Isolation and chemical synthesis of a major, novel biliary bile acid in the common wombat (Vombatus ursinus): 15α-hydroxyliothocholic acid

Genta Kakiyama, * Hideyuki Tamegai, * Takashi Iida,1* Kuniko Mitamura, † Shigeo Ikegawa, ‡ Takaaki Goto, ‡ Naryasu Mano, § Junichi Goto, § Peter Holz, ** Lee R. Hagey, †† and Alan F. Hofmann1,1‡

Department of Chemistry, †† College of Humanities and Sciences, Nihon University, Sakurajosui, Setagaya, Tokyo 156-8550, Japan; Faculty of Pharmaceutical Sciences, † Kinki University, Kowakae, Higashi-Osaka, 577-8502, Japan; Graduate School of Pharmaceutical Sciences, § Tohoku University, Aobayama, Sendai 980-8578, Japan; Healesville Sanctuary, ** Healesville, Victoria 3777, Australia; and Division of Gastroenterology, †† Department of Medicine, University of California, San Diego, La Jolla, CA 92093-0663

Abstract The major bile acids present in the gallbladder bile of the common Australian wombat (Vombatus ursinus) were isolated by preparative HPLC and identified by NMR as the taurine N-acylamidates of chenodeoxycholic acid (CDCA) and 15α-hydroxyliothocholic acid (3α,15α-dihydroxy-5β-cholan-24-oic acid). Taurine-conjugated CDCA constituted 78% of biliary bile acids, and (taurine-conjugated) 15α-hydroxyliothocholic acid constituted 11%. Proof of structure of the latter compound was obtained by its synthesis from CDCA via a Δ14 intermediate. The synthesis of its C-15 epimer, 15β-hydroxyliothocholic acid (3α,15β-dihydroxy-5β-cholan-24-oic acid), is also reported. The taurine conjugate of 15α-hydroxyliothocholic acid was synthesized and shown to have chromatographic and spectroscopic properties identical to those of the compound isolated from bile. It is likely that 15α-hydroxyliothocholic acid is synthesized in the wombat hepatocyte by 15α-hydroxylation of lithocholic acid that was formed by bacterial 7α-dehydroxylation of CDCA in the distal intestine. Thus, the wombat appears to use 15α-hydroxylation as a novel detoxification mechanism for CDCA. —Kakiyama, G., H. Tamegai, T. Iida, K. Mitamura, S. Ikegawa, T. Goto, N. Mano, J. Goto, P. Holz, L. R. Hagey, and A. F. Hofmann. Isolation and chemical synthesis of a major, novel biliary bile acid in the common wombat (Vombatus ursinus): 15α-hydroxyliothocholic acid. J. Lipid Res. 2007. 48: 2682–2692.

Supplementary key words bile acid synthesis • detoxification of bile acids • bile acid metabolism in marsupials • 15α-hydroxylation • lithocholic acid

Bile acids are amphipathic end products of cholesterol metabolism that mediate numerous physiological functions in the liver, biliary tract, and intestine. Bile acids are of two types: C24 bile acids [with a C5 (isopentanoic acid) side chain] and C27 bile acids [with a C8 (isooctanoic acid) side chain]. The C24 and C27 bile acids, together with C27 bile alcohols, are the predominant chemical metabolites of cholesterol and are the major chemical form in which cholesterol is eliminated in most vertebrates (1). All primary C24 bile acids have a hydroxyl group at position C-3 (from cholesterol) and at C-7, as cholesterol 7α-hydroxylation is the rate-limiting step in bile acid biosynthesis. Thus, chenodeoxycholic acid (CDCA; 3α,7α-dihydroxy-5β-cholan-24-oic acid) is the root C24 bile acid.

In the majority of vertebrates, hydroxylation of CDCA (or an intermediate in the synthesis of CDCA) occurs at an additional site on the steroid nucleus. The sites of such hydroxylation have been identified at C-1, C-4, C-5, C-6, C-12, C-15, and C-16 (for original references, see Ref. 2). We have suggested that the ability to form such trihydroxy bile acids by the hepatocyte may have evolved as a response to the acquisition of a bacterial flora in the cecum mediating dehydroxylation at C-7. Bacterial dehydroxylation of CDCA results in the formation of lithocholic acid, a 3α-monohydroxy secondary bile acid (3). Lithocholic acid is toxic when it accumulates in the enterohepatic circulation of bile acids (4–12), causing segmental bile duct obstruction and destructive cholangitis in the mouse (12) and bile duct hyperplasia and choledocholithiasis in the rat (6, 7). On the other hand, 7-dehydroxylation of a trihydroxy bile acid results in the formation of a dihydroxy bile acid; the dihydroxy bile acids formed by bacterial 7-dehydroxylation of the common natural bile acids are known to be less toxic than lithocholic acid when they accumulate in the enterohepatic circulation.

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We report here the structure of a novel dihydroxy bile acid, 15α-hydroxylicholithocholic acid, which is a major biliary bile acid in the wombat (Vombatus ursinus), a common Australian marsupial. We have confirmed its structure by direct synthesis of this bile acid from CDCA. The 15α-hydroxylicholithocholic acid occurs in bile as its taurine conjugate, which was also synthesized and shown to have identical chromatographic and spectroscopic properties as the compound isolated from bile.

METHODS

Biological material

Wombat bile was obtained by aspiration of the gallbladder at autopsy from two wombats that died as a result of being struck by automobiles. The wombat cadavers had been transported to the Healesville Sanctuary (Victoria, Australia), where the autopsy disclosed no tissue abnormalities other than those caused by trauma. Bile samples were diluted in three volumes of isopropanol and mailed to the laboratory of T.I. for analysis.

