# A novel primary bile acid in the Shoebill stork and herons and its phylogenetic significance

**L. R. Hagey,[1,*] C. D. Schteingart,[†] H-T. Ton-Nu,[†] and A. F. Hofmann[†]**

Zoological Society of San Diego,* San Diego, CA 92112; and Department of Medicine,† University of California, San Diego, San Diego, CA 92093

**Abstract The Shoebill stork, an enigma phylogenetically, was found to contain as its dominant biliary bile acid 16α-hydroxychenodeoxycholic acid, a heretofore undescribed bile acid. The bile acid occurred as its taurine *N*-acyl amidate; structure was established by nuclear magnetic resonance (NMR) and mass spectrometry (MS). A search for this novel bile acid in other Ciconiiformes showed that it constituted >92% of biliary bile acids in five of nine herons in the Ardidae, but was absent in all other families (Ciconiidae, Threskiornithidae, Scopidae, Phoenicopteridae). The presence of this biochemical trait in the Shoebill stork and certain herons suggests that these birds are closely related.**—Hagey, L. R., C. D. Schteingart, H-T. Ton-Nu, and A. F. Hofmann. **A novel primary bile acid in the Shoebill stork and herons and its phylogenetic significance. *J. Lipid Res.* 2002.** 43: **685–690.**

**Supplementary key words** 16α-hydroxylation • bile acid metabolism • Ciconiiformes

The Shoebill stork (*Balaeniceps rex*), also termed the whale-bill or whale-head stork, is a large, dark blue-gray wading bird, with a wide thick bill shaped as its name implies (**Fig. 1**). The avian family, Balaenicipitidae, which contains the Shoebill stork as its only member, is part of the order Ciconiiformes, a heterogeneous group of large long-legged wading birds. The phylogenetic assignment of the shoebill stork has long been a subject of controversy among avian biologists. Based on morphological similarities, the Shoebill stork has been thought to be related to other storks (1), to the herons (2), or even to the pelican, a completely different order (3). A recent study of mitochondrial DNA from a large number of Ciconiiformes has also concluded that the Shoebill stork is closely related to the pelicans (4).

Bile acids are the water-soluble, amphipathic end products of cholesterol metabolism. They are synthesized in the liver, conjugated with glycine or taurine, and are secreted into the intestine where they facilitate the absorption of dietary lipids. A major fraction is reabsorbed from the intestine, taken up by the liver, and resecreted into bile; this movement between liver and the intestine is termed the enterohepatic circulation. During enterohepatic cycling, the hydroxyl groups of bile acids undergo biotransformation by intestinal bacteria, the major changes being dehydroxylation at C-7 and dehydrogenation of one or more hydroxyl groups. These "secondary" bile acids are absorbed in part and also cycle enterohepatically together with the "primary" bile acids formed in the liver (5). Although the steady state composition of biliary bile acids is the result of hepatic biosynthesis and bacterial modification, it is relatively constant in the adult animal and is influenced little by diet or gender (6).

In contrast to most small molecules found in vertebrates, bile acids are strikingly diverse in their pattern of hydroxylation and side chain structure (7). Bile acid structure shows a pattern of progressive molecular development in the course of vertebrate evolution (8). The parallel evolution between vertebrate species and bile acid molecular structures suggests that bile acid structure may provide useful phylogenetic information. Here, we characterize the biliary bile acids of the Shoebill stork and other birds of the order Ciconiiformes.

## METHODS

Bile samples were obtained from deceased birds housed at the San Diego Zoo, and were provided by the Pathology Laboratory of the San Diego Zoo. Gallbladder samples were obtained by puncture and aspiration. Bile samples were diluted in several volumes of reagent grade isopropanol immediately after collection to prevent bacterial degradation and to precipitate biliary proteins. The protocol had been approved by the Committee on Animal Studies of the University of California, San Diego.

Conjugated bile acids were analyzed by reverse phase HPLC using a $C_{18}$ column and a methanol phosphate buffer system (9) based on that described by Rossi et al. (10), and by TLC. Bile acids were isolated by collecting the HPLC eluant or by scraping se-

Abbreviations: GC-MS, gas chromatography-mass spectrometry; NMR, nuclear magnetic resonance; MS, mass spectrometry; SIMS, Secondary ion mass spectrometry; SC, side chain.

