Study on the Bile Salt from Megamouth Shark. I. The Structures of a New Bile Alcohol, 7-Deoxyxycymnol, 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 sccynol sulfates, (24R,25S)- and (24R,25R)-(−)3x,7x,12x,24,26-pentahydroxy-5β-cholen-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 (24R)-5β-cholen-3x,12x,24,26,27-pentol, based on chemical and spectral data. On the basis of the physicochemical data, the new salts were established as (24R,25S)- and (24R,25R)-(−)3x,12x,24,26-tetrahydroxy-5β-cholen-27-yl sodium sulfate.

**Key words** bile salt; 7-deoxyxycymnol; sodium 7-deoxyxycymnol sulfate; sodium sccynol sulfate; megamouth shark

There have been many studies on the structures and pharmacological effects of bile components, especially on the bile 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. They contain various C-24 bile acids, such as ursodeoxycholic acid, chenodeoxycholic acid and cholic acid. In contrast, less work has been carried out on chemical elucidation of sodium bile alcohol sulfates and bile alcohols, such as sccynol, chimaerol, cyprinol and petromyzonol, which are major components in biles of fishes, such as shark (Isurus paucus and Heterodontus japonicus) and carp (Cyprinus carpio L.). These bile salts are also used as a crude drug to treat dyspnea due to disorder of the throat or pharynx, eye disease, etc., and as an anesthetic.

The name sccynol was given by Hammarsten (1898) to an alcohol in the bile of shark *Scyliorhinus ocellatus.* 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 sccynol sulfate, (24R,25S)- and (24R,25R)-(−)3x,7x,12x,24,26-pentahydroxy-5β-cholen-27-yl sodium sulfate (2 and 3), in the bile of *Lamna ditropis,* Chlamydoselachus anguineus *Garman* and *Glyphis glaucus,* but only one sodium sccynol sulfate (2) in the bile of *Rhizoprionodon acutus.*

We also found a cofactor bile salt, sodium chimaerol sulfate, in the bile of *Lamna ditropis* and *Rhizoprionodon acutus.* These findings imply that the chemistry of Chlamydoselachus anguineus Garman supports the view that the living sharks are survivors of a group ancestral to the elasmobranches containing sccynol, and are noteworthy, because chimaerol could be a biochemical precursor of sccynol 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. 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 sccynol 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_{54}H_{44}Na_{12}O_{16}. 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 sccynol 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 sccynol sulfates, 2 and 3, indicated that the former sulfates are the sodium sulfate salts of a bile alcohol with a 5β-cholastane 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 I, deoxycholic acid and sccynol are shown in Table 1. From a detailed comparison of the data with those of deoxycholic acid, the signals of I for C_{1}, C_{2}, and C_{3} 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-sccynol. Thus, I is represented as 7-deoxyxycymnol, (24R)-5β-cholastane-3x,12x,24,26,27-pentol. This was confirmed by analyses of the NMR (\(^{1}H, \(^{13}\)C noise-decoupled, DEPT (distortionless enhancement by polarization transfer), \(^{1}H-^{1}H\) correlation spectroscopy (COSY), \(^{1}H-^{13}\)C COSY and HMBC (heteronuclear multiple bond connectivity)) spectra and 1

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

The deshielding of the C\textsubscript{27} carbon and proton of the new sodium bile alcohol sulfates with comparison of those of 1 indicated that the hydroxyl group at C\textsubscript{27} in these sulfates was indeed esterified with SO\textsubscript{3}Na (Table 2), as in sodium scymnol sulfates of Lanna ditropsis\textsuperscript{10} Thus, the sulfates are concluded to be sodium 7-deoxyscymlol sulfate, (24R,25S) and (24R,25R)-3\textalpha{},12\textalpha{},24,26,27-tetrahydroxy-5\textbeta{}-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-deoxyscymlol 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 Lanna ditropsis and together with 2 in Rhizopronodon acutus\textsuperscript{10,11}

