Identification of a novel bile acid in swans, tree ducks, and geese: \(3\alpha,7\alpha,15\alpha\)-trihydroxy-5\(\beta\)-cholan-24-oic acid

Genta Kakiyama, Takashi Iida, Takaaki Goto, Nariyasu Mano, Junichi Goto, Toshio Nambara, Lee R. Hagey, Claudio D. Schteinart, and Alan F. Hofmann

Department of Chemistry, College of Humanities and Sciences, Nihon University, Sakurajou, Setagaya, Tokyo 156-8550, Japan; Graduate School of Pharmaceutical Sciences, Tohoku University, Aobayama, Sendai 980-8578, Japan; Department of Medicine, University of California, San Diego, La Jolla, CA 92093-0813; and Department of Chemistry, Ferring Research Institute, San Diego, CA 92121

Abstract By HPLC, a taurine-conjugated bile acid with a retention time different from that of taurocholate was found to be present in the bile of the black-necked swan, Cygnus melanocoryphus. The bile acid was isolated and its structure, established by \(^1\)H and \(^1^3\)C NMR and mass spectrometry, was that of the taurine N-acyl amide of \(3\alpha,7\alpha,15\alpha\)-trihydroxy-5\(\beta\)-cholan-24-oic acid. The compound was shown to have chromatographic and spectroscopic properties that were identical to those of the taurine conjugate of authentic \(3\alpha,7\alpha,15\alpha\)-trihydroxy-5\(\beta\)-cholan-24-oic acid, previously synthesized by us from ursodeoxycholic acid. By HPLC, the taurine conjugate of \(3\alpha,7\alpha,15\alpha\)-trihydroxy-5\(\beta\)-cholan-24-oic acid was found to be present in 6 of 6 species in the subfamily Dendrocyginae (tree ducks) and in 10 of 13 species in the other two subfamilies in the Anatidae family. It was also not present in species from the other two families of the order Anseriformes.

C\(_24\) bile acids are found in most mammals and are present in bile as N-acyl amides (conjugates) of taurine or glycine (4). Individual C\(_24\) bile acids are distinguished by their pattern of hydroxylation on the C\(_{19}\) nucleus or the C\(_5\) side chain. The default hydroxylation pattern of bile acids is at carbon 3 (C-3) (because bile acids are formed from cholesterol) and at C-7 (because hydroxylation at C-7 (catalyzed by cholesterol 7α-hydroxylase or sterol 7-hydroxylase) is believed to be an essential step in the biosynthesis of all bile acids) (5). Thus, chenodeoxycholic acid (\(3\alpha,7\alpha\)-dihydoxy) may be considered the root C\(_24\) bile acid (6).

In most mammals, the majority of biliary bile acids are trihydroxy bile acids (4, 7). Hydroxylation at a third nuclear site, presumably mediated by cytochrome P450 hydroxylases, varies considerably between species. Hydroxylation at C-12 (cholic acid) and at C-6 (hyocholic acid and the muricholic acid epimers) has long been known (7). The third most common site of hydroxylation is probably at C-16, a hydroxylation site discovered many years ago in snakes by Haslewood (7) but now known to occur frequently in avian species (8). Hydroxylation at C-1 also has been reported. In the Australian opossum, hydroxylation at C-1 is in the \(\alpha\)-configuration (9), whereas in certain fruit doves and pigeons, C-1 hydroxylation is in the \(\beta\)-configuration (10).

Identification of novel bile acids is based on nuclear magnetic resonance spectroscopy and mass spectrometry, but chemical synthesis is highly desirable for confirmation of the assigned structure. We recently reported the synthesis of \(3\alpha,7\alpha,16\alpha\)-trihydroxy-5\(\beta\)-cholan-24-oic acid (11). In the process of that work, we were able to synthesize \(3\alpha,7\alpha,15\alpha\)-trihydroxy-5\(\beta\)-cholan-24-oic acid and \(3\alpha,7\alpha,15\beta\)-trihydroxy-5\(\beta\)-cholan-24-oic acid. It seemed likely to us that bile acids hydroxylated at C-1 should occur in nature, because hydroxylation at C-15 of a bile acid analog sulfonate during enterohepatic cycling in the hamster had already been observed (12). We report here the isolation...
of a new natural bile acid, 3α,7α,15α-trihydroxy-5β-cholan-24-oic acid, from the bile of the black-necked swan, where it occurs as its taurine conjugate. We also show that this novel bile acid is present in the biliary bile acids of swans, tree ducks, and geese.

