from Sigma Chemical Company. Trypsin (TPCK-treated) was from Cooper Biomedical. All other chemicals were obtained from Aldrich Chemical Company except for bulk solvents which were obtained from Fisher.

Analytical chemistry

Melting points were determined on a Thomas Hoover melting point apparatus and are uncorrected. Fourier transfrom infrared spectra (FTIR) were obtained in KBr

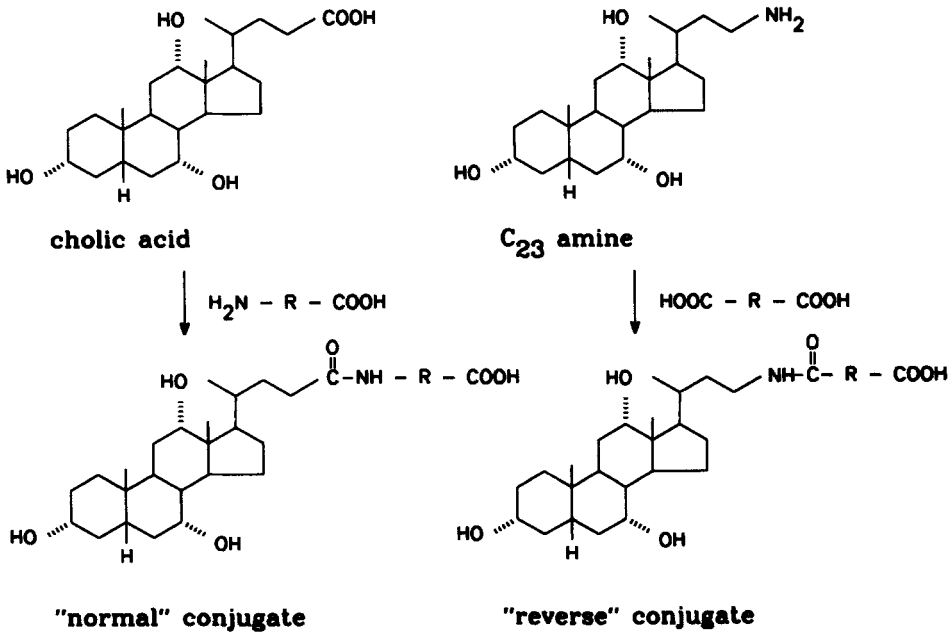

Fig. 1. Normal and reverse conjugates of cholic acid and its amine derivative.

disks on a Perkin-Elmer 1605 fourier transform infrared spectrophotometer. Absorption frequencies are given as reciprocal centimeters. Proton nuclear magnetic resonance (PNMR) spectra were obtained on a Varian-Gemini 200 MHz superconducting spectrometer. Chemical shifts are given in parts per million relative to a tetramethylsilane internal standard.

Chromatography

Analytical reversed-phase high performance liquid chromatography (RP-HPLC) was performed using a Whatman Partisil 10 ODS-3 (C_{18}) column (4.6 mm × 25 cm; 10 μm particle size) with a flow rate of 1 ml/min. The mobile phase consisted of methanol–water 75:25 (v/v), containing 2.5 mM KH_2PO_4 and 20 mM NaOH, adjusted to pH 6.0 with 85% H_3PO_4 (24). Preparative RP-HPLC was performed using a glass column (1.3 × 30 cm) packed with Bakerbond Wide-Pore C_{18} prepscale support (40 μm particle size) using the same solvent system used for the analytical column. Conjugated bile acids and analogues were detected by ultraviolet spectrophotometry (205 nm) using a Beckman Model 163 variable wavelength detector. Thin-layer chromatography was carried out using precoated 0.25 mm silica gel plates (Macherey-Nagel) in the solvent systems described below. Bile acids and analogues were detected by spraying with a 4% ethanolic solution of phosphomolybdic acid and heating at 110°C for 5 to 10 min. Amine derivatives were visualized by spraying with 0.2% ninhydrin in ethanol–acetic acid 14:1 (v/v). Solvent systems used were: SS1, chloroform–methanol–acetic acid 80:10:10; SS2, chloroform–methanol–acetic acid 80:5:5; SS3, n-butanol–triethylamine–water 8:3:3.

Critical micelle concentration

The procedure described by DeVendittis et al. (25) was used, with some modifications. The fluorescence of 8-anilino-1-naphthalene-sulfonic (ANS) acid was followed as a function of bile acid or analog concentration. Measurements were made on a Perkin-Elmer LS50 luminescence spectrofluorometer. A stirred cuvette maintained at 25°C containing 1 ml 26 μM ANS, 100 mM NaOH, 50 mM NaCl was titrated with 100 mM bile acid or analog in 26 μM ANS, 100 mM NaOH, 50 mM NaCl. Fluorescence was monitored using an excitation wavelength of 370 nm and an emission wavelength range of 400–600 nm. After adjusting for volume changes during the titration, the bile acid or analog concentration was plotted against relative fluorescent intensity. The break point in the resulting curve was taken as the CMC.

