3,4-Seco-12 α-hydroxy-5 β-cholan-3,4,24-trioic Acid, a Novel Secondary Bile Acid: Isolation from the Bile of the Common Ringtail Possum (Pseudocheirus peregrinus) and Chemical Synthesis

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Abstract

The major bile acids present in gallbladder bile of the common ringtail possum (Pseudocheirus peregrinus), an Australian marsupial, were isolated by preparative HPLC and identified by NMR and by comparison with synthetic standards. The major compound present (52%) was 3α,12α-dihydroxy-7-oxo-5β-cholan-24-oic acid (7-oxodeoxycholic acid), about three fourths conjugated with taurine. Also present was 3α,7β,12α-trihydroxy-5β-cholan-24-oic acid (20%; ursodeoxycholic acid) largely in unconjugated form. In addition, 3,4-seco-12α-hydroxy-5β-cholan-3,4,24-trioic acid was present in unconjugated form and constituted 8% of biliary bile acids. Proof of the structure of this novel 3,4-seco acid was obtained by its chemical synthesis from deoxycholic acid via an intermediary 3β,4β-dihydroxy derivative that was then oxidatively cleaved with sodium periodate. As all primary bile acids have a hydroxyl or oxo substituent at C-7, the absence of such in the seco-bile acid suggests that it is a secondary bile acid, synthesized by bacterial enzymes present in the intestine.

Keywords: Common ringtail possum; Secondary bile acids; 3α,7β,12α-Trihydroxy-5β-cholan-24-oic acid; 3α,12α-Dihydroxy-7-oxo-5β-cholan-24-oic acid; 3,4-Seco-12α-hydroxy-5β-cholan-3,4,24-trioic acid; Bile acid metabolism

Introduction

Bile acids (C24 and C27) and bile alcohols (C27) are the end products of cholesterol metabolism that have multiple physiological functions. After their synthesis bile acids and bile alcohols are made water soluble by “conjugation” with glycine or taurine for bile acids and with sulfate for bile alcohols. In the liver, bile acids stimulate bile flow and solubilize biliary cholesterol. In the small intestine, bile acids solubilize dietary lipids, and in the large intestine, modulate water and electrolyte movement [1,2]. In addition, in the past decade, bile acids have been shown to also possess potent and important signaling properties [3]. Bile acids modulate the expression of multiple genes via the nuclear receptor FXR (farnesoid X receptor) that is activated by ligands employed were of analytical reagent grade. The authentic standards that were used to clarify the structures of the natural bile acids are summarized in Figure 2. Evidence for the assignments of each of the structures is presented in the text. Conjugated 3α,7β,12α-trihydroxy-5β-cholan-24-oic acid [8] and 3α,12α-dihydroxy-7-oxo-5β-cholan-24-oic acid [9] were conjugated with taurine as described previously [7]. All other chemicals and reagents employed were of analytical reagent grade.

Experimental Procedures

Biological material

A bile sample from the common ringtail possum was obtained at necropsy by the Pathology Department of the Zoological Society of San Diego (CA, USA). So far, all attempts to obtain a bile sample from a second possum have failed, but in our experience, biliary bile acid composition is generally quite similar in animals of a given species. The bile was dispersed in 4 volumes of reagent grade 2-propanol and stored at refrigerator temperature until analysis.

Materials and reagents

The authentic standards that were used to clarify the structures of the natural bile acids are summarized in Figure 2. Evidence for the assignments of each of the structures is presented in the text. Conjugated 3α,7β,12α-trihydroxy-5β-cholan-24-oic acid [8] and 3α,12α-dihydroxy-7-oxo-5β-cholan-24-oic acid [9] were conjugated with taurine as described previously [7]. All other chemicals and reagents employed were of analytical reagent grade.