METHODS

Biological material

Bile from the black-necked swan, Cygnus melanocoryphus, and from most other species mentioned in this study was obtained by aspiration from the gallbladder of deceased birds during autopsies by the Pathology Department of the Zoological Society of San Diego. Bile from the barnacle goose was provided by Sea World (San Diego CA), and bile from two other goose species was provided by Dr. Valentine Lance of San Diego State University. Bile samples were dispersed in 4 volumes of reagent-grade isopropanol and kept at 4°C until analysis.

Biliary bile acid analysis for screening of individual species was performed by HPLC as described (4).

Materials and reagents

Taurocholate (3α,7α,12α-trihydroxy-5β-cholan-24-yl taurine) and taurochenodeoxycholate (3α,7α-dihydroxy-5β-cholan-24-yl taurine) were from our laboratory collection. 3α,7α,15α-Trihydroxy-5β-cholan-24-oic acid was prepared in five steps from the methyl ester diacetate of ursodeoxycholic acid, according to the procedures described in a previous paper (11).

HPLC analysis of gallbladder bile of the black-necked swan

The apparatus used in this work consisted of a Jasco Gulliver Series HPLC system (two PU-2080 intelligent high-pressure pumps, an HG-980-31 solvent mixing module, and an HG-980-50 degasser; Tokyo, Japan) equipped with a Shimadzu C-RSA data-processing system (Kyoto, Japan). An Alltech 2000ES evaporative light-scattering detection (ELSD) system (Deerfield, IL) was used under the following conditions: flow rate of purified compressed air as a nebulizing gas, 1.91/min; temperature of the heated drift tube, 75°C; gain, 16. A Capcell Pak type MG II column (5 μm, 250 mm × 3.0 mm inner diameter; Shiseido, Tokyo, Japan) was used as an analytical C18 reverse-phase column. The column temperature was kept at 37°C using a Sugai u-620 type 30 V column heater (Wakayama, Japan). A mixture (43:68, v/v) of 15 mM ammonium acetate (pH 7)-methanol mixture (35:65, v/v) was used under the following conditions: flow rate of purified compressed air as a nebulizing gas, 1.91/min; temperature of the heated drift tube, 75°C; gain, 16. A Capcell Pak type MG II column (5 μm, 250 mm × 3.0 mm inner diameter; Shiseido, Tokyo, Japan) was used as an analytical C18 reverse-phase column. The column temperature was kept at 37°C using a Sugai u-620 type 30 V column heater (Wakayama, Japan). A mixture (43:68, v/v) of 15 mM ammonium acetate (pH 5.4)-methanol was used as the mobile phase. The flow rate was kept constant at 0.4 ml/min.

The gallbladder bile of the black-necked swan in isopropanol (100 μl) was centrifuged for 10 min at 10,000 rpm. The supernatant liquid was applied to a preconditioned Sep-Pak® C18 cartridge (360 mg; Waters, Milford, MA). After the cartridge was washed with water (1 ml) and 20% methanol (1 ml), the bile acid fraction was eluted with 90% methanol (2 ml). The solvent was evaporated under reduced pressure, and the residue was dissolved in methanol (100 μl). An aliquot (1 μl) of the sample solution was injected into the HPLC-ELSD system.

Isolation of compound A by HPLC-ELSD

A Capcell Pak type MG II column (5 μm, 250 mm × 10 mm inner diameter) was used as a preparative C18 reverse-phase column. The ELSD system was set to the following conditions: drift tube temperature, 70°C; gas (air) flow rate, 1.5 l/min; gain, 1; splitting ratio, 30:1. Aqueous methanol (70%) containing 0.01% trifluoroacetic acid (pH 2.7) was used as the mobile phase at a flow rate of 2.0 ml/min.

Measurement of mass spectra

Low-resolution mass spectra (LR-MS) were recorded on a JEOL JMS-303 mass spectrometer with electron ionization (EI) at 70 eV using the positive ion mode (PIM). Liquid chromatography-mass spectrometry (LC-MS) was performed and high-resolution mass spectra (HR-MS) were obtained on a JEOL JMS-LC10A magnetic mass spectrometer equipped with electrospray ionization (ESI) using the negative ion mode (NIM). Chromatographic separation was carried out on a YMC Pack ProC18 (3 μm, 100 × 2.0 mm inner diameter; 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 0.16 ml/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 or 200 V/250 V.

