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

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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-hydroxylithocholic acid (3α,15α-dihydroxy-5β-cholan-24-oic acid). Taurine-conjugated CDCA constituted 78% of biliary bile acids, and (taurine-conjugated) 15α-hydroxylithocholic acid constituted 11%. Proof of structure of the latter compound was obtained by its synthesis from CDCA via a Δ^{14} intermediate. The synthesis of its C-15 epimer, 15β-hydroxylithocholic acid (3α,15β-dihydroxy-5β-cholan-24-oic acid), is also reported. The taurine conjugate of 15α-hydroxylithocholic 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-hydroxylithocholic 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 lithocholic acid.—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-hydroxylithocholic acid. *J. Lipid Res.* 2007.** 48: **2682–2692.**

Supplementary key words bile acid synthesis • detoxification of bile acids • bile acid metabolism in marsupials • 15-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: C_{24} bile acids [with a C_5 (isopentanoic acid) side chain] and C_{27} bile acids [with a C_8 (isooctanoic acid) side chain]. The C_{24} and C_{27} bile acids, together with C_{27} 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 C_{24} 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 C_{24} 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-hydroxylithocholic 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-hydroxylithocholic 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. EI low-resolution (LR) mass spectra were determined on a JEOL JMS-303 mass spectrometer at 70 eV. High-resolution (HR) mass spectra were measured using a JEOL LCmate double-focusing magnetic mass spectrometer equipped with an EI or fast-atom bombardment (FAB) probe under the positive ion mode. HR-MS 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. Reverse-phase TLC was carried out on precoated RP-18F_{254S} plates using methanol-water-acetic acid mixtures (90:10:1, v/v/v) as the developing solvent. Sep-Pak Vac tC_{18} 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-2085 high-pressure pumps, an MX-2080-32 solvent mixing module, and an HG-980-50 degasser; Tokyo, Japan) equipped with a Shimadzu C-R8A data-processing system (Kyoto, Japan). A Capcell Pak-type MGII column [250 mm × 3.0 mm inner diameter (ID); particle size, 5 μm; Shiseido, Tokyo, Japan] was used for analytical purposes. The column temperature was kept at 37°C 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-amidated, and taurine-amidated bile acids, an isocratic elution mode was used according to the procedure of Roda et al. (13). The mobile phase used 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.

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 MGII 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.

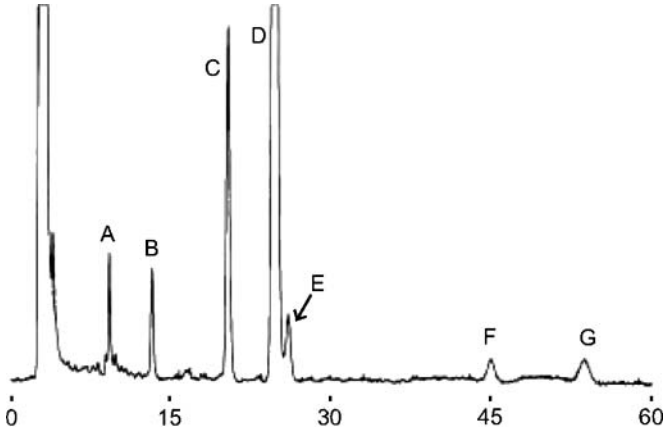

Fig. 1. HPLC-evaporative light-scattering detection profile of the bile acids of the common wombat. For each peak (A–G), the compound, its retention time, and its percentage of total biliary bile acids are given. Peak A, unknown (9.2 min), 2.3%; peak B, unknown (13.1 min), 2.3%; peak C, 3α,15α-dihydroxy-5β-chol-24-oyl taurine (20.1 min), 11.2%, peak D, chenodeoxycholyl taurine (24.0 min), 77.9%; peak E, unknown (26.8 min), 2.1%; peak F, unknown (44.6 min), 1.7%; peak G, lithocholyl taurine (53.3 min), 2.5%.

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 C_{18} 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% CH_3CN (0 min) → 80% CH_3CN (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, ±4 kV; capillary voltage, ±30 V; tube lens offset voltage, ±100 V.

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

The synthetic scheme used to prepare 15α- and 15β-hydroxylithocholic 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 CH_2Cl_2. The combined CH_2Cl_2 extract was washed with 10% HCl and then with water to neutrality, dried with Drierite, and evaporated to an oily residue, which, when treated with CH_2Cl_2-methanol, crystallized as colorless crystals: yield, 3.6 g (86%); mp 119–123°C [literature value (14) mp 120–121°C]. IR (KBr) $\nu_{max}cm^{-1}$; 1,738 (C=O). ^{1}H-NMR ($CDCl_3$) δ; 0.54 (3H, s, 18-CH_3), 0.87 (3H, s, 19-CH_3), 0.93 (3H, d, J = 6.2 Hz, 21-CH_3), 1.29 (3H, t, J = 7.0 Hz, $COOCH_2\underline{CH_3}$), 3.67 (3H, s, -$COOCH_3$), 4.16 (2H, m, $COO\underline{CH_2}CH_3$), 4.58 (1H, brm, 3β-H), 5.09 (1H, m, 7-H). ^{13}C-NMR ($\underline{C}DCl_3$) δ; 11.8 (C-18), 14.2 ($COOCH_2\underline{CH_3}$), 18.4 (C-21), 24.3 (C-19), 51.4 ($COO\underline{CH_3}$), 63.5 ($COO\underline{CH_2}CH_3$), 77.3 (C-3), 115.2 (C-7), 137.2 (C-8), 154.6 ($\underline{C}OOCH_2CH_3$), 174.7 (C-24). LR-MS (EI) *m/z*: 370 (M-CtOH, 100%), 355 (M-CtOH-CH_3, 49%), 255 [M-CtOH-CH_3-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%).

