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Study on the Bile Salt Sodium Scymnol Sulfate, from *Rhizoprionodon acutus*

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Sodium scymnol sulfate, which is a major component in bile of *Rhizoprionodon acutus*, was isolated by 3 steps of chromatography to afford 753 mg of the bile salt from 5 gallbladders (65 g), and its structure was determined to be 3α,7α,12α,24,26-pentahydroxy-5β-cholestan-27-yl sodium sulfate, on the basis of chemical and physical data.

Keywords——bile; sodium scymnol sulfate; *Rhizoprionodon*; scymnol; anhydroscymnol; dehydrocholic acid

Bile salts containing sodium scymnol sulfate were first isolated from the bile of the shark *Scymnus borealis* (*Somniosus microcephalus* SCHN.) by Hammarsten in 1898.[1] The structure of the bile alcohol scymnol prepared from bile salts of Hammarsten's original collection was chemically studied by Bridgewater *et al.*[2] and Cross,[3] and it was confirmed synthetically to be 5β-cholestane-3α,7α,12α,24,26,27-hexol (**3**). The structure of the native salt was concluded to be sodium scymnol sulfate, 3α,7α,12α,24,26-pentahydroxy-5β-cholestan-27-yl sodium sulfate (**1**), on the basis of chemical data.[2,3] However to our knowledge, there has been no report describing in detail the isolation of the native bile salt and no confirmation of the structure of the bile alcohols and native salts by modern techniques. So, we would like now to report a study on the isolation and structural elucidation of the bile salt, sodium scymnol sulfate, from *Rhizoprionodon acutus*.

Isolation of sodium bile alcohol sulfate from the gall-bladder of *Rhizoprionodon acutus* was achieved by 3 steps of column chromatography (Amberlite XAD-2, Sephadex LH-20 and high performance liquid chromatography (HPLC)), as summarized in Chart 1. The details of

lyophilized bile of *Rhizoprionodon acutus* (10.25 g)
| extracted with 1) *n*-hexane (100 ml × 3)
| and 2) MeOH (100 ml × 3)
fraction I (3.67 g)
| 1) dissolved in H_2O
| 2) Amberlite XAD-2 c.c., eluted with 1) H_2O (400 ml)
| and 2) EtOH (400 ml)
fraction II (1.96 g)
| Sephadex LH-20 c.c., eluted with i) $CHCl_3$–MeOH (1 : 1) (300 ml)
| and ii) MeOH (500 ml)
fraction III (1.06 g)
| HPLC: YMC-Pack A-324 (ODS)
| mobile phase CH_3CN–H_2O (1 : 3)
colorless amorphous powder (753 mg)
( ) indicates yield.
c.c., column chromatography.

Chart 1. Isolation Procedure for Sodium Scymnol Sulfate

the isolation processes are described in the experimental section.

The structural elucidation of the isolated sodium bile alcohol sulfate, compound **1**, was carried out as follows. From direct atomic absorption analysis, **1** has a sodium atom in the molecule. Its secondary ion mass spectrum (SIMS) showed the ion peak at m/z 654, indicating that the molecular weight of the free alkoxy hydrogen sulfate is 547. From these data, the molecular formula of **1** was determined to be $C_{27}H_{47}NaSO_9$. Reaction of **1** with CrO_3 yielded the acidic compound **2**. This compound was identified as dehydrocholic acid by direct comparison of its physical data with those of an authentic sample. In its infrared (IR) spectrum there was no unique absorption due to the carbonyl function at 1700—1550 cm^{-1}, but absorption bands at 3420 and 1230 cm^{-1} were assignable to alcohol and sulfate ester