1H and 13C NMR

NMR spectra were recorded at 25°C in CD3OD in a 5 mm tube on a JEOL EX-270 instrument (270 and 68.8 MHz for 1H and 13C, respectively) or a JEOL ECA-600 instrument (600 and 149.4 MHz for 1H and 13C, respectively); 1H and 13C chemical shifts were expressed in ppm. Complete 1H and 13C resonance assignments were made using a combination of two-dimensional homonuclear (1H-1H COSY), heteronuclear (1H-13C) shift-correlated techniques, which include 1H-13C homonuclear correlation spectroscopy (COSY), 1H-13C heteronuclear correlation spectroscopy (HETCOR), 1H detected heteronuclear multiple quantum correlation (HMOC), and 31P detected heteronuclear multiple bond correlation (HMBG) experiments. These two-dimensional NMR spectra were recorded using standard pulse sequences and parameters recommended by the manufacturer. One-dimensional 13C distortionless enhancement by polarization transfer (DEPT; 135°, 90°, and 45°) spectra were also measured to determine the exact 1H signal multiplicity and to differentiate between CH3, CH2, CH, and C based on their proton environments.

Conjugation of 3α,7α,15α-trihydroxy-5β-cholan-24-oic acid with taurine

To a magnetically stirred solution of authentic 3α,7α,15α-trihydroxy-5β-cholanoic acid (15 mg, 0.04 mmol) (1) in dry N,N-dimethylformamide (1.5 ml), powdered taurine (15 mg, 0.12 mmol), diethyl phosphorocyanide (15 μl), and anhydrous triethylamine (15 μl) were successively added, and the reacting suspension was stirred at room temperature for 60 min (13). The reaction mixture was adjusted to pH 12 with 1 M NaOH and extracted with ether to remove the triethylamine. After adjusting the pH to 8 with 1 M HCl, the resulting solution was diluted with water (9 ml), passed through a preconditioned Sep-Pak® C18 cartridge (5 g), and eluted successively with water (10 ml), 20% methanol (10 ml), and 60% methanol (20 ml). The last fraction, which contained the desired taurine conjugate, was evaporated under reduced pressure. Recrystallization of the residue from methanol-ethyl acetate gave an analytically pure sample of 3α,7α,15α-trihydroxy-5β-cholan-24-yl taurine (sodium salt) as colorless crystals: yield, 16 mg (80%); melting point, 163–166°C. LR-MS (EI-PID) m/z: 446 (M-3H2O-CH3, 2%), 572 (100%), 357 (36%), 354 (73%), 339 (57%), 300 (48%), 291 [M-side chain (S.C.)-H2O; 3%], 271 (21%), 272 (M-S.C.-H2O; 13%), 254 (M-S.C.-3H2O; 48%), 253 (86%), 250 (M-S.C.-ring D; 3%), 214 (M-S.C.-ring D-2H2O; 10%), 196 (M-S.C.-ring D-3H2O; 3%). HR-MS
RESULTS

Isolation of 3α,7α,15α-trihydroxy-5β-cholan-24-oic acid from swan bile and proof of structure

As shown in Fig. 1, HPLC-ELSD analysis of the bile acid fraction obtained from the gallbladder bile of the black-necked swan showed two major peaks, which were designated compounds A (74%) and C (25%), accompanied by a trace amount of compound B (1%).

Major peaks A and C were subjected to LC-ESI-MS analysis with selected ion monitoring (SIM) under the NIM. Figure 2 shows SIM chromatograms for peaks A and C and authentic taurocholate and taurochenodeoxycholate. The deprotonated molecules, [M-H]−, obtained were as follows: a) m/z 514.3, a trihydroxy C24 bile acid; b) m/z 496.3, a disubstituted (hydroxyl, oxo) C24 bile acid (not shown in Fig. 2); and c) m/z 498.3, a dihydroxy C24 bile acid. All of these structures were assumed to be conjugated in Nacyl linkage with taurine, based on the m/z number of the deprotonated molecules and the known dominance of taurine conjugation in vertebrate bile acids (4).