Synthesis of 3α-hydroxy-5β-chol-7-en-24-oic acid (5a) and its methyl ester (5b)

The 3α-cathyloxy-Δ^7 ester **4** (2.65 g) was hydrolyzed using 5% methanolic KOH (35 ml), followed by acidification with H_2SO_4 and recrystallization from methanol. The Δ^7-unsaturated acid **5a** was isolated as colorless needles: yield, 1.98 g (92%); mp 183–185°C [literature value (14) mp 183–184°C]. IR (KBr) $\nu_{max}cm^{-1}$; 1,708 (C=O), 3,352 (OH). ^{1}H-NMR ($CDCl_3$) δ; 0.55 (3H, s, 18-CH_3), 0.86 (3H, s, 19-CH_3), 0.95 (3H, d, J = 6.2 Hz, 21-CH_3), 3.63 (1H, brm, 3β-H), 5.10 (1H, m, 7-H). ^{13}C-NMR ($CDCl_3$) δ; 11.9 (C-18), 18.4 (C-21), 24.5 (C-19), 71.4 (C-3), 115.4 (C-7), 137.2 (C-8), 179.3 (C-24). LR-MS (EI) *m/z*: 374 (M, 25%), 356 (M-H_2O, 100%), 341 (M-H_2O-CH_3, 69%), 302 (16%), 255 [M-H_2O-S.C., 32%], 228 (M-H_2O-part of ring D, 14%), 213 (M-H_2O-S.C.-ring D, 30%). HR-MS (EI), calculated for 374.2821 [M]; found *m/z*, 374.2799.

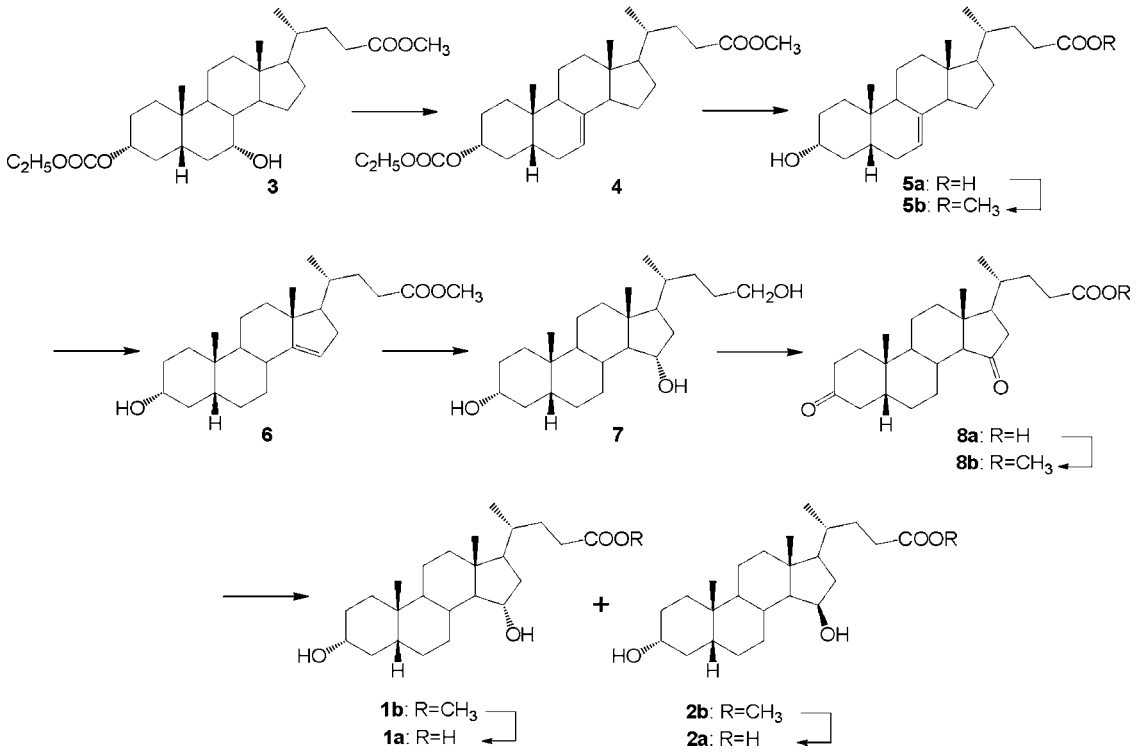

Fig. 2. Synthetic route to epimeric 3α,15ξ-dihydroxy-5β-cholan-24-oic acids (**1** and **2**) from chenodeoxycholic acid 3-cathylate methyl ester (**3**).

