Study on the Bile Salt from Megamouth Shark. I. The Structures of a New Bile Alcohol, 7-Deoxyscymnol, and Its New Sodium Sulfates

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New sodium bile alcohol sulfates were obtained from the bile of megamouth shark by chromatography on silica gel and Sephadex LH-20, together with two sodium scymnol sulfates, (24*R*,25*S*)- and (24*R*,25*R*)-(+)-3α,7α,12α,24,26-pentahydroxy-5β-cholestan-27-yl sodium sulfate (2 and 3). On hydrolysis in pyridine–dioxane, the new salts afforded a new bile alcohol (1), whose structure was determined to be (24*R*)-5β-cholestane-3α,12α,24,26,27-pentol, based on chemical and spectral data. On the basis of the physicochemical data, the new salts were established as (24*R*,25*S*)- and (24*R*,25*R*)-(+)-3α,12α,24,26-tetrahydroxy-5β-cholestan-27-yl sodium sulfate.

Key words bile salt; 7-deoxyscymnol; sodium 7-deoxyscymnol sulfate; sodium scymnol sulfate; megamouth shark

There have been many studies on the structures and pharmacological effects of bile components, especially on the biles of bear and cow, which are important crude drugs used as a sedative for biliary calculus, as an antiphlogistic for liver, and as a remedy for jaundice, *etc.* in traditional Chinese medicine.[1] They contain various C-24 bile acids, such as ursodeoxycholic acid,[2] chenodeoxycholic acid[3] and cholic acid.[4] In contrast, less work has been carried out on chemical elucidation of sodium bile alcohol sulfates and bile alcohols, such as scymnol,[5] chimaerol,[6] cyprinol[7] and petromyzonol,[8] which are major components in biles of fishes, such as shark (*Isurepsis glauca* and *Heterodontus japonicus*) and carp (*Cyprinus carpio* L.). These biles are also used as a crude drug to treat dyspnea due to disorder of the throat or pharynx, eye disease, *etc.*, and as an analgetic.[1]

The name scymnol was given by Hammarsten (1898) to an alcohol in the bile of shark *Scymnus borealis*.[9] During the course of our studies on the bile salts of sharks, *Rhizoprionodon acutus* and *Lamna ditropis*, we have found that there are two main constituents, sodium scymnol sulfates, (24*R*,25*S*)- and (24*R*,25*R*)-(+)-3α,7α,12α,24,26-pentahydroxy-5β-cholestan-27-yl sodium sulfate (**2** and **3**), in the bile of *Lamna ditropis*, *Chlamydoselachus anguines* GARMAN and *Glyphis glaucus*, but only one sodium scymnol sulfate (**2**) in the bile of *Rhizoprionodon acutus*.[10] We also found a cofactor bile salt, sodium chimaerol sulfate, in the bile of *Lamna ditropis* and *Rhizoprionodon acutus*.[11] These findings imply that the chemistry of *Chlamydoselachus anguines* GARMAN supports the view that the living sharks are survivors of a group ancestral to the elasmobranches containing scymnol, and are noteworthy, because chimaerol could be a biochemical precursor of scymnol through C-terminal hydroxylation.

Megamouth shark was first found in Hawaii in 1976 and so far only 6 males have been caught. Recently, a female megamouth shark was stranded in Hakata Bay.[12] To our knowledge, there is no report describing the components of bile of megamouth shark, so we studied the bile salts in order to compare the bile salts of megamouth shark with those of other sharks. Here we report the isolation and structures of bile salts from megamouth shark.

Isolation of two new sodium bile alcohol sulfates as a mixture, together with two sodium scymnol sulfates, **2** and **3**, from the gall-bladder of megamouth shark was achieved by 2 steps of column chromatography (silica gel and Sephadex LH-20). The details of the isolation processes are described in the experimental section.

The structure elucidation of the sodium bile alcohol sulfates was carried out as follows. From direct atomic absorption analysis, it was confirmed that the sulfates have a sodium atom in the molecule. The positive FAB-mass spectrum showed the ion peak at *m/z* 555, indicating that their molecular weights are 554. From these data and elemental analysis, the molecular formulae were determined to be $C_{27}H_{47}NaO_8S$. Reaction of the sulfates with CrO_3 yielded only the acidic compound **4**. This compound was identified as 3,12-dioxocholan-24-oic acid by direct comparison of its physical data with those of an authentic sample. The IR spectrum, which resembled that of sodium scymnol sulfate, showed absorption bands at 3450 and 1150 cm^{-1}, which are assignable to alcohol and sulfate ester functions. A detailed comparison of the ^{13}C-NMR data of the sulfates with those of sodium scymnol sulfates, **2** and **3**, indicated that the former sulfates are the sodium sulfate salts of a bile alcohol with a 5β-cholestane skeleton.

