Novel Bile Acids from Bear Bile Powder and Bile of Geese

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Two new bile acids, tauroselocholic acid (1) and tauroansocholic acid (2), a new natural bile acid, cygnocholic acid (3) were respectively isolated from bear bile powder *Selenaretos thibetanus* Cuvier and bile of geese *Anser anser domesticus*, together with seven known compounds. By spectrum analysis of MS, 1D and 2D NMR, the structures of the new compounds were elucidated as 3α,7α, 9α-trihydroxy-5β-cholan-24-oic acid *N*-[2-sulfoethyl] amide (1), 3α,5,7α-trihydroxy-5β-cholan-24-oic acid *N*-[2-sulfoethyl] amide (2) and 3α,7α,15α-trihydroxy-5β-cholan-24-oic acid (3).

Key words *Selenaretos thibetanus*; *Anser anser domesticus*; bile acid

Bile acids, as the water-soluble amphipathic end products of cholesterol metabolism, are important due to their roles in elimination of cholesterol and absorption of lipids and fatsoluble vitamins in the intestine.[1)] C24 bile acids found in most mammals are present in bile as *N*-acyl amidates (conjugates) of taurine or glycine.[2)] All primary C24 bile acids have a hydroxyl group at C-3 which is from cholesterol and at C-7, as cholesterol 7α-hydroxylation is the rate-limiting step in bile acid biosynthesis. Thus, chenodeoxycholic acid (CDCA; 3α,7α-dihydroxy-5β-cholan-24-oic acid) is the root C24 bile acid.[3)] Chenodeoxycholic acid and ursodeoxycholic acid (UDCA; 3α,7α-dihydroxy-5β-cholanoic acid) are widely used for the treatment of cholesterol gall-stones and ursodeoxycholic acid has been introduced for the therapy of cholestatic liver diseases.[4—9)] In the previous study, several bile acids including CDCA, CA, TCDCA, TUDCA, TCA were obtained from bile of *Ursus arctos* by Yamaguchi *et al.*[10)] In order to search for new natural bioactive constituents from animal bile and provide chemical standards for the research on its quality control, two kinds of bile from different animals including *Selenaretos thibetanus* Cuvier and *Anser anser domesticus* were chemically investigated in our research group. This paper describes the isolation and structural determination of two novel bile acids, tauroselocholic acid (**1**), tauroansocholic acid (**2**), and a new natural bile acid, cygnocholic acid (**3**) which had been synthesized by Iida *et al.*,[11)] together with seven known compounds from these two kinds of bile.

Results and Discussion

Compound **1** was isolated as colorless syrup, and it showed positive Gregory Pascoe reaction, suggesting it to be a bile acid. Its molecular formula $C_{26}H_{45}NO_7S$ was established by the pseudo-molecular ion peak at *m*/*z* 514.2860 $[M-H]^-$ in the HR-ESI-MS and confirmed by the NMR data analysis. The IR spectrum indicated the presence of hydroxyl group (3420 cm^{-1}), secondary amide group (1647, 1551 cm^{-1}) and sulfonic acid group (1210 cm^{-1}).

The ^{1}H-NMR data (Table 1) exhibited signals of two singlet methyl groups [δ_H 0.69 (3H, s), 0.90 (3H, s)], one doublet methyl group [δ_H 0.95 (3H, d, J=6.5 Hz)], two hydroxymethines [δ_H 3.35, 3.88 (1H, m)], and an AB coupling system of a taurine group [δ_H 2.95, 3.55 (2H, t, J=7.0 Hz)]. Its ^{13}C-NMR spectra (distortionless enhancement by polarization transfer (DEPT)) exhibited three methyls, twelve methylenes, seven methines and four quaternary carbons. Analysis of the ^{13}C-NMR spectrum showed three hydroxylated carbons at δ 68.2 (CH), 73.1 (CH) and 75.8 (q C), and one amide carbonyl at δ 176.6. The above data showed high similarity with those of the known compound, 3α,7α-dihydroxy-5β-cholanoic acid *N*-[2-sulfoethyl] amide (**8**)[10)] except for an additional hydroxyl group, which implied **1** to has 3α-hydroxyl and 7α-hydroxyl substituents. Interpretation of the ^{1}H–^{1}H correlation spectroscopy (COSY) spectrum led to the determination of partial structures I, II and III (Fig. 2). Partial structure I were connected to II by heteronuclear multiple bonding connectivity (HMBC) correlations (Fig. 2) from H_3-19 to C-5, C-9, C-10, from H_3-18 to C-12, C-13, C-14, C-17, and from H-11β to C-8. The structural moiety I was also connected to III by HMBC correlations (Fig. 2) from H_2-23 and H_2-25 to C-24. Analysis of ^{13}C-NMR spectra through DEPT of **1** also showed that C-9 was a non-protonated carbon. Thus, the additional hydroxyl group was determined at C-9, which was confirmed by HMBC correlations (Fig. 2) from H_2-1 and H_3-19 to C-9 (δ 75.8).