The corresponding Δ^7 ester **5b**, prepared from **5a** by the usual methanol and *p*-toluenesulfonic acid method, was recrystallized from methanol as colorless thin plates: yield, 60%; mp 88–92°C [literature value (14) mp 104–105°C]. IR (KBr) $\nu_{max}cm^{-1}$; 1,614 (C=C), 1,734 (C=O), 3,017 (=C-H), 3,337 (OH). ^{1}H-NMR ($CDCl_3$) δ; 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, 3β-H), 3.67 (3H, s, -$COOCH_3$), 5.10 (1H, m, 7-H). ^{13}C-NMR ($CDCl_3$) δ; 11.9 (C-18), 18.4 (C-21), 24.4 (C-19), 51.4 ($COOCH_3$), 71.3 (C-3), 115.4 (C-7), 137.2 (C-8), 174.7 (C-24). LR-MS (EI) *m/z*: 388 (M, 27%), 370 (M-H_2O, 100%), 316 (M-H_2O-ring A, 14%), 355 (M-H_2O-CH_3, 75%), 255 (M-H_2O-S.C., 42%), 228 (M-H_2O-S.C.-part of ring D, 18%), 213 (M-H_2O-S.C.-ring D, 41%). 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 17:44:39 (%). The mixture was chromatographed on a column of 25% $AgNO_3$-impregnated silica gel (30 g) (15). Elution with hexane-EtOAc (4:1 → 7:3, v/v) provided three well-separated fractions. The less polar fraction was identified as methyl 3α-hydroxy-5β-chol-8(9)-en-24-oate (**9**; **Fig. 3**), which resisted crystallization attempts: yield, 36 mg (12%); viscous oil [literature value (16)] mp 116–117°C). IR (KBr) $\nu_{max}cm^{-1}$; 1,737 (C=O), 3,420 (OH). ^{1}H-NMR ($CDCl_3$) δ; 0.87 (3H, s, 18-CH_3), 0.94 (3H, d, J = 6.2 Hz, 21-CH_3), 0.99 (3H, s, 19-CH_3), 3.59 (1H, brm, 3β-H), 3.66 (3H, s, -$COOCH_3$). ^{13}C-NMR ($CDCl_3$) δ; 19.3 (CH_3), 23.6 (CH_3), 28.6 (CH_3), 51.3 ($COOCH_3$), 70.9 (C-3), 130.1 and 131.7 (C-8 and C-9, respectively, or vice versa), 174.6 (C-24). LR-MS (EI) *m/z*: 388 (M, 27%), 370 (M-H_2O, 100%), 355 (M-H_2O-CH_3, 59%), 316 (79%), 273 (M-S.C., 18%), 255 (M-H_2O-S.C., 83%), 229 (M-H_2O-S.C.-part of ring D, 17%), 213 (M-H_2O-S.C.-ring D, 26%). HR-MS (EI), calculated for $C_{25}H_{40}O_3$, 388.2977; found *m/z*, 388.2981.

Continued elution gave a homogeneous viscous oil, which was characterized as methyl 3α-hydroxy-5β-chol-8(14)-en-24-oate (**10**; Fig. 3): yield, 115 mg (38%). IR (KBr) $\nu_{max}cm^{-1}$; 1,739 (C=O), 3,393 (OH). ^{1}H-NMR ($CDCl_3$) δ; 0.80 (3H, s, 18-CH_3), 0.83 (3H, s, 19-CH_3), 0.94 (3H, d, J = 6.2 Hz, 21-CH_3), 3.64 (1H, brm, 3β-H), 3.66 (3H, s, -$COOCH_3$). ^{13}C-NMR ($CDCl_3$) δ; 18.0 (C-18), 18.6 (C-21), 23.6 (C-19), 51.4 ($COOCH_3$), 127.0 (C-8), 141.5 (C-14), 174.7 (C-24). LR-MS (EI) *m/z*: 388 (M, 35%), 370 (M-H_2O, 100%), 355 (M-H_2O-CH_3, 78%), 316 (13%), 255 (M-H_2O-S.C., 40%), 229 (M-H_2O-S.C.-part of ring D, 23%), 215 (46%), 213 (M-H_2O-S.C.-ring D, 39%). HR-MS (EI), calculated for $C_{25}H_{40}O_3$, 388.2977; found *m/z*, 388.2993.

The most polar fraction was crystallized from aqueous ethanol to give the desired methyl 3α-hydroxy-5β-chol-14-en-24-oate (**6**) as colorless amorphous solids; yield, 92 mg (31%); mp 140–141°C [literature value (14) mp 140–141°C]. IR (KBr) $\nu_{max}cm^{-1}$; 1,740 (C=O), 3,048 (=C-H), 3,255 (OH). ^{1}H-NMR ($CDCl_3$) δ; 0.89 (3H, s, 18-CH_3), 0.92 (3H, d, J = 5.9 Hz, 21-CH_3), 0.93 (3H, s, 19-CH_3), 3.62 (1H, brm, 3β-H), 3.67 (3H, s, -$COOCH_3$), 5.15 (1H, brs, 15-H). ^{13}C-NMR ($CDCl_3$) δ; 16.8 (C-18), 18.5 (C-21), 23.1 (C-19), 51.5 ($COOCH_3$), 71.9 (C-3), 116.9 (C-15), 155.4 (C-14), 174.8 (C-24). LR-MS (EI) *m/z*: 388 (M, 9%), 370 (M-H_2O, 21%), 355 (M-H_2O-CH_3, 11%), 273 (M-S.C., 57%), 255 (M-H_2O-S.C., 100%), 208 (part of ring C+ring D+CH_3+S.C., 13%). HR-MS (EI), calculated for $C_{25}H_{40}O_3$, 388.2977; found *m/z*, 388.2961.