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HPLC-ELSD analysis of gallbladder bile of the common ringtail possum

The Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC) apparatus used was a Jasco LC-2000 plus HPLC system, which consisted of two PU-2085 high-pressure pumps, an MX-2080-32 solvent mixing module, a DG-980 degasser, and a CO-2060 column heater with a ChromNAV data processing system (Tokyo, Japan). A Capcell Pack type C18 AQ RP-column (3.0 mm x 150 mm I.D.; particle size, 5 μm; Shiseido, Tokyo, Japan) was employed and kept at 37°C. An Alltech 2000ES Evaporative Light-Scattering Detector (ELSD; Deerfield, IL, USA) was used under the following conditions: The flow rate of purified compressed air used as a nebulizing gas was 2.2 L/min and the temperature of the heated drift was 80.9°C. The mobile phase used was a mixture of 15 mM-ammonium acetate/acetic acid buffer solution (pH 5.0), methanol (38:62, v/v); the flow rate was kept at isocratic conditions of 0.4 mL/min during the analysis.

Isolation of major biliary bile acids of common ringtail possum by preparative HPLC

The isopropanol solution of the common ringtail possum was evaporated under a stream of N2, and the residue was dissolved in water (1.5 mL). The aqueous solution was centrifuged for 10 min at 2000 rpm, and the supernatant solution was recovered; the procedure was repeated three times for the residue. The total volume of the combined supernatant solution (4.5 mL) was adjusted to 15 mL by diluting with water. The solution was passed through a preconditioned Sep-Pak tC18 cartridge (5 g; Waters, Milford, MA, USA). After the cartridge was washed with successively with water (50 mL) and then with 15 mM ammonium acetate/acetic acid buffer solution (pH 5.0) containing methanol (50 mL; 10%,v/v), and the resulting mixture was stirred overnight at room temperature. The reaction mixture was adjusted to pH 12 with 10% NaOH and then to pH 8 with 10% HCl. The solution was diluted with water (18 mL) and passed through a preconditioned Sep-Pak tC18E cartridge (10 g). The isocratic elution mode was used over a mixed solvent system of water, followed by vacuum freeze-drying. Figure 3 shows the HPLC-ELSD result that was obtained; the identities of individual peaks A ~ H were as follows: Rf value, -2.0 kV; ion source temperature, 80°C; desolvating plate temperature, 250°C; absolute ring-lens voltage, -15 V; mass range, m/z 50-1000; nebulizing gas, nitrogen gas (N2).

1H and 13C NMR analysis of isolated compounds

NMR spectra were recorded at 23°C in CDCl3 or pyridine-d4 on a JEOL ECA-500 instrument using 500.2 MHz for 1H and 125.8 MHz for 13C. The 1H and 13C resonance assignments were made using a combination of Two-Dimensional (2D) homonuclear (1H-1H) and heteronuclear (1H-13C) shift-correlated techniques, which include 1H-1H COSY correlation, 1H Nuclear Overhauser And Exchange Spectroscopy (NOESY), 1H detected heteronuclear multiple quantum coherence (HMOC; 1H-13C coupling), and 13C detected heteronuclear multiple bond connectivity (HMBC; long-range 1H-13C coupling) experiments. These 2D-NMR spectra were recorded using standard pulse sequences and parameters recommended by the manufacturer. The 13C distortion less enhancement by polarization transfer (DEPT; 135°, 90°, and 45°) spectra were also measured to determine the exact 13C signal multiplicity and to differentiate between CH2, CH, and C based on their proton environments.

Synthesis of reference standards

Chemical synthesis of 3a,7β,12α-trihydroxy-5β-cholan-24-oyl taurine: To a magnetically stirred solution of unconjugated 3α,7β,12α-trihydroxy-5β-cholan-24-oic acid (10.4 mg, 25 μmol) [8] in N,N-dimethylformamide (2 mL) was added successively taurine (80 μmol), 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride hydroxide (DMT-MM; 40 μmol) and trimethylamine (50 μL). The resulting mixture was stirred overnight at room temperature. The reaction mixture was adjusted to pH 12 with 10% NaOH and then to pH 8 with 10% HCl. The solution was diluted with water (18 mL) and passed through a preconditioned Sep-Pak tC18E cartridge (5 g). After the cartridge was washed with water (50 mL), the desired taurine-conjugated bile acid was eluted with 50% aqueous methanol. After evaporation of the solvent, the residue was redissolved in methanol and combined supernatant liquids were filtered with a Mini-Uni Pre membrane filter (pore size, 0.45 μm; Whatman, NJ, USA).