Peak C was readily identifiable as the common 3α,7α-dihydroxy primary bile acid conjugate, taurochenodeoxycholate. Identification was made by direct comparison of the HPLC-ELSD retention time, the m/z value (498.4) of the deprotonated molecule, and the retention time and fragmentation pattern during the LC-ESI-MS measurement, using an authentic standard.

The largest peak, A, had a retention time less than that of authentic taurocholate. Figure 3 shows the negative ion LC-ESI-MS fragmentation pattern of the isolated compound A, which gave a deprotonated m/z of 514.5. A fragment ion with strong intensity was observed at m/z 79.9, corresponding to SO3−, supporting the presence of an Nacylamido linkage with taurine in the side chain. However, no other significant ion serving to characterize the structure of compound A, particularly the position of three hydroxyl groups, appeared in the spectrum.

To identify the location and orientation of the hydroxyl groups, compound A was isolated by preparative HPLC-ELSD and analyzed by 1H and 13C NMR. The chemical shifts, spectral pattern, and signal multiplicity of the 1H

Fig. 1. Reverse-phase HPLC-evaporative light-scattering detection profile of the bile acids of the black-necked swan. For each peak (A–C), the compound, its retention time, and its percentage of total biliary bile acids are given. A: 3α,7α,15α-Trihydroxy-5β-cholan-24-oyl taurine (10.89 min), 74%. B: 7-Deoxy-hydroxy-oxo derivative of compound A (16.54 min), 1%. C: Taurochenodeoxycholate (19.07 min), 25%.

Fig. 2. Negative ion liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) analysis with a selected ion monitoring mode of authentic taurocholate and synthetic 3α,7α,15α-trihydroxy-5β-cholan-24-oyl taurine (A), authentic taurochenodeoxycholate (B), compound A (C), and compound C (D).
and $^{13}$C signals suggested that carbons 3, 7, and 15 were the sites of hydroxylation and that all of the hydroxyl groups were in the $\alpha$-configuration.

Table 1 shows $^1$H and $^{13}$C NMR spectral data for compound A, together with those of authentic $3\alpha,7\alpha,15\alpha$-trihydroxy-$5\beta$-cholan-24-oyl acid. Complete $^1$H and $^{13}$C NMR spectral assignments of the synthetic taurine conjugate of $3\alpha,7\alpha,15\alpha$-trihydroxy-$5\beta$-cholan-24-oic acid were also made on the basis of several two-dimensional NMR techniques: COSY, long-range COSY, NOESY, HETCOR, HMQC, and HMBC (Table 1). Differentiation of CH$_3$, CH$_2$, CH, and C was made with the $^{13}$C DEPT spectra. The

**Table 1.** $^1$H and $^{13}$C NMR data for synthetic $3\alpha,7\alpha,15\alpha$-trihydroxy-$5\beta$-cholan-24-oyl taurine and compound A

| No. | Type | $^{13}$C | $^1$H | $^{13}$C | $^1$H |
|-----|------|---------|-------|---------|-------|
| 1   | CH$_2$ | 36.48  | 1.73  | 0.98    | 36.48 |
| 2   | CH$_2$ | 31.23  | 1.35  | 1.61    | 31.23 |
| 3   | CH    | 72.72  | 3.38  | 1.64    | 72.72 |
| 4   | CH$_2$ | 40.07  | 2.18  | 1.35    | 40.06 |
| 5   | CH    | 42.95  | 4.01  | 1.66    | 42.96 |
| 6   | CH$_2$ | 34.08  | 1.55  | 2.10    | 34.05 |
| 7   | CH    | 68.96  | 4.01  | 68.95   | 4.00  |
| 8   | CH    | 40.52  | 1.66  | 40.51   | 1.66  |
| 9   | CH    | 35.37  | 1.84  | 35.36   | 1.84  |
| 10  | C     | 35.88  | 35.88 | 35.88   | 35.88 |
| 11  | CH$_2$ | 21.82  | 1.52  | 1.32    | 21.81 |
| 12  | CH    | 41.62  | 1.26  | 1.95    | 41.62 |
| 13  | C     | 44.85  | 1.45  | 44.85   | 1.45  |
| 14  | CH    | 59.43  | 1.45  | 59.44   | 1.45  |
| 15  | CH    | 72.88  | 3.97  | 72.86   | 3.97  |
| 16  | CH$_2$ | 41.11  | 1.78  | 41.11   | 1.78  |
| 17  | CH    | 55.62  | 1.42  | 55.60   | 1.42  |
| 18  | CH$_3$ | 13.51  | 0.72  | 13.51   | 0.73  |
| 19  | CH$_3$ | 23.34  | 0.92  | 23.36   | 0.93  |
| 20  | CH    | 36.31  | 0.95  | 36.31   | 0.95  |
| 21  | CH$_2$ | 18.62  | 1.39  | 18.63   | 1.39  |
| 22  | CH$_2$ | 33.00  | 1.29, 1.75 | 33.01 |
| 23  | CH$_2$ | 34.00  | 2.10, 2.46 | 34.09 |
| 24  | C     | 176.35 | 176.43| 176.43 |
| 25  | CH$_3$ | 36.62  | 3.57  | 36.62   | 3.58  |
| 26  | CH$_5$ | 51.48  | 2.95  | 51.48   | 2.95  |

