Bile Acids of Fel Ursi

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The first direct separation of Fel Ursi using octadecyl silica (ODS) open column chromatography has been accomplished and provided to provide several pure, free and conjugated bile acids, the structures of which were substantiated by spectroscopic methods.

Key words *Ursus arctos*; Fel Ursi; bile acid; direct separation

Fel Ursi is prepared by drying the bile of *Ursus arctos* L. and analogous species, and has, since olden times, been utilized as an Asian folk medicine. Pharmacological tests have shown various actions of the substance: the aqueous solution of Fel Ursi and a major component, tauroursodeoxycholic acid (TUDCA; synthetic), as characteristic of Fel Ursi, showed an antispasmodic action. Ursodeoxycholic acid (UDCA), a free type of TUDCA, is next to deoxycholic acid (DCA) and stronger than cholic acid (CA) in its bile activity.[1] UDCA is specific to bear, and no other bile can be used as a substitute, therefore Fel Ursi is a valuable crude drug. There are many qualitative and quantitative analyses: Uji and Takiura checked the alkaline hydrolysate of conjugated bile acids by GLC and showed that Fel Ursi is constituted of UDCA, chenodeoxycholic acid (CDCA), DCA and CA, and that the kind and amount of these acids vary according to the bile specimen.[2] After Sjövall reported developing a quantitative method of the conjugated bile acids by paper chromatography (PPC),[3] many other papers followed and almost all of them remarked on the preparation of static phase and developing solvent, that is, they were analyzed by color comparator after coloration of the developed spot and the eluted fraction with sulfuric acid.[4] These reports were concerned with the bile of man and other animals, while the quantitative analysis of the bile acids in Fel Ursi was reported by Hara *et al.*[5] as above-mentioned and the method using TLC-field flame ionizing detector (FID).[6] Concerning the separation of the commercially conjugated bile acids using HPLC, Yokota *et al.* reported the result of analysis of the phenanthlene derivatives.[7] Separation methods using a 3α-hydroxysteroid dehydrogenase fixed enzymatic column[8] and applying phosphate buffer[9] and IR were also used by Yokota *et al.*[10] As stated, surprisingly few examples have been reported of the direct separation and quantitative analysis of conjugated bile acids using HPLC or reversed column chromatography without the addition of enzyme, acid or alkali, followed by one of structure determination by one of various spectroscopic means. All of the specimens referred to that were used in TLC and HPLC were synthesized.[11] Therefore, we wanted to establish a direct separation method using open-column chromatography with reversed silica gel, without the addition of acid or alkali to confirm the component chemical structure for the efficacy of Fel Ursi.

Fel Ursi (9.58 g) prepared from gall bile drained from live wild bears artificially bred and raised in Hei Long Jian province in China was extracted with MeOH to afford an extract (7.86 g), a part (5.86 g) of which was was passed through ion exchange resin IR-120 and subjected to Bondapak C_{18} with 40—100% MeOH, gradiently increasing MeOH, to afford Y-1-b (**5**, 1.66 g), Y-1-a (**4**, 1.37 g) and Y-2 (**1**, 136 mg). The still mixed fraction (fraction 3) was further separated with silica gel with $CHCl_3$: MeOH : water = from 8 : 2 : 0.2 to 7 : 3 : 0.2) to give Y-2 (**1**, 33 mg), Y-3 (**2**, 9 mg), Y-1 (**4**+**5**, 155 mg) and Y-4 (**3**, 82 mg).

Y-2 (**1**) obtained as colorless needles, mp 113—117 °C, $[\alpha]_D$ +16.6° (MeOH), showed a quasi molecular ion at *m/z* 391 $[M-H]^-$ in the FAB-MS and absorptions due to hydroxyl at 3403 cm^{-1} and carbonyl at 1706 cm^{-1} in the IR spectrum. Based on the ^{13}C-NMR data illustrated in Table 2, **1** was estimated as a CA derivative having two hydroxy groups. The locations of these two groups were deduced to be at C-3 and C-7 according to the report related to the ^{13}C-NMR spectra of the hydroxylated bile acids by Iida *et al.*[12] The proton signals of a multiplet (1H, W_H=25.0 Hz) at δ 3.80 and a broad singlet (1H, W_H=10.0 Hz) at δ 4.03, both of which were adjacent to the hydroxy group, were assigned to be β-axial proton at C-3 and β-equatorial proton at C-7, respectively. Consequently, Y-2 (**1**) was identified as CDCA.