Structural confirmation was carried out in the following way. As depicted in Chart 1, the sulfates afforded only compound **1**, in good yield, on hydrolysis with pyridine–dioxane. The ^{13}C-NMR data of **1**, deoxycholic acid and scymnol are shown in Table 1. From a detailed comparison of the data with those of deoxycholic acid, the signals of **1** for C_1 to C_{21} were assigned. The remaining 6 signals, [34.1(t), 33.1(t), 73.2(d), 50.1(d), 62.1(t), 62.9(t)] are ascribable to C_{22} to C_{27} on the basis of a comparison with those of 5β-scymnol.[10] Thus, **1** is represented as 7-deoxyscymnol, (24*R*)-5β-cholestane-3α,12α,24,26,27-pentol. This was confirmed by analyses of the NMR (^1H, ^{13}C noise-decoupled, DEPT (distortionless enhancement by polarization transfer), ^1H–^1H correlation spectroscopy (COSY), ^1H–^{13}C COSY and HMBC (heteronuclear multiple bond connectivity)) spectra and

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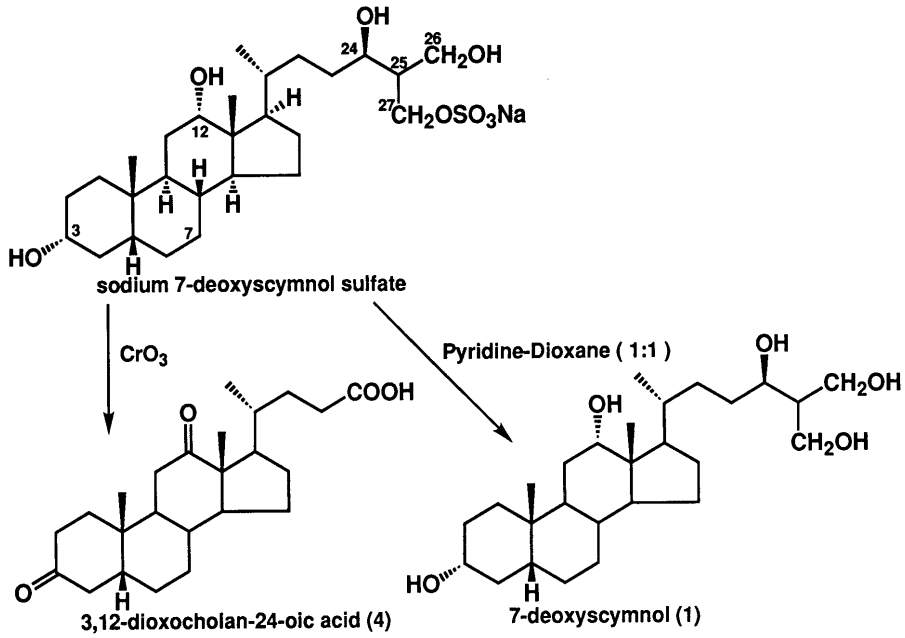

Chart 1. Preparation of 7-Deoxyscymnol (**1**) and 3,12,-Dioxocholan-24-oic Acid (**4**) from Sodium 7-Deoxyscymnol Sulfate

Table 1. ^{13}C-NMR Data (δ_C) for 7-Deoxyscymnol (**1**), Deoxycholic Acid and 5β-Scymnol in CD_3OD

Position	1	Deoxycholic acid	5β-Scymnol[10]
1	37.3 t	37.2 t	37.2 t
2	31.9 t	31.8 t	31.9 t
3	73.3 d	73.3 d	73.5 d
4	38.0 t	38.0 t	41.1 t
5	44.4 d	44.4 d	43.6 d
6	29.2 t	29.2 t	36.5 t
7	28.3 t	28.2 t	69.8 t
8	38.3 d	38.2 d	41.7 d
9	35.6 d	35.6 d	28.5 d
10	36.1 s	36.1 s	36.6 s
11	30.7 t	30.7 t	30.3 t
12	74.9 d	74.8 d	74.8 d
13	48.3 s	48.3 s	48.1 s
14	50.1 d	50.0 d	43.8 d
15	25.7 t	25.6 t	25.0 t
16	29.6 t	29.4 t	29.5 t
17	49.1 d	48.9 d	50.0 d
18	14.0 q	14.0 q	13.8 q
19	24.5 q	24.5 q	24.0 q
20	37.8 t	37.5 t	37.8 t
21	18.8 q	18.4 q	18.9 q
22	34.1 t	33.1 t	34.0 t
23	33.1 t	32.8 t	33.0 t
24	73.2 d	178.9 s	73.0 d
25	50.1 d		49.0 d
26	62.1 t		62.0 t
27	62.9 t		62.7 t

