

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

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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 ¹H and ¹³C NMR and mass spectrometry, was that of the taurine *N*-acyl amidate of $3\alpha,7\alpha,15\alpha$ -trihydroxy- 5β -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- 5β -cholan-24-oic acid, previously synthesized by us from ursodeoxycholic acid. By HPLC, the taurine conjugate of $3\alpha,7\alpha,15\alpha$ -trihydroxy- 5β -cholan-24-oic acid was found to be present in 6 of 6 species in the subfamily Dendrocygninae (tree ducks) and in 10 of 13 species in the subfamily Anserinae (swans and geese) but not in other subfamilies in the Anatidae family. It was also not present in species from the other two families of the order Anseriformes. **Key words:** $3\alpha,7\alpha,15\alpha$ -Trihydroxy- 5β -cholan-24-oic acid is a new primary bile acid that is present in the biliary bile acids of swans, tree ducks, and geese and may be termed 15α -hydroxy-chenodeoxycholic acid.—Kakiyama, G., T. Iida, T. Goto, N. Mano, J. Goto, T. Nambara, L. R. Hagey, C. D. Schteingart, and A. F. Hofmann. **Identification of a novel bile acid in swans, tree ducks, and geese: $3\alpha,7\alpha,15\alpha$ -trihydroxy- 5β -cholan-24-oic acid.** *J. Lipid Res.* 2006. 47: 1551–1558.

Supplementary key words Anseriformes • bile acid evolution • bile acid conjugation

Bile acids are the amphipathic end products of cholesterol metabolism. In the small intestine, luminal bile acids promote lipid absorption by forming mixed micelles with dietary lipids (1) and also have antimicrobial effects (2). In addition, plasma bile acids appear to promote thermogenesis by interacting with a G protein-coupled receptor present on the surface of adipocytes in brown adipose tissue (3).

C_{24} bile acids are found in most mammals and are present in bile as *N*-acyl amidates (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 7α -hydroxylase) is believed to be an essential step in the biosynthesis of all bile acids] (5). Thus, chenodeoxycholic acid ($3\alpha,7\alpha$ -dihydroxy) 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 α -configuration (9), whereas in certain fruit doves and pigeons, C-1 hydroxylation is in the β -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- 5β -cholan-24-oic acid (11). In the process of that work, we were able to synthesize $3\alpha,7\alpha,15\alpha$ -trihydroxy- 5β -cholan-24-oic acid and $3\alpha,7\alpha,15\beta$ -trihydroxy- 5β -cholan-24-oic acid. It seemed likely to us that bile acids hydroxylated at C-15 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

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of a new natural bile acid, $3\alpha,7\alpha,15\alpha$ -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 autopsy 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 ($3\alpha,7\alpha,12\alpha$ -trihydroxy- 5β -cholan-24-oil taurine) and taurochenodeoxycholate ($3\alpha,7\alpha$ -dihydroxy- 5β -cholan-24-oil taurine) were from our laboratory collection. $3\alpha,7\alpha,15\alpha$ -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-R8A 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, 1.9 l/min; temperature of the heated drift tube, 75°C; gain, 16. A Capcell Pak type MG II column (5 μ m, 250 mm \times 3.0 mm inner diameter; Shiseido, Tokyo, Japan) was used as an analytical C_{18} 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 buffer (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 μ l) was centrifuged for 10 min at 10,000 rpm. The supernatant liquid was applied to a preconditioned Sep-Pak® C_{18} 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 μ l). An aliquot (1 μ l) 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 μ m, 250 mm \times 10 mm inner diameter) was used as a preparative C_{18} 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-LCmate double-focusing magnetic mass spectrometer equipped with electrospray ionization (ESI) using the negative ion mode (NIM). Chromatographic separation was carried out on a YMC Pack ProC₁₈ column (3 μ m, 100 \times 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 23°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) and heteronuclear (¹H-¹³C) shift-correlated techniques, which include ¹H-¹H homonuclear correlation spectroscopy (COSY), long-range ¹H-¹H COSY, ¹H-¹H nuclear Overhauser effect spectroscopy (NOESY), ¹H-¹³C heteronuclear correlation spectroscopy (HETCOR), ¹H detected heteronuclear multiple quantum correlation (HMQC), and ¹H detected heteronuclear multiple bond correlation (HMBC) 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 $3\alpha,7\alpha,15\alpha$ -trihydroxy- 5β -cholan-24-oic acid with taurine