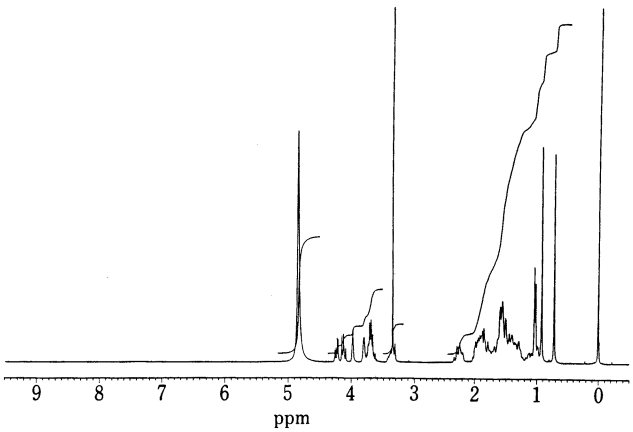


Fig. 1. ^{1}H-NMR Spectrum of **1** at 270 MHz in Methanol-d_4

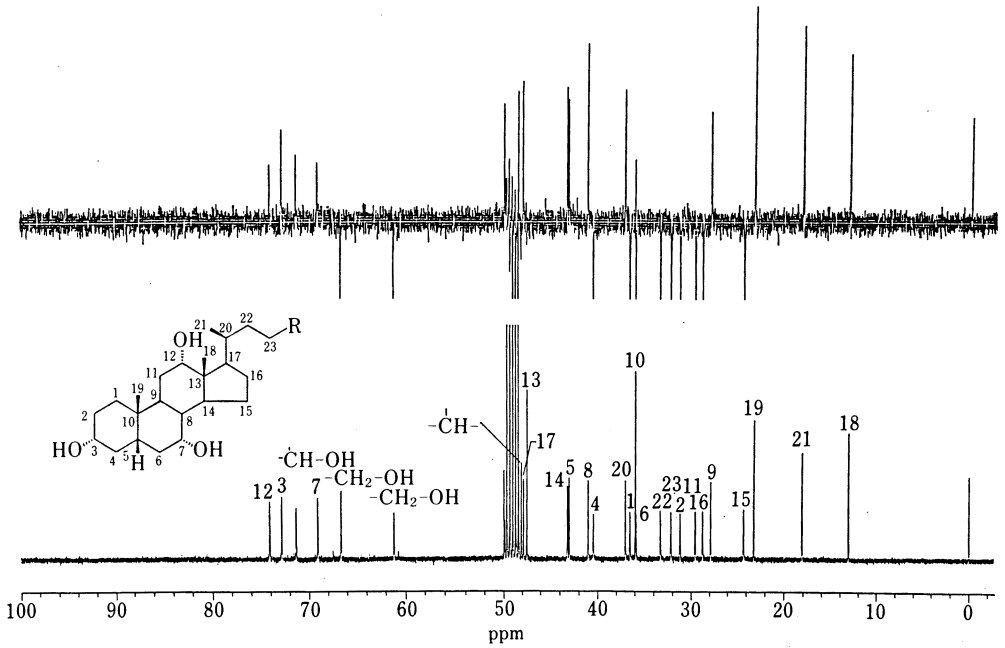


Fig. 2. ^{13}C-NMR Spectra of **1** at 25.5 MHz in Methanol-d_4 Obtained by Off-Resonance and INEPT Methods

Quaternary resonances are suppressed. CH_3 and CH sites give positive intensities, while CH_2 sites give negative intensities.

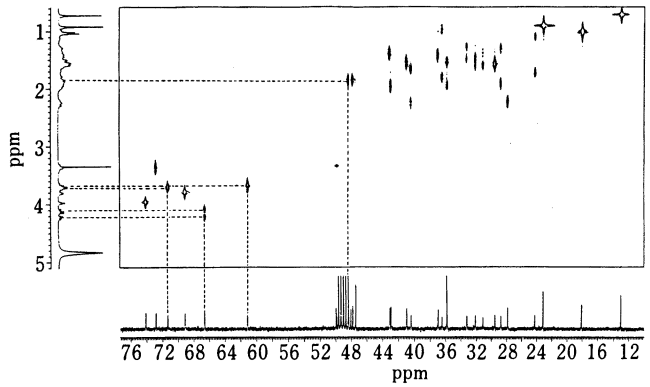


Fig. 3. Computer Map of ^{1}H-Shift-Correlated Spectrum of **1** at 270 MHz in Methanol-d_4