δ_C values in ppm. Multiplicities of carbon signals were determined by means of the DEPT method and are indicated as d, t and q. Assignments were based on ^{1}H–^{1}H and ^{1}H–^{13}C COSY, DEPT, and HMBC experiments.

observed nuclear Overhauser effects (NOEs) (Fig. 1). It is biologically and physiologically probable that the *R* configuration at C_{24} of 7-deoxyscymnol is the same as those of natural 5β-ranol, 5β-chimaerol and 5β-scymnol.

The deshielding of the C_{27} carbon and proton of the new sodium bile alcohol sulfates with comparison of those of **1** indicated that the hydroxyl group at C_{27} in these sulfates was indeed esterified with SO_3Na (Table 2), as in sodium scymnol sulfates of *Lamna ditropis*.[10] Thus, the sulfates are concluded to be sodium 7-deoxyscymnol sulfate, (24*R*,25*S*) and (24*R*,25*R*)-3α,12α,24,26-tetrahydroxy-5β-cholestan-27-yl sodium sulfate.

It is very interesting in connection with the biological evolution of the shark that in the bile of megamouth shark there are minor sodium 7-deoxyscymnol sulfates together with the main constituents, sodium scymnol sulfates, **2** and **3**, whereas sodium chimaerol sulfate is found together with the two sodium scymnol sulfates, **2** and **3**, in *Lamna ditropis* and together with **2** in *Rhizoprionodon acutus*.[10,11] We carefully analyzed the biles of these three sharks, *Rhizoprionodon acutus*, *Lamna ditropis* and megamouth shark, and confirmed that sodium 7-deoxyscymnol sulfates were not present in the biles of the former two sharks, and sodium chimaerol sulfate was not present in the last. This is noteworthy, because it suggests that in megamouth shark sodium 7-deoxyscymnol sulfate is a secondary metabolic product formed from sodium scymnol sulfate by 7-dehydroxylation, just as the C-24 bile acid, 7-deoxycholic acid, is formed from cholic acid.

In summary, we have obtained new sodium bile alcohol sulfates from the bile of megamouth shark and prepared a new bile alcohol from these bile salts. The bile alcohol was identified as 7-deoxyscymnol, (24*R*)-5β-cholestane-3α,12α,24,26,27-pentol and the bile salts as (24*R*,25*S*)- and (24*R*,25*R*)-(+)-3α,12α,24,26-tetrahydroxy-5β-cholestan-27-yl sodium sulfate, respectively, on the basis of their physicochemical data.

Experimental

Melting points were determined on a Yanaco micro melting point apparatus and are uncorrected. IR spectra were taken on a JASCO IR-700 grating IR spectrometer. Optical rotation was measured with a JASCO DIP-140. Mass spectra (MS) were recorded on a JMS-AX505W instrument and FAB-MS was obtained on a JMS-SX102 machine, using glycerin as the matrix. NMR spectra were recorded on JEOL GX-500

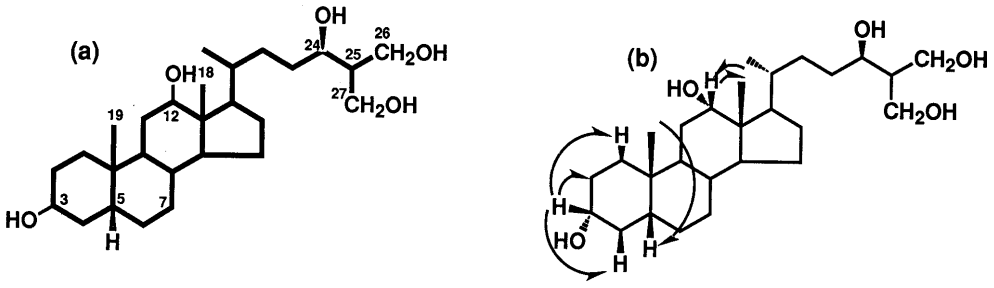

Fig. 1. Results of 2D-Correlation and NOE Experiments on 7-Deoxyscymnol (**1**)

(a) Heavy lines indicate the connectivities assigned on the basis of ^{1}H–^{1}H COSY and HMBC. (b) Arrows denote irradiated protons (tail)/observed protons (head) in NOE difference experiments.