To a magnetically stirred solution of authentic $3\alpha,7\alpha,15\alpha$ -trihydroxy- 5β -cholan-24-oic 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 μ l), and anhydrous triethylamine (15 μ l) were successively added, and the resulting 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® C_{18} 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 $3\alpha,7\alpha,15\alpha$ -trihydroxy- 5β -cholan-24-oil taurine (sodium salt) as colorless crystals: yield, 16 mg (80%); melting point, 163–166°C. LR-MS (EI-PIM) *m/z*: 446 (M-3H₂O-CH₃; 2%), 372 (100%), 357 (36%), 354 (73%), 339 (57%), 300 (48%), 291 [M-side chain (S.C.)-H₂O; 3%], 271 (21%), 272 (M-S.C.-2H₂O; 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

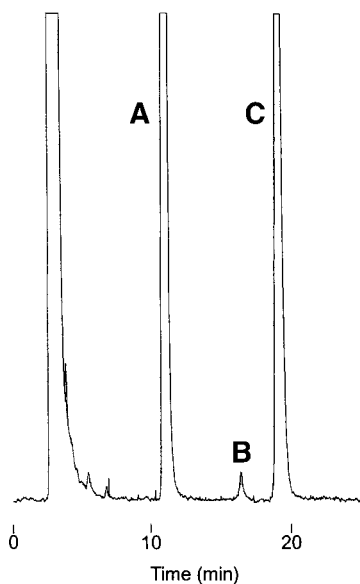


Fig. 1. Reverse-phase HPLC-ELSD detection profile of the bile acids of the black-necked swan. For each peak (A–C), the compound, its retention time, and its percentage of total biliary bile acids are given. A: $3\alpha,7\alpha,15\alpha$ -Trihydroxy- 5β -cholan-24-oyl taurine (10.89 min), 74%. B: 7-Deoxy-hydroxy-oxo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

(ESI-NIM), calculated for $C_{26}H_{44}NO_7S$ $[M-H]^-$: 514.2838; found, m/z 514.2801.

RESULTS

Isolation of $3\alpha,7\alpha,15\alpha$ -trihydroxy- 5β -cholan-24-oyl 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 C_{24} bile acid; b) m/z 498.3, a disubstituted (hydroxyl, oxo) C_{24} bile acid (not shown in **Fig. 2**); and c) m/z 498.3, a dihydroxy C_{24} bile acid. All of these structures were assumed to be conjugated in N -acyl 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 $3\alpha,7\alpha$ -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 SO_3^- , supporting the presence of an N -acylamido 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 ^{13}C NMR. The chemical shifts, spectral pattern, and signal multiplicity of the 1H