Synthesis of 3α,15α,24-trihydroxy-5β-cholane (7)

To a stirred solution of the Δ^{14} ester (**6**) (30 mg) in dry tetrahydrofuran (THF) (900 μl), a solution of BH_3/THF (1.0 mol/l, 390 μl) was added gradually, and the mixture was stirred at room temperature under a stream of N_2. After 5 h, 3 N NaOH (300 μl) and then 30% H_2O_2 (300 μl) were added with ice-bath cooling, and the mixture was stirred overnight at room temperature. The resulting solution was acidified with 10% HCl, and the reaction product was extracted with EtOAc. The combined EtOAc layers were washed with saturated brine and evaporated to dryness. The oily residue was chromatographed on a reverse-phase C_{18}-bonded silica gel (10 g; particle size, 50 μm). Elution with methanol-water (7:3, v/v) afforded the title compound **7**, which was recrystallized from EtOAc-hexane as colorless amorphous solids: yield, 21 mg (75%); mp 102–105°C. IR (KBr) $\nu_{max}cm^{-1}$; 3,340 (OH). ^{1}H-NMR ($CDCl_3$) δ; 0.68 (3H, s, 18-CH_3), 0.92 (3H, d, J = 7.0 Hz, 21-CH_3), 0.94 (3H, s, 19-CH_3), 3.61 (2H, t, J = 6.6

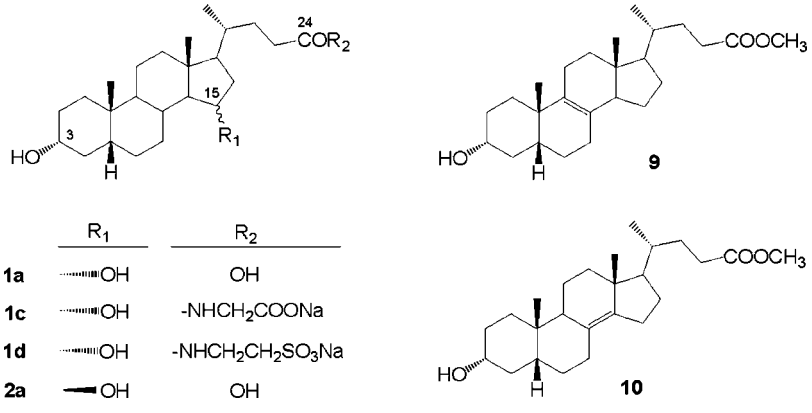

Fig. 3. Left: Chemical structures of epimeric 3α,15ξ-dihydroxy-5β-cholan-24-oic acids (**1a** and **2a**) and the glycine (**1c**) and taurine (**1d**) *N*-acylamidates (conjugates) of **1a**. Right: Chemical structures of methyl 3α-hydroxy-5β-chol-8(9)-en-24-oate (**9**) (above) and 3α-hydroxy-5β-chol-8(14)-en-24-oate (**10**) (below), side products formed by the attempted acid-catalyzed dehydration procedure of Yamasaki and colleagues (14, 22) on compound **5b**.

Hz, 24-H_2), 3.63 (1H, brm, 3β-H), 3.94 (1H, brm, 15β-H). ^{13}C-NMR ($CDCl_3$) δ; 13.3 (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-H_2O), 342 (96%, M-2H_2O), 327 (M-2H_2O-CH_3, 44%), 288 (M-H_2O-ring A, 30%), 273 (M-H_2O-S.C., 86%), 255 (M-2H_2O-S.C., 100%) 217 (ring A+B+C, 62%). HR-MS (FAB^+), calculated for $C_{24}H_{42}O_3Na$ [M+Na], 401.3032; found *m/z*, 401.3045.

Synthesis of 3,15-dioxo-5β-cholan-24-oic acid (8a) and its methyl ester (8b)

Jones reagent (500 μl) was added gradually to a solution of the 3α,15α,24-triol **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 C_{18}-bonded 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) $\nu_{max}cm^{-1}$; 1,705 (C=O, ketone), 1,736 (C=O, carboxyl group). ^{1}H-NMR ($CDCl_3$) δ; 0.78 (3H, s, 18-CH_3), 1.02 (3H, d, J = 5.9 Hz, 21-CH_3), 1.03 (3H, s, 19-CH_3). ^{13}C-NMR ($CDCl_3$) δ; 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%), 259 (M-S.C.-part of ring D, 48%), 232 (M-S.C.-ring D, 15%), 215 (16%), 197 (S.C.+ring D+CH_3, 100%). HR-MS (EI), calculated for $C_{24}H_{36}O_4$ [M], 388.2614; found *m/z*, 388.2587.

The free acid **8a**, reesterified with methanol and *p*-toluenesulfonic 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) $\nu_{max}cm^{-1}$; 1,713 (C=O, ketone), 1,735 (C=O, ester). ^{1}H-NMR ($CDCl_3$) δ; 0.78 (3H, s, 18-CH_3), 1.00 (3H, d, J = 6.2 Hz, 21-CH_3), 1.03 (3H, s, 19-CH_3), 3.67 (3H, s, $COOCH_3$). ^{13}C-NMR ($CDCl_3$) δ; 12.9 (C-18), 18.5 (C-21), 22.5 (C-19), 51.6 ($COO\underline{C}H_3$), 174.1 (C-24), 212.8 (C-3), 214.9 (C-15). LR-MS (EI) *m/z*: 402 (M, 45%), 387 (M-CH_3, 34%), 371 (20%) 329 (16%), 287 (M-S.C., 100%), 259 (M-S.C.-part of ring D, 54%), 211 (S.C.+CH_3+ring D, 33%). HR-MS (EI), calculated for $C_{25}H_{38}O_4$ [M^+], 402.2770; found *m/z*, 402.2762.