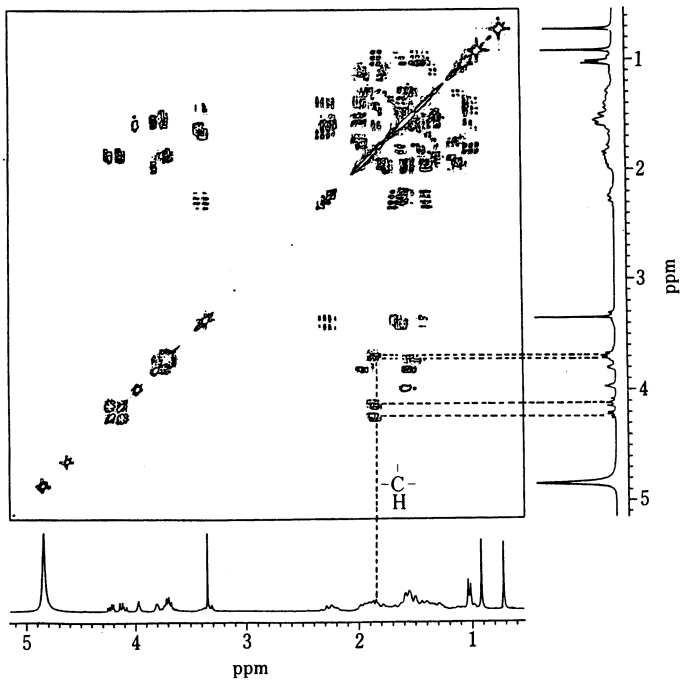


Fig. 4. 2D-COSY NMR Spectrum of **1** at 270 MHz in Methanol-d_4

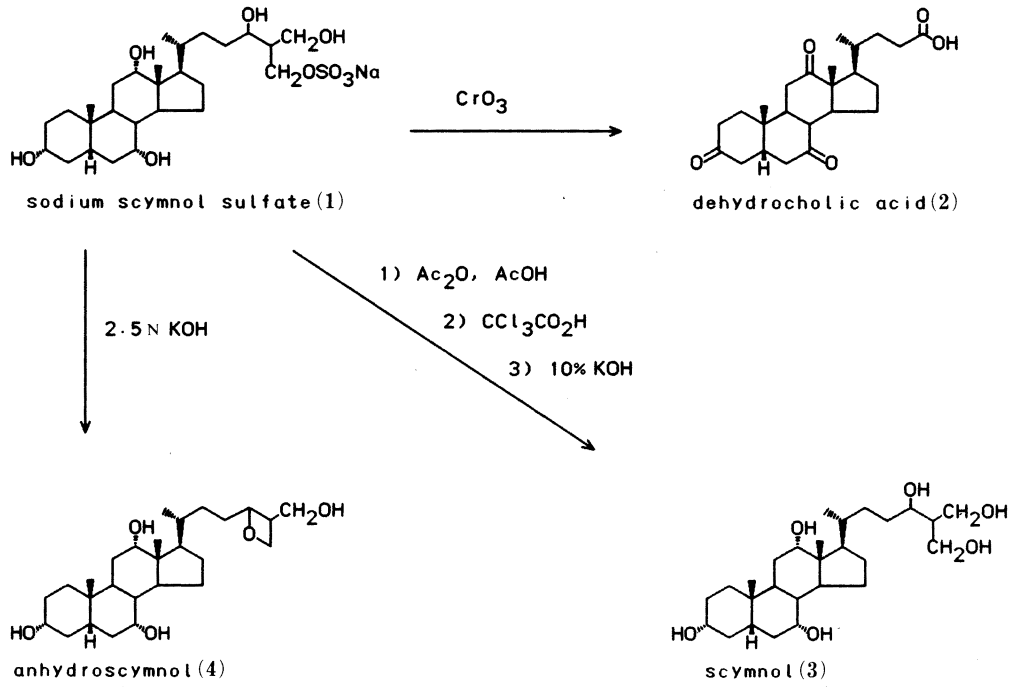
functions, respectively. The results indicate that compound **1** is the sodium sulfate salt of a bile alcohol with a 5β-cholestane skelton. The nuclear magnetic resonance (NMR) [^{1}H and ^{13}C noise decoupled and INEPT (insensitive nuclei enhanced by polarization transfer), two-dimensional ^{1}H shift-correlated (2D-COSY), and C–H-shift COSY] spectra are shown in Figs. 1—4. From a detailed comparison of the data with those of cholic acid, the marked signals in the ^{13}C-NMR spectrum of **1** (Fig. 2) were assigned to the C_1 to C_{23} partial structure. The remaining 4 signals [71.4 (d), 66.7 (t), 61.2 (t) and 48.3 (d)] are ascribable to –$\dot{C}$H–O, –CH_2–OSO_3Na, –CH_2–O and –$\dot{C}$H*– based on detailed analyses of the noise-decoupled and INEPT NMR spectra. The 2D-COSY and C–H-shift COSY NMR spectral data indicate that –$\dot{C}$H*– is bonded to the other three carbons. Thus, the partial structure R is represented as below. As a result, **1** is suggested to be