Aqueous solubility

Bile acids or analogs (100 μmol) were suspended in 4 ml 0.15 M NaCl (pH to 1.0 with HCl for acids; pH to 12.0 with NaOH for amines). The tubes were incubated at 25°C or 37°C for 35 days. The tubes were then centrifuged at 750 *g* for 1 h and filtered to remove any residual crystals. The concentrations of bile acids in the supernatant were then determined colorimetrically after reaction with sulfuric acid as described by Fini et al. (26). Individual standard curves were prepared for each compound.

Dissociation constants

Dissociation constants (pKa) were determined as described by Fini and Roda (27) by potentiometric titration of 2.0 mM solutions of the bile acid or analog in 80% dimethyl sulfoxide (DMSO). Titrations were performed at 25°C using 20 mM NaOH in 80% DMSO as titrant. Measurements were made using a Fisher Accumet Model 805 pH meter equipped with a glass combination electrode. The apparent pKa_{DMSO} value was taken as the midpoint of the titration curve and was converted to aqueous pKa using the formula: $pKa = -0.80 + 0.67\ (pKa_{DMSO})$ (27).

Chemical stability

The stability of conjugated bile acids and analogs to base hydrolysis was determined by incubating approximately 5 mg bile acid or analog in 2 N NaOH at 95°C for various times or at 121°C (autoclave) for 2 h. The solutions were then extracted twice with ethyl acetate, acidified, and extracted twice more. The combined ethyl acetate layers were dried down and a sample was analyzed by TLC. Duplicate plates were sprayed with either phosphomolybdic acid reagent or ninhydrin reagent.

Enzymatic stability

The stability of bile acids and analogs to hydrolysis by bacterial conjugated bile acid hydrolase was assessed by incubating each of them (0.4 mM final concentration) for various times in the presence of 55 units of *Clostridium perfringens* cholylglycine hydrolase (Sigma Chemical Co.) in a final volume of 5 ml using the buffer and reaction conditions recommended by the supplier. Samples (0.5 ml) taken at various time points up to 24 h were extracted at both low and high pH with ethyl acetate as described above and, after drying down the combined organic layers, subjected to TLC analysis and detection. Sensitivity to the digestive enzymes trypsin (200 units/ml), carboxypeptidases A and B (15 units/ml), crude pancreatin (1 mg/ml), and proteinase K (20 units/ml) was assessed by incubation with conjugated bile acids or analogs (1 μmol) in 1 ml 20 mM sodium phosphate, 150 mM sodium chloride, pH 7.0, for up to 24 h. Time samples were removed at intervals and treated as described above before being subjected to TLC analysis.

Syntheses

Briefly, the free bile acids were converted to their corresponding nornitriles as described by Schteingart and Hofmann (23). Each nornitrile was then reduced with lithium aluminum hydride and the resulting C_{23}-amines were reacted with succinic anhydride to give the reverse amide analogs. The structurally isomeric β-alanine conjugates were prepared as controls for chemical and physical properties. Specific reaction conditions and product characterization are as follows (**Fig. 2**).

Formylated bile acids (28). Ursodeoxycholic acid (3α,7β-dihydroxy-5β-cholan-24-oic acid; **1a)** (10 g) or cholic acid (3α,7α,12α-trihydroxy-5β-cholan-24-oic acid; **1b)** (10 g) was dissolved in 50 ml 90% formic acid in a 250-ml round-bottom flask. The temperature was raised to 45°C and 12–14 drops of 70% perchloric acid was added. The temperature was maintained at 45–50°C with stirring for 1.5 h after which the reaction mix was cooled to room temperature and 25–30 ml of acetic anhydride was slowly added, while maintaining the temperature below 60°C. The reaction was allowed to cool to room temperature and poured into 500–600 ml of ice-cold deionized water with stirring. The white precipitate was collected by filtration and washed with 100–200 ml of cold water.

3α,7β-Diformyl-5β-cholan-24-oic acid (**2a**). The product was recrystallized once from ethanol–water to give short (5–10 mm), colorless, needle-like crystals. Yield: 98%. MP: 169–170°C. IR: 1200 cm^{-1}. PNMR ($CDCl_3$) δ: 0.69 (s, 3H, Me-18), 0.93 (d, J = 6.7 Hz, 3H, Me-21), 0.99 (s, 3H, Me-19), 7.99 (d, J = 1.3 Hz, 1H, H-3), 8.03 (d, J = 1.3 Hz, 1H, H-7).