Individual, major bile acids were isolated by preparative HPLC, which consisted of a Hitachi L-7100 pump, a Refraction Index (RI)-102 detectors, and a type 30V column heater. For simultaneous separation of unconjugated and glycine- and taurine-amidated bile acids, RP-HPLC separation was carried out by isocratic elution modes on a Capcell Pak type C18 AQ RP-column (10 mm x 250 mm I.D.; particle size, 5 μm) using a mixture of 15 mM ammonium acetate/acetic acid buffer solution (pH 5.0) and methanol (35:65, v/v) as the mobile phase at a flow rate of 3.0 mL/min. The 65% methanol fractions, which contained each of the compounds A, B, C, D, and F, were collected by evaporation of the solvent from each of the fractions, the residues were dried by lyophilization. The residues were then dissolved in methanol and combined supernatant liquids were filtered with a Mini-Uni Pre membrane filter (pore size, 0.45 μm; Whatman, NJ, USA).

HR-LC/ESI-MS analysis of isolated compounds

High-resolution liquid chromatography-mass spectra with an electrospray ionization (HR-LC/MS) were carried out using a JEOL Accu TOF LC-plus liquid chromatography-mass spectrometer equipped with an ESI source and coupled to an Agilent 1200 series binary pump (Agilent Technologies Inc., Santa Clara, CA, USA) operated in the negative mode. HR-LC/ESI-MS of isolated compounds were carried out in the following injection mode, using a mixture of 5 mM ammonium acetate/acetic acid buffer solution (pH 4.4) and methanol (33:67, v/v) on a Capcell Pak type AQ RP-column (150 mm x 3.0 mm I.D.; particle size, 3 μm); the flow rate was kept at isocratic conditions of 0.2 mL/min during the analysis. The ionization conditions were as follows: Rf value, -2.0 kV; ion source temperature, 80°C; desolvating plate temperature, 250°C; absolute ring-lens voltage, -15 V; mass range, m/z 50-1000; nebulizing gas, nitrogen gas (N2).
Chemical synthesis of 3α,12α-dihydroxy-7-oxo-5β-cholan-24-oic acid: 3α,12α-Dihydroxy-7-oxo-5β-cholan-24-oic acid (10.3 mg, 25 μmol) [9] was converted to its taurine conjugate by the method as described for the preparation of 3α,7β,12α-trihydroxy-5β-cholan-24-oic acid: m.p., 212-214°C (colorless amorphous solid from methanol-EtOAc); yield, 11 mg (82%). 1H-NMR (pyridine-d5) δ: 0.64 (3H, s, 18-CH3), 1.06 (3H, d, J=6.3 Hz, 21-CH3), 1.11 (3H, s, 19-CH3), 3.42 (2H, m, -CH2SO3H), 3.70 (1H, brm, 3β-H), 4.10-4.15 (3H, m, 12β-H and -NHCH2-). HR-LC/ESI-MS, calculated for C26H42NO7S, 512.2682 [M-H]-; found, m/z 512.2678.

Chemical synthesis of 3,4-seco-12α-acetoxy-5β-cholan-3,4,24-trioic acid 24-methyl ester (3): The synthetic scheme used to prepare 3,4-seco-12α-hydroxy-5β-cholan-3,4,24-trioic acid (1), starting from deoxycholic acid (3α,12α-dihydroxy-5β-cholan-24-oic acid; DCA) via methyl 3β,4β-dihydroxy-12α-acetoxy-5β-cholan-24-oate (2) is shown in Figure 4.