$$R = -\underset{\underset{OH}{|}}{CH}-\underset{\underset{CH_2OSO_3Na}{|}}{CH^*}-CH_2OH$$

sodium scymnol sulfate (**1**), 3α,7α,12α,24,26-pentahydroxy-5β-cholestan-27-yl sodium sulfate, as designated by Haslewood *et al.*[2]

This was confirmed in the following way. As depicted in the scheme, alkaline degradation of **1** with aqueous potassium hydroxide gave **4**, and on nonaqueous acid treatment of the O-acetylated derivative of **1** with trichloroacetic acid, followed by alkaline hydrolysis with aqueous potassium hydroxide it afforded **3** in moderate yield. From physicochemical data, **4** and **3** were determined to be anhydroscymnol (24,26-epoxy-5β-cholestane-3α,7α,12α,27-tetrol) and scymnol (5β-cholestane-3α,7α,12α,24,26,27-hexol), respectively.[2,3] As noted above, the reactivity of this compound is the same as that of sodium scymnol sulfate reported previously.[1-3] It was observed that the signals at 71.4 (d), 66.7 (t) and 61.2 (t) ppm, which are assigned to C_{24}, C_{26} and C_{27} in the carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum of **1**, are appeared at 72.4, 62.7 and 62.0 ppm in that of **3** and at 88.3 (d), 72.4 (t) and 64.6 (t) ppm in that of **4**. This indicates that in compound **1** the hydroxyl group at C_{27} is indeed esterified with SO_3Na. The formation of the trimethylene oxide ring of **4** can be accounted for by elimination of the OSO_3Na group due to the nucleophilic attack of hydroxyl oxygen at C_{24} of **1** during alkaline degradation.

In summary, we have established an isolation procedure, which could be applicable to other bile salts, for sodium scymnol sulfate from the bile of *Rhizoprionodon acutus*, and confirmed that its structure is 3α,7α,12α,24,26-pentahydroxy-5β-cholestan-27-yl sodium sulfate. Stereochemical studies on sodium scymnol sulfate (**1**), scymnol (**3**) and anhydroscymnol (**4**) will be reported shortly.[4]



Experimental

Melting points were determined on a Yanaco micro melting point apparatus and are uncorrected. IR spectra were taken on a JASCO IRA-2 grating infrared spectrometer. Optical rotation was measured with a Jasco DIP-140.

Low- and high-resolution mass spectra (MS) were recorded on Hitachi M-80A and JEOL JMS D-100 instruments. SIMS were obtained on a Hitachi M-80B machine, using diethanolamine as the matrix. NMR spectra were recorded on JEOL FX-90, GX-270, GX-400 and GX-500 spectrometers using tetramethylsilane (TMS) as an internal standard.