[1] To whom correspondence should be addressed.
e-mail: lhagey@ucsd.edu

**Fig. 1.** Shoebill stork (*Balaeniceps rex*). The photograph was provided by the Zoological Society of San Diego and is used with their permission.

lected silica gel bands from a TLC plate after using a double development system (11). Bile acids were deconjugated chemically (1 N NaOH, 130°C, 4 h) and isolated by solvent extraction.

Gas chromatography-mass spectrometry (GC-MS) analyses of compounds A–E was performed using a Series II model 5890 gas chromatograph equipped with a Hewlett-Packard 5970 Series Mass Selective detector. Compounds were chromatographed as their TMSi methyl ester derivatives. Separation was carried out on a 30 m SPB-35 (Supelco, Bellefonte, PA) capillary column at 272°C isothermal with 7 psi of helium carrier gas. Relative retention times and fragmentation spectra of peaks obtained by GC-MS were compared with those of known standards for identification.

Secondary ion mass spectrometry (SIMS) was performed at the University of California, San Francisco, on a Kratos MS-50 mass spectrometer equipped with a cesium ion source operated at approximately 100 $\mu A/cm^2$ beam flux in the negative mode. Glycerol was used as liquid matrix on a copper probe tip.

Evidence for hydroxylation at the 16 position and in the α-configuration was obtained using $^1$H- and $^{13}$C-NMR. Proton nuclear magnetic resonance spectra of the methyl ester per acetyl derivatives of the bile acids obtained by deconjugation of peaks A and C were recorded in $Cl_3CD$ at 360 MHz. $^{13}$C-NMR spectra of the methyl ester derivatives of C and D were recorded in $Cl_3CD$ at 125 MHz. Multiplicities were determined with the APT sequence. Chemical shifts are given in ppm relative to tetramethylsilane; for $^{13}$C-NMR, the central peak of the signal of $Cl_3CD$ was used as reference δ 77.0 ppm.

## RESULTS

HPLC analysis of the conjugated bile acids in gallbladder bile from the Shoebill stork (**Fig. 2**) showed six major peaks (A–F). The retention times for five of the six peaks did not correspond to those of known bile acids. To identify these unknown compounds, the HPLC eluant containing each major peak of the HPLC chromatogram was collected and analyzed using SIMS. The quasimolecular ions obtained were as follows: *A*) *m/z* 530, a tetrahydroxy $C_{24}$ bile acid; *B*) *m/z* 512, a trisubstituted (dihydroxy, oxo) $C_{24}$ bile acid; *C*) *m/z* 514; *D*) *m/z* 514, trihydroxy $C_{24}$ bile acids; *E*) *m/z* 496, a disubstituted (hydroxy, oxo) $C_{24}$ bile acid; and *F*) *m/z* 498, 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 and the known dominance of taurine conjugation in vertebrate bile acids (8).

All bile acids have a 3 hydroxyl group since they are formed from cholesterol. All primary bile acids described to date also have a 7-hydroxyl substituent (or its oxo derivative) since cholesterol 7α-hydroxylase is considered to be the rate-limiting enzyme in bile acid biosynthesis. Therefore, we reasoned that the unknown bile acids had a third hydroxyl group either on the steroid nucleus or the side chain. To identify this position, material from the largest peak, C, was