We carefully analyzed the biles of these three sharks, Rhizopronodon acutus, Lanna ditropsis and megamouth shark, and confirmed that sodium 7-deoxyscymlol 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-deoxyscymlol sulfate is a secondary metabolic product formed from sodium scymnol sulfate by 7-dehydroxylation, just as the C-24 bile acid, 7-deoxycymlol, 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-deoxyscymlol, (24R)-5\textbeta{}-cholestan-3\textalpha{},12\textalpha{},24,26,27-pentol and the bile salts as (24R,25S) and (24R,25R)-(-)-3\textalpha{},12\textalpha{},24,26,27-tetrahydroxy-5\textbeta{}-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-AX506E instrument and FAB-MS was obtained on a JMS-SX102 machine, using glycerin as the matrix. NMR spectra were recorded on JEOL GX-500.
and 2-400 spectrometers using tetrakisdimethylsilane (TMS) as an internal standard. Chemical shifts are recorded in $\delta$ values (ppm) and coupling constants in hertz (Hz). Multiplicities of $^{13}$C-NMR signals were determined by means of the DEPT method. $^1$H, $^13$C COSY, $^1$H-$^13$C COSY, HMBC, and NOE difference spectra were obtained with the JOELO 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 $^{13}$Deoxyxynoll Sulfates from the Bile of Megamouth Shark**
The lyophilized bile (5.85 g) of megamouth shark was extracted with $n$-hexane (100 ml x 2) and then MeOH (120 ml x 2). The MeOH extract (4.80 g) was chromatographed on silica gel with the lower layer of CHCl$_3$-MeOH-H$_2$O (65:32:10) to afford fraction I (240 mg) and sodium silycmol sulfates (997 mg). Fraction I (240 mg) was applied to a Sephadex LH-20 column and elution with CHCl$_3$ and MeOH (1:1) afforded sodium and potassium 7-deoxyxynoll sulfates (229 mg) (FAB mass $m/z$: 571 (C$_2$H$_5$KO$_2$S$^+$H$^+$), 555 (C$_2$H$_5$NaO$_2$S$^+$H$^+$)). Purification of the bile salts (229 mg) by HPLC yielded 220 mg of sodium 7-deoxyxynoll sulfates. Purification of the sodium silycmol sulfates (300 mg) by HPLC yielded 35 mg of (24R,25R)-(+)$\cdot$3-ethyl-12a,24,26-pentahydroxy-5$\beta$-cholest-27-$\gamma$-sulfate (3) and 27 mg of (24R,25S)-(-)$\cdot$3-ethyl-12a,24,26-pentahydroxy-5$\beta$-cholest-27-$\gamma$-sulfate (2). Removal of inorganic salts in each bile salt purified as above was carried out by ODS (Sep Pak$^R$ C18, Millipore) column chromatography with H$_2$O and MeOH. The conditions for HPLC were as follows: column, YMC-Pack A-324 (ODS) 10 x 300 mm; flow rate, 3 ml/min; mobile phase, 31.5% CH$_3$CN-0.1% sodium phosphate buffer (pH 6.70); detector, RI. Sodium 7-deoxyxynoll sulfates gave the following physical data. Sodium 7-deoxyxynoll sulfates: White amorphous powder. Anal. Calcd for C$_2$H$_5$NaO$_2$S: C, 58.48; H, 8.48. Found: C, 58.66; H, 8.53. FAB mass $m/z$: 555 (C$_2$H$_5$NaO$_2$S$^+$H$^+$), 475 (M+Na$^+$-SO$_3$$^-$). $\nu_{\text{max}}$ (KBr) cm$^{-1}$: 3450, 3330, 2952, 1646, 1601, 1467, 1349, 1150. $^{13}$C-NMR (in CD$_3$OD, 125.7 MHz) $\delta$ (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).

**Oxidation of Sodium 7-Deoxyxynoll Sulfate with CrO$_3$**
A solution of 10 mg of sodium CrO$_3$ in 50 μl of H$_2$O was added to a solution of 5 mg of sodium 7-deoxyxynoll 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$_2$O and extracted with 2 ml of ethyl acetate and sodium 7-deoxyxynoll sulfates (1:1) twice. The organic layer was washed with 1 ml of H$_2$O and then brine, and dried over MgSO$_4$. The solvent was evaporated, and the residue was recrystallized from aqueous ethanol to give 4 colorless long needles (2 mg) (mp 188°C) (lit. $^{13}$ mp 189°C). This product was identified as 3,12-dioxocholest-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. $^{13}$

References