Chemistry: standard analytical methods

Melting point (mp) values were determined on a micro hot-stage apparatus and are uncorrected. Infrared (IR) spectra were obtained in KBr discs on a Jasco FT-IR 4100 spectrometer (Tokyo, Japan). $^1$H- and $^{13}$C-NMR spectra were obtained on a JEOL JNM-EX 270 FT instrument at 270 and 68.8 MHz, respectively; in this report, only values for critical functionalities are given. El low-resolution (LR) mass spectra were determined on a JEOL JMS-903 mass spectrometer at 70 eV. High-resolution (HR) mass spectra were measured using a JEOL LCmate double-focusing mass spectrometer equipped with an EI or FAB probe under the positive ion mode. Normal-resolution (LR) spectra were obtained using a JEOL JLCmate double-focusing magnetic mass spectrometer equipped with an EI or fast-atom bombardment (FAB) probe under the positive ion mode. HRMS spectra were also obtained on a JEOL JMS-700 mass spectrometer with an EI or FAB probe under the positive ion mode. Normal-phase TLC was performed on precoated silica gel plates (0.25 mm layer thickness; E. Merck, Darmstadt, Germany) using hexane-ethyl acetate-acetic acid mixtures (40:60:1–60:40:1, v/v/v) as the developing solvent. Sep-Pak Vac tC18 cartridges (adsorbent weight, 5 g) for solid-phase extraction were purchased from Waters Associates (Milford, MA); they were washed successively with 50 ml of methanol and 50 ml of distilled water before use.

Bile acid standards

CDCA was kindly supplied by Mitsubishi Pharma (Osaka, Japan). Cholyl taurine, chenodeoxycholyl taurine, ursodeoxycholyl taurine, deoxycholyl taurine, and lithocholyl taurine were from our laboratory collection.

HPLC with an evaporative light-scattering detector

The apparatus used was a Jasco HPLC system (two PU-980 high-pressure pumps, an HG-980-31 solvent mixing module, and an HG-980-50 degasser). Reverse-phase chromatographic separation was carried out by stepwise gradient elution on a Capcell Pak C_{18}-type MGI column (5 μm, 250 mm × 10 mm ID) using methanol-water mixtures as the mobile phase. The mobile phase was gradually increased at a flow rate of 2 ml/min as follows: 48% (0–15 min) → 50% (15.1–30 min) → 52% (30.1–45 min) → 54% (45.1–60 min). The 52% methanol fraction, which contained compound C (the unknown bile acid), was evaporated under a nitrogen stream at 35°C.

Isolation of a major, unidentified bile acid from the bile of wombats

The bile of the wombats was diluted with isopropanol (~10 ml) and filtered, and the filtrate was evaporated under a nitrogen stream at 35°C. The residue was dissolved in methanol-water (1:9) (5 ml) and then applied to a preconditioned Sep-Pak tC_{18} cartridge (400 mg; Waters). After the cartridge was washed successively with water (2 ml) and 20% methanol (2 ml), the bile acid fraction was eluted with methanol. The methanol eluate was evaporated under a nitrogen stream at 35°C; the residue was then dissolved in 200 μl of methanol, and its individual bile acids were isolated by preparative HPLC. The apparatus consisted of a Jasco Gulliver series HPLC system (two PU-980 high-pressure pumps, an HG-980-31 solvent mixing module, and an HG-980-50 degasser). Reverse-phase chromatographic separation was carried out by stepwise gradient elution on a Capcell Pak C_{18}-type MGI column (5 μm, 250 mm × 10 mm ID) using methanol-water mixtures as the mobile phase. The methanol composition was gradually increased at a flow rate of 2 ml/min as follows: 48% (0–15 min) → 50% (15.1–30 min) → 52% (30.1–45 min) → 54% (45.1–60 min). The 52% methanol fraction, which contained compound C (the unknown bile acid), was evaporated under a nitrogen stream at 35°C. The compound was subjected to preparative HPLC. For the analyses of individual bile acids, a Shimadzu C-RSA data-processing system (Kyoto, Japan) was used to monitor peak areas. The retention times of the compounds were determined using a Sugai u-620-type 30V column heater (Wakayama, Japan). An Alltech 2000ES evaporative light-scattering detector (Deerfield, IL) was used; the flow rate of purified compressed air as a nebulizing gas was 1.6 l/min, and the temperature of the heated drift tube was 82°C.

For simultaneous separation of unconjugated, glycine- and taurine-amidated, and taurine-amidated bile acids, an isocratic elution mode was used according to the procedure of Roda et al. (13). The mobile phase was 65% (v/v) aqueous methanol containing 15 mM ammonium acetate adjusted to pH 5.4 with acetic acid, and the flow rate was kept at 400 μl/min. Figure 1 shows the HPLC result that was obtained; the identities of individual peaks are discussed in Results.
LC-ESI-MS of wombat bile components

LC-MS analyses of wombat bile components were performed on a JEOL JMS-LCmate double focusing magnetic mass spectrometer equipped with an ESI probe using the negative ion mode. Chromatographic separation was carried out on a YMC Pack Pro C18 column (3 μm, 100 × 2.0 mm ID; YMC, Kyoto, Japan) using a 20 mM ammonium acetate (pH 7)-methanol mixture (35:65, v/v) as the mobile phase at a flow rate of 200 μl/min. The mass detector was set to the following conditions: needle voltage, −2.5 kV; orifice 1 temperature, 150°C; desolvating plate temperature, 250°C; ring lens voltages, 30 V/100 V.