Y-3 (**2**) obtained as a colorless powder, $[\alpha]_D$ +33.2° (MeOH), showed a quasi molecular ion peak at *m/z* 431 $[M+Na]^+$ in the FAB-MS and the IR absorption bands due to hydroxy at 3411 cm^{-1} and due to carbonyl group at 1708 cm^{-1}. The ^{13}C-NMR spectrum (Table 2) showed three hydroxylated carbon signals which were assigned to C-3, C-7 and C-12.[11] The proton signals at δ 3.75 (1H, m, W_H=18.3 Hz) and 4.09 (1H, br s, W_H=6.1 Hz) were ascribable to 3-Hβ and 7-Hβ, respectively. Moreover, the signal at δ 4.25 (1H, br s, W_H=13.3 Hz) could be assigned to 12-Hβ (equatorial). Therefore, Y-3 (**2**) was identified as CA.

Y-4 (**3**) obtained as colorless needles, mp 213—216 °C, $[\alpha]_D$ +23.1° (MeOH), showed a molecular ion peak at *m/z* 538 $[M+Na]^+$ in the positive FAB-MS and the IR absorptions due to hydroxy at 3413 cm^{-1} and amide group at 1647 cm^{-1}. The ^1H-NMR spectrum showed signals due to three methine protons adjacent to the hydroxy group at δ 3.75 (1H, m, W_H=18.4 Hz), 4.07 (1H, br s, W_H=8.4 Hz) and 4.20 (1H, br s, W_H=6.7 Hz), a methylene group adjacent to the amine group at δ 3.51 (2H, t, J=6.4 Hz), a methylene group neighboring the sulfonic acid at δ 4.18 (2H, t, J=5.8 Hz) and an amino proton at δ 8.70 (s). Newly appearing carbon signals at δ 36.3 and 51.6 were assigned to two methylene carbons next to the amino group and sulfonic acid group, respectively, and the signal at δ 176.6 in **2** was shifted higher to δ 174.6 by comparing with those of **2**. Therefore, Y-4 (**3**) was determined to be taurocholic acid (TCA).

Y-1-a (**4**), obtained as colorless needles, mp 132—135 °C,

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Table 1. Bile Acids of Fel Ursi

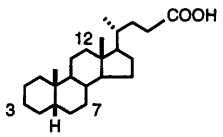
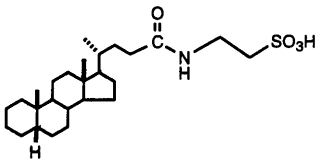
	CDCA (1)	CA (2)	TCDCA (4)	TUDCA (5)	TCA (3)
OH	3α,7α	3α,7α,12α	3α,7α	3α,7β	3α,7α,12α
Conjugated	—	—		Taurine	
Yield	3.83%	0.13%	19.3%	23.2%	1.60%
Structure					

Table 2. ^{13}C-NMR Data for Y-2 (1), Y-3 (2), Y-4 (3), Y-1-a (4) and Y-1-b (5)

	Y-2 (1)	Y-3 (2)	Y-4 (3)	Y-1-a (4)	Y-1-b (5)
C-1	36.3	36.2	36.1	36.7	35.7
2	31.8	31.6	31.5	31.7	31.4
3	71.8	71.9	71.8	71.8	71.0
4	40.1	41.0	40.8	40.0	38.8
5	42.6	42.5	42.6	42.6	43.3
6	35.8	35.8	35.7	35.8	38.8
7	67.6	67.7	67.6	67.6	70.6
8	40.2	40.6	40.5	40.2	44.1
9	33.3	27.4	27.2	33.3	39.8
10	35.6	35.3	35.2	35.6	34.5
11	21.1	29.6	29.5	21.0	21.7
12	41.0	72.4	72.4	40.9	40.6
13	42.8	46.9	46.8	42.7	43.8
14	50.8	42.7	42.3	50.8	56.6
15	24.1	23.2	23.6	24.0	27.6
16	28.6	28.1	27.1	28.3	29.0
17	56.4	47.3	46.9	56.2	55.5
18	12.1	13.0	12.9	12.1	12.5
19	23.2	23.2	23.1	23.2	23.8
20	35.8	36.0	35.9	35.8	35.9
21	18.6	17.6	17.5	18.9	18.9
22	31.8	32.0	32.2	32.3	32.4
23	31.8	32.1	33.6	33.6	33.8
24	176.6	176.6	174.6	174.0	174.4
1′			36.3	36.2	36.5
2′			51.6	51.7	51.7