To a magnetically stirred solution of compound 2 (31 mg, 55 μmol), prepared from DCA in 4 steps [10], in acetone (2 mL) was added a solution of sodium periodate (NaIO4, 50 mg) dissolved in water (1.5 mL). After the mixture was stirred at room temperature for 2 h, the reaction product was extracted with EtOAc. The combined extract was washed with water to neutrality, dried with Drierite, and evaporated to an oily residue. To a solution of the residue dissolved in acetone (2 mL) was added three drops of Jones reagent, and the mixture was stirred at room temperature for 30 min. After adding a few drops of 2-propanol, the reaction product was extracted with CH2Cl2. The combined organic layer was washed with water, dried with Drierite, and evaporated to give an oily residue. Chromatography of the residue on a column of silica gel (1.0 g) and elution with hexane-EtOAc-acetic acid (150:50:1, v/v/v) afforded the title compound (3) which recrystallized from EtOAc-hexane as colorless amorphous crystals: m.p., 145-148°C; yield, 19 mg (58%). 1H-NMR (pyridine-d5) δ: 0.64 (3H, s, 18-CH3), 0.81 (3H, d, J=5.7 Hz, 21-CH3), 0.94 (3H, s, 19-CH3), 1.91 (3H, s, -OCOCH3), 3.58 (3H, s, -COOCH3), 5.21 (1H, m, 12β-H). HR-LC/ESI-MS, calculated for C27H41O8, 493.2801 [M-H]-; found, m/z 493.2796.

Chemical synthesis of 3,4-seco-12α-hydroxy-5β-cholan-3,4,24-trioic acid (1): A solution of compound 3 (19 mg, 31 μmol) in 10% methanolic KOH (2 mL) was refluxed for 1 h. Most of the solvent was evaporated under reduced pressure, and the residue was dissolved in water and then acidified with 10% H2SO4 with ice-bath cooling. The precipitated solid was filtered, washed with water, and dried. The crude product was recrystallized from methanol as colorless amorphous crystals. M.p., 217-220°C; yield, 11 mg (65%). 1H-NMR (pyridine-d5) δ: 0.71 (3H, s, 18-CH3), 0.99 (3H, s, 19-CH3), 1.17 (3H, d, J=6.3 Hz, 21-CH3), 4.16 (1H, m, 12β-H). HR-LC/ESI-MS, calculated for C24H37O7, 437.2539 [M-H]-; found, m/z 437.2532.
Results

Biliary bile acid composition

Figure 3 shows a representative HPLC-ELSD chromatogram of the bile acid composition in the gallbladder bile of the common ringtail possum. Table 1 gives the RRTs of each peak (A ~ H) observed using HPLC-ELSD as well as their HR-LC/ESI-MS data. Identification of the major peaks was made by a direct comparison with authentic reference compounds prepared in our laboratory. Thus, peak E (16.5% of total bile acids) was found to be cholyl taurine (3α,7α,12α-trihydroxy-5β-cholan-24-oyl taurine); peak G (2.4%) was unconjugated cholic acid (3α,7α,12α-trihydroxy-5β-cholan-24-oic acid; CA); and peak H (1.3%) was chenodeoxycholyl taurine (3α,7α-dihydroxy-5β-cholan-24-oyl taurine).

The remaining compounds A ~ D and F were each isolated as a single peak by preparative HPLC-RI, and their HR-LC/ESI-MS m/z values were measured. Peaks A (m/z 514.2844) gave the deprotonated molecular ion [M-H] - of C24H37O5 corresponding to a C24 trihydroxy-taurine-conjugated bile acid. Peak C (m/z 407.2828) and 13C NMR signal patterns of the synthetic trioic acid (1) were in good agreement with those of compound F isolated from a mixture of the biliary bile acids of the common ringtail possum. Table 2 shows the 1H and 13C NMR spectral data for naturally occurring compounds as well as that of synthetic 3,4-seco-12α-dihydroxy-5β-cholan-3,4,24-trioic acid (1). The 'H and 13C NMR spectral patterns of both the compounds were essentially identical. Thus, the 18-, 19-, and 21-CH signals in the both 'H NMR spectra were observed at 0.71 (singlet; s), 0.99 (s) and 1.17 (doublet) ppm, along with the 12β-H at 4.16 ppm (multiplet). Furthermore, these compounds showed three characteristic signals arising from carboxyl groups at 176.4, 176.5 and 177.2 ppm and at 72.3 ppm due to the 12β-H bearing a 12α-hydroxyl group in the 13C NMR spectra.