LC-ESI-MS/MS of compound C

Negative ion LC-ESI-MS/MS spectra of compounds C and 1d (see below) were obtained on a Finnigan LTQ (Thermo Fisher Scientific, Inc., Waltham, MA) equipped with a Paradigm MS4 HPLC system (AMR, Inc., Tokyo, Japan). Chromatographic separation was carried out on a TSKgel ODS-100V (5 μm, 150 × 2.0 mm ID) using 5 mM ammonium acetate (pH 6)-acetonitrile mixtures as the mobile phase. A linear gradient was used: 30% CH3CN (0 min) → 80% CH3CN (30 min); the flow rate was kept constant at 200 μl/min. The mass detector was set as follows: capillary temperature, 270°C; sheath gas flow rate, 50 arbitrary units; auxiliary gas flow rate, 5 arbitrary units; source voltage, 64 kV; capillary voltage, 630 V; tube lens offset voltage, 6100 V.

Synthesis of methyl 3α-cathyl-5β-chol-7-en-24-oate (4)

The synthetic scheme used to prepare 15α- and 15β-hydroxy-lithocholic acid (and their methyl esters) is shown in Fig. 2 (compounds 1–8).

To a magnetically stirred solution of methyl chenodeoxycholate 3-cathylate (3; 4.4 g) in pyridine (50 ml), phosphoryl chloride (15 ml) was slowly added with ice bath cooling. After being allowed to stand overnight at room temperature, the mixture was poured into ice water (0°C) and extracted with CH2Cl2. The combined CH2Cl2 extract was washed with 10% HCl and then with water to neutrality, dried with Drierite, crystallized as colorless crystals: yield, 3.6 g (86%); mp 119–123°C [literature value (14) mp 120–121°C]. IR (KBr) νmax cm⁻¹: 1738 (C=O). 1H-NMR (CDCl3) δ: 0.54 (3H, s, 18-CH3), 0.87 (3H, s, 19-CH3), 0.93 (3H, d, J = 6.2 Hz, 21-CH3), 1.29 (3H, t, J = 7.0 Hz, COOCH2CH3), 3.67 (3H, s, -COOCH3), 4.16 (2H, m, COOCH2CH3), 4.58 (1H, brm, 3β-H), 5.09 (1H, m, 7-H). 13C-NMR (CDCl3) δ: 11.8 (C-18), 14.2 (COOCH2CH3), 18.4 (C-21), 24.3 (C-19), 51.4 (COOCH3), 63.5 (COOCH2CH3), 77.3 (C-3), 115.2 (C-7), 137.2 (C-8), 154.6 (COOCH2CH3), 174.7 (C-24). LR-MS (EI) m/z: 370 (M-CtOH, 100%), 355 (M-CtOH-CH3, 49%), 255 [M-CtOH-CH3-side chain (S.C.)-ring D, 32%], 228 (M-CtOH-S.C.-part of ring D, 11%), 213 (M-CtOH-S.C.-ring D, 21%).

Fig. 2. Synthetic route to epimeric 3α,15α-dihydroxy-5β-cholan-24-oic acids (1 and 2) from chenodeoxycholic acid 3-cathylate methyl ester (3).
The corresponding Δ7 ester 5b, prepared from 5a by the usual method and p-toluene sulfonic acid method, was recrystallized from methanol as colorless thin plates: yield, 60%; mp 88–92°C [lit. value (14) mp 104–105°C]. IR (KBr) v_{max} = 3391 cm\(^{-1}\); 1,614 (C=C), 1,734 (C=O), 3,017 (C–H), 3,327 (OH). \(^1\)H-NMR (CDCl\(_3\)) \(\delta\) 0.54 (3H, s, 18-CH\(_3\)), 0.85 (3H, s, 19-CH\(_3\)), 0.94 (3H, d, \(J = 5.9\) Hz, 21-CH\(_3\)), 3.62 (1H, brm, 21-H), 3.67 (3H, s, -COOCH\(_3\)), 5.10 (1H, m, 7-H). \(^13\)C-NMR (CDCl\(_3\)) \(\delta\) 18.4 (C-21), 24.4 (C-19), 51.4 (COO\(_3\)), 137.2 (C-8), 174.7 (C-24). LR-MS (EI) \(m/z\) 388 (M, 27%), 370 (M-H\(_2\)O, 100%), 355 (M-H\(_2\)O-CH\(_3\), 78%), 255 (M-H\(_2\)O-S.C., 13%). HR-MS (EI), calculated for C\(_{25}\)H\(_{40}\)O\(_3\): 388.2977; found \(m/z\), 388.2974.

### Synthesis of methyl 3α-hydroxy-5β-chol-14-en-24-oate (6)

A solution of the Δ\(^7\) ester 5b (300 mg) in dry CHCl\(_3\) (10 ml) was prepared, and dry HCl gas was bubbled through it for 2 h in an ice bath. The CHCl\(_3\) solution was washed successively with water, with 5% NaHCO\(_3\) solution, and then again with water to neutrality. It was then dried with Drierite and evaporated to an oily residue, which, according to capillary GC analysis, consisted of a mixture of three components in an approximate ratio of 7:3:4.

Synthesis of methyl 3α-hydroxy-5β-chol-8(9)-en-24-oate (9)

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Fig. 3. Left: Chemical structures of epimeric 3α,15α-dihydroxy-5β-cholan-24-oic acids (1a and 2a) and the glycine (1c) and taurine (1d) N-acyl amides (conjugates) of 1a. Right: Chemical structures of methyl 3α-hydroxy-5β-chol-8(9)-en-24-oate (9) (above) and 3α-hydroxy-5β-chol-8(14)-en-24-oate (10) (below), side products formed by the attempted acid-catalyzed dehydration procedure of Yamasaki and colleagues (14, 22) on compound 5b.
Hz, 24-H2), 3.63 (1H, brm, 38-H), 3.94 (1H, brm, 15b-H).
13C-NMR (CDCl3) 6; 13.5 (C-18), 18.4 (C-21), 23.4 (C-19), 63.5 (C-24), 71.8 (C-3), 73.8 (C-15). LR-MS (EI) m/z 360 (45%, M-H2O), 342 (96%, M-2H2O), 327 (M-2H2O-CH3, 44%), 288 (M-H2O-ring A, 30%), 273 (M-H2O-S-C., 86%), 255 (M-H2O-S-C., 100%) 217 (ring A+B+C, 62%). HRMS (FAB+) calculated for C24H36O4Na [M+Na]+, 401.3032; found m/z, 401.3045.