$[\alpha]_D$ +16.3°, (MeOH) showed a quasi molecular ion peak at *m/z* 498 $[M-H]^-$ in the negative FAB-MS and the IR absorption bands due to hydroxy group at 3401 cm^{-1}, carbonyl group at 1645 cm^{-1} and sulfonic acid group at 1170 cm^{-1}. The ^{1}H-NMR spectrum exhibited signals due to a proton adjacent to the 3α-hydroxy group at δ 3.79 (m, W_H=16.7 Hz), a proton adjacent to the 7α-hydroxy group at δ 4.02 (br s, W_H=10.0 Hz), and two methylene groups originating from a taurine combination at δ 3.52 (2H, t, *J*=6.4 Hz, α-methylene to amino group) and 4.24 (2H, m, W_H=16.7 Hz, α-methylene to sulfonic acid group), suggesting that **4** was taurochenodeoxycholic acid (TCDCA). Furthermore, a comparative study of the ^{13}C-NMR spectrum (Table 2) of **4** with that of **1** revealed a new occurrence of signals at δ 36.2 and 51.7, which supported the above deduced structure.

Y-1-b (**5**), obtained as colorless needles, mp 178—183 °C, $[\alpha]_D$ +40.9° (MeOH), showed a quasi molecular ion peak at *m/z* 498 $[M-H]^-$ in the negative FAB-MS and the IR absorption bands due to hydroxy group at 3394 cm^{-1} and amide group at 1646 cm^{-1}. The ^{13}C-NMR spectrum (Table 2) of **5** showed two hydroxylated carbon signals. Other signals except for those of C-6, 7, 8, 9, 14, 15 were nearly identical with those of **4**, therefore, **5** was regarded as an epimer at C-7, that is, 7β-OH. The proton signals due to H-3 at δ 3.78 (m, W_H=16.7 Hz) and H-7 at δ 3.84 (m, W_H=13.3 Hz) were assigned to the 3α and 7α-protons, respectively. Two methylene signals at δ 3.54 and 4.22 could be assigned to α-methylene adjacent to the amino group and α-one to sulfonic acid group, respectively. Consequently, Y-1-b (**5**) was determined to be an epimer at C-7 of **4**, namely, TUDCA.

Thus, we isolated several free and conjugated bile acid derivatives directly using Bondapak column chromatography after treatment with ion exchange IR-120, using no acid or alkali, or derivatizing, and verified their structures by measurement of FAB-MS, ^{1}H-NMR and ^{13}C-NMR spectral data. It was apparent that Fel Urci used this time was almost entirely composed of taurine-conjugated TCDCA (19.3%) and TUDCA (23.2%). This method will be broadly applicable to the separation of animal bile.

Experimental

Melting points (uncorrected) were measured using Boetius micro-melting point apparatus. Optical rotations were determined on a JASCO DIP-1000 KUY polarimeter (*l*=0.5). IR spectra were obtained with a Hitachi 270-30 type spectrophotometer. FAB-MS were obtained in a glycerol matrix in the positive ion mode using a JEOL JMS-DX300 or JMS-DX 303HF, and EI-MS on a JEOL JMS-01SG or JMS-DX303HF. NMR spectra were measured in pyridine-d_5 on a JEOL α-500 spectrometer and chemical shifts were referenced to tetramethylsilane (TMS). Column chromatography was carried out with Silica gel 60 (40—63 μm, Merck) and Bondapak C_{18} (Merck). TLC was performed on a precoated Silica gel 60F_{254} (Merck) and RP-18 F_{254S} (Merck).

Crude Material The crude material of Fel Ursi was prepared from the gall bile drained from live wild bears artificially bred and raised in Hei Long Jian province.

Isolation The dried crude material (9.58 g) was extracted with MeOH and a part (5.86 g) of the resulting extract (7.86 g) was passed through ion exchange resin IR-120 and subjected to Bondapak C_{18} column chromatography with 40—100% MeOH, gradiently, to afford eight fractions (frs. 1—8); fr. 2 [Y-1-b (**5**), 1.66 g], fr. 3 (560 mg), fr. 4 [Y-1-a (**4**), 1.37 g], fr. 6, 7 [Y-2 (**1**), 169 mg] and fr. 8 (101 mg). Fraction 3 was further chromatographed using silica gel with $CHCl_3$–MeOH–water (from 8:2:0.2 to 7:3:0.2) to give eight fractions: fr. 2 [Y-2 (**1**), 44 mg], fr. 3 [Y-3 (**2**), 9 mg], fr. 6 (Y-1-a+Y-1-b, 170 mg) and fr. 8 [Y-4 (**3**), 82 mg].