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

tert-Butylamineborane complex (30 mg) was added to a stirred solution of the 3,15-dioxo ester **8b** (30 mg) in CH_2Cl_2 (3 ml), and the mixture was refluxed overnight. After cooling the mixture, 10% HCl (900 μl) was added, and the solution was stirred for 30 min. The CH_2Cl_2 layer was washed with 5% $NaHCO_3$ 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,15β-dihydroxy-5β-cholanoate (**2b**), which recrystallized from aqueous methanol as colorless amorphous solids: yield, 17 mg (56%); mp 64–67°C. IR (KBr) $\nu_{max}cm^{-1}$; 1,739 (C=O), 3,409 (OH). ^{1}H-NMR ($CDCl_3$) δ; 0.92 (3H, d, J = 5.7 Hz, 21-CH_3), 0.93 (3H, s, 19-CH_3), 0.96 (3H, s, 18-CH_3), 3.64 (1H, brm, 3β-H) 3.67 (3H, m, -$COOCH_3$), 4.19 (1H, t, J = 5.7 Hz, 15α-H). ^{13}C-NMR ($CDCl_3$) δ; 14.6 (C-18), 18.2 (C-21), 23.3 (C-19), 51.5 ($COO\underline{C}H_3$), 70.2 (C-15), 71.7 (C-3), 174.6 (C-24). LR-MS (EI) *m/z*: 388 (M-H_2O, 59%), 370 (M-2H_2O, 42%), 355 (M-2H_2O-CH_3, 22%), 273 (M-H_2O-S.C., 34%), 255 (M-2H_2O-S.C., 33%), 217 (ring A+B+C, 43%), 208 (100%). HR-MS (EI), calculated for $C_{25}H_{40}O_3$, 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 attempts: yield, 10 mg (33%). IR (KBr) $\nu_{max}cm^{-1}$; 1,737 (C=O), 3,350 (OH). ^{1}H-NMR ($CDCl_3$) δ; 0.68 (3H, s, 18-CH_3), 0.90 (3H, d, J = 5.7 Hz, 21-CH_3), 0.93 (3H, s, 19-CH_3), 3.62 (1H, brm, 3β-H), 3.66 (3H, m, -$COOCH_3$), 3.94 (1H, brm, 15β-H). ^{13}C-NMR ($CDCl_3$) δ; 13.3 (C-18), 18.1 (C-21), 23.4 (C-19), 51.5 ($COO\underline{C}H_3$), 71.7 (C-3), 73.6 (C-15), 174.6 (C-24). LR-MS (EI) *m/z*: 388 (M-H_2O, 72%), 370 (M-2H_2O, 42%), 355 (M-2H_2O-CH_3, 50%), 273 (M-H_2O-S.C., 98%), 255 (M-2H_2O-S.C., 88%), 217 (ring A+B+C, 41%), 208 (100%). HR-MS (EI), calculated for $C_{25}H_{40}O_3$, 388.2977 [M]; found *m/z*, 388.2969.

Synthesis of 3α,15α-dihydroxy-5β-cholan-24-oic acid (1a) and its 15β-epimer (2a)

The 3α,15α-dihydroxy ester **1b** (50 mg) was refluxed in 5% methanolic KOH (1 ml) for 1 h. Solvent was removed by evaporation, and the residue was dissolved in water and acidified with 10% H_2SO_4 with stirring and ice-bath cooling. The precipitate was collected by filtration, washed with water, and recrystallized from EtOAc as colorless amorphous solids of 3α,15α-dihydroxy acid **1a** (analytically pure): yield, 46 mg (95%); mp 206–208°C. IR (KBr) $\nu_{max}cm^{-1}$; 1,703 (C=O), 3,245 (OH). ^{1}H NMR (CD_3OD) δ; 0.71 (3H, s, 18-CH_3), 0.94 (3H, d, J = 5.9 Hz, 21-CH_3), 0.96 (3H, s, 19-CH_3), 3.54 (1H, brm, 3β-H), 3.84 (1H, brm, 15β-H). ^{13}C NMR (CD_3OD) δ; 13.7 (C-18), 18.6 (C-21), 21.8 (C-11), 24.0 (C-19), 27.6 (C-6), 28.3 (C-7), 31.2 (C-2), 31.9 (C-22), 32.2 (C-23), 35.7 (C-10), 36.1 (C-20), 36.6 (C-1), 36.9 (C-8), 37.2 (C-4), 41.6 (C-16), 41.7 (C-12), 42.0 (C-5), 43.5 (C-9) 44.9 (C-13), 54.8 (C-17), 64.0 (C-14), 72.4 (C-3), 74.2 (C-5) 178.0 (C-24). LR-MS (EI) *m/z*: 374 (M-H_2O, 36%), 356 (M-2H_2O, 100%), 341 (M-2H_2O-CH_3, 43%), 302 (M-H_2O-ring A, 32%), 273 (M-H_2O-S.C, 48%), 255 (M-2H_2O-S.C., 60%), 217 (ring A+B+C, 51%). HR-MS (FAB^+), calculated for $C_{24}H_{40}O_4Na$, 415.2824 [M+Na]; found *m/z*, 415.2788.