Synthesis of 3,15-dioxo-5β-chol-24-ol (8a) and its methyl ester (8b)

Jones reagent (500 μl) was added gradually to a solution of the 3α,15α,24-riol 7 (30 mg) in acetone (4 ml) under 10°C, and the mixture was stirred for 30 min at room temperature. Methanol (500 μl) was added, and the oxidation product was extracted with EtOAc. The combined EtOAc extracts were washed with saturated brine, dried over Drierite, and evaporated to dryness. The oily residue was chromatographed using a column of C18bonded silica gel (10 g; particle size, 50 μm). Elution with methanol-water (7:3, v/v) afforded the title dioxo acid 8a, which was recrystallized from EtOAc-hexane as colorless amorphous solids: yield, 22 mg (72%); mp 165–166°C. IR (KBr) νmax cm−1: 1,705 (C=O, ketone), 1,736 (C=O, COOH); 342 (M, 61%), 370 (19%), 318 (M-ring A, 10%), 287 (M-S.C., 100%), 217 (ring A+B+C, 62%). HRMS (EI), calculated for C24H19O5Na [M+Na]+, 388.2977; found m/z, 388.2969.

Synthesis of 3α,15α-dihydroxy-5β-chol-24-ol (1a) and its 15β-epimer (2a)

The 3α,15α-dihydroxy-5β-chol-24-ol (1a) (analytically pure): yield, 46 mg (95%); mp 206–208°C. IR (KBr) νmax cm−1: 1,703 (C=O, COOH); 342 (M, 61%), 370 (19%), 318 (M-ring A, 10%), 287 (M-S.C., 44%), 255 (M-S-C-part of ring D, 48%), 232 (M-S-C-ring D, 15%), 215 (16%), 197 (S-C++-dihydrid+CH3, 100%). HRMS (EI), calculated for C25H40O4 [M], 388.2614; found m/z, 388.2587.

The free acid 8a, recrystallized with methanol and p-toluene-sulfonic acid and processed by the usual work-up, yielded the corresponding methyl ester. Recrystallization from EtOAc-hexane gave the dioxo ester 8b as colorless amorphous solids: yield, 91%; mp 110–112°C. IR (KBr) νmax cm−1: 1,713 (C=O, ester), 1,735 (C=O, ketone), 1,753 (C=O, ester). 1H-NMR (CDCl3) 6: 0.78 (3H, s, 18-CH3), 1.02 (3H, d, J = 5.9 Hz, 21-CH3), 1.03 (3H, s, 19-CH3). 13C-NMR (CDCl3) 6; 13.0 (C-18), 18.5 (C-21), 22.5 (C-19), 177.9 (C-24), 212.9 (C-3), 214.9 (C-15). LR-MS (EI) m/z: 388 (M, 61%), 370 (19%), 318 (M-ring A, 10%), 287 (M-S.C., 44%), 255 (M-S-C-part of ring D, 48%), 232 (M-S-C-ring D, 15%), 215 (16%), 197 (S-C++-dihydrid+CH3, 100%). HRMS (EI), calculated for C25H40O4Na [M+Na]+, 415.2824; found m/z, 415.2788.

Synthesis of methyl 3α,15α-dihydroxy-5β-chol-24-olate (1b) and its 15β-epimer (2b)

Test Butylamineborene complex (30 mg) was added to a stirred solution of the 3,15-dioxo ester 8b (30 mg) in CH2Cl2 (3 ml), and the mixture was refluxed overnight. After cooling the mixture, 10% HCl (300 μl) was added, and the solution was stirred for 30 min. The CH2Cl2 layer was washed with 5% NaHCO3 and water, dried over Drierite, and evaporated to dryness. The oily residue, which consisted essentially of two components on normal-phase TLC, was chromatographed on a column of silica gel (10 g). Elution with EtOAc-hexane (1:1–3:2, v/v) provided two well-separated fractions. The less polar fraction was identified as methyl 3α,15α-dihydroxy-5β-cholanoate (2b), which recrystallized from aqueous methanol as colorless amorphous solids: yield, 17 mg (56%); mp 64–67°C. IR (KBr) νmax cm−1: 1,739 (C=O), 3,409 (OH). 1H-NMR (CDCl3) 6; 0.92 (3H, d, J = 5.7 Hz, 21-CH3), 0.93 (3H, s, 19-CH3), 0.96 (3H, s, 18-CH3), 3.63 (1H, brm, 3β-H) 3.67 (3H, m, COOCH3), 4.19 (1H, t, J = 5.7 Hz, 15α-H). 13C-NMR (CDCl3) 6; 14.6 (C-18), 18.2 (C-21), 23.3 (C-19), 51.5 (COOCH3), 70.2 (C-15), 71.7 (C-3). LR-MS (EI) m/z: 388 (M-H2O, 59%), 370 (M-2H2O, 42%), 355 (M-2H2O-CH3, 22%), 273 (M-H2O-S-C., 34%), 255 (M-2H2O-S-C., 39%), 217 (ring A+B+C, 43%), 208 (100%). HRMS (EI), calculated for C25H38O7Na, 388.2977 [M]; found m/z, 388.2976.