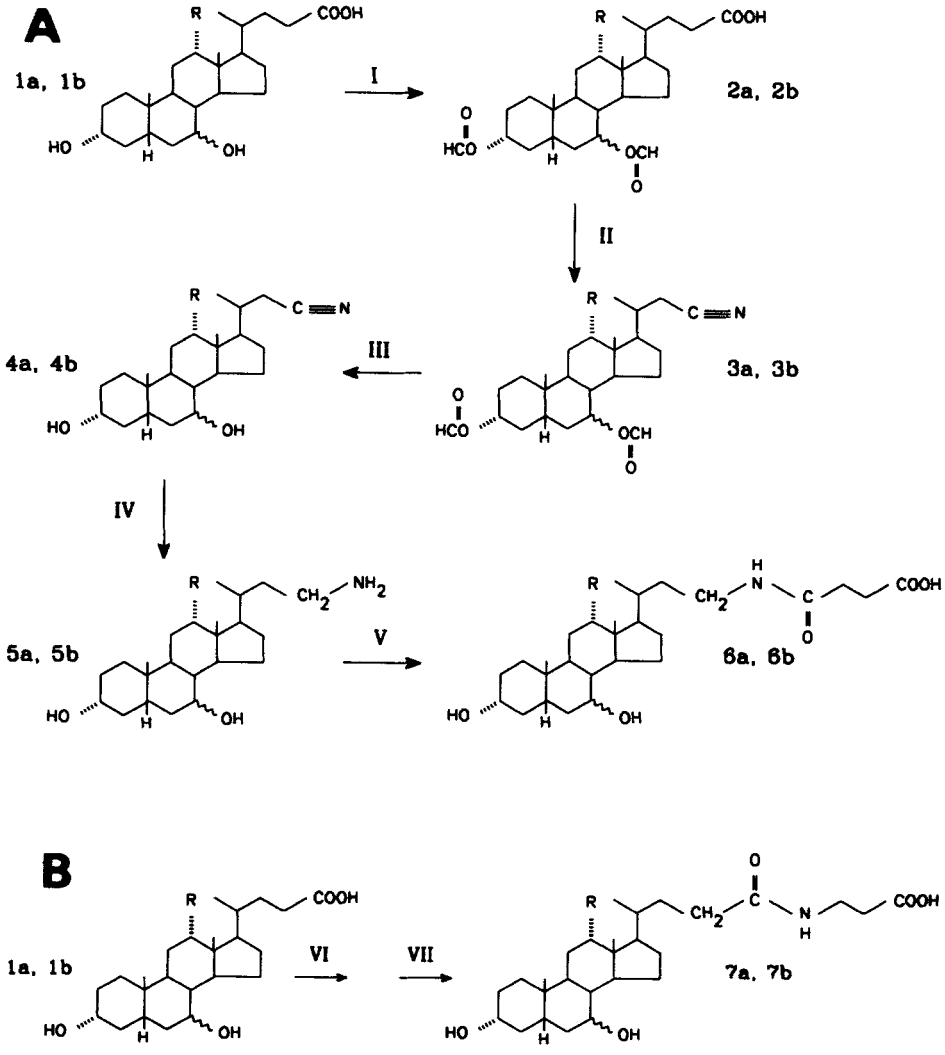

Fig. 2. Synthesis of conjugated bile acids and analogs. a: R = H, ~ = equatorial (β-OH); b: R = OH, ~ = axial (α-OH). A: Reverse succinyl amide synthesis. **I:** HCOOH, $(Ac)_2O$, H^+; **II:** $NaNO_2$, F_3CCOOH, $(F_3CCO)_2$; **III:** NaMeO, MeOH; **IV:** $LiAlH_4$; **V:** succinic anhydride, pyridine. B: β-alanine conjugate synthesis. **VI:** Et_3N, EEDQ, $H_2N(CH_2)_2CO(OEt)$; **VII:** EtOH, K_2CO_3.

3α,7α,12α-Triformyl-5β-cholan-24-oic acid (**2b**). This was recrystallized in the same manner as **1a.** Yield: 98%. MP: 166–167°C. IR: 1200 cm^{-1}. PNMR ($CDCl_3$) δ: 0.76 (s, 3H, Me-18), 0.85 (d, J = 10.18, 3H, Me-21), 0.95 (s, 3H, Me-19), 4.72 (m, 1H, H-3), 5.08 (bs, 1H, H-7), 5.28 (bs, 1H, H-12), 8.03 (s, 1H, CHO-3), 8.11 (s, 1H, CHO-7), 8.17 (s, 1H, CHO-12).