The 3α,15β-dihydroxy ester **2b** (50 mg) was hydrolyzed with 5% methanolic KOH (1 ml) and processed as described for the preparation of **1a** to yield the crude acid. Recrystallization from EtOAc gave the 3α,15β-dihydroxy acid **2a** as colorless amorphous solids: yield, 44 mg (91%); mp 119–121°C [literature value (17) mp 185.5–186.5°C]. IR (KBr) $\nu_{max}cm^{-1}$; 1,710 (C=O), 3,321 (OH). ^{1}H NMR (CD_3OD) δ; 0.94 (3H, s, 18-CH_3), 0.95 (3H, d, J = 7.6 Hz, 21-CH_3), 0.98 (3H, s, 19-CH_3), 3.53 (1H, brm, 3β-H), 4.16 (1H, brm, 15α-H). ^{13}C NMR (CD_3OD) δ ; 15.2 (C-18), 18.8 (C-21), 21.8 (C-11), 24.0 (C-19), 26.8 (C-7), 28.3 (C-6), 31.2 (C-16), 32.0 (C-23), 32.3 (C-2), 33.0 (C-20), 35.9 (C-10), 36.4 (C-8), 36.6 (C-22), 37.3 (C-1), 42.1 (C-4), 42.2 (C-9), 42.9 (C-12), 43.6 (C-5 and C-13), 57.6 (C-17), 62.4 (C-14), 70.6 (C-15), 72.5 (C-3), 178.2 (C-24). LR-MS (FAB) *m/z*: 374 (M-H_2O, 39%), 356 (M-2H_2O, 54%), 341 (M-2H_2O-CH_3, 27%), 302 (M-H_2O-ring A, 11%), 273 (M-H_2O-S.C., 34%), 263 (38%), 255 (M-2H_2O-S.C., 54%), 217 (M-H_2O-S.C.-ring D, 100%). HR-MS (FAB^+), calculated for $C_{24}H_{40}O_4Na$, 415.2824 [M+Na]; found *m/z*, 415.2802.

Synthesis of 3α,15α-dihydroxy-5β-cholan-24-oyl 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), diethylphosphorocyanide (30 μl), and triethylamine (125 μl) were added, and the mixture was stirred at room temperature for 1 h. The reaction mixture 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 tC_{18} 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 glycine conjugate of the title compound (**1c**), after the solvent was evaporated under reduced pressure. The residue was recrystallized from methanol-Et_2O in the form of colorless amorphous solids: yield, 22 mg (61%); mp 170–173°C. IR (KBr) $\nu_{max}cm^{-1}$; 1,593 (C=O), 3,394 (OH). ^{1}H-NMR (CD_3OD) δ; 0.71 (3H, s, 18-CH_3), 0.96 (3H, d, J = 5.4 Hz, 21-CH_3), 0.96 (3H, s, 19-CH_3), 3.53 (1H, brm, 3β-H), 3.74 (2H, s, -$NHCH_2$-) 3.84 (1H, brm, 15β-H). ^{13}C-NMR (CD_3OD) 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 (-CH_2N-), 44.9 (C-13), 54.9 (C-17), 64.1 (C-14), 72.5 (C-3), 74.2 (C-15), 176.2, 176.3 (C-24, -COONa). LR-MS (FAB^+) *m/z*: 494 (M+Na, 100%). HR-MS (FAB^+), calculated for $C_{26}H_{42}O_5NNa_2$, 494.2858 [M+Na]; found *m/z*, 494.2832.

Synthesis of 3α,15α-dihydroxy-5β-cholan-24-oyl taurine (1d; sodium salt)

A suspension of **1a** (50 mg), powdered taurine (40 mg), diethylphosphorocyanide (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 tC_{18} 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-Et_2O as colorless amorphous solids of **1d**: yield, 40 mg (59%); mp 157–160°C. IR (KBr) $\nu_{max}cm^{-1}$; 1,636 (C=O), 3,421 (OH). LR-MS (FAB^+) *m/z*: 544 (M+Na, 100%). HR-MS (FAB^+), calculated for $C_{26}H_{44}O_6NSNa_2$, 544.2685 [M+Na]; found *m/z*, 544.2665.

RESULTS AND DISCUSSION

Synthesis of 15α-hydroxylithocholic acid

Figure 1 shows a representative HPLC-evaporative light-scattering detection chromatogram of the bile acid composition in the gallbladder bile of the wombat. Identification of the major peaks was made by a direct comparison with authentic reference compounds available in our laboratory. The largest peak, designated D (78% of total bile acids), was proven to be taurine-conjugated CDCA. The structure of the second most abundant component, peak C (11%), was tentatively identified as 15ξ-hydroxylithocholyl taurine (3α,15ξ-dihydroxy-5β-cholan-24-oyl taurine), based on the HPLC and LC-MS data (see below). Wombat bile also contained minor components labeled A, B, E, F, and G (total amounts, less than ~11%). Peak G (2.5%) was identified as taurine-conjugated lithocholic acid. LC-ESI-MS under negative ion mode exhibited a signal arising from a deprotonated molecule $[M-H]^-$ at *m/z* 498 for peak A, *m/z* 496 for peak B, *m/z* 498 for peak E, and *m/z* 480 for peak F. However, definitive identification of the minor conjugated bile acids present in wombat bile is beyond the scope of the present paper.