The more polar fraction was characterized as the desired 3α,15α-dihydroxy ester 1b, which resisted crystallization at attempts: yield, 10 mg (33%). IR (KBr) νmax cm−1: 1,737 (C=O), 3,350 (OH). 1H-NMR (CDCl3) 6; 0.68 (3H, s, 18-CH3), 0.90 (3H, d, J = 5.7 Hz, 21-CH3), 0.93 (3H, s, 19-CH3), 3.62 (1H, brm, 3β-H), 3.94 (3H, m, COOCH3), 3.94 (1H, brm, 15b-H). 13C-NMR (CDCl3) 6; 13.3 (C-18), 18.1 (C-21), 23.4 (C-19), 51.5 (COOCH3), 71.7 (C-3), 73.6 (C-15), 174.6 (C-24). LR-MS (EI) m/z: 388 (M-H2O, 72%), 370 (M-2H2O, 42%), 355 (M-2H2O-CH3, 50%), 273 (M-H2O-S-C., 98%), 255 (M-2H2O-S-C., 88%), 217 (ring A+B+C, 41%), 208 (100%). HRMS (EI), calculated for C25H38O7Na, 388.2977 [M]; found m/z, 388.2969.

Synthesis of 3α,15α-dihydroxy-5β-chol-24-acyl glycine (1c; sodium salt)

To a magnetically stirred solution of 3α,15α-dihydroxy acid 1a (30 mg) in dry dimethylformamide (3 ml), glycine methyl ester hydrochloride (25 mg), diethylphosphorylorancian (30 μl), and triethylamine (125 μl) was added, and the mixture was stirred at room temperature for 1 h. The reaction was extracted with EtOAc, and the combined extracts were washed with saturated brine and evaporated to dryness. The residue was then
refluxed in 5% methanolic NaOH (4 ml) for 1 h, and the solution was adjusted to pH 9 by 10% HCl. Most of the solvent was evaporated under reduced pressure, and the residue was dissolved in water (5 ml). The aqueous solution was loaded onto a Sep-Pak Vac C18 cartridge, which was washed successively with water (20 ml), 30% methanol (20 ml), and 40% methanol (20 ml). Elution with 50% methanol (60 ml) gave the glycin conjugate of the title compound (1e), after the solvent was evaporated under reduced pressure. The residue was recrystallized from methanol-Et2O in the form of colorless amorphous solids: yield, 22 mg (61%); mp 157–160°C.

However, definitive identification of the minor conjugated molecule [M-H] was not achieved. After several attempts, we decided to undertake the synthesis of the two stereoisomeric 3a,15b-dihydroxy-5β-cholanoic acid (1a and 2a) and the glycin (1c) and taurine (1d) conjugates of 1a as authentic reference specimens (Fig. 3).

The formation of 3α,15β-dihydroxy-5β-cholanoic acid (2a) from lithocholic acid by fungal enzymes was reported previously by two groups (17, 19–21). However, the preparation of the corresponding 15α-hydroxy epimer (1a) has hitherto been unreported. On attempting to repeat the preparation of 2a from lithocholic acid with Penicillium species ATCC 12556 (21), we encountered difficulties in isolating sufficient 2a to be adequate for chemical epimerization to 1a.

Our aim, therefore, was to develop an alternative synthesis of 1a and 2a, as summarized in Fig. 2. A key intermediate in our work was methyl 3α-hydroxy-Δ13β-cholenoate (6). To prepare this compound, the 3α-cathylxy-7α-hydroxy ester 3, prepared from CDCA (14), was converted to the 3α-cathylxy-Δ2-ester 4 by an elimination reaction with phosphorly chloride. Subsequent alkaline hydrolysis of 4, followed by the usual esterification of the resulting 3α-hydroxy-Δ7 acid 5a, gave the corresponding 3α-hydroxy-Δ7 ester 5b.

The most promising method reported in the literature for the preparation of the unsaturated Δ14 ester 6 from 5b on a substantial scale appeared to be the procedure of Yamasaki and colleagues (14, 22), in which acid-catalyzed isomerization of a double bond in 5b with dry HCl gas was reported to give 6, together with the Δ10(14) isomer 9. On repeating this work using modern instrumentation (NMR, MS, HPLC, and capillary GC) that was unavailable at the time of their efforts, we encountered difficulties that led us to investigate the reaction in detail. We found that the reaction product consisted of a mixture of three unsaturated compounds, Δ8 (9), Δ8(14) (10), and Δ14 (6) isomers, in an approximate ratio of 20:40:40 (%) as estimated by capillary GC. (The structure of compound 6 is shown in Fig. 2, and those of compounds 9 and 10 are shown in Fig. 3.) The three positional isomers were not resolved by conventional silica gel column chromatography but were efficiently separated by chromatography on 25% AgNO3-impregnated silica gels (15); compounds were eluted in the order Δ8 before Δ8(14) before Δ14 isomer. The individual isomers were distinguished by their 1H- and 13C-NMR spectra. The 13C-NMR spectra revealed that compounds 9 and 10 had a tetrasubstituted double bond, whereas compound 6 was trisubstituted. The chemical shifts of 13C signals in 6 and 10 were in good agreement with those reported in the literature (23). The remaining tetrasubstituted alkene 9, there-

RESULTS AND DISCUSSION

Synthesis of 3α,15α-dihydroxy-5β-cholane-24-oxytaurine (1d; sodium salt)