d, doublet; brm, broad multiplet; m, multiplet; s, singlet; t, triplet.

$^a$ Measured at 600 and 149.4 MHz for $^1$H and $^{13}$C, respectively.

$^b$ Measured at 270 and 68.8 MHz for $^1$H and $^{13}$C, respectively.
correlation of the $^1$H and $^{13}$C signals by the HETCOR spectrum is illustrated in Fig. 4. As expected, the spectral pattern, chemical shifts, and signal multiplicity of the major $^1$H and $^{13}$C signals of compound A were in good agreement with those of synthetic $3\alpha,7\alpha,15\alpha$-trihydroxy-5$\beta$-cholan-24-oyl taurine. A particularly noteworthy feature is that in compound A, the CH signals at the 3, 7, and 15 positions resonate at 72.72, 68.95, and 72.86 ppm, respectively, whereas the neighboring protons appear at 3.41 ($3\alpha$-H, broad multiplet), 4.00 ($7\alpha$-H, multiplet), and 3.97 (15$\alpha$-H, broad multiplet) ppm. These $^1$H and $^{13}$C resonances for the 5$\beta$-steroid moiety were in good agreement with those reported for sodium $3\alpha,7\alpha,15\alpha$-trihydroxy-5,26-bishomo-5$\beta$-cholestane-26-sulfonate (12) but not for methyl $3\alpha,7\alpha,16\alpha$-trihydroxy-5$\beta$-cholane-24-oate triacetate (8). (Further discussion of the NMR spectra of C-15 hydroxy bile acids is available in references 8 and 12).

In addition, $^{13}$C signals were present at 36.62 and 51.48 ppm; these were assigned to CH$_2$N and CH$_2$S, respectively, and the adjacent protons appeared at 3.57 (25-H$_2$, t) and 2.95 (26-H$_2$, t). These $^1$H and $^{13}$C data for compound A also agreed very well with those reported for 3$\alpha$-sulfo-7$\beta$-(2-acetamido-2-deoxy-$\alpha$-D-glucopyranosyl)-5-cholen-24-oyl taurine (14), again providing evidence for the presence of the N-acylamido linkage with taurine in the side chain.

In the SIM chromatogram (Fig. 2A, C), the synthetic taurine amidate of authentic $3\alpha,7\alpha,15\alpha$-trihydroxy-5$\beta$-cholan-24-oic acid had the same retention time as compound A isolated from the bile of the black-necked swan. Thus, compound A was identified as the taurine conjugate of $3\alpha,7\alpha,15\alpha$-trihydroxy-5$\beta$-cholan-24-oyl taurine (Fig. 5), a natural bile acid N-acylamidate not reported previously.

This new bile acid may be named 15$\alpha$-hydroxy-chenodeoxycholic acid (CDCA) (15). For conversational purposes only, we suggest the trivial name "cygnocholic acid," in keeping with the longstanding tradition of assigning bile acid trivial names according to their biological source (15).

Peak B showed a deprotonated molecule at $m/z$ 496.4 by LC-ESI-MS, suggesting that this compound is the taurine conjugate of $3\alpha$-hydroxy-15-oxo-cholanoate, or less likely, 3-oxo-15$\alpha$-hydroxy-cholanoate (or both). Such a com-

Fig. 4. $^1$H-$^{13}$C Heteronuclear correlation spectroscopy (HETCOR) spectrum of synthetic $3\alpha,7\alpha,15\alpha$-trihydroxy-5$\beta$-cholan-24-oyl taurine.