Formylated nitriles. In a 100-ml round-bottom flask, 5 g of (**2a**) or (**2b**) was dissolved in a mixture of 17.5 ml trifluoroacetic acid and 4.7 ml trifluoroacetic anhydride at 4°C. To this mixture was added 0.84 g sodium nitrite in small aliquots over a period of 1 h. After the last addition the reaction mixture was stirred at 4°C for 1 h. The flask was then transferred to a 38°C bath and stirred for 1 h. The reaction was then removed from the bath and to it was added 300 ml 2 M NaOH and 100 g ice. Diethyl ether (100 ml) was added and the entire mixture was transferred to a separatory funnel. The aqueous layer was extracted with 100 ml of ether three times. The combined ether layers were washed three times with 100 ml 1 N NaOH and then three times with deionized water. The ether layer was then dried over sodium sulfate and evaporated to give a amber resin containing 3α,7β-diformyl-24-nor-5β-cholan-23-nitrile (**3a**) or 3α,7α,12α-triformyl-24-nor-5β-cholan-23-nitrile (**3b**) along with partial deformylation products. The resin was taken directly to the next step without further workup. For yields see the next step.

Nitriles. The resin from the preceding step was dissolved in 100 ml methanol and a 3-fold molar excess of sodium methoxide was added. The reaction was refluxed for 45 min. After cooling to room temperature, approximately 300 ml water was added; the mixture was transferred to a separatory funnel and extracted with 150 ml ethyl acetate three times. The organic layer was washed with 20% NaCl solution to neutrality, dried over sodium sulfate, and evaporated to yield the nornitrile as a fine, white, crystalline powder. The powder was lyophilized to remove any traces of water before the next step.

3α,7β-Dihydroxy-24-nor-5β-cholan-23-nitrile (**4a**). Yield: 86%. MP: 228–229°C. IR: 2247 cm^{-1}. PNMR ($CDCl_3$) δ: 0.67 (s, 3H, Me-18), 0.92 (s, 3H, Me-19), 1.14 (d, J = 6.7 Hz, 3H, Me-21), 2.29 (m, 2H, H-22), 3.54 (m, 2H, H-3 and H-7).

3α,7α,12α-Trihydroxy-24-nor-5β-cholan-23-nitrile (**4b**). Yield: 70%. MP: 161–165°C. IR: 2250 cm^{-1}. PNMR ($CDCl_3$) δ: 0.69 (s, 3H, Me-18), 0.88 (s, 3H, Me-19), 1.22 (d, J = 5.3 Hz, 3H, Me-21), 3.45 (m, 1H, H-3), 3.83 (bs, 1H, H-7), 3.93 (bs, 1H, H-12).

Amines. The dry nornitrile (**4a** or **4b**) (8.3 mmol) was dissolved in 300 ml freshly distilled, dry tetrahydrofuran in a 1-liter round-bottom flask. With magnetic stirring, 25 ml of 1 M lithium aluminum hydride in tetrahydrofuran was slowly added. The reaction mixture was then refluxed for 24–30 h. After cooling to room temperature, ice-cold deionized water was slowly added in small portions until no more gas evolution was observed. The crude amine mixture was transferred to a separatory funnel with 200 ml of 0.1 N NaOH. This mixture was extracted a total of three times with 200-ml portions of ethyl acetate. The combined ethyl acetate layers were concentrated to approximately 300 ml and then back-extracted four times with 150-ml portions of 0.1 N HCl. The combined aqueous layers were stirred while 2 M NaOH was slowly added until a milky white precipitate formed. This suspension was then extracted three times with 150-ml portions of ethyl acetate. The pooled organic phases were then washed three times with 100-ml portions of deionized water, dried over sodium sulfate, and rotary-evaporated to dryness.

3α,7β-Dihydroxy-24-nor-5β-cholan-23-amine (**5a**) (norursodeoxycholanylamine, NUDCN). Yield: 67%. MP: 139–141. IR: 1654 cm^{-1}. PNMR [$(CD_3)_2SO$] δ: 0.61 (s, 3-H, Me-18), 0.87 (d, J = 11.31 Hz, 6-H, Me-19 and Me-21), 2.49 (m, 2-H, H-22), 3.30 (b, 2-H, H-23).

3α,7α-12α-Trihydroxy-24-nor-5β-cholan-23-amine (**5b**) (norcholanylamine, NCN). Yield: 54%. MP: 124–127°C. IR: 1654 cm^{-1}. PNMR (CD_6SO) δ: 0.59 (s, 3H, Me-18), 0.81 (s, 3H, Me-19), 0.93 (t, 3H, Me-21).