In order to determine the position of the three carboxyl groups in compound F, the HMBC spectrum was measured as shown in Figure 5. The three peaks occurred at 176.4, 176.5, and 177.2 ppm were correlated with 1α-/1β-H2. Similar couplings were also observed between the 5β-H and 3β-H and 2α-/2β-H2 or the 5β-H and 4α-/4β-H2. The remaining penultimate carboxyl group in the 13C NMR spectra.

The structure of the unknown compound F (7.9%) was then subjected to further analysis. By HR-LC/ESI-MS analysis, peak F showed m/z 437.2539, indicating the deprotonated molecule ion [M-H] - of C24H37O5. This observation strongly suggested that the unknown F was a novel bile acid having three carboxyl groups, i.e., a seco bile acid. Table 2 shows the 1H and 13C NMR spectral data for naturally occurring compound F as well as that of synthetic 3,4-seco-12α-dihydroxy-5β-cholan-3,4,24-trioic acid (1). The 'H and 13C NMR spectral patterns of both the compounds were essentially identical. Thus, the 18-, 19-, and 21-CH signals in the both 'H NMR spectra were observed at 0.71 (singlet; s), 0.99 (s) and 1.17 (doublet) ppm, along with the 12β-H at 4.16 ppm (multiplet). Furthermore, these compounds showed three chemical shifts arising from carboxyl groups at 176.4, 176.5 and 177.2 ppm and at 72.3 ppm due to the 12β-H bearing a 12α-hydroxyl group in the 13C NMR spectra.

Discussion

Chemical aspects

Chemical synthesis of an authentic sample of 3,4-seco-12α-hydroxy-5β-cholan-3,4,24-trioic acid (1) was attained in 2 steps starting from methyl 3β,4β-dihydroxy-12α-acetoxy-5β-cholanoate (2), which was obtained in 4 steps from deoxycholic acid (DCA) (Figure 4) [10]. Oxidative cleavage of compound 2 with sodium periodate [11,12] and subsequent treatment of the resulting product with Jones reagent resulted in simultaneous dicarboxylation at C-3 and C-4 to give 3,4-seco-12α-acetoxy-5β-cholan-3,4,24-trioic acid 24-methyl ester (3). Alkaline hydrolysis of the 3 followed by acidification afforded the desired 3,4,24-trioic acid (1). The RRT on the HPLC as well as the 1H and 13C NMR signal patterns of the synthetic trioic acid (1) were in good agreement with those of compound F isolated from a mixture of the biliary bile acids of the common ringtail possum.
Our study shows that biliary bile acids in the common ringtail possum differ from those of most mammals in at least two ways (Table 3). First, a seco-bile acid was present. As all primary bile acids have a hydroxyl- or oxo-substituent at C-7, it is likely that the seco-bile acid is a secondary bile acid, formed by bacterial enzymes from Deoxycholic Acid (DCA) in the intestine. Second, the majority of bile acids (80%) appear to be secondary bile acids that have been generated from primary bile acids by bacterial enzymes.

The intermediates in the formation of the seco-bile acid are unknown. The four rings of the bile acid structural platform are generally considered to be stable in vertebrates. However, environmental bacteria have at least two pathways for opening the B ring [14]. We can speculate that the opening of the A ring occurs by an enzymatic pathway that is coprophagic, producing two types of feces, one of which is eaten (see below). This behavioral characteristic is also observed in rabbits and both genera have biliary bile acids that are predominantly secondary.

Table 3: Retention time (min), percentage composition (%), observed HR-LC/ESI-MS (M+H) data, calculated mass data, and estimated structure.

| Peak | Retention time (min) | Percentage composition (%) | Observed HR-LC/ESI-MS (M+H) data | Calculated mass data | Estimated structure |
|------|---------------------|----------------------------|-----------------------------------|---------------------|-------------------|
| A    | 4.8                 | 2.1                        | 514.2844                          | 514.2839            | C₃H₅NO₅S         |
| B    | 5.5                 | 38.9                       | 512.2656                          | 512.2682            | C₃H₅NO₅S         |
| C    | 7.0                 | 18.2                       | 407.2828                          | 407.2798            | C₃H₅O             |
| D    | 9.2                 | 12.7                       | 405.2625                          | 405.2641            | C₃H₅O             |
| E    | 11.7                | 16.5                       | 514.2839                          | C₃H₅NO₅S            | C₃H₅O             |
| F    | 13.6                | 7.9                        | 437.2539                          | C₃H₅O              |
| G    | 21.8                | 2.4                        | 407.2803                          | 407.2798            | C₃H₅O             |
| H    | 22.5                | 1.3                        | 498.2998                          | 498.2889            | C₃H₅O             |