Identification of 15α-hydroxylithocholic acid (3α,15α-dihydroxy-5β-cholan-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,15ξ-dihydroxy-5β-cholan-24-oic acids (**1a** and **2a**) and the glycine (**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-Δ^{14}-5β-cholenoate (**6**). To prepare this compound, the 3α-cathyloxy-7α-hydroxy ester **3**, prepared from CDCA (14), was converted to the 3α-cathyloxy-Δ^7 ester **4** by an elimination reaction with phosphoryl chloride. Subsequent alkaline hydrolysis of **4**, followed by the usual esterification of the resulting 3α-hydroxy-Δ^7 acid **5a**, gave the corresponding 3α-hydroxy-Δ^7 ester **5b.**

The most promising method reported in the literature for the preparation of the unsaturated Δ^{14} 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 $\Delta^{8(14)}$ 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, Δ^8 (**9**), $\Delta^{8(14)}$ (**10**), and Δ^{14} (**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% $AgNO_3$-impregnated silica gels (15); compounds were eluted in the order Δ^8 before $\Delta^{8(14)}$ before Δ^{14} isomer. The individual isomers were distinguished by their ^{1}H- and ^{13}C-NMR spectra. The ^{13}C-NMR spectra revealed that compounds **9** and **10** had a tetrasubstituted double bond, whereas compound **6** was trisubstituted. The chemical shifts of ^{13}C signals in **6** and **10** were in good agreement with those reported in the literature (23). The remaining tetrasubstituted alkene **9**, there-

fore, was deduced to have a double bond between C-8 and C-9 in the 5β-steroid nucleus. The ^{13}C 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-Δ^{14} ester **6** with B_2H_6/THF and subsequent treatment of the resulting alkylborane with H_2O_2/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*-butylamineborane complex yielded a mixture of 3α,15α-dihydroxy (**1b**) and 3α,15β-dihydroxy (**2b**) esters. The reduction was stereoselective, 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 ^{1}H- and ^{13}C-NMR. In the ^{1}H-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-1,3-diaxial interaction between the 18-methyl and 15β-hydroxyl 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 ^{1}H-NMR data are consistent with those reported for epimeric 3α,7α,15ξ-trihydroxy bile acids (25) and a 3α,12α,15β-trihydroxy analog (21). On the other hand, the ^{13}C-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-cholanoic 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 ^{13}C-NMR spectral data are completely consistent with each other. Polymorphism in crystal habit is well known for C_{24} bile acids (26).

Synthesis of the taurine and glycine conjugates of 15α-hydroxylithocholic acid

N-Acylamidation of the C-24 carboxyl group in **1a** with glycine methyl ester hydrochloride or freshly powdered taurine was effectively attained using diethyl phosphocyanide 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 taurine (**1d**) conjugates (as the sodium salts) were recovered efficiently by applying a reverse-phase prepacked cartridge (Sep-Pak Vac tC_{18}) 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* 480, 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 ^{1}H-NMR spectra of isolated compound C, and **Table 1** compiles the ^{1}H and ^{13}C signal assignments of compound C and **1d**. The ^{1}H- and ^{13}C-NMR spectral patterns of both compounds were essentially identical. Particularly noteworthy were signals occurring at 2.97 ppm (t, J = 6.4 Hz, -C$\underline{H}_2$S-) and 3.60 ppm (t, J = 6.4 Hz, -C$\underline{H}_2$N-) by ^{1}H-NMR, indicating the presence of a taurine-conjugate moiety (2, 27). As mentioned above, the ^{1}H 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-hydroxylithocholyl taurine.

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 taurine conjugate of CDCA, the major bile acid of the animal, undergoes bacterial deconjugation and 7-dehydroxylation to form lithocholic