A suspension of 1a (50 mg), powdered taurine (40 mg), diethylphosphorocyanid (40 μl), and triethylamine (75 μl) in dry dimethylformamide (5 ml) was stirred at room temperature. After 1 h, the mixture was adjusted to pH 12–14 with 1 M NaOH and then to pH 8–9 with 10% HCl. The resulting solution was diluted with water (45 ml), passed through a Sep-Pak Vac C18 cartridge (50 ml), and washed successively with water (20 ml) and 20% methanol (20 ml). Elution with 50% methanol (60 ml) gave the desired taurine conjugate 1d, after evaporation of the solvent under reduced pressure. The residue was recrystallized from methanol-Et2O as colorless amorphous solids of 1d: yield, 40 mg (59%); mp 207–209°C. IR (KBr) νmax cm⁻¹: 1,593 (C=O), 3,394 (OH). 1H-NMR (CD3OD) δ: 0.71 (3H, s, 18-CH3), 0.96 (3H, d, J = 5.4 Hz, 21-CH3), 0.96 (3H, s, 19-CH3), 3.53 (1H, brm, NH2), 2.34 (1H, brm, 15β-H). 13C-NMR (CD3OD) δ: 13.7 (C-18), 18.8 (C-21), 21.8 (C-11), 24.0 (C-19), 27.7 (C-6), 28.3 (C-7), 31.2 (C-2), 33.0 (C-22), 34.1 (C-23), 35.7 (C-10), 36.4 (C-20), 36.6 (C-1), 37.0 (C-8), 37.2 (C-4), 41.6 (C-16), 41.7 (C-12), 42.0 (C-5), 43.6 (C-9), 44.5 (CH2-NH2), 44.9 (C-15), 54.9 (C-17), 64.1 (C-14), 72.5 (C-3), 74.2 (C-15), 176.2, 176.3 (C-24, COONa). HR-MS (FAB⁺) m/z 494 (M+Na, 100%). HRMS (FAB⁺), calculated for C26H42O5NNa2, 494.2858 [M+Na]; found m/z, 494.2832.

Synthesis of 3α,15α-dihydroxy-5β-cholane-24-oxytaurine (1d; sodium salt)

A suspension of 1a (50 mg), powdered taurine (40 mg), diethylphosphorocyanid (40 μl), and triethylamine (75 μl) in dry dimethylformamide (5 ml) was stirred at room temperature. After 1 h, the mixture was adjusted to pH 12–14 with 1 M NaOH and then to pH 8–9 with 10% HCl. The resulting solution was diluted with water (45 ml), passed through a Sep-Pak Vac C18 cartridge (50 ml), and washed successively with water (20 ml) and 20% methanol (20 ml). Elution with 50% methanol (60 ml) gave the desired taurine conjugate 1d, after evaporation of the solvent under reduced pressure. The residue was recrystallized from methanol-Et2O as colorless amorphous solids of 1d: yield, 40 mg (59%); mp 207–209°C. IR (KBr) νmax cm⁻¹: 1,593 (C=O), 3,394 (OH). 1H-NMR (CD3OD) δ: 0.71 (3H, s, 18-CH3), 0.96 (3H, d, J = 5.4 Hz, 21-CH3), 0.96 (3H, s, 19-CH3), 3.53 (1H, brm, NH2), 2.34 (1H, brm, 15β-H). 13C-NMR (CD3OD) δ: 13.7 (C-18), 18.8 (C-21), 21.8 (C-11), 24.0 (C-19), 27.7 (C-6), 28.3 (C-7), 31.2 (C-2), 33.0 (C-22), 34.1 (C-23), 35.7 (C-10), 36.4 (C-20), 36.6 (C-1), 37.0 (C-8), 37.2 (C-4), 41.6 (C-16), 41.7 (C-12), 42.0 (C-5), 43.6 (C-9), 44.5 (CH2-NH2), 44.9 (C-15), 54.9 (C-17), 64.1 (C-14), 72.5 (C-3), 74.2 (C-15), 176.2, 176.3 (C-24, COONa). HR-MS (FAB⁺) m/z 494 (M+Na, 100%). HRMS (FAB⁺), calculated for C26H42O5NNa2, 494.2858 [M+Na]; found m/z, 494.2832.

Identification of 15α-hydroxylithocholic acid (3α,15α-dihydroxy-5β-cholane-24-oic acid) in the biliary bile acids of the wombat was mentioned in a review by J. S. Pyrek (18) many years ago, but details were never published because of his untimely death. To verify the structure, in particular the position and the stereochemical configuration of hydroxyl groups as well as the nature of the N-acylamidated moiety in the side chain at C-24, we decided to undertake the synthesis of the two stereoisomeric 3α,15β-dihydroxy-5β-cholanoic acid (1a and 2a) and the glycin (1c) and taurine (1d) conjugates of 1a as authentic reference specimens (Fig. 3).
fore, was deduced to have a double bond between C-8 and C-9 in the 5β-steroid nucleus. The 13C chemical shifts of the olefinic carbons at C-8 (130.1 ppm) and C-9 (131.7 ppm) in 9 were similar to those reported (128.3 and 134.9 ppm, respectively) for 3α-hydroxy-5α-cholest-8-ene (24).

Hydroboration of the 3α-hydroxy-Δ14 ester 6 with B₂H₆/THF and subsequent treatment of the resulting alkylborane with H₂O₂/NaOH resulted in simultaneous hydroxylation at C-15 and C-24 to give 5β-cholane 3α,15α,24-triol (7). Attempted selective oxidation of the hydroxyl group at the C-24 position in 7 with pyridinium chlorochromate, pyridinium dichromate, or Swern oxidation was unsuccessful, yielding complicated mixtures, even under milder conditions than are conventionally used. Hence, 7 was completely oxidized with Jones reagent to give the 3,15-dioxo acid 8a.