Fig. 5. Structure of $3\alpha,7\alpha,15\alpha$-trihydroxy-5$\beta$-cholan-24-oyl taurine.
In this work, we isolated an unknown conjugated bile acid from the bile of the black-necked swan and show that it is the taurine conjugate of a new natural bile acid, 3α,7α,15α-trihydroxy-5β-cholan-24-oic acid, a bile acid previously synthesized by us. We also found that this new trihydroxy bile acid is present in the biliary bile acids of tree ducks, swans, and geese, in which it occurs as its taurine conjugate.

### DISCUSSION

In this work, we isolated an unknown conjugated bile acid from the bile of the black-necked swan and show that it is the taurine conjugate of a new natural bile acid, 3α,7α,15α-trihydroxy-5β-cholan-24-oic acid, a bile acid previously synthesized by us. We also found that this new trihydroxy bile acid is present in the biliary bile acids of tree ducks, swans, and geese, in which it occurs as its taurine conjugate.
In 1991, Pyrek (20) (now deceased) reported in a review article on mass spectrometry of natural products that 3α,15α-dihydroxy-5β-cholan-24-oic acid was present in the biliary bile acids of the wombat. This compound, which has not been synthesized to date, would be formed by bacterial 7-dehydroxylation of the primary, trihydroxy bile acid reported in this paper. We analyzed one sample of wombat bile by HPLC and found compounds with retention times corresponding to that of the taurine conjugate of 3α,7α,15α-trihydroxy-5β-cholan-24-oic acid (5% of total biliary bile acids) and consistent with that predicted for the taurine conjugate of 3α,15α-dihydroxy-5β-cholan-24-oic acid (56% of total biliary bile acids). Proof of structure of the compound tentatively identified as the taurine conjugate of 3α,15α-dihydroxy-5β-cholan-24-oic acid is beyond the scope of this paper.

Hydroxylation of a bile acid analog sulfonate at C-15 in the hamster has been reported previously, as noted above (12). In addition, Lund et al. (21) reported the formation from cholesterol of C21 bile acids hydroxylated at C-15 in either the 15α- or 15β-configuration by the isolated perfused female rat liver. Mouse cytochrome P450 2A6 when mutated (phenylalanine-209 to asparagine) acquires the ability to 15α-hydroxylate corticosterone (22). Xenobiotics may also undergo 15α-hydroxylation. For example, desogestrel, an orally active progestogen, is converted to a 15α-hydroxy metabolite in rats (23).

The biological forces responsible for the evolution of C-15 hydroxy bile acids are not known. Perhaps specific cytochrome P450 hydroxylases for the detoxification of plant toxins evolved and were adapted for bile acid biosynthesis. We have speculated elsewhere that the formation of trihydroxy bile acids by the hydroxylation of CDCA or a precursor of CDCA was desirable, at least in vertebrates possessing a cecum, because of the marked hepatotoxicity of lithocholic acid, the bacterial metabolite of CDCA (24).

In Fig. 6, we depict changes in biliary bile acid composition in relation to a reasonable evolutionary scheme for the order Anseriformes. The scheme for evolution is based on current views of the phylogenetic relationships derived from morphological studies (17) or analysis of mitochondrial DNA (18). The oldest family, Anhimidae, has allochenodeoxycholic acid as its major biliary bile acid, suggesting evolution from reptiles in which such bile acids occur (7). This was followed by the development of enzymes for the formation of 5β bile acids (A/B ring juncture cis), with the result that allochenodeoxycholic acid was replaced by chenodeoxycholic acid, the root bile acid in most vertebrates. Hydroxylation at 15α occurs transiently in tree ducks, swans, and geese and is eventually replaced by hydroxylation at C-23. The reason for the changes in the position of hydroxylation with evolution are not known, although C-23 conjugated bile acids have been shown to be more resistant to bile acid

![Fig. 6](image_url)

**Fig. 6.** Biliary bile acid composition denoted by nuclear substituents in relation to an evolutionary scheme for the order Anseriformes based on morphological studies (17) and mitochondrial DNA analysis (18). 15α-Hydroxylation arose in the subfamilies of Anatidae but was replaced by 23(R)-hydroxylation in later evolving families. *Cereopsis* is an exception, but its evolutionary position is controversial.
deconjugation than bile acids with an unsubstituted side chain (25).