Succinyl amides. The free amine (**5a** or **5b**) (3.3 mmol) was dissolved in anhydrous tetrahydrofuran (120 ml) containing 0.32 ml pyridine (4 mmol). Succinic anhydride (0.4 g; 4 mmol) was slowly added and the mixture was stirred for 3 h at room temperature. Excess reagent was destroyed by adding approximately 30 ml water and the solvent was evaporated to leave a beige resin. Ester side products were hydrolyzed by dissolving the residue in 100 ml 2 M aqueous sodium hydroxide and incubating at 58°C for 1 h. The solution was acidified with 6 N HCl and extracted three times with ethyl acetate (150 ml each). The combined ethyl acetate layers were back extracted three times with 7% aqueous sodium bicarbonate (150 ml each). These aqueous layers were again acidified and extracted with ethyl acetate as above. The ethyl acetate layers were extracted several times with 20% sodium chloride until the aqueous layer was neutral, and then three times with water. The ethyl acetate was evaporated off and the crude amide was dissolved in 25 ml 70% ethanol and applied to a Dowex 1 column equilibrated with 70% ethanol. After washing the column with 1 l of 70% ethanol, the amide was eluted with 1 l of 1 M acetic acid in 70% ethanol. Fractions (40 ml) were collected and portions were analyzed by TLC to determine the presence of amide. Positive fractions showing no impurities were pooled, evaporated to dryness, and recrystallized from ethanol–water. When further purification was required, the material was subjected to preparative RP-HPLC.

N-(3α,7β-Dihydroxy-24-nor-5β-cholanyl)-succinamic acid (**6a**) ("succinylnorursodeoxycholanylamide," SNUDCN). Yield: 54%. MP: 103–105°C. IR: 1560 cm^{-1}, 1652 cm^{-1}, 1725 cm^{-1}. PNMR (CD_6SO) δ: 0.61 (s, 3H, Me-18), 0.89 (d, J = 6.7 Hz, 6H, Me-19 and Me-21), 2.32 (m, 4H,

H-25 and H-26), 7.75 (t, 1H, H-23).

N-(3α,7α,12α-Trihydroxy-24-nor-5β-cholanyl)-succinamic acid (**6b**) (succinylnorcholanylamide, SNCN) Yield: 42%. MP: 178–180°C. IR: 1656 cm^{-1}. PNMR (CD_6SO) δ: 0.59 (s, 3H, Me-18), 0.81 (s, 3H, Me-19), 0.95 (d, J = 6.7 Hz, 3H, Me-21), 2.34 (m, 4H, H-25 and H-26), 7.74 (t, 1H, H-23).

β-Alanyl conjugates. Ursodeoxycholic acid and cholic acid were converted to their β-alanine amide derivatives by the method of Tserng, Hachey, and Klein (29). The crude product was purified by preparative reversed-phase HPLC.

N-(2-Carboxyethyl)-3α,7β-dihydroxy-5β-cholan-24-amide (**7a**) (ursodeoxycholyl-β-alanine, AUDCA). Yield: 82%. MP: 154–157°C. IR: 1604 cm^{-1}, 1707 cm^{-1}. PNMR (CD_6SO) δ: 0.60 (s, 3H, Me-18), 0.87 (d, J = 3.77 Hz, 6H, Me-19 and Me-21), 2.34 (t, 2H, H-23), 2.49 (t, 2H, H-26), 7.85 (t, 1H, H-25).

N-(2-Carboxyethyl)-3α,7α,12α-trihydroxy-5β-cholan-24-amide (**7b**) (cholyl-β-alanine, ACA). Yield: 48%. MP: 222–224°C. IR: 1604 cm^{-1}, 1707 cm^{-1}. PNMR (CD_6SO) δ: 0.58 (s, 3H, Me-18), 0.81 (s, 3H, Me-19), 0.92 (d, J = 6.7 Hz, 3H, Me-21), 2.35 (t, 2H, H-23), 2.50 (t, 2H, H-26), 7.84 (t, 1H, H-24).

RESULTS AND DISCUSSION

Yields

Although C_{23} and C_{24} amines and amine derivatives have appeared in a variety of reports (30–35), this is the first report of the use of these amine derivatives for synthesis of "reverse amide" analogs of conjugated bile acids. The synthesis of these amines through nitrile intermediates is one of at least three possible routes of synthesis. To our knowledge, the earliest description in the literature of C_{23} amine derivatives of bile acids featured them as intermediates during the side chain degradation of bile acids to produce steroids (33). This process used a Curtius reaction whereby the bile acid was degraded to the amine via the azide–isocyanate sequence. A second possible route would be the amidation of a 24-nor-bile acid chloride with ammonia, followed by reduction to the 24-nor-23-amine. Bellini, Quaglio, and Guarneri (31, 32) used this approach to synthesize C_{24} amines, substituting a variety of amines as well as ammonia in the initial amidation step. The third route, which we used here, is through a nitrile intermediate. We used the nitrile route rather than the Curtius reaction route because of higher yields obtained in trial reactions. The amidation–reduction reaction is a viable alternative, depending on the relative yields of the [nitrile→amine] reaction versus the [nitrile→nor-bile acid→amine] reactions.