After esterification of 8a in the usual manner, reduction of the resulting 3,15-diketone 8b with tert-butylamine borane complex yielded a mixture of 3α,15α-dihydroxy (1b) and 3α,15β-dihydroxy (2b) esters. The reduction was stereo-selective, as the 3β-hydroxy isomers (i.e., 3β,15α- and 3β,15β-dihydroxyls) were not formed, in agreement with our previous work on this topic (25). The two stereoisomeric pairs were cleanly separated by column chromatography on silica gel to give 1b and 2b in isolated yields of 56% and 33%, respectively. The stereochemical nature of the 15-hydroxyl group in 1b and 2b was confirmed by 1H- and 13C-NMR. In the 1H-NMR spectra, the 18-methyl proton signal (0.96 ppm) in 2b is deshielded to a large extent by ~0.3 ppm, compared with that (0.64 ppm) of the parent compound methyl lithocholate, probably owing to quasi-3,3-diaxial interaction between the 18-methyl and 15β-hydroxy groups, whereas the corresponding 18-methyl signal (0.68 ppm) in 1b is barely shifted. The 3β-H in both 1b and 2b showed essentially identical chemical shifts (3.62 and 3.64 ppm, respectively) and signal multiplicity (a broad multiplet). The chemical shifts and the signal multiplicity for the 15-proton signal in both isomers differed considerably from each other; the 15β-H in 1b appeared at 3.94 ppm as a broad multiplet, whereas the 15α-H in 2b resonated at 4.19 ppm as a triplet (J = 5.4 Hz). The 1H-NMR data are consistent with those reported for epimeric 3α,7α,15β-trihydroxy bile acids (25) and a 3α,12α,15β-trihydroxy analog (21). On the other hand, the 13C-NMR chemical shifts of the C-24 free acid (2a) (see below) of 3α,15β-dihydroxy epimer 2b agreed completely with the previously described product obtained by microbiological transformation of lithocholic acid (17, 19, 20).

Alkaline hydrolysis of 1b followed by acidification afforded the desired 3α,15α-dihydroxy-5β-cholanic acid (1a) in an overall yield of 2.3% starting from 3. The free 3α,15β-hydroxy acid 2a was also similarly obtained from the ester 2b. The mp (119–121°C) of the synthetic 2a differed markedly from that (186–187°C) reported by Kulprecha et al. (17). Although the reason for this discrepancy is unclear, the 13C-NMR spectral data are completely consistent with each other. Polymorphism in crystal habit is well known for C24 bile acids (26).

Synthesis of the taurine and glycyne conjugates of 15α-hydroxy-lithocholic acid

N-Acylamidation of the C-24 carboxyl group in 1a with glycine methyl ester hydrochloride or freshly powdered tauroine was effectively attained using diethyl phosphorocyanide as a condensing reagent and triethylamine as a catalyst (27). After the condensation reaction, the resulting solutions were adjusted to appropriate pH with the treatment of acid and/or base (see Methods), and the desired glycine (1c) and tauroine (1d) conjugates (as the sodium salts) were recovered efficiently by applying a reverse-phase prepacked cartridge (Sep-Pak Vac tC18) for solid-phase extraction.

Proof of structure of peak C in the HPLC result of wombat bile

As shown in Fig. 4, LC-ESI-MS/MS of peak C under negative ion mode gave only one peak, corresponding to that of the deprotonated molecule [M-H]⁻ at m/z 498. When the collision-induced dissociation spectrum of the signal as a precursor ion was measured, an intense peak appeared at m/z 489, corresponding to the elimination of one molecule of water from the [M-H]⁻, accompanied by minor fragment ions at m/z 432, 416, 386, 355, and 327. An essentially identical collision-induced dissociation spectrum was obtained for synthetic 1d.

Compound C was isolated from a mixture of bile acids in wombat bile by preparative HPLC. Figure 5 shows the 1H-NMR spectra of isolated compound C, and Table 1 compiles the 1H and 13C signal assignments of compound C and 1d. The 1H- and 13C-NMR spectral patterns of both compounds were essentially identical. Particularly noteworthy were signals occurring at 2.97 ppm (t, J = 6.4 Hz, -CH₂S-) and 3.60 ppm (t, J = 6.4 Hz, -CH₂N-) by 1H-NMR, indicating the presence of a taurine-conjugate moiety (2, 27). As mentioned above, the 1H signals occurring at 3.53 and 3.83 ppm, owing to the 3β-H and 15β-H, respectively, also provide the confirmatory evidence for the structure of compound C as 15α-hydroxy-lithocholyl tauroine.

Biological aspects

Because of its geological history, the continent of Australia has a unique fauna. It is generally considered that Australia separated from what became Antarctica some 60 million years ago. Marsupials, which had originated in the land mass becoming South America, migrated via a land bridge connecting South America to the land mass eventually becoming Antarctica and, in turn, via a land bridge to the land mass that became Australia (28). Among these marsupials, the Australian opossum Trichosurus vulpecula was shown by Lee, Lester, and Pyrek (29) to have a novel bile acid, 1α,3α,7α-trihydroxy-5β-cholan-24-oic acid.