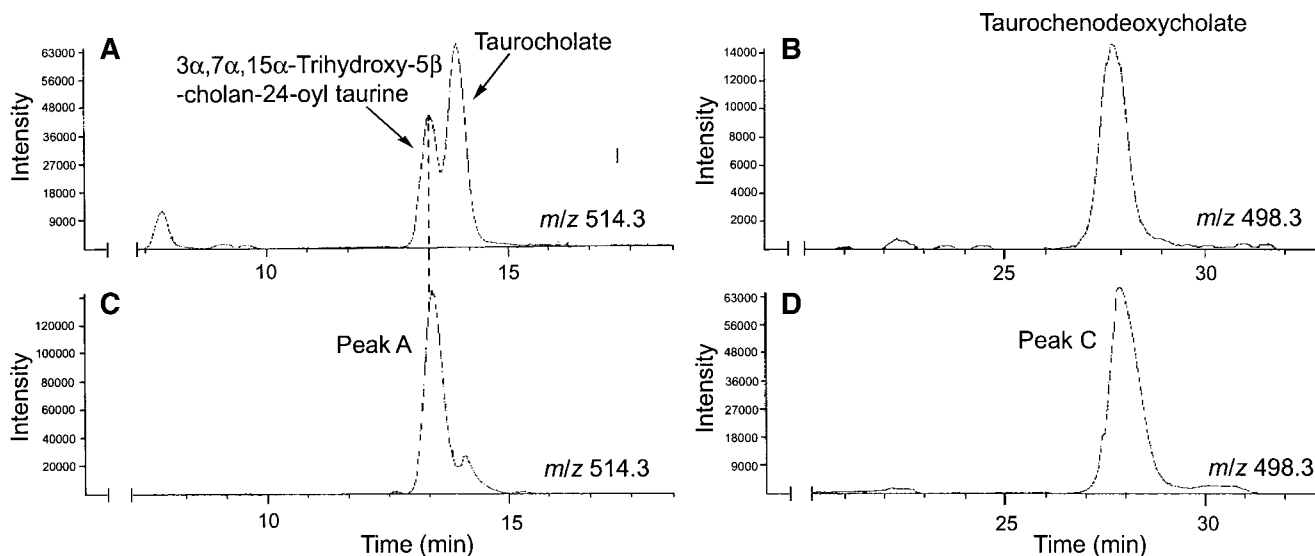


Fig. 2. Negative ion liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) analysis with a selected ion monitoring mode of authentic taurocholate and synthetic $3\alpha,7\alpha,15\alpha$ -trihydroxy- 5β -cholan-24-oyl taurine (A), authentic taurochenodeoxycholate (B), compound A (C), and compound C (D).

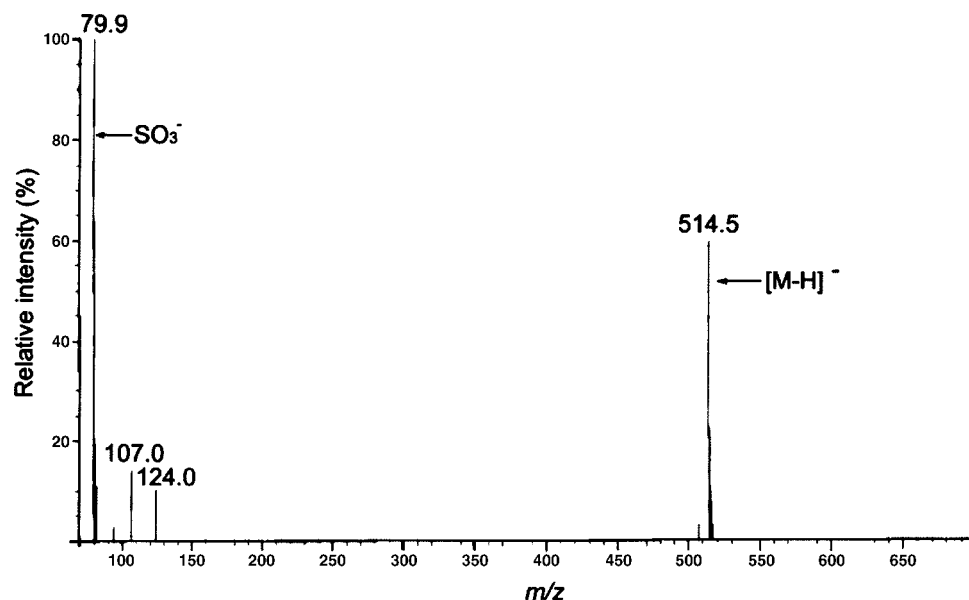


Fig. 3. Negative ion LC-ESI-MS fragmentation pattern of compound A.

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 α -configuration.

Table 1 shows ^1H and ^{13}C NMR spectral data for compound A, together with those of authentic $3\alpha,7\alpha,15\alpha$ -trihydroxy- 5β -cholan-24-oic acid. Complete ^1H and ^{13}C

NMR spectral assignments of the synthetic taurine conjugate of $3\alpha,7\alpha,15\alpha$ -trihydroxy- 5β -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. ^1H and ^{13}C NMR data for synthetic $3\alpha,7\alpha,15\alpha$ -trihydroxy- 5β -cholan-24-oic acid and compound A