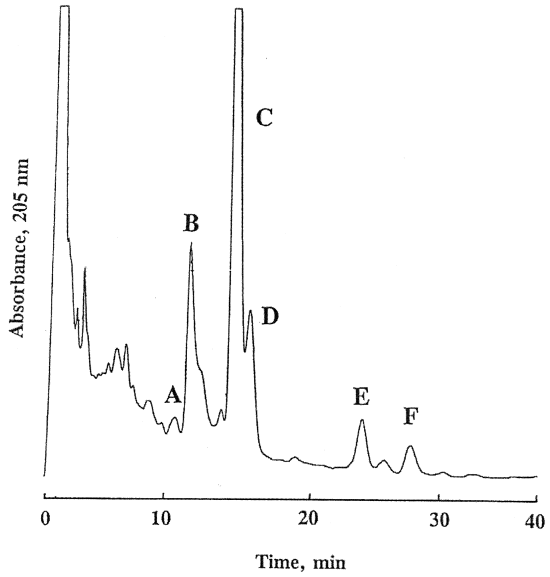



**Fig. 2.** Reversed phase HPLC of the biliary bile acids of the Shoebill stork. For each peak (A–F), the compound, its relative retention time, and its percent of total biliary acids are given. A: 3α,7α,12α,16α-tetrahydroxy-5β-cholan-24-oyl-taurine (0.306) 1.2%. B: dihydroxy-oxo-derivative of C (0.310) 9.3%. C: 3α,7α,16α-trihydroxy-5β-cholan-24-oyl-taurine (0.392) 68.9%. D: 3α,12α,16α-trihydroxy-5β-cholan-24-oyl-taurine (0.409) 7.4%. E: 7-deoxy-oxo-derivative of C (0.590) 9.1%. F: chenodeoxycholyl-taurine (0.670) 4.1%.

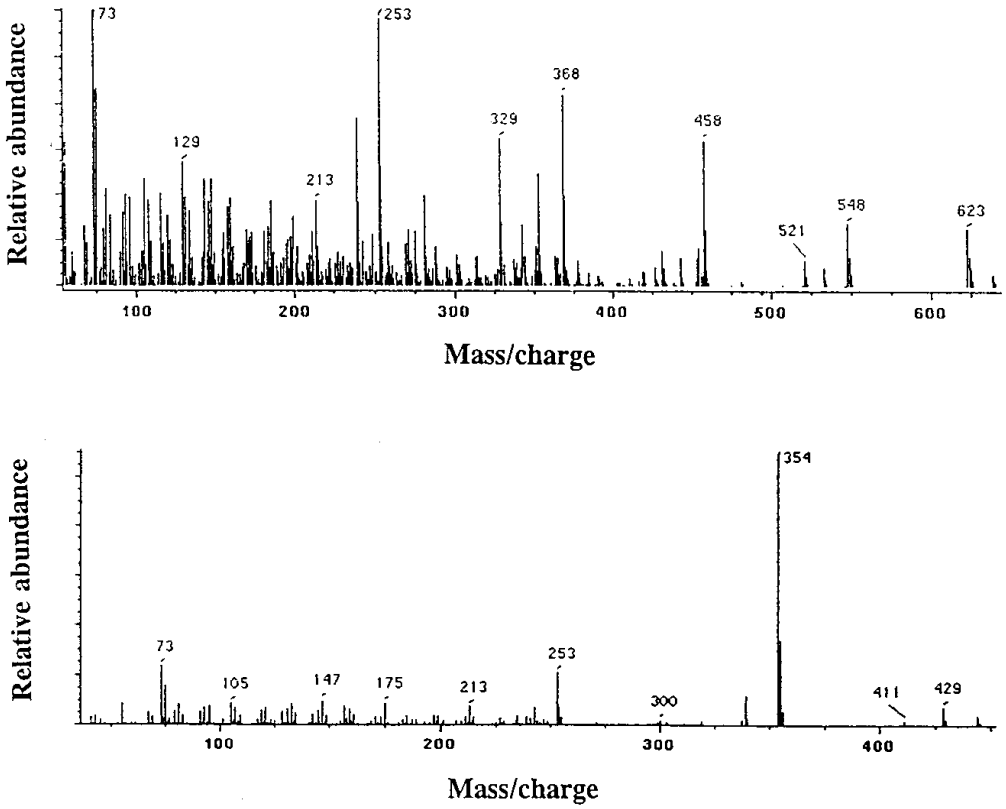


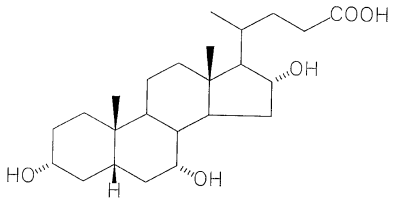

**Fig. 3.** A: Gas chromatography-mass spectrometry (GC-MS) fragmentation pattern of the methyl ester per trimethylsilyl (TMSi) derivative of compound C. B: GC-MS fragmentation pattern of the methyl ester per TMSi derivative of the lactone of compound C.