With this report, the number of additional hydroxylation sites on the cholanic acid nucleus in primary bile acids is now seven (6α, 6β, 12α, 1α, 1β, 15α, and 16α). Additional sites of hydroxylation, such as C-2 or C-4, occur during experimental cholestasis (26); hydroxylation at C-4 also occurs during fetal development in human (27). Hydroxylation at C-5 of C23 (C24 nor) bile acids has also been reported to occur in the hamster (28). Thus, it seems likely that natural trihydroxy bile acids hydroxylated at C-2, at C-4, or at C-5 in addition to C-3 and C-7 will be identified as primary bile acids in vertebrates in the future.

The authors thank Dr. Joseph Steinbach for his aid in creating graphics and Drs. Melvyn Korman (Monash Medical Center) and Dr. Peter Holz (Zoos Victoria, Australia) for providing a sample of wombat bile for us.

REFERENCES

1. Hofmann, A. F., and K. J. Mysels. 1988. Bile salts as biological surfactants. Colloids and Surfaces. 30: 145–173.
2. Hofmann, A. F., and L. Eckmann. 2006. How bile acids confer gut mucosal protection against bacteria. Proc. Natl. Acad. Sci. USA. 103: 4335–4334.
3. Yamamoto, M., S. M. Houten, C. Matakai, M. A. Christoflolette, B. W. Kim, H. Sato, N. Messaddeq, J. W. Haney, O. Ezaki, T. Kodama, et al. 2006. Bile acids induce energy expenditure by promoting intracellular thyroid hormone activation. Nature. 439: 402–403.
4. Moschetta, A., F. Xu, L. R. Hagey, G. P. van Berge-Henegouwen, K. J. van Erpecum, J. F. Brouwers, J. C. Cohen, M. Bierman, H. H. Hobbs, J. H. Steinbach, et al. 2005. A phylogenetic survey of biliary lipids in vertebrates. J. Biol. Chem. 280: 3825–3835.
5. Russell, D. W. 2003. The enzymes, regulation, and genetics of bile acid biosynthesis. Ann. Rev. Biochem. 72: 137–174.
6. Hofmann, A. F., C. D. Schteingart, and L. R. Hagey. 1995. Species differences in bile acid metabolism. In Bile Acids and Liver Diseases (International Falk Workshop). G. Paumgartner and U. Beuers, editors. Kluwer Academic Publishers, Boston. 3–30.
7. Haslewood, G. A. D. 1978. The Biological Importance of Bile Salts. North Holland Publishing Co., Amsterdam.
8. Hagey, L. R., C. D. Schteingart, H-T. Ton-Nu, and A. F. Hofmann. 2002. A novel primary bile acid in the Shoebill stork and herons and its phylogenetic significance. J. Lipid Res. 43: 685–690.
9. Lee, S. P., R. Lester, and J. S. Pyrek. 1987. Vulpecholic acid (1α,3α,7α-trihydroxy-5β-cholanoic acid-24-oic acid): a novel bile acid from a marsupial, Trichosurus vulgaris. J. Lipid Res. 28: 19–31.
10. Hofmann, F. A., L. R. Hagey, H-T. Ton-Nu, and C. D. Schteingart. 1994. Biliary bile acids of fruit pigeons and doves (Columbiformes): presence of 1β-hydroxydeoxycholic acid and conjugation with glycine as well as taurine. J. Lipid Res. 35: 2041–2048.
11. Iida, T., M. Hikosaka, G. Kakiyama, K. Shiraishi, C. D. Schteingart, L. R. Hagey, H-T. Ton-Nu, A. F. Hofmann, N. Mano, J. Goto, et al. 2002. Potential bile acid metabolites. XXV. Synthesis and chemical properties of stereoisomeric 3α,7α,16- and 3α,7α,15-trihydroxy-5β-cholanoic acid in bile acids. J. Lipid Res. 43: 599–604.
12. Hofmann, A. F., J. Sjövall, G. Kurz, A. Radominska, C. D. Schteingart, G. S. Tint, Z. R. Vlahcevic, and K. D. R. Setchell. 1992. A proposed nomenclature for bile acids. J. Lipid Res. 33: 599–604.
13. Hylomen, P. B. 1985. Metabolism of bile acids in intestinal microflora. In Sterols and Bile Acids. H. Danielsson and J. Sjövall, editors. Elsevier, Amsterdam. 331–342.