No.	Type	$3\alpha,7\alpha,15\alpha$ -Trihydroxy- 5β -cholan-24-oic acid ^a		Compound A ^b		
		^{13}C	^1H	^{13}C	^1H	
			α	β		
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 (brm)	72.72	3.41 (1H, brm, β -H)
4	CH_2	40.07	2.18	1.64	40.06	
5	CH	42.95		1.35	42.96	
6	CH_2	34.08	1.55	2.10	34.05	
7	CH	68.96		4.01 (m)	68.95	4.00 (1H, m, β -H)
8	CH	40.52		1.66	40.51	
9	CH	35.37	1.84		35.36	
10	C	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			44.85	
14	CH	59.43	1.45		59.44	
15	CH	72.88		3.97 (brm)	72.86	3.97 (1H, brm, β -H)
16	CH_2	41.11	1.78	1.88	41.11	
17	CH	55.62	1.42		55.60	
18	CH_3	13.51	0.72 (s)		13.51	0.73 (3H, s)
19	CH_3	23.34	0.92 (s)		23.36	0.93 (3H, s)
20	CH	36.31		1.39	36.31	
21	CH_3	18.62	0.95 (d, J 6.6 Hz)		18.63	0.95 (3H, d, J 5.7 Hz)
22	CH_2	33.00	1.29, 1.75 (each, m)		33.01	
23	CH_2	34.00	2.10, 2.46 (each, m)		34.09	
24	C	176.35			176.43	
25	CH_2N	36.62	3.57 (t, J 7.8 Hz)		36.62	3.58 (2H, t, J 7.0 Hz)
26	CH_2S	51.48	2.95 (t, J 7.8 Hz)		51.48	2.95 (2H, t, J 7.0 Hz)

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

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

^b Measured at 270 and 68.8 MHz for ^1H and ^{13}C , respectively.