isolated, deconjugated, and the methyl ester per-TMSi derivative formed. The fragmentation pattern is shown in **Fig. 3A**. Major fragments included: *m/z* 623 (3.6%) M-15; *m/z* 548 (4.1%) M-90; *m/z* 533 (1.2%) M-90-15; *m/z* 521 (1.7%) M-87-15; *m/z* 458 (9.2%) M-90-90; *m/z* 443 (1.8%) M-90-90-15; *m/z* 433 (2.3%) M-SC-90; *m/z* 368 (12.3%) M-90-90-90; *m/z* 353 (7.2%) M-90-90-90-15; *m/z* 343 (4.0%) M-SC-90-90; *m/z* 329 (9.5%) M-129-90-90; *m/z* 281 (5.8%) M-87-90-90-90; *m/z* 253 (17.1%) M-SC-90-90-90; *m/z* 239 (10.8%) M-129-90-90-90; *m/z* 213 (5.4%) M-230-90-90. The value of 129 is derived from the side chain plus C-17; 87 is C-22–C-24 of the side chain; 230 is the side chain plus the D-ring.

Under the conditions used during GC-MS analysis, a considerable amount of a second compound, a lactone, (Fig. 3B) was generated as an artifact. This lactone provided a powerful clue to the identity of compound C. The large peak at *m/z* 354 [formally, M-$CH_3OSi(CH_3)_3$-TMSOH-TMSOH] suggested that this artifact could be a lactone produced by cyclization of the 24-carboxyl group to a hydroxyl group on a nearby position (16, 17, or 20). High resolution mass spectrometry of a sample of the methyl ester of the bile acid isolated from C showed a set of fragments for the expected trihydroxy methyl ester, and another set corresponding to the loss of the methanol moiety, as would be expected for a lactone.

By analysis of the $^1$H-NMR spectrum of the methyl ester peracetate, and the $^{13}$C-NMR spectrum of the methyl ester derivative, it was determined that the third hydroxyl group was located in position 16α. Proton assignments were as follows: 0.702 (s, 3H, Me-18); 0.928 (s, 3H, Me-19); 0.960 (d, J = 6.5 Hz, 3H, Me-21); 1.997 (s, 3H, $CH_3COO^-$); 2.035 (s, 3H, $CH_3COO^-$); 2.049 (s, 3H, $CH_3COO^-$); 3.649 (s, 3H, COOMe); 4.591 (m, 1H, H-3); 4.813 (m, 1H, H-7); and 4.939 (bt, J = 6.5 Hz,1H, H-16) for a structure of methyl-3α,7α,16α-triacetyloxy-5β-cholan-24-oate. $^{13}$C-NMR chemical shifts for the methyl ester of 3α,7α,16α-trihydroxy-5β-cholan-24-oic acid were as follows: C-1 35.29; C-2 30.66; C-3 72.03; C-4 39.60[c]; C-5 41.54; C-6 34.22[b]; C-7 68.41; C-8 38.92; C-9 32.61; C-10 35.08[b]; C-11 20.27; C-12 39.99[c]; C-13 44.04; C-14 47.43; C-15 36.25; C-16 76.81; C-17 66.11; C-18 13.14; C-19 22.80; C-20 33.72; C-21 18.60; C-22 30.80[a]; C-23 30.95[a]; C-24 175.07; and $CH_3$- 51.55. (Superscripts denote peaks that may be exchanged.) Key signatures for the 16α hydroxylation pattern were the downfield displacement of C-17 to 66.3 ppm and the upfield shift of C-14 to 47.4 ppm from their chemical shifts in methyl chenodeoxycholate (55.7 and 50.3 ppm, respectively) (12). Thus, peak C was the taurine conjugate of 3α,7α,16α-trihydroxy-5β-cholan-24-oic acid (**Fig. 4**).