14. Livezey, B. C. 1996. A phylogenetic analysis of geese and swans (Anseriformes: Anserinae) including selected fossil species. Syst. Biol. 45: 415–450.
15. Hofmann, A. F., J. Sjo¨vall, G. Kurz, A. Radominska, C. D. Schteingart, G. S. Tint, Z. R. Vlahcevic, and K. D. R. Setchell. 1992. A proposed nomenclature for bile acids. J. Lipid Res. 33: 599–604.
16. Verhoeven, C. H. J., S. F. M. Krebbers, G. N. Wagenars, and R. M. E. Vos. 1998. In vitro and in vivo metabolism of desogestrel in several species. Drug Metab. Dispos. 26: 927–936.
17. Hofmann, A. F., and L. R. Hagey. 1998. Bile acids and biliary disease: peaceful coexistence versus deadly warfare. In Gut and Liver. H. E. Blum, J. C. Bode, and R. B. Sartor, editors. Kluwer Academic Publishers, Boston, MA. 85–103.
18. Merrill, J. R., C. D. Schteingart, L. R. Hagey, Y. Peng, H-T. Ton-Nu, E. Flick, M. Jirsa, and A. F. Hofmann. 1996. Hepatic biotransformation in rodents and physicochemical properties of 23R,3β-hydroxychenodeoxycholic acid, a natural α-hydroxy bile acid. J. Lipid Res. 37: 98–112.
19. Marschall, H. U., M. Wagner, K. Bodin, G. Zollner, P. Fickert, J. Gumhold, D. Silbert, A. Fuchsbichler, J. Sjo ¨vall, and M. Trauner. 1998. In vitro and in vivo metabolism of desogestrel in several species. Drug Metab. Dispos. 26: 927–936.
20. Hofmann, A. F., and L. R. Hagey. 1998. Bile acids and biliary disease: peaceful coexistence versus deadly warfare. In Gut and Liver. H. E. Blum, J. C. Bode, and R. B. Sartor, editors. Kluwer Academic Publishers, Boston, MA. 85–103.
21. Hofmann, A. F., and L. R. Hagey. 1998. Bile acids and biliary disease: peaceful coexistence versus deadly warfare. In Gut and Liver. H. E. Blum, J. C. Bode, and R. B. Sartor, editors. Kluwer Academic Publishers, Boston, MA. 85–103.
22. Hofmann, A. F., and L. R. Hagey. 1998. Bile acids and biliary disease: peaceful coexistence versus deadly warfare. In Gut and Liver. H. E. Blum, J. C. Bode, and R. B. Sartor, editors. Kluwer Academic Publishers, Boston, MA. 85–103.
23. Hofmann, A. F., and L. R. Hagey. 1998. Bile acids and biliary disease: peaceful coexistence versus deadly warfare. In Gut and Liver. H. E. Blum, J. C. Bode, and R. B. Sartor, editors. Kluwer Academic Publishers, Boston, MA. 85–103.
24. Hofmann, A. F., and L. R. Hagey. 1998. Bile acids and biliary disease: peaceful coexistence versus deadly warfare. In Gut and Liver. H. E. Blum, J. C. Bode, and R. B. Sartor, editors. Kluwer Academic Publishers, Boston, MA. 85–103.
25. Hofmann, A. F., and L. R. Hagey. 1998. Bile acids and biliary disease: peaceful coexistence versus deadly warfare. In Gut and Liver. H. E. Blum, J. C. Bode, and R. B. Sartor, editors. Kluwer Academic Publishers, Boston, MA. 85–103.
26. Hofmann, A. F., and L. R. Hagey. 1998. Bile acids and biliary disease: peaceful coexistence versus deadly warfare. In Gut and Liver. H. E. Blum, J. C. Bode, and R. B. Sartor, editors. Kluwer Academic Publishers, Boston, MA. 85–103.
27. Hofmann, A. F., and L. R. Hagey. 1998. Bile acids and biliary disease: peaceful coexistence versus deadly warfare. In Gut and Liver. H. E. Blum, J. C. Bode, and R. B. Sartor, editors. Kluwer Academic Publishers, Boston, MA. 85–103.
28. Hofmann, A. F., and L. R. Hagey. 1998. Bile acids and biliary disease: peaceful coexistence versus deadly warfare. In Gut and Liver. H. E. Blum, J. C. Bode, and R. B. Sartor, editors. Kluwer Academic Publishers, Boston, MA. 85–103.