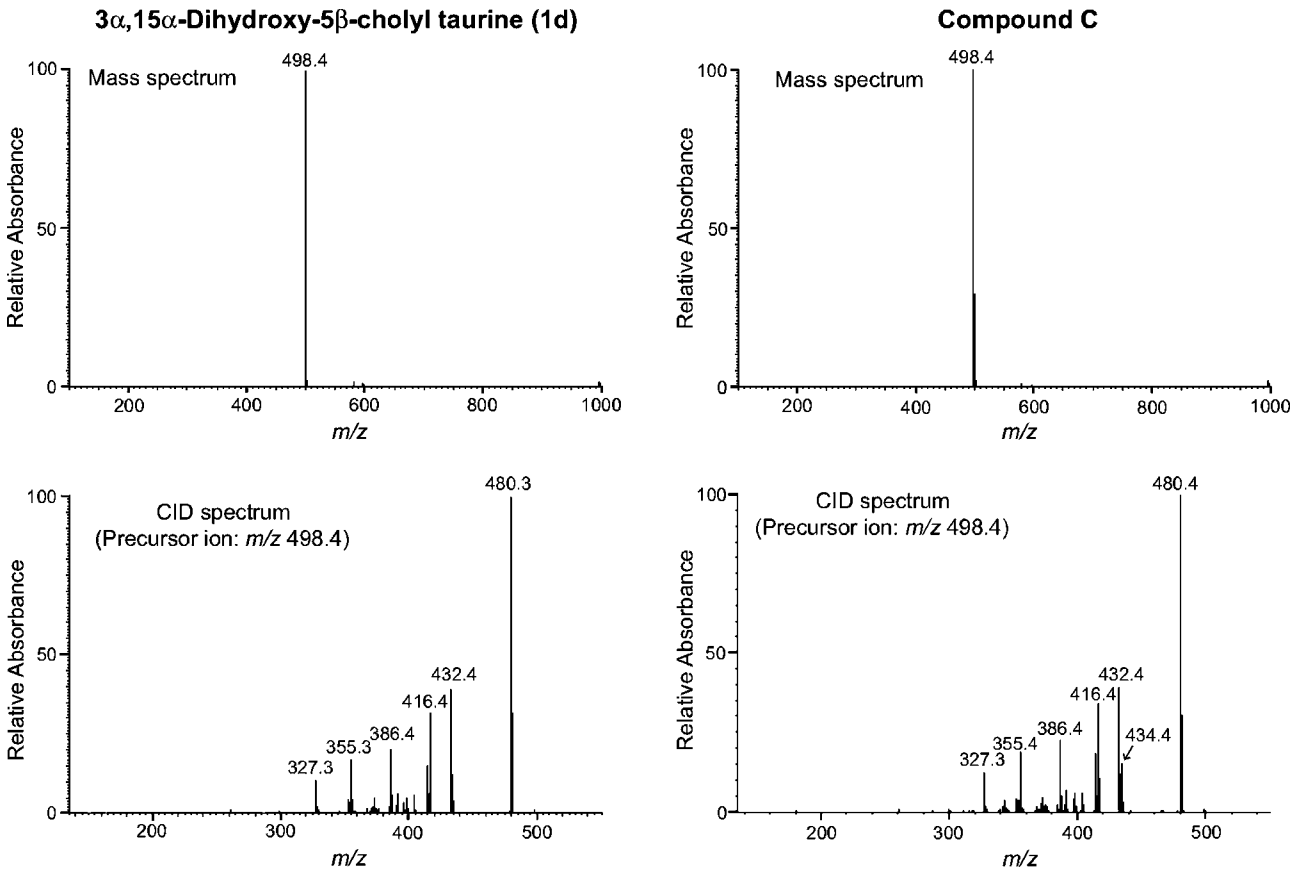

Fig. 4. Negative ion LC-ESI-MS/MS fragmentation pattern of synthetic **1d** (left) and compound C (right). CID, collision-induced dissociation.

acid (31). 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),

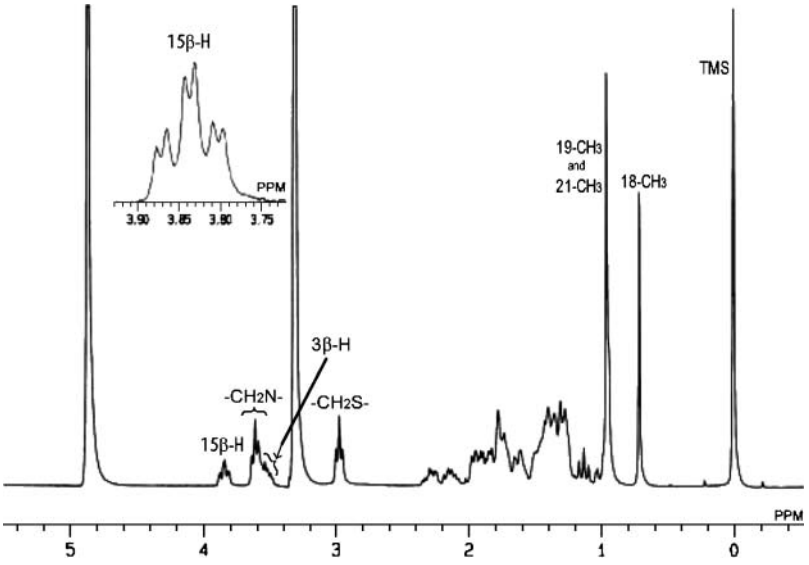

Fig. 5. ^{1}H-NMR spectrum of compound C isolated from wombat bile. TMS, tetramethylsilane.

TABLE 1. ^{1}H- and ^{13}C-NMR data for synthetic **1d** and compound C

		Synthetic **1d**		Compound C	
No.	Type	^{13}C	^{1}H	^{13}C	^{1}H
1	CH_2	36.84[a]		36.96[a]	
2	CH_2	31.21		31.23	
3	CH	72.47	3.53 (brm)	72.49	3.53 (brm)
4	CH_2	37.19		37.21	
5	CH	43.52		43.56	
6	CH_2	28.32		28.33	
7	CH	27.65		27.67	
8	CH	36.94		36.96	
9	CH	41.96		41.97	
10	C	35.72		35.74	
11	CH_2	21.81		21.83	
12	CH	41.71[b]		41.72[b]	
13	C	44.92		44.94	
14	CH	64.02		64.04	
15	CH	74.18	3.84 (m)	74.19	3.83 (m)
16	CH_2	41.62[b]		41.63[b]	
17	CH	54.82		54.84	
18	CH_3	13.67	0.71 (s)	13.69	0.71 (s)
19	CH_3	43.52	0.96 (s)	24.02	0.96 (s)
20	CH	36.31		36.33	
21	CH_3	18.7	0.95 (d)	18.70	0.95 (d)
22	CH_2	33.01		33.01	
23	CH_2	33.91		33.94	
24	C	176.74		176.80	
25	CH_2N	36.57[a]	3.62 (t, 6.7Hz)	36.58[a]	3.60 (t, 6.4Hz)
26	CH_2S	51.3	2.98 (t, 6.5Hz)	51.35	2.97 (t, 6.4Hz)

brm, broad multiplet; d, doublet; dd, double doublet; m, multiplet; s, singlet; t, triplet.
[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 toxicity of lithocholic acid when its precursor, CDCA, is administered chronically (46–49).

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 geese, 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 pregnanoic 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 15-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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