Peak A showed a *m/z* value of 530, which corresponded to a taurine conjugated $C_{24}$ tetrahydroxy bile acid. Because C-12 is a common site of bile acid hydroxylation in vertebrates, we thought it likely that this compound contained hydroxyl groups on carbon atoms 3, 7, 12, and 16. To identify the location and orientation of the fourth hy-




16α-Hydroxychenodeoxycholic acid

**Fig. 4.** Structure of 16α-hydroxychenodeoxycholic acid.

droxyl group and to confirm the positions of the other three hydroxyl groups, the compound was isolated and the methyl ester peracetyl derivative examined by $^{1}$H-NMR. Proton assignments were as follows: 0.701 (s, 3H, Me-18), 0.804 (s, 3H, Me-19), 0.953 (d, J = 6.5 Hz, 3H, Me-21), 1.990 (s, 3H, $CH_3COO^-$), 2.090 (s, 3H, $CH_3COO^-$), 2.060 (s, 3H, $CH_3COO^-$), 2.108 (s, 3H, $CH_3COO^-$), 3.648 (s, 3H, COOMe), 4.602 (m, 1H, H-3), 4.899 (m, 1H, H-7), 4.939 (bt, J = 6.5 Hz, 1H, H-16), and 5.027 (m, 1H, H-12), for a structure of methyl-3α,7α,12α,16α-tetraacetyloxy-5β-cholan-24-oate.

This analysis confirmed that peak A was the taurine conjugate of 3α,7α,12α,16α-tetrahydroxy-5β-cholan-24-oic acid. The structure of the methyl ester derivative of the bile acid obtained by deconjugation of peak D was subsequently shown by $^{13}$C-NMR to be 3α,12α,16α-trihydroxy-5β-cholan-24-oic acid, the 7-deoxy metabolite of compound C. $^{13}$C-NMR chemical shifts for the methyl ester of 3α,12α,16α-trihydroxy-5β-cholan-24-oic acid were as follows: C-1 35.12; C-2 30.30; C-3 71.72; C-4 36.39; C-5 42.02; C-6 27.02; C-7 26.15; C-8 35.31; C-9 33.45[b]; C-10 31.09; C-11 28.23; C-12 72.81; C-13 48.23; C-14 45.32; C-15 36.59; C-16 76.66; C-17 57.57; C-18 14.23; C-19 23.04; C-20 33.50[b]; C-21 17.60; C-22 31.06[a]; C-23 30.56[a]; C-24 175.04; and $CH_3$-51.55.

Peak B (Fig. 1) was a dihydroxy-oxo bile acid with substituents at C-3,C-7, and C-16, presumably formed by bacterial dehydrogenation of a hydroxyl group of compound C during enterohepatic cycling. Peak E was a hydroxy-oxo-bile acid with substituents at C-3 and C-16. This compound is presumably a dehydrogenated 7-deoxy metabolite of compound C formed by bacterial enzymes during enterohepatic cycling. Peak F was readily identifiable as the common dihydroxy- primary bile acid, chenodeoxycholic acid (3α,7α-dihydroxy-5β-cholan-24-oic acid). Identification was by HPLC retention time, the *m/z* value of 498 by SIMS, and the retention time and fragmentation pattern during GC-MS.

The 16α-hydroxychenodeoxycholic acid was present as a major biliary bile acid in several heron species. **Figure 5** is the HPLC profile of biliary bile acids from a Boat-billed heron (*Cochlearius cochlearius*). The major peak (C) of this bird was also shown to be the taurine conjugate of 3α,7α, 16α-trihydroxy-5β-cholan-24-oic acid; the remaining large peak (F) was the taurine conjugate of chenodeoxycholic acid. The other peaks identified in the Shoebill stork (A, B, D, E) were not present in this heron or in the biliary bile acids of the other herons that were examined.

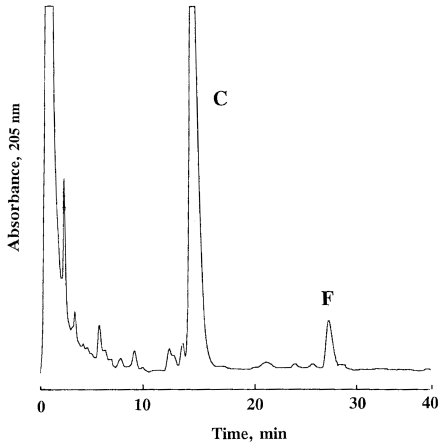



**Fig. 5.** Reversed phase HPLC of the biliary bile acids of the Boat-billed heron. Peak description according to Fig. 1 and is as follows: (C) 3α,7α,16α-trihydroxy-5β-cholan-24-oyl-taurine (0.392) 81.5%; (F) chenodeoxycholyl-taurine (0.670) 18.5%.