The H_3-19/H-5β correlation and the absence of the H_3-18/H-14α correlation in NOESY spectrum (Fig. 3) of compound **1** established *cis* type junction of rings-A/B and *trans*

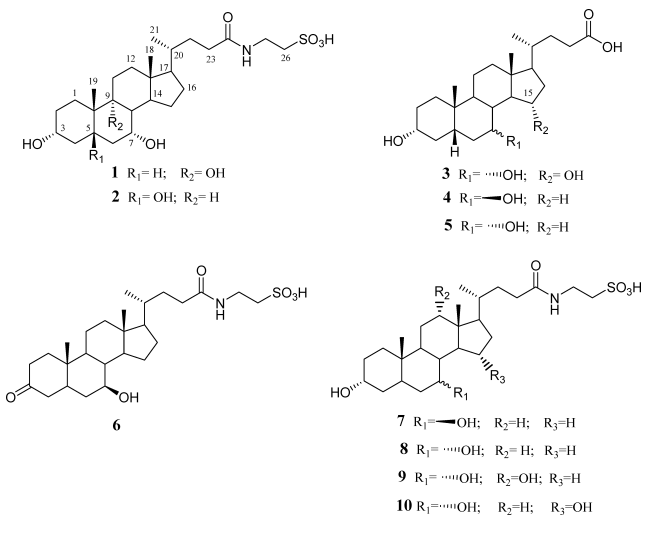

Fig. 1. Structures of Compounds **1**—**10**

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Table 1. ^{1}H-NMR Data (in CD_3OD, 500 MHz) for **1**—**3**, δ in ppm

Number	**1**			**2**			**3**		
	Type	^{1}H		Type	^{1}H		Type	^{1}H	
		α	β		α	β		α	β
1	CH_2	1.62	1.68	CH_2	1.53	1.32	CH_2	1.83	1.01
2	CH_2	1.20	1.55	CH_2	1.34	1.62	CH_2	1.35	1.63
3	CH	—	3.34	CH	—	3.82	CH	—	3.39
4	CH_2	1.25	1.85	CH_2	1.65	1.84	CH_2	2.16	1.63
5	CH	—	1.35	C	—	—	CH	—	1.35
6	CH_2	1.71	1.61	CH_2	2.52	1.67	CH_2	1.55	2.07
7	CH	—	3.88	CH	—	3.82	CH	—	4.01
8	CH	—	1.40	CH	—	1.48	CH	—	1.66
9	C	—	—	CH	1.85	—	CH	1.86	—
10	C	—	—	C	—	—	C	—	—
11	CH_2	1.30	1.45	CH_2	1.48	1.36	CH_2	1.52	1.29
12	CH_2	1.07	2.00	CH_2	1.16	2.00	CH_2	1.30	1.97
13	C	—	—	C	—	—	C	—	—
14	CH	1.17	—	CH	1.43	—	CH	1.52	—
15	CH_2	1.35	1.83	CH_2	1.15	1.73	CH	—	3.97
16	CH_2	1.55	1.45	CH_2	1.91	1.32	CH_2	1.78	1.88
17	CH	1.10	—	CH	1.16	—	CH	1.46	—
18	CH_3	0.69		CH_3	0.68		CH_3	0.73	
19	CH_3	0.90		CH_3	0.87		CH_3	0.92	
20	CH	—	1.38	CH	—	1.43	CH	—	1.44
21	CH_3	0.95		CH_3	0.96		CH_3	0.94	
22	CH_2	1.75	1.25	CH_2	1.77	1.30	CH_2	1.78	1.32
23	CH_2	2.18	2.05	CH_2	2.24	2.09	CH_2	2.33	2.21
24	C	—	—	C	—	—	C	—	—
25	CH_2	3.55		CH_2	3.57				
26	CH_2	2.95		CH_2	2.95				