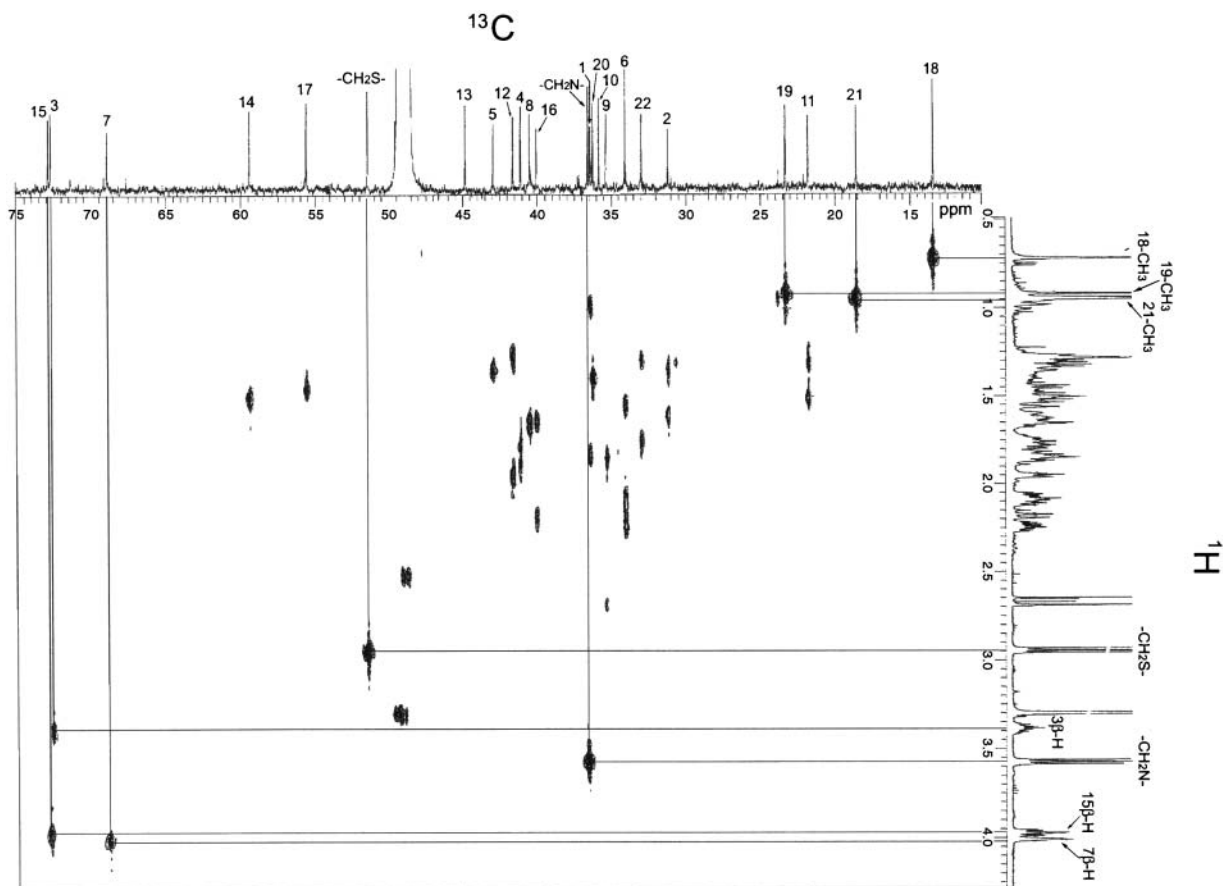


Fig. 4. ^1H - ^{13}C Heteronuclear correlation spectroscopy (HETCOR) spectrum of synthetic $3\alpha,7\alpha,15\alpha$ -trihydroxy- 5β -cholan-24-oyl taurine.

correlation of the ^1H 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 ^1H and ^{13}C signals of compound A were in good agreement with those of synthetic $3\alpha,7\alpha,15\alpha$ -trihydroxy- 5β -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 ($3\alpha\text{-H}$, broad multiplet), 4.00 ($7\alpha\text{-H}$, multiplet), and 3.97 ($15\alpha\text{-H}$, broad multiplet) ppm. These ^1H and ^{13}C resonances for the 5β -steroid moiety were in good agreement with those reported for sodium $3\alpha,7\alpha,15\alpha$ -trihydroxy-5,26-bishomo- 5β -cholestane-26-sulfonate (12) but not for methyl $3\alpha,7\alpha,16\alpha$ -trihydroxy- 5β -cholan-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_2N and CH_2S , respectively, and the adjacent protons appeared at 3.57 (25-H_2 , t) and 2.95 (26-H_2 , t). These ^1H and ^{13}C data for compound A also agreed very well with those reported for 3α -sulfo- 7β -(2-acetamido-2-deoxy- α -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- 5β -

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- 5β -cholan-24-oic acid (Fig. 5), a natural bile acid *N*-acylamidate not reported previously.

This new bile acid may be named 15α -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 3α -hydroxy-15-oxo-cholanoate, or less likely, $3\text{-oxo-}15\alpha$ -hydroxy-cholanoate (or both). Such a com-

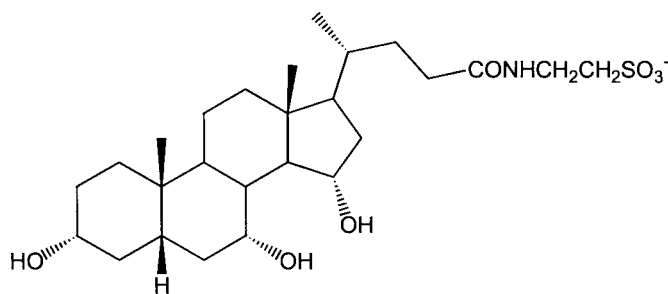


Fig. 5. Structure of $3\alpha,7\alpha,15\alpha$ -trihydroxy- 5β -cholan-24-oyl taurine.

TABLE 2. Biliary bile acids of subfamily Dendrocygninae of the family Anatidae (order Anseriformes) based on HPLC analysis

Trivial Name	Latin Name	Pattern of Hydroxylation ^a	
		3 α ,7 α	3 α ,7 α ,15 α
Indian whistling tree duck	<i>Dendrocygna arcuata</i>	99.6	0.4
White-faced whistling tree duck	<i>Dendrocygna viduata</i>	99.2 \pm 0.5	0.8 \pm 0.5
Plumed whistling tree duck	<i>Dendrocygna eytoni</i>	97.6	2.4
Java tree duck	<i>Dendrocygna javanica</i>	94.7	5.3
Black-bellied tree duck	<i>Dendrocygna autumnalis</i>	99.4, 100.0, 41.4	0.6, 0, 58.6
Fulvous whistling duck	<i>Dendrocygna bicolor</i>	98.0	2.0

All bile acids were conjugated with taurine.