**Table 1** summarizes the biliary bile acids that were present in the Shoebill stork and in 9 other herons and egrets. In five of these birds, 16α-hydroxychenodeoxycholic acid was the dominant bile acid. In the remaining four birds, the dominant trihydroxy bile acid was cholic acid, in which a hydroxyl group is present at C-12.

To determine whether 16α-hydroxychenodeoxycholic acid was present in other families of the order Ciconiiformes, biliary bile acids from representative species of these families were examined by HPLC (**Table 2**). There is only one member of the Scopidae family, the Hammerhead stork, whose bile was different from the other birds. 16α-Hydroxychenodeoxycholic acid was not detected, instead the bile was dominated by chenodeoxycholic acid (97.4%). Among the Threskiornithidae (spoonbills and ibises), only one bird, the Roseate spoonbill, contained the novel bile acid, and the proportion was less than 10%. In the Ciconiidae family (storks), 16α-hydroxychenodeoxycholic acid was not present in the biliary bile acids, which consisted predominantly of cholic acid and chenodeoxycholic acid. Biliary bile acids from this group of birds also contained additional bile acids of currently unknown structure.

## DISCUSSION

The present work indicates that the dominant biliary bile acid of the Shoebill stork and certain herons is the 16α-hydroxy derivative of chenodeoxycholic acid. This compound is likely to be a primary bile acid. Hydroxylation of bile acids at C-16 was described previously in primitive snakes (boas and pythons) by the late G. A. D. Haslewood (13) but 16α-hydroxychenodeoxycholic acid has not been reported previously. Trihydroxy- $C_{24}$ bile acids occurring in other vertebrates are commonly hydroxylated at either C-12 (cholic acid) or C-6 (muricholic acid, hyocholic acid) in addition to the default hydroxylations at C-3 and C-7; hydroxylation at C-1 has also been reported (14, 15). Formation of

TABLE 1. Biliary bile acid composition by HPLC of the Shoebill stork and representative members of the family Ardeidae (herons)

| Common Name | Latin Name | Bile Acid Composition, %[a,c] | | | |
|---|---|---|---|---|---|
| | | 3α7α[b] | 3α7α16α | 3α7α12α | 3α7α12α16α |
| Balaenicipitae | | | | | |
| Shoebill stork | *Balaeniceps rex* | 5.6 | 92.8 | 0.0 | 1.6 |
| Ardeidae | | | | | |
| Cattle Egret | *Ardeola ibis* | 2.0 | 98.0 | 0.0 | 0.0 |
| Great Blue heron | *Ardea herodias* | 5.9 | 94.1 | 0.0 | 0.0 |
| Little Blue heron | *Hydranessa caerula* | 7.8 | 92.2 | 0.0 | 0.0 |
| Boat-billed heron | *Cochlearius cochlearius* | 7.2 | 92.8 | 0.0 | 0.0 |
| Great egret | *Egretta alba* | 24.2 | 75.8 | 0.0 | 0.0 |
| Snowy egret | *Egretta thula* | 55.0 | 33.6 | 11.4 | 0.0 |
| Black-capped night heron | *Nycticorax nycticorax* | 42.0 | 0.0 | 58.0 | 0.0 |
| Goliath heron | *Ardea goliath* | 45.8 | 0.0 | 54.2 | 0.0 |
| Whistling heron | *Syrigma sibilatrix* | 32.4 | 0.0 | 67.6 | 0.0 |

[a] Gallbladder bile acid composition has been normalized to 100%. Bile acids reported usually comprise >95% of total bile acids.

[b] Abbreviations: 3α7α, chenodeoxycholic acid; 3α7α16α, 16α-hydroxychenodeoxycholic acid; 3α7α12α, cholic acid; 3α7α12α16α, 16α-hydroxycholic acid.