*Measured on a capcell pak type C₁₈ AQ reversed-phase column, eluting with a mixture of 15 mM-ammonium acetate/acetate buffer (pH 5.0) and methanol (38:62, v/v).

Table 1: HPLC and HR-ESI-MS data of the bile salts present in the biliary bile of the common ringtail possum.

| No.  | Carbon | Compound A | Compound C | Compound B | Compound D | Compound F |
|------|--------|------------|------------|------------|------------|------------|
| 1-10 | C₁₅    | 35.56      | 35.60      | 34.56      | 34.57      | 35.13      |
| 11-20| C₁₆    | 31.23      | 31.24      | 30.67      | 30.69      | 29.70      |
| 21-30| C₁₇    | 70.94      | 70.96      | 70.23      | 70.22      | 176.41     |
| 31-40| C₁₈    | 38.29      | 38.33      | 38.39      | 38.43      | 177.18     |
| 41-50| C₁₉    | 43.21      | 43.25      | 46.81      | 46.32      | 48.63      |
| 51-60| C₂₀    | 38.80      | 38.78      | 45.69      | 45.70      | 25.19      |
| 61-70| C₂₁    | 70.62      | 70.63      | 211.43     | 211.37     |
| 71-80| C₂₂    | 44.42      | 44.47      | 49.77      | 49.78      | 36.09      |
| 81-90| C₂₃    | 32.53      | 32.57      | 36.39      | 36.42      | 39.63      |
| 91-100| C₂₄   | 33.99     | 34.01      | 34.88      | 34.89      | 36.61      |
| 101-110| C₂₅  | 29.99     | 30.04      | 30.20      | 30.27      | 30.27      |

s: Singlet; d: Doublet; m: Multiplet; brm: Broad Multiplet. *Chemical shifts were expressed as δ ppm relative to TMS. Values in parentheses refer to coupling constants (J in Hz). Measured in pyridine-d₅ at 500.2 MHz in ¹H NMR and at 125.8 MHz in ¹³C NMR.

Table 2: ¹H and ¹³C NMR signal assignments of isolated and synthetic compounds.

Biological aspects

The common ringtail possum (Pseudocheirus peregrinus) is an Australian marsupial (Figure 1). It lives in a variety of habitats (forests, dense scrub and suburban gardens) and eats a variety of leaves of both native and introduced plants, as well as flowers and fruits. The possum is coprophagic, producing two types of feces, one of which is eaten (see below). This behavioral characteristic is also observed in rabbits and both genera have biliary bile acids that are predominantly secondary.

Our study shows that biliary bile acids in the common ringtail possum differ from those of most mammals in at least two ways (Table 3). First, a seco-bile acid was present. As all primary bile acids have a hydroxyl- or oxo-substituent at C-7, it is likely that the seco-bile acid is a secondary bile acid, formed by bacterial enzymes from Deoxycholic Acid (DCA) in the intestine. Second, the majority of bile acids (80%) appear to be secondary bile acids that have been generated from primary bile acids by bacterial enzymes.

The intermediates in the formation of the seco-bile acid are unknown. The four rings of the bile acid structural platform are generally considered to be stable in vertebrates. However, environmental bacteria have at least two pathways for opening the B ring [14]. We can speculate that the opening of the A ring occurs by an enzymatic pathway that parallels the Baeyer-Villiger oxidation reaction. In this reaction a bile acid with a 3-oxo functional group in the A ring is converted to a pair of regioisomers –3-oxa-4-one-4α-homo- and 3-one-4-oxa-4α-homo. Each of these regioisomers could serve as a precursor for the synthesis of the 3,4-seco bile acid. Other instances where the A ring has been...
Figure 5: HMBC NMR spectrum of compound F.