^aOnly one bird in each species was analyzed, with the exception of the white-faced whistling tree duck (n = 5), with values shown as means \pm SD, and the black-bellied tree duck (n = 3), for which values of individual birds are given. Phocacholic acid (3 α ,7 α ,23R-trihydroxy-5 β -cholan-24-oic acid) was not present in any species.

pound could be formed by bacterial enzymes during the enterohepatic cycling of bile acids, as intestinal bacteria are well known to affect deconjugation, dehydroxylation at C-7, and dehydrogenation at any of the nuclear hydroxy groups (16).

Occurrence of 3 α ,7 α ,15 α -trihydroxy-5 β -cholan-24-oic acid in birds

The black-necked swan is a member of the subfamily Anserinae of the family Anatidae. The family Anatidae in turn is a member of the order Anseriformes. [For a review of the phylogeny of Anserinae, see Livezey (17) and Donne-Goussé, Laudet, and Hänni (18).] In the order Anseriformes, there are two additional families, Anhimidae (three species of screamers) and Anserindae (only one

species, the magpie goose). The 15 α -hydroxy bile acid was not present in the biliary bile acids of the Southern screamer (*Chauna torquata*) or in the magpie goose (unpublished observation).

The family Anatidae can be divided into 10 subfamilies. The 15 α -hydroxy compound was present in 6 of 6 species in the subfamily Dendrocygninae (tree ducks) and in 10 of 13 species in the subfamily Anserinae (swans and geese). HPLC analyses are presented in **Tables 2** and **3**. The 15 α -hydroxy compound was present in the greatest proportion in the whooper swan, a common swan of Europe. The proportion of the 15 α -hydroxy compound was highly variable in those species in which multiple birds were examined, suggesting marked polymorphism in the cytochrome P450 enzyme responsible for hydroxylation at C-15 (19). No sample from the closely related subfamily Thalassorninae (white-backed duck) was available to us.

The 15 α -hydroxy compound was not present in the remaining seven subfamilies in the family Anatidae. These subfamilies comprise Stictonettinae (freckled ducks), Plecotropterinae (spur-wing geese), Tadorninae (transition species between geese and ducks), Anatinae (dabbling and diving ducks), Merginae (eiders, scooters, and mergansers), and Oxyurinae (stiff-tail ducks). In these species, the third site of hydroxylation was at C-23(R), the α -carbon of the side chain.

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, 3 α ,7 α ,15 α -trihydroxy-5 β -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.

TABLE 3. Biliary bile acids of subfamily Anserinae of the family Anatidae (order Anseriformes)

Trivial Name	Latin Name	Pattern of Hydroxylation ^a		
		3 α ,7 α	3 α ,7 α ,15 α	3 α ,7 α ,23R
Cape barren goose	<i>Cereopsis novaehollandiae</i>	100.0	0	0
Coscoroba swan	<i>Coscoroba coscoroba</i>	96.6	3.4	0
Black swan	<i>Cygnus atratus</i>	91.7	8.3	0
Whooper swan	<i>Cygnus cygnus</i>	50.4	49.6	0
Black-necked swan ^b	<i>Cygnus melanocoryphus</i>	84.6	15.4	0
Canada goose	<i>Branta canadensis</i>	89.5, 93.2	0, 0	10.5, 6.8
Dusky Canada goose	<i>Branta canadensis occidentalis</i>	85.6	3.8	10.6
Barnacle goose	<i>Branta leucopsis</i>	79.8	17.6	2.6
Red-breasted goose	<i>Branta ruficollis</i>	89.6, 82.3	6.0, 13.7	4.4, 4.0
Nene goose	<i>Branta sandvicensis</i>	85.5	4.0	10.5
Greater white-fronted goose	<i>Anser albifrons</i>	96.9	0	3.1
Snow goose	<i>Anser caerulescens</i>	99.4	0	0.6
Swan goose	<i>Anser cygnoides</i>	92.3	2.2	5.5
Bar-headed goose	<i>Anser indicus</i>	86.7, 86.8	4.8, 6.8	8.5, 6.4

All bile acids were conjugated with taurine.