Material——Gall-bladders (65 g), obtained from five *Rhizoprionodon acutus* (*ca.* 8 kg) collected in November 1986 near Suruga Bay, Shizuoka Prefecture, Japan, were homogenized and the mixture was freeze-dried. This material (10.25 g) was used as a source.

Isolation of Sodium Scymnol Sulfate——After defatting the material with refluxing *n*-hexane (100 ml × 3), it was extracted with methanol (100 ml × 3) under reflux for 1 h. The concentrate (3.67 g) was dissolved in H_2O (80 ml), and applied to an Amberlite XAD-2 column (3.0 × 16.0 cm). The column was eluted with H_2O (400 ml) and then with ethanol (400 ml). The ethanol eluate (1.95 g) was applied to a Sephadex LH-20 column (3.0 × 32.0 cm) in chloroform and methanol (1 : 1). The column was eluted it with chloroform and methanol (1 : 1) (200 ml), then with methanol in batches of 50 ml. Concentration of the methanol eluate gave a white gum (1.06 g). Purification of this material (120 mg) by HPLC yielded 85.6 mg of sodium scymnol sulfate as a white amorphous powder. The conditions for HPLC were as follows: column, YMC-Pack A-324 (ODS) 10 × 300 mm; flow rate, 2 ml/min; mobile phase 25% CH_3CN–0.1 N sodium phosphate buffer (pH 6.43); detector, refractive index (RI). Compound **1** gave the following physical data. White amorphous powder. $[\alpha]_D^{20}$ 21.75° (c=0.5, MeOH). *Anal.* Calcd for $C_{27}H_{47}NaO_9S$: C, 56.82; H, 8.30; Na, 4.03; S, 5.62. Found: C, 56.99; H, 8.79; Na, 4.23; S, 5.62. SIMS m/z: 654 [$C_{27}H_{47}SO_9$. HN ($C_2H_6O)_2$], 574 [$C_{27}H_{47}O_6$. HN ($C_2H_6O)_2$]. IR ν_{max}^{KBr} cm^{-1}: 3420, 2950, 1470, 1380, 1230, 1070, 980, 910, 810. ^{1}H-NMR (in CD_3OD) δ: 4.22 (1H, dd, J=4.5, 10.0 Hz), 4.11 (1H, dd, J=6.7, 10.0 Hz), 4.00 (1H, br s), 3.80 (1H, m), 3.80—3.62 (3H, m), 3.45—3.30 (1H, m), 2.35—2.15 (2H, m), 2.05—1.02 (23H, m), 1.02 (3H, d, J=6.3 Hz), 0.92 (3H, s), 0.72 (3H, s). ^{13}C-NMR (in CD_3OD) δ: 74.1 (d), 72.9 (d), 71.4 (d), 69.1 (d), 66.7 (t), 61.2 (t), 48.3 (d), 47.8 (d), 47.5 (s), 43.1 (d), 43.0 (d), 41.0 (d), 40.3 (t), 37.0 (d), 36.5 (t), 35.9 (s), 35.8 (t), 33.3 (t), 32.1 (t), 31.2 (t), 29.5 (t), 28.8 (t), 27.9 (d), 24.3 (t), 23.2 (q), 18.1 (q), 13.1 (q).

Oxidation of 1 with CrO_3——A solution of 220 mg of chromic anhydride in 1 ml of H_2O was added to a solution of 114 mg of **1** in 1 ml of acetic acid at 0 °C. The mixture was stirred for 5 h at 25 °C, then diluted with 20 ml of H_2O and extracted twice with 20 ml of ethyl acetate. The organic layer was washed with 10 ml of H_2O and then brine, and dried over anhydrous $MgSO_4$. The solvent was removed in a rotary evaporator under reduced pressure, and the residue was recrystallized from aqueous ethanol to give **2** as colorless long needles (90 mg) (mp 233—235 °C) (lit.,[5] mp 237 °C). This product was identified as dehydrocholic acid by comparison of the physical data (mp, IR and NMR) with those of an authentic sample obtained from Wako Co.