Figure 6: 1H-1H COSY NMR spectrum of compound F.
opened are found in 3,4-sectoterpenoids [15] and in steroids degraded by the thermophilic fungus Myceliophthora thermophila [16] as well as in steroids mediated by Steroidobacter denitrificans [17].

We propose the following sequence of events to explain the biliary bile acid composition of the possum. The dominant primary acid synthesized is cholic acid which is conjugated with taurine in the liver. In the intestine, cholytaurine undergoes bacterial deconjugation. The liberated cholic acid is absorbed in part but a fraction in the intestine undergoes oxidation at C-7 by bacterial dehydrogenases to form 7-oxodeoxycholic acid (7-oxo-DCA: 3α,12α-dihydroxy-7-oxo-5β-cholan-24-oic acid), a fraction of which is absorbed. In the hepatocyte, the 7-oxo-DCA undergoes partial reduction to form cholic acid [18]. In addition, in the intestine, some of the 7-oxo compound is reduced by bacterial enzymes to form ursolic acid (3α,7β,12α-trihydroxy-5β-cholan-24-oic acid), which in turn is absorbed. As a hydrophilic bile acid, it may well be incompletely unconjugated during passage through the hepatocyte [19]. We cannot exclude the possibility that 7-oxo-DCA may also be a primary bile acid.

Ursolic acid has been reported to be a major bile acid (10%) in a patient with cholesterol gallstones [20] as well as in a mouse model of cystic fibrosis where it constituted 25% of biliary bile acids [21]. In both instances, ursolic acid was considered to be a secondary bile acid. Ursolic acid has also been reported to be present in the urine [22] and feces [23] of healthy subjects.

The possum is known to engage in coprophagy [24,25]. Feces consist of two types of pellets, the one containing undigested residue, and the other, a “soft” pellet contains cecal content that is likely to include bile acids. The possum ingests the soft pellets, and as a result, colonic content including bile acids is exposed to the vast absorptive surface and the microbiome of the small intestine. Bile acid metabolism in the possum appears to be similar to that of the rabbit whose bile contains predominantly DCA [13].

Our paper confirms previous work attesting the diversity of bile acid structures to be found in Australian marsupials. The 1α-hydroxy derivative (1α-OH-CDCA) of Chenodeoxycholic acid (CDCA) was shown to be the major bile acid in the Australian opossum Trichosurus vulpecula (Lesson), and dubbed vulpecholic acid [26,27]. This bile acid was also identified in the biliary bile acids of the spotted cuscus (Phalanger maculatus), and 1β-hydroxy-CDCA was identified in the biliary bile acids of the feather-tailed glider (Acrobates pygmaeus) [6].

Hydroxy-oxo bile acids have also been identified in the biliary bile acids of Australian marsupials, just as observed by us in the biliary bile acids of the possum. In the Queensand kaola (Pseudocheirus peregrinus) and the spotted-tailed quoll (Dasyurus maculatus), 7-oxo-lithocholic acid (3α-hydroxy-7-oxo-5β-cholan-24-oic acid) is a dominant biliary bile acid [6].

**Phylogenetic aspects**

It is generally believed that the marsupials (Metatheria) are an old and relatively less advanced lineage that split away from their sister group of placental mammals (Eutheria) at some point deep in geologic time. The structures of bile salts found in the bile of marsupials do not support this idea. Primitive extant mammals still alive today (Petaurungulates) utilize a mixture of C<sub>24</sub> bile alcohols. The supposedly older and even more primitive marsupials should also feature a similar suite of bile salts. Instead, what is found is a series of derived C<sub>27</sub> bile acids. It is apparent that the switch from bile alcohols to bile acids, and the utilization of taurine for conjugation had already occurred in marsupials far earlier in time than the last common precursor of marsupials and eutherian mammals (estimated to be more than 100 million years ago). Currently, the marsupial lineage is an active site of bile acid evolution, with different species exhibiting new and structurally unique bile salts as noted above [1,2,5,6].

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