[c] Bile acids were amidated with the amino acid taurine.

a trihydroxy- bile acid occurs by a cytochrome P450 hydroxylase acting either on chenodeoxycholic acid or an early intermediate in its biosynthesis (16). The 3α,12α,16α-trihydroxy bile acid is likely to be formed by bacterial 7-dehydroxylation of the 3α,7α,12α,16α-tetrahydroxy- bile acid during enterohepatic cycling (17).

16α-Hydroxychenodeoxycholic acid was also the dominant bile acid of several herons. The presence of a common bile acid profile and a number of similarities in the musculoskeletal system (1) suggest that the Shoebill stork and at least some herons are closely related. A study on heron nuclear and mitochondrial DNA by Sheldon et al. (18) placed the Boat-billed heron at the root of the family. This bird had a very high proportion of 16α-hydroxy-bile acids, whereas more recently evolved species had 12-hydroxy bile acids. These data suggest that 16α-hydroxylation is the primitive condition within this family.

Biliary bile acid composition has also been used by us to suggest that the flamingo (Phoenicopteridae) belongs to the order Anseriformes, rather than to the order Ciconiiformes (19). The flamingo has biliary bile acids rich in 3α,7α,23R-trihydroxy-5β-cholan-24-oic acid, a bile acid common in geese and ducks.

In summary, these analyses of biliary bile acids of representative species of the order Ciconiiformes have identified a novel bile acid, 16α-hydroxychenodeoxycholic acid, that is present in the Shoebill stork and certain herons. If bile acid structure provides valid information on evolu-

TABLE 2. Biliary bile acid composition by HPLC of the Shoebill stork and representative members of the families Scopidae, Threskiornithidae (ibises and spoonbills), and Ciconiidae (storks)

| Common Name | Latin Name | Bile Acid Composition, %[a,c] | | | |
|---|---|---|---|---|---|
| | | 3α7α[b] | 3α7α16α | 3α7α12α | unknown |
| Balanencipitae | | | | | |
| Shoebill stork | *Balaenceps rex* | 5.6 | 94.4 | 0.0 | 0.0 |
| Scopidae | | | | | |
| Eastern Hammerkop | *Scopus umbretta* | 97.4 | 0.0 | 2.6 | 0.0 |
| Threskiornithidae | | | | | |
| Roseate Spoonbill | *Ajaia ajaia* | 84.3 | 5.5 | 10.2 | 0.0 |
| Black-headed ibis | *Threskiornis melanocephalus* | 89.3 | 0.0 | 10.7 | 0.0 |
| White spoonbill | *Platelea leucorodia* | 83.7 | 0.0 | 16.3 | 0.0 |
| Hadada ibis | *Hagedashia hagedash* | 72.7 | 0.0 | 27.3 | 0.0 |
| Puna ibis | *Plegadis ridgewayi* | 53.6 | 0.0 | 46.4 | 0.0 |
| Scarlet ibis | *Eudocimus ruber* | 24.5 | 0.0 | 75.5 | 0.0 |
| American white ibis | *Eudocimus albus* | 9.3 | 0.0 | 90.7 | 0.0 |
| Ciconiidae | | | | | |
| Marabou stork | *Leptoptilos crumeniferus* | 55.5 | 0.0 | 39.7 | 4.8 |
| Lesser adjutant stork | *Leptoptilos javanicus* | 33.6 | 0.0 | 63.3 | 3.1 |
| African open-bill stork | *Anastomus lamelligerus* | 82.6 | 0.0 | 10.5 | 6.9 |
| Black stork | *Ciconia nigra* | 4.9 | 0.0 | 80.0 | 15.1 |
| Woolly-neck stork | *Ciconia episcopus* | 20.0 | 0.0 | 46.3 | 33.7 |
| Painted stork | *Mycteria leucocephala* | 19.6 | 0.0 | 36.2 | 44.2 |
| Milky stork | *Mycteria cinerea* | 2.6 | 0.0 | 41.8 | 55.6 |

Abbreviations as in Table 1.

tionary relationships, these species are phylogenetically related.