We obtained overall yields from free bile acid precursors to the succinyl conjugates of norursodeoxychol-23-amine and norchol-23-amine of 30.5% and 15.6%, respectively. The lower yields obtained for the trihydroxy derivatives are presumably the result of the higher solubility of the intermediates at each step compared with the dihydroxy analogs and the consequent lower efficiency of the extraction, washing, and crystallization steps. In contrast, the low aqueous solubility of ursodeoxycholic acid derivatives resulted in very clean crystallizations and extractions.

Melting points

The melting points for the reverse amides, SNUDCN and SNCN (103–105°C and 178–180°C, respectively), were each lower than those of their structural isomers, AUDCA and ACA, (154–157°C and 222–224°C, respectively) (**Table 1**). One explanation for this is the more stable hydrogen bonding possible between the interdigitated side chains of two molecules of the normal conjugates. These hydrogen bonding differences may be of physiological consequence, for instance, when comparing effects of the normal and reverse conjugates that are exerted through interactions with cellular membranes.

Dissociation constants

The acid dissociation constants (pKa) observed for the β-alanyl and reverse succinyl conjugates were approximately 0.5 units higher than those of the corresponding glycine conjugates (Table 1). This effect is expected due to the lengthening of the side chain by one methylene group and the consequent reduction in the influence exerted by the amide bond on the dissociation of the carboxyl group. With both the dihydroxy- and trihydroxy-conjugates, the reverse amides had a slightly higher pKa than the "normal" β-alanyl conjugates. Although the differences were small and perhaps insignificant, they are explainable, again due to the field effects of the amide bond on the dissociation properties of the carboxyl group. The orientation of the amide electropositive (nitrogen) and electronegative (carbonyl) groups is expected to influence the ionization of the nearby carboxyl group. We predict that the difference in pKa for normal versus reverse amides would be more pronounced with fewer methylene groups separating the amide bond from the terminal carboxyl group (e.g., glycine conjugate vs. reverse malonyl amide).

Critical micelle concentration

While the CMC values for AUDCA and SNUDCN were similar to one another but both lower than that of GUDCA, the CMCs for GCA, ACA, and SNCN were all similar (Table 1). This may be due to the lower polarity (higher hydrophobicity) of ursodeoxycholic acid and its conjugates and the more significant effect of the presence of additional methylene groups on the side chain. The higher polarity of the trihydroxy cholic acid is apparently affected to a lesser extent by the addition of methylene

TABLE 1. Physical and chemical properties of conjugated bile acids and analogs

Compound	Melting Point	pK_a[a]	Critical Micelle Concentration[b]	Solubility[c]	Retention Time[d]	R_f[e] 1	R_f[e] 2	R_f[e] 3
	°C		mM	μM				
Free acids								
CA	195–197	ND[f]	10–12	225	ND	0.64	0.25	
UDCA	198–200	ND	11–13	57	ND		0.49	
Glycine amides								
GCA	132–135	3.94	6–7	580	1.00	0.18	0.02	
GUDCA	118–120	3.99	7–9	105	0.66		0.14	
β-Alanine amides								
ACA	222–224	4.43	10–11	224	1.29	0.28	0.04	
AUDCA	154–157	4.49	6–7	150	0.81		0.18	
Succinyl amides								
SNCN	178–180	4.62	10–11	260	1.37	0.28	0.04	
SNUDCN	103–105	4.52	6–7	172	0.90		0.18	
Free amines								
NCN	124–127	ND	ND	3000	ND	0.02		0.32
NUDCN	139–141	ND	ND	12	ND	0.03		0.63

[a] Extrapolated from titrations in 80% (w/v) dimethyl sulfoxide at 25°C.
[b] Determined as described by DeVendittis et al. (25) at 25°C.
[c] Solubility at 25°C of uncharged species (pH = 1.0 for acids; pH = 12.0 for bases).
[d] Reversed-phase HPLC retention times relative to glycocholic acid.
[e] Relative mobility on silica gel TLC plates in solvent system 1 (chloroform–methanol–acetic acid 80:10:10), 2 (chloroform–methanol–acetic acid 80:5:5), or 3 (1-butanol–triethylamine–water 8:3:3).
[f] Not determined.

groups to the side chain. In either case, no significant difference was seen between the β-alanyl conjugates and their reverse amide structural isomers, indicating that the rearrangement of the amide bond of these bile acid has little effect on this micellar property.