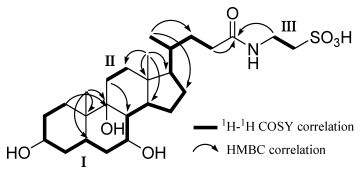

Fig. 2. ^{1}H–^{1}H COSY and Key HMBC Correlations of **1**

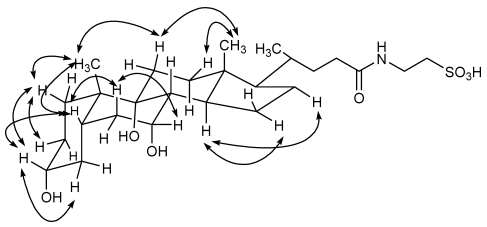

Fig. 3. Selected NOESY Correlations of **1**

type junction of rings-C/D. The H_3-18/H-8β and H_3-19/H-8β correlations justified the *trans* type junction of rings-B/C, which also established the α arrangement of 9-OH. The observed NOESY correlations (Fig. 3) between H-3/H-1β, H-3/H-4β, H_3-18/H-2β, H_3-18/H-11β, H_3-18/H-12β, H-6β/H-8, H-14/H-15α, H-14/H-16α, H_3-21/H-22α and H_3-21/H-23α confirmed the relative configurations of **1**. The structure of compound **1** was thus determined as 3α,7α,9α-trihydroxy-5β-cholan-24-oic acid *N*-[2-sulfoethyl] amide, and named tauroselocholic acid.

Compound **2**, obtained as white amorphous powder, was an isomer of **1** by a pseudo-molecular ion peak $[M-H]^-$ at *m*/*z* 514.2844 in the HR-ESI-MS spectrum. Its IR spectrum was very similar to that of **1**. The ^{1}H-NMR spectrum indicated the presence of three methyl groups at δ 0.68 (3H, s), 0.87 (3H, s) and 0.96 (3H, d, J=6.5), two hydroxymethines [δ_H 3.82, 3.82 (1H, m)], and an AB coupling system of a taurine group [δ_H 2.95, 3.57 (2H, t, J=7.0 Hz)]. Its ^{13}C-NMR spectra exhibited three methyls, twelve methylenes, seven methines and four quaternary carbons including three hydroxylated carbons at δ 68.6 (CH), 68.7 (CH) and 76.1 (q C), and one amide carbonyl at δ 176.6. The above data were closely related to those of known compound methyl-3α,5,7α-trihydroxy-24-nor-5β-chlan-23-oate,[12] except that signal of one methyl (δ_H 3.67, δ_C 51.3) disappeared, and additional signals of a taurine group (δ_H 2.95; δ_C 51.4 and δ_H 3.57; δ_C 36.6) and one methylene ($\delta_{H\alpha}$ 1.77, $\delta_{H\beta}$ 1.30; δ_C 33.2) appeared, suggesting **2** to be taurine conjugated 3α,5β,7α-trihydroxy C24 bile acid. A sequence of connectives through H-20β, H_2-22 and H_2-23, in turn, was observed in ^{1}H–^{1}H COSY, and the signal of H_2-25 correlated H_2-26. The connection C23–C24–C25 was supported by the HMBC correlations from H_2-23 and H_2-25 to C-24. Therefore, the structure of **2** was identified as 3α,5,7α-trihydroxy-5β-cholan-24-oic acid *N*-[2-sulfoethyl] amide and the trivial name tauroansocholic acid was given.