Table 2. ^{13}C-NMR Data (δ_C) for Side Chains of Sodium 7-Deoxyscymnol Sulfates (SDS) and Sodium 5β-Scymnol Sulfates, **2** and **3**, in CD_3OD

Position	SDS	2	3
20	37.7 d	37.8 d	37.8 d
21	18.8 q	18.9 q	18.9 q
22	34.0 t	34.1 t	34.1 t
23	33.1 t, 33.0 t	33.1 t	33.0 t
24	72.4 d, 72.2 d	72.4 d	72.2 d
25	48.2 d, 48.7 d	48.2 d	48.6 d
26	61.5 t, 62.0 t	61.5 t	62.0 t
27	68.3 t, 67.4 t	68.4 t	67.6 t

δ_C values in ppm. Multiplicities of carbon signals were determined by means of the DEPT method and are indicated as d, t and q. Assignments were based on ^{1}H–^{1}H and ^{1}H–^{13}C COSY, DEPT, and HMBC experiments.

and α-400 spectrometers using tetramethylsilane (TMS) as an internal standard. Chemical shifts are recorded in δ values (ppm) and coupling constants in hertz (Hz). Multiplicities of ^{13}C-NMR signals were determined by means of the DEPT method. ^{1}H–^{1}H COSY, ^{1}H–^{13}C COSY, HMBC and NOE difference spectra were obtained with the JEOL standard pulse sequences and data processing was performed with standard software.

Material Gall-bladder, obtained from a female megamouth shark (*ca.* 790 kg) stranded in November 1994 at Hakata Bay, Fukuoka Prefecture, Japan, was homogenized and the homogenate was freeze-dried (25.85 g).

Isolation of Sodium 7-Deoxyscymnol Sulfates from the Bile of Megamouth Shark The lyophilized bile (5.85 g) of megamouth shark was extracted with *n*-hexane (100 ml × 2) and then MeOH (120 ml × 2). The MeOH extract (4.80 g) was chromatographed on silica gel with the lower layer of $CHCl_3$–MeOH–H_2O (65 : 32 : 10) to afford fraction I_a (240 mg) and sodium scymnol sulfates (997 mg). Fraction I_a (240 mg) was applied to a Sephadex LH-20 column and elution with $CHCl_3$ and MeOH (1 : 1) afforded sodium and potassium 7-deoxyscymnol sulfates (229 mg) (FAB mass *m/z*: 571 $(C_{27}H_{47}KO_8S+H)^+$, 555 $(C_{27}H_{47}NaO_8S+H)^+$). Purification of the bile salts (229 mg) by HPLC yielded 220 mg of sodium 7-deoxyscymnol sulfates. Purification of the sodium scymnol sulfates (300 mg) by HPLC yielded 53 mg of (24*R*,25*R*)-(+)-3α,7α,12α,24,26-pentahydroxy-5β-cholestan-27-yl sulfate (**3**) and 27 mg of (24*R*,25*S*)-(+)-3α,7α,12α,24,26-pentahydroxy-5β-cholestan-27-yl sodium sulfate (**2**).[10] Removal of inorganic salts in each bile salt purified as above was carried out by ODS (Sep Pak® C18, Millipore) column chromatography with H_2O and MeOH. The conditions for HPLC were as follows: column, YMC-Pack A-324 (ODS) 10 × 300 mm; flow rate, 3 ml/min; mobile phase, 31.5% CH_3CN–0.1 N sodium phosphate buffer (pH 6.70); detector, RI. Sodium 7-deoxyscymnol sulfates gave the following physical data. Sodium 7-deoxyscymnol sulfates: White amorphous powder. *Anal.* Calcd for $C_{27}H_{47}NaO_8S$: C, 58.48; H, 8.48. Found: C, 58.66; H, 8.53. FAB mass *m/z*: 555 $(C_{27}H_{47}NaO_8S+H)^+$, 475 $(M+Na+H-SO_3)^+$. IR ν_{max} (KBr) cm^{-1}: 3450, 3330, 2952, 1646, 1601, 1467, 1349, 1150. ^{13}C-NMR (in CD_3OD, 125.7 MHz) δ (ppm): 74.9 (C-12), 73.3 (C-3), 72.4 and 72.2 (C-24), 68.3 and 67.4 (C-27), 62.0 and 61.5 (C-26), 50.0 (C-14), 49.2 (C-17), 48.6 (C-13), 48.7 and 48.2 (C-25), 44.5 (C-5), 38.2 (C-8), 38.0 (C-4), 37.7 (C-20), 37.3 (C-1), 36.1 (C-10), 35.6 (C-9), 34.0 (C-22), 33.1 and 33.0 (C-23), 31.9 (C-2), 30.7 (C-11), 29.5 (C-16), 29.2 (C-6), 28.2 (C-7), 25.7 (C-15), 24.5 (C-19), 18.8 (C-21), 14.1 (C-18).