The wombat, another Australian marsupial, is an herbivore and has a voluminous large intestine (30). Based on studies of fecal bile acid composition in numerous mammals, it is highly likely that in the distal intestine of the wombat, the tauroine conjugate of CDCA, the major bile acid of the animal, undergoes bacterial deconjugation and 7-dehydroxylation to form lithocholic acid.
We hypothesize that lithocholic acid is absorbed and returned to the liver, where it undergoes 15α-hydroxylation to form 15α-hydroxylithocholic acid. If this hypothesis is correct, the wombat resembles other rodents [mouse (9–12, 32), rat (33–38), and guinea pig (39, 40)], species in which lithocholic acid is efficiently hydroxylated at C-6, C-7, or both during hepatocyte transport. Sulfation (36) and glucuronidation (35, 37) of lithocholic acid also occur in these species, but to a much smaller extent. N-Acylamidation (mostly with taurine) of the dihydroxy and trihydroxy metabolites of lithocholic acid occurs, but their sulfates and glucuronides have not been reported. In contrast, in human (40, 41) and nonhuman primates [chimpanzee (42), baboon (43, 44),

![Fig. 4. Negative ion LC-ESI-MS/MS fragmentation pattern of synthetic 1d (left) and compound C (right). CID, collision-induced dissociation.](image1)

![Fig. 5. 1H-NMR spectrum of compound C isolated from wombat bile. TMS, tetramethylsilane.](image2)
TABLE 1. $^1$H- and $^{13}$C-NMR data for synthetic Id and compound C

<table>
<thead>
<tr>
<th>No.</th>
<th>Type</th>
<th>Synthetic Id</th>
<th>Compound C</th>
</tr>
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<tr>
<td>1</td>
<td>CH$_2$</td>
<td>36.84$^a$</td>
<td>36.96$^a$</td>
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<tr>
<td>2</td>
<td>CH$_3$</td>
<td>32.12</td>
<td>32.23</td>
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<tr>
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<td>CH</td>
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<td>72.49 (3.55 (brm)</td>
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</tr>
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<td>51.3 (2.98 (t, 6.3Hz)</td>
<td>51.35 (2.97 (t, 6.4Hz)</td>
</tr>
</tbody>
</table>

$^a$, $^b$ May be reversed.

and rhesus monkey (45)], lithocholic acid is not hydroxylated but is excreted into bile as such and in part in sulfated form. The extent of sulfation is much less in the rhesus monkey and baboon than in human, explaining the toxicology of lithocholic acid when its precursor, CDCA, is administered chronically (46–48).

The proportion of 15α-hydroxylithocholic acid in the biliary bile acids of the wombat was more than four times greater than that of lithocholic acid, its probable precursor. The proportion of a given bile acid within the biliary bile acids depends on the size of its individual pool divided by the sum of the total bile acid pool. The size of the bile acid pool of any individual bile acid depends, in turn, on the magnitude of its input and of its intestinal conservation; intestinal conservation, which is responsible for the development of a recirculating bile acid pool, may be considered to amplify the input (3). For primary bile acids, input is from biosynthesis (from cholesterol); for secondary bile acids, input is from the absorption of newly formed molecules from the distal intestine. Intestinal conservation occurs by active and passive absorption. In the steady state, input is balanced by loss attributable to fecal excretion or biotransformation or both. For primary bile acids, biotransformation involves mostly bacterial 7-dehydroxylation; for secondary bile acids, biotransformation involves hepatocyte hydroxylation, as reported here. It is likely that the far larger pool of 15α-hydroxylithocholic acid compared with that of its precursor is the result of greater intestinal conservation of the dihydroxy bile acid, as well as its lack of conversion to any other bile acid. In the rabbit, the pool of deoxycholic acid is >20 times larger than that of cholic acid, its precursor (50).

For trihydroxy bile acids, bacterial 7α-dehydroxylation generates a 3α,X-dihydroxy bile acid, where X denotes the additional hydroxylation site on CDCA. Thus, 7-deoxydihydroxy bile acids may arise either by hepatic hydroxylation of lithocholic acid or by bacterial 7-dehydroxylation of a trihydroxy bile acid (or both). The 7α-deoxy derivatives of 1β,3α,7α-trihydroxy acid (1β,3α-dihydroxy) have been reported (51–53), as has that of 3α,4β,7α-trihydroxy acid (3α,4β-dihydroxy) (54, 55), but they are not known to be present in biliary bile acids in appreciable proportions. The 7-deoxy derivatives of hyocholic acid (3α,6α-dihydroxy, hyodeoxycholic acid) and muricholic acid (3α,6β, murideoxycholic acid) are well known biliary bile acids, as is deoxycholic acid. As yet, the 7-deoxy derivatives of bile acids hydroxylated at C-5 or C-16 have not been reported.

We previously reported that 15α-hydroxylation occurs in swans, tree ducks, and greese, in whom 3α,7α,15α-trihydroxy-5β-cholan-24-oic acid is a primary bile acid (2). We think it highly unlikely that 15α-hydroxylithocholic acid arose by bacterial 7α-dehydroxylation of this primary, trihydroxy bile acid, as it was not present in the biliary bile acids of the wombat. Additional supporting evidence for 15α-hydroxylithocholic acid originating from the hydroxylation of lithocholic acid is the concurrent occurrence of lithocholic acid (in taurine-conjugated form) itself in the biliary bile acids of the wombat.

Because metabolic studies were not performed, we cannot exclude the highly unlikely possibility that 15α-hydroxylithocholic acid is a primary bile acid (i.e., that it is formed from cholesterol in the hepatocyte). However, all primary bile acids (and bile alcohols) reported to date in healthy vertebrates have a hydroxy or oxo group at C-7.

Hydroxylation at C-15 of a bile acid sulfonate analog occurs in the hamster (56), and hydroxylation at C-15 of pregnanolic acids (C$_21$ steroids that may also be considered bile acid homologs with a two carbon side chain) occurs in the rat (57). Thus, the enzyme(s) mediating 15α-hydroxylation appear(s) to have evolved in parallel in multiple vertebrate species. The CYP isoforms involved in the hydroxylation of estradiol at C-15 have been identified as CYP 1A1, 1B1, and 3A4 (58). Whether these CYP isoforms and/or others are involved in the formation of 15α-hydroxylithocholic acid in the wombat remains to be determined.

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