Solubility

As expected, all the trihydroxy compounds had greater aqueous solubility of their uncharged forms than their corresponding dihydroxy compounds (Table 1). The glycine conjugate of cholic acid was relatively more soluble than either the β-alanine conjugate or the reverse succinyl conjugate. In contrast, the glycine conjugate of ursodeoxycholic acid was relatively less soluble than either the β-alanyl or the reverse succinyl conjugates. In both cases, the β-alanyl conjugates of each bile acid were slightly less soluble than their corresponding reverse conjugates.

Chromatographic behavior

The β-alanyl- and the reverse succinyl-amides both migrated faster than their corresponding glycine conjugates with all TLC solvent systems tested (Table 1). None of the systems resolved the structural isomers from each other. When chromatographed using reversed-phase HPLC, however, these compounds were resolved, with the reverse succinyl amides being retained longer than the β-alanyl conjugates. The most hydrophobic dihydroxy compound tested, SNUDCN, was still eluted earlier than the most polar trihydroxy conjugate, GCA. When comparing each 3α,7β-dihydroxy compound with the 3α,7α,12α-trihydroxy compound with identical side chain, the former always had lower retention indices than the latter, as is normally the case with ursodeoxycholic acid versus cholic acid and their natural derivatives.

Chemical stability

When tested for sensitivity to base hydrolysis under conditions routinely used for the hydrolysis of natural bile acid conjugates (121°C, 2 h, 2 M NaOH), both the normal and the reverse conjugates were found to be completely hydrolyzed. Thin-layer chromatographic analysis of the reverse conjugate hydrolysates demonstrated the presence of a ninhydrin-positive compound co-migrating with the norcholan-23-amine precursor (data not shown). To exclude the possibility that the reverse conjugates were more sensitive than normal conjugates to base hydrolysis, reactions were also performed under milder conditions for variable time periods (2 M NaOH, 90°C, 0.5 h–24 h). After TLC analysis of the reaction mixes, no significant difference was noted in the rate of hydrolysis of the two species (data not shown).

Susceptibility to enzymatic hydrolysis

Both reverse amide derivatives (SNUDCN and SNCN) were resistant to hydrolysis by the cholylglycine hydrolase from *Clostridium perfringens* (**Fig. 3**). Under the conditions