Acid Hydrolysis of Sodium 7-Deoxyscymnol Sulfates with Pyridine–Dioxane A mixture of 80.6 mg of sodium 7-deoxyscymnol sulfates and 1.0 ml of pyridine–dioxane (1 : 1) was refluxed for 12 h. The solvent was removed *in vacuo*, and the concentrate was chromatographed on octadecyl silica (ODS) (Sep-Pak® C18, Millipore) with MeOH–H_2O (6 : 4 and 8 : 2) to afford 44.6 mg of **1**. Upon crystallization of this product from MeOH and H_2O, colorless plates (23 mg) were obtained. The physical properties of **1** are as follows. Compound **1**: colorless plates. mp 192.5—194.0 °C. $[\alpha]_D^{25}$ 54.6° (*c* = 0.7, methanol). *Anal.* Calcd for $C_{27}H_{48}O_5 \cdot H_2O$: C, 68.94; H, 10.64. Found: C, 68.89; H, 10.80. FAB mass *m/z*: 453 $(C_{27}H_{48}O_5+H)^+$, 435 $(C_{27}H_{48}O_5-H_2O+H)^+$, 417 $(C_{27}H_{48}O_5-2H_2O+H)^+$, 399 $(C_{27}H_{48}O_5-3H_2O+H)^+$, 381 $(C_{27}H_{48}O_5-4H_2O+H)^+$, 363 $(C_{27}H_{48}O_5-5H_2O+H)^+$. IR ν_{max} (KBr) cm^{-1}: 3360, 2930, 2860, 1447, 1374, 1039. ^{1}H-NMR (in CD_3OD, 500 MHz) δ (ppm): 3.96 (1H, m, 12-H), 3.77 (2H, dd, *J* = 11.0, 5.0 Hz, 27-H), 3.72 (1H, ddd, *J* = 10.5, 5.8, 3.6 Hz, 24-H), 3.67 (2H, dd, *J* = 11.0, 6.3 Hz, 26-H), 3.52 (1H, m, 3-H), 1.95—1.72 (6H, m, 16, 9, 6, 17, 4, 1-H), 1.67 (1H, m, 25-H), 1.64—1.54 (4H, m, 15, 2, 14, 23-H), 1.54—1.34 (9H, m, 11, 4, 22, 23, 7, 8, 2, 20, 5-H), 1.34—1.22 (3H, m, 16, 6, 22-H), 1.22—1.10 (1H, m, 7-H), 1.10—0.94 (1H, m, 15-H), 1.02 (3H, d, *J* = 6.5 Hz, 21-H), 0.99—0.94 (1H, m, 1-H), 0.93 (3H, s, 19-H), 0.71 (3H, s, 18-H). ^{13}C-NMR spectral data are given in Table 1.

Oxidation of Sodium 7-Deoxyscymnol Sulfate with CrO_3 A solution of 10 mg of chromic anhydride in 50 μl of H_2O was added to a solution of 5 mg of sodium 7-deoxyscymnol sulfates in 50 μl of acetic acid at 0 °C. The mixture was stirred for 5 h at 25 °C, then diluted with 1 ml of H_2O and extracted with 2 ml of ethyl acetate and *n*-BuOH (1 : 1) twice. The organic layer was washed with 1 ml of H_2O and then brine, and dried over $MgSO_4$. The solvent was evaporated, and the residue was recrystallized from aqueous ethanol to give **4** as colorless long needles (2 mg) (mp 188 °C) (lit.,[13] mp 189 °C). This product was identified as 3,12-dioxocholan-24-oic acid by comparison of the physical data (mp, IR, NMR) with those of an authentic sample prepared from 7-deoxycholic acid (Wako Co.) in the reported manner.[13]

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