Y-2 (CDCA, **1**): Colorless needles, mp 113—117 °C (from aqueous MeOH); $[\alpha]_D^{28}$ +16.6° (*c*=0.1, MeOH). IR ν_{max}^{KBr} cm^{-1}: 3403, 1706, 1558, 1066. Negative FAB-MS *m/z*: 391 $[M-H]^-$. ^{1}H-NMR (pyridine-d_5) δ: 0.70 (3H, s, 18-Me), 0.97 (3H, s, 19-Me), 1.00 (3H, d, *J*=4.9 Hz, 21-Me), 3.80 (1H, m, W_H=25.0 Hz, 3β-H), 4.03 (1H, m, W_H=10.0 Hz, 7β-H).

Y-3 (CA, **2**): A colorless powder, $[\alpha]_D^{27}$ +33.0° (*c*=0.1, MeOH). IR ν_{max}^{KBr} cm^{-1}: 3411, 1708, 1552. Positive FAB-MS *m/z*: 431 $[M+Na]^+$. ^{1}H-NMR (pyridine-d_5) δ: 0.81 (3H, s, 18-Me), 1.00 (3H, s, 19-Me), 1.24 (3H, d, *J*=

6.1 Hz, 21-Me), 3.75 (1H, m, W_H=18.3 Hz, 3β-H), 4.09 (1H, br s, W_H=6.1 Hz, 7β-H), 4.25 (1H, br s, W_H=13.3 Hz, 12β-H).

Y-4 (TCA, **3**): Colorless needles, mp 213—216 °C (from aqueous MeOH); $[\alpha]_D^{27}$ +23.1° (c=0.4, MeOH). IR ν_{max}^{KBr} cm^{-1}: 3413, 1647, 1637, 1558. Positive FAB-MS *m/z*: 538 $[M+H]^+$. ^{1}H-NMR (pyridine-d_5) δ: 0.77 (3H, s, 18-Me), 0.99 (3H, s, 19-Me), 1.16 (3H, d, J=6.1 Hz, 21-Me), 3.51 (2H, t, J=6.4 Hz, –CH_2–NH–), 3.75 (1H, m, W_H=18.4 Hz, 3β-H), 4.07 (1H, br s, W_H=8.4 Hz, 7β-H), 4.18 (2H, t, J=5.8 Hz, –CH_2–SO_3H), 4.20 (1H, br s, W_H=6.7 Hz, 12β-H), 8.70 (1H, s, –NH–).

Y-1-a (TCDCA, **4**): Colorless needles, mp 132—135 °C (from aqueous MeOH); $[\alpha]_D^{27}$ +16.3° (c=0.2, MeOH). IR ν_{max}^{KBr} cm^{-1}: 3401, 1645, 1550, 1170. Negative FAB-MS *m/z*: 498 $[M-H]^-$. ^{1}H-NMR (pyridine-d_5) δ: 0.65 (3H, s, 18-Me), 0.91 (3H, d, J=6.1 Hz, 21-Me), 0.95 (3H, s, 19-Me), 3.52 (2H, t, J=6.4 Hz, –CH_2–NH–), 3.79 (1H, m, W_H=16.7 Hz, 3β-H), 4.02 (1H, br s, W_H=10.0 Hz, 7β-H), 4.24 (2H, m, W_H=16.7 Hz, –CH_2–SO_3H).

Y-1-b (TUDCA, **5**): Colorless needles, mp 178—183 °C (from aqueous MeOH); $[\alpha]_D^{28}$ +40.9° (c=0.1, MeOH). IR ν_{max}^{KBr} cm^{-1}: 3394, 1646, 1554. Negative FAB-MS *m/z*: 498 $[M-H]^-$. ^{1}H-NMR (pyridine-d_5) δ: 0.69 (3H, s, 18-Me), 0.97 (3H, d, J=6.1 Hz, 21-Me), 0.97 (3H, s, 19-Me), 3.54 (2H, br s, W_H=13.3 Hz, –CH_2–NH–), 3.78 (1H, m, W_H=16.7 Hz, 3β-H), 3.84 (1H, br s, W_H=13.3 Hz, 7α-H), 4.22 (2H, m, W_H=16.7 Hz, –CH_2–SO_3H).

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