The molecular formula of **3** was determined as $C_{24}H_{40}O_5$ by HR-ESI-MS at *m*/*z* 431.2751 $[M+Na]^+$. The IR spectrum indicated the presence of the hydroxyl group (3402 cm^{-1}). The NMR data (Tables 1, 2) were in good agreement with those of the known compound 3α,7α,15α-trihydroxy-5β-cholan-24-oyl taurine (**10**),[13] except for signals due to absence of taurine group at the side chain. Compound **3** was further determined as 3α,7α,15α-trihydroxy-5β-cholan-24-

Table 2. ^{13}C-NMR Data (in CD_3OD, 125 MHz) for **1**—**3**, δ in ppm

Number	1	2	3
1	47.3	31.1	36.5
2	30.7	30.9	31.2
3	73.1	68.6	72.7
4	29.7	44.9	40.0
5	42.8	76.1	42.9
6	43.7	45.9	34.1
7	68.2	68.7	69.0
8	43.8	40.1	40.5
9	75.8	36.7	35.4
10	40.2	41.2	35.9
11	22.4	21.9	21.8
12	41.4	40.8	41.6
13	44.6	43.5	44.9
14	57.6	51.4	59.6
15	27.9	24.7	72.9
16	30.7	29.2	41.1
17	56.4	57.2	55.6
18	12.6	12.1	13.5
19	17.0	16.6	23.4
20	36.6	36.9	36.2
21	19.0	18.9	18.5
22	33.2	33.2	32.2
23	34.2	34.2	32.0
24	176.6	176.6	178.2
25	36.8	36.6	—
26	51.5	51.4	—

oic acid by interpretation of 2D NMR spectrum, and had been named as cygnocholic acid by Kakiyama *et al.*[13]

Seven known compounds among six compounds from bear bile powder, ursodeoxycholic acid (**4**),[14] chenodeoxycholic acid (**5**),[10] 2-[[(5β,7α)-7-hydroxy-3,24-dioxocholan-24-yl]amino]-ethanesulfonic acid (**6**),[15] tauroursodeoxycholic acid (**7**),[10] taurochenodeoxycholic acid (**8**),[10] taurocholic acid (**9**),[10] and 3α,7α,15α-trihydroxy-5β-cholan-24-oyl taurine (**10**)[13] from geese bile, were identified by comparing their NMR data with those reported.

Experimental

General Procedures Optical rotations were measured on a Perkin-Elmer-243B digital polarimeter. IR spectra were measured on a NEXUS-470 FTIR (Nicolet) spectrometer, KBr pellets, in cm^{-1}. NMR spectra were recorded on a VARIAN INOVA 500 NMR spectrometer with chemical shifts shown as δ-values (ppm) and TMS as an internal standard. ESI-MS were measured on a QSTAR (ABI, U.S.A.) mass spectrometer and HR-ESI-MS on a Bruker APEX II FT-ICR-MS mass spectrometer. Semi-preparative HPLC was carried out using a Waters 600 Pump with 600 controller (Waters C18 Nova-Pak column, 300×7.8 mm, 5 μm), with ELSD detector (Alltech). All solvents used were of analytical grade (Beijing Chemical and Industry Factory). Silica gel (Qingdao Mar. Chem. Ind. Co., Ltd.), Sephadex LH-20 gel (Pharmacia) and C_{18} reverse-phased silica gel (150—200 mesh, Merck, performed by applying a N_2 pressure of 0.12 MPa) were used for column chromatography.

Biological Materials Bear bile powder was prepared by drying the bile drained from live wild bears *Selenaretos thibetanus* Cuvier, artificially bred and raised in Hei Bao Medicine Group in China. The gallbladders from geese *Anser anser domesticus* were provided by He Wang Breeding Center of Landaise geese, Beijing, China.