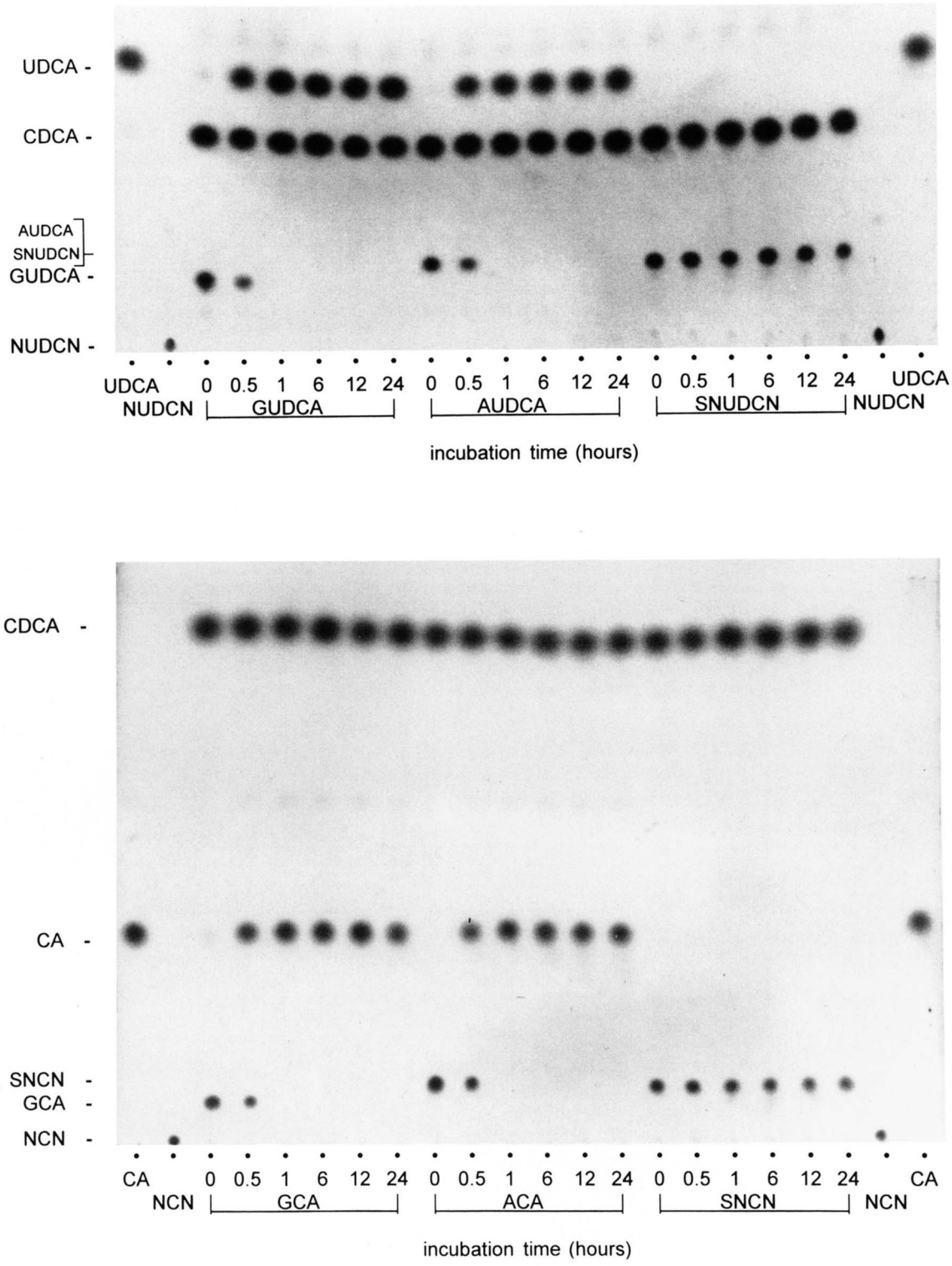

Fig. 3. Hydrolysis of cholic acid conjugates and analogs by C. perfringens cholylglycine hydrolase. Phosphomolybdic acid was used to visualize compounds on the plates shown. Parallel plates were sprayed with ninhydrin (not shown). Chenodeoxycholic acid (CDCA) was added to all samples prior to extraction as an internal standard. Top: The solvent system was chloroform–methanol–acetic acid 80:5:5. Compounds tested were ursodeoxycholylglycine (GUDCA), ursodeoxycholyl-β-alanine (**7a;** AUDCA), or the reverse succinyl amide (**6a;** SNUDCN). Standard lanes contained GUDCA, AUDCA, SNUDCN, and nor-cholanylamine (**5a;** NUDCN). Bottom: The solvent system was chloroform–methanol–acetic acid 80:10:10. Compounds tested were cholylglycine (GCA), cholyl-β-alanine (**7b;** ACA), or the reverse succinyl amide (**6b;** SNCN). Standard lanes contained GCA, ACA, SNCN, and norcholanylamine (**5b;** NCN).

used, the glycine and β-alanine derivatives of ursodeoxycholic and cholic acids were completely hydrolyzed within 1 h; whereas, even after 24 h, there was no detectable hydrolysis of the reverse amide conjugates, as judged by the absence of either phosphomolybdic acid- or ninhydrin-reactive material near the origin. Neither the normal nor the reverse-conjugated bile acids were hydrolyzed to a measurable extent by pancreatin, trypsin, proteinase K, or the carboxypeptidases A and B (data not shown). We are currently extending these analyses to determine whether conjugated bile acid hydrolases from other bacterial sources are similarly ineffective in the hydrolysis of these conjugate bile acid analogs.

Significance

The reverse amide conjugated bile acid analogs described herein differ from normal bile acid conjugates in several parameters that make them potentially suitable as therapeutic bile acids. Most notable of these are their resistance to hydrolysis by bacterial conjugated bile acid hydrolases and their higher acid dissociation constants. As discussed above, this latter property, while being mainly due to the additional methylene group in the case of the compounds discussed, is expected to hold for the reverse amide isomers of glycine-conjugated bile acids. This property should result in increased passive diffusion in the gastrointestinal tract compared to the normal conjugates, while the presence of the amide bond and attached anionic side chain should maintain the ability of the compound to be recognized by the ileal and hepatic transport systems.

The resistance of the analogs to conjugated bile acid hydrolase, conferred by the reversal of the amide bond, is dramatic, at least in the case for the enzyme from *Clostridium perfringens*. As there are numerous other gastrointestinal bacteria that produce enzymes of this type, it remains to be determined whether the analogs are universally stable, and whether other amidases may be present in the intestinal lumen that can hydrolyze these substrates. As the reactions required for amide bond inversion involve relatively simple chemistry, this manner of protecting a conjugated bile acid from side chain hydrolysis is of potential utility for any bile acid where increased biological stability is required. The ability of these reverse amides to undergo enterohepatic circulation is currently under investigation.

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