^aOnly one bird was available for each species, with the exception of the black-necked swan (n = 9), the red-breasted goose (n = 2), the bar-headed goose (n = 2), and the Canada goose (n = 2). Individual values for the latter three species are given.

^bFor the black-necked swan, in seven of nine birds, the proportion of 15 α -hydroxy-chenodeoxycholic acid ranged from 0 to 4%; the eighth bird contained 58.7%; and the ninth bird, which was used for isolation of the 15-hydroxy compound, contained 74%. The mean value for all nine birds are given.

In 1991, Pyrek (20) (now deceased) reported in a review article on mass spectrometry of natural products that $3\alpha,15\alpha$ -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 $3\alpha,7\alpha,15\alpha$ -trihydroxy- 5β -cholan-24-oic acid (5% of total biliary bile acids) and consistent with that predicted for the taurine conjugate of $3\alpha,15\alpha$ -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 $3\alpha,15\alpha$ -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 C_{21} 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

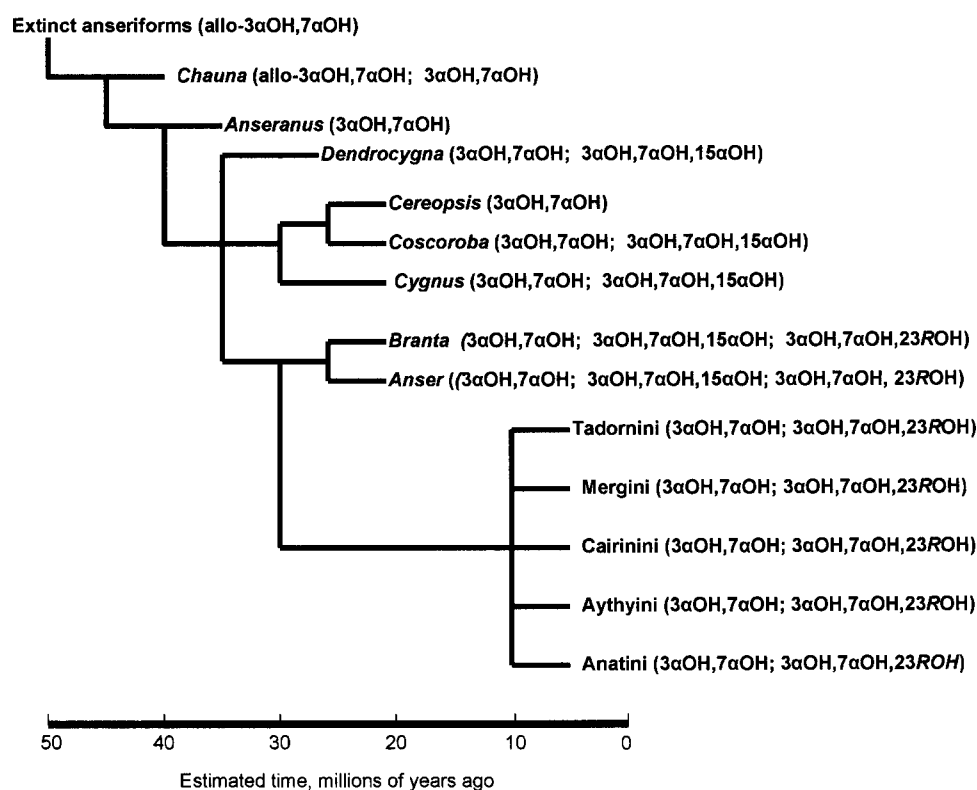


Fig. 6. Biliary bile acid composition denoted by nuclear substituents in relation to an evolutionary scheme for the order Anseriformes based on morphological studies (17) and mitochondrial DNA analysis (18). 15α -Hydroxylation arose in the subfamilies of Anatidae but was replaced by 23(*R*)-hydroxylation 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 cholanoic acid nucleus in primary bile acids is now seven (6 α , 6 β , 12 α , 1 α , 1 β , 15 α , and 16 α). 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. ■

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