Extraction and Isolation Bear bile powder (190 g) was dissolved in water and successively partitioned with EtOAc and *n*-BuOH. The EtOAC extract (8 g) was subjected to silica gel chromatography column (CC) and eluted with PE–Me_2CO (20 : 1—1 : 5) to yield four fractions (Frs. 1—4). Fr. 3 was further subjected to silica gel CC and eluted with $CHCl_3$–EtOAC (3 : 2) to obtain **4** (10.0 mg) and **5** (6.0 mg). The *n*-BuOH extract (170 g) was subjected to silica gel CC and eluted with EtOAc–MeOH–H_2O (8 : 2 : 0.5) to yield Frs. 1—6. Fr. 3 was further subjected to silica gel CC and eluted with $CHCl_3$–MeOH (4 : 1) followed by purification on Sephadex LH-20 ($CHCl_3$–MeOH, 1 : 1) to afford **6** (10.0 mg). Fr. 5 was subjected to silica gel vacuum liquid chromatography and eluted with $CHCl_3$–MeOH (10 : 1) to obtain Frs. 5-I—IV. Fr. 5-III further using ODS (MeOH–H_2O, 6 : 4) afforded **7** (224 mg) and **8** (354 mg). Fr. 5-IV was re-subjected to ODS (MeOH–H_2O, 4 : 6) to yield **1** (7.0 mg) and **9** (90 mg).

The gallbladders from geese were cut and the bile (5.0 l) was partitioned with EtOAc and *n*-BuOH. The *n*-BuOH extract (200 g) was subjected to silica gel column and eluted with $CHCl_3$–MeOH (5 : 1—2 : 1) to yield Frs. 1—3. Further isolation of Fr. 3 by ODS (MeOH–H_2O, 4 : 6) provided Frs. 3-I and II. Further purification of Fr. 3-I by prep. HPLC (MeOH/H_2O (0.5‰ CF_3COOH), 65 : 35) gave **2** (6 mg). Fr. 3-II was subjected to silica gel column and eluted with EtOAc–MeOH–H_2O (8 : 2 : 0.5) to yield **10** (40 mg). The EtOAc extract (30 g) was subjected to silica gel column and eluted with PE–Me_2CO (20 : 1—1 : 5) to yield Frs. 1—7. Fr. 5 (600 mg) was applied to silica gel CC with $CHCl_3$–EtOAc (1 : 3) elution to afford **3** (10.0 mg).

Compound **1**: Colorless syrup; $[\alpha]_D^{20}$ +21.3° (c=0.16, MeOH); IR (KBr) ν_{max} cm^{-1}: 3420, 2931, 2869, 1647, 1551, 1453, 1210, 1047; ^{1}H-NMR (CD_3OD, 500 MHz), see Table 1; ^{13}C-NMR (CD_3OD, 125 MHz), see Table 2. HR-ESI-MS m/z 514.2860 $[M-H]^-$ (Calcd for $C_{26}H_{44}NO_7S$: 514.2844).

Compound **2**: Colorless syrup; $[\alpha]_D^{20}$ +14.8° (c=0.14, MeOH). IR (KBr) ν_{max} cm^{-1}: 3425, 2928, 2859, 1732, 1635, 1160, 1037. ^{1}H-NMR (CD_3OD, 500 MHz), see Table 1; ^{13}C-NMR (CD_3OD, 125 MHz), see Table 2. HR-ESI-MS m/z 514.2857 $[M-H]^-$ (Calcd for $C_{26}H_{44}NO_7S$: 514.2844).

Compound **3**: Colorless syrup; $[\alpha]_D^{20}$ +7.6° (c=0.08, MeOH). IR (KBr) ν_{max} cm^{-1}: 3402, 2931, 2869, 1731, 1452, 1371, 1080, 983 cm^{-1}. ^{1}H-NMR (CD_3OD, 500 MHz), see Table 1; ^{13}C-NMR (CD_3OD, 125 MHz), see Table 2. HR-ESI-MS m/z 431.2751 $[M+Na]^+$ (Calcd for $C_{24}H_{40}O_5Na$: 431.2768).

Present chemical investigation has resulted in the isolation and characterization of two new bile acids and a new natural bile acid together with seven known compounds. It will be worthwhile to investigate the biological potential of these molecules. Moreover, in contrast to most small molecules found in vertebrates, bile acids are strikingly diverse in their pattern of hydroxylation and side chain structure.[16] Bile acid structure shows a pattern of progressive molecular development in the course of vertebrate evolution.[17] The sites of an additional hydroxylation on the cholanoic acid nucleus in primary bile acids have been identified at 6α, 6β, 12α, 1α, 1β, 15α and 16α.[13] With this report, two new additional sites of hydroxylation on the cholanoic acid nucleus in primary bile acids were discovered at C-9 and C-5. These bile acid structures may provide the useful information for phylogenesis.

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