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## REFERENCES

1. Feduccia, A. 1977. The whalebill is a stork. *Nature.* **266:** 719–720.
2. Cracraft, J. 1981. Toward a phylogenetic classification of the recent birds of the world (class Aves). *Auk.* **98:** 681–714.
3. Hedges, S. B., and C. G. Sibley. 1994. Molecules vs morphology in avian evolution: the case of the "palecaniform" birds. *Proc. Natl. Acad. Sci. USA.* **91:** 9861–9865.
4. Van Tuinen, M., D. B. Butvil, J. A. Kirsch, and S. B. Hedges. 2001. Convergence and divergence in the evolution of aquatic birds. *Proc. R. Soc. Lond. B.* **268:** 1345–1350.
5. Hofmann, A. F. 1994. Bile acids. *In* The Liver: Biology and Pathobiology. I. M. Arias, J. L. Boyer, N. Fausto, W. B. Jakoby, D. Schachter, D. A. Shafritz, editors. Raven Press, Ltd., New York. 677–718.
6. Hagey, L. R., M. A. Gavrilkina, and A. F. Hofmann. 1997. Age-related changes in biliary bile acid composition of bovids. *Canadian J. Zoology.* **75:** 1193–1201.
7. 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, U. Beuers, editors. Kluwer Academic Publishers, Boston. 3–30.
8. Hagey, L. R. 1992. Bile acid biodiversity in vertebrates: chemistry and evolutionary implication. Ph.D. Dissertation. University of California, San Diego, San Diego, CA.
9. Hagey, L. R., C. D. Schteingart, S. S. Rossi, H-T. Ton-Nu, and A. F. Hofmann. 1998. An *N*-acyl glycyltaurine conjugate of deoxycholic acid in the biliary bile acids of the rabbit. *J. Lipid Res.* **39:** 2119–2124.
10. Rossi, S. S., J. L. Converse, and A. F. Hofmann. 1987. High pressure liquid chromatographic analysis of conjugated bile acids in human bile: simultaneous resolution of sulfated and unsulfated lithocholyl amidates and the common conjugated bile acids. *J. Lipid Res.* **28:** 589–595.
11. Elferink, R. P. O., J. De Haan, K. J. Lambert, L. R. Hagey, A. F. Hofmann, and P. L. M. Jansen. 1989. Selective hepatobiliary transport of nordeoxycholate side chain conjugates in mutant rates with a canalicular transport defect. *Hepatology.* **9:** 861–865.
12. Iida, I., T. Tamura, T. Matsumoto, F. C. Chang. 1983. Carbon-13 NMR spectra of hydroxylated bile acid sterioisomners. *Organic Magnetic Resonance.* **21:** 305–309.
13. Haslewood, G. A. D. 1978. The Biological Importance of Bile Salts. North Holland Publishing Co., Amsterdam.
14. Lee, S. P., R. Lester, and J. St.Pyrek. 1987. Vulpecholic acid (1α,3α, 7α-trihydroxy-5β-cholan-24-oic acid): A novel bile acid from a marsupial, *Trichosurus vulpecula. J. Lipid Res.* **28:** 19–31.
15. Hagey, L. R., C. D. Schteingart, H-T. Ton-Nu, and A. F. Hofmann. 1994. Biliary bile acids of fruit pigeons and doves (Columbiformes): presence of 1-β-hydroxychenodeoxycholic acid and conjugation with glycine as well as taurine. *J. Lipid Res.* **35:** 2041–2048.
16. Bjorkhem, I. 1985. Mechanism of bile acid biosynthesis in mammalian liver. *In* Sterols and Bile Acids. H. Danielsson, and J. Sjövall, editors. Elsevier, Amsterdam. 231–272.
17. Hylemon, 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.
18. Sheldon, F. H., C. E. Jones, and K. G. McCracken. 2000. Relative patterns and rates of evolution in heron nuclear and mitochondrial DNA. *Mol. Biol. Evol.* **17:** 437–450.
19. Hagey, L. R., C. D. Schteingart, H-T. Ton-Nu, S. S. Rossi, D. Odell, and A. F. Hofmann. 1990. β-Phocacholic acid in bile: biochemical evidence that the flamingo is related to an ancient goose. *The Condor.* **92:** 593–597.