Acid Hydrolysis of 1 with Trichloroacetic Acid——A mixture of 420 mg of **1** and 6 ml of acetic acid and acetic anhydride (1 : 1) was refluxed for 3 h. The solvent was removed *in vacuo*, and the residue was dissolved in 4 ml of dry dioxane. To this solution was added 2 ml of freshly distilled trichloroacetic acid. The mixture was left for 7 d at room temperature, then diluted with 20 ml of aqueous 1 M $BaCl_2$ to yield 164 mg of $BaSO_4$. After removal of the precipitate by filtration, the filtrate was concentrated under reduced pressure. A solution of 1 g of potassium hydroxide in 10 ml of methanol was added to the concentrate and the mixture was refluxed for 3 h. The excess methanol was removed and the residue was diluted with 30 ml of H_2O, then neutralized with diluted hydrochloric acid, and extracted twice with 50 ml of ethyl acetate and *n*-butanol (1 : 1). The organic phase was washed with saturated aqueous NaCl and dried with $MgSO_4$. The concentrate was chromatographed with $CHCl_3$–MeOH–H_2O (60 : 40 : 10) as an eluent to afford 207.8 mg of **3**. Upon crystallization of this product from ethyl acetate and methanol, colorless plates were obtained. Crystallographical analysis of **3** is in progress to determine the absolute structure, especially the configuration around C_{24}. The physical properties of **3** are as follows. mp 183—184 °C (190 °C)[1] (colorless plates). $[\alpha]_D^{25}$ 40.40° (c=0.5, MeOH) (34 ± 2° (c=0.9, ethanol)).[1] *Anal.* Calcd for $C_{27}H_{48}O_6 \cdot H_2O$: 66.63; H, 10.36. Found: C, 66.47; H, 9.93. High-resolution MS: 432.3223 (error −1.4 mMU) for $C_{27}H_{44}O_4$($C_{27}H_{48}O_6 - 2H_2O$). IR ν_{max}^{KBr} cm^{-1}: 3400, 2950, 1480, 1380, 1080, 1040, 980, 920. ^{1}H-NMR (in CD_3OD) δ: 3.95 (1H, br), 3.80—3.50 (6H, m), 3.48 (1H, m), 2.40—2.15 (2H, m), 2.10—1.10 (23H, m), 1.07 (3H, d, J=6.01 Hz), 0.91 (3H, s), 0.71 (3H, s). ^{13}C-NMR (in CD_3OD) δ: 74.8 (d), 73.5 (d), 73.0 (d), 69.8 (d), 62.7 (t), 62.0 (t), 50.0 (t), 49.0 (d), 48.1 (s), 43.8 (d), 43.6 (d), 41.7 (d), 41.1 (t), 37.8 (d), 37.2 (t), 36.6 (s), 36.5 (t), 34.0 (t), 33.0 (t), 31.9 (t), 30.3 (t), 29.5 (t), 28.5 (d), 25.0 (t), 24.0 (q), 18.9 (q), 13.8 (q). From these data, this compound was identified as 5β-cholestane-3α,7α,12α,24,26,27-hexol (scymnol).

Alkaline Degradation of 1 with Potassium Hydroxide——In a sealed tube, 500 mg of **1** in 50 ml of 2.5 N aqueous potassium hydroxide was heated at 120 °C for 16 h. After saturation of the resultant solution with sodium chloride, the products were extracted with 100 ml of ethyl acetate and *n*-butanol (1 : 1) three times. The organic layer was washed with water and saturated sodium chloride, and the solvent was distilled off under reduced pressure. The concentrate was subjected to silica gel chromatography with the lower layer of $CHCl_3$–MeOH–H_2O (70 : 30 : 10) as an eluent to provide 361 mg of **4**. Recrystallization of compound **4** from aqueous ethanol gave 216 mg of colorless needles (mp 228—230 °C) (lit. 228—231 °C).[2] Compound **4** has the following physical properties. $[\alpha]_D^{20}$ 39.6° (c=1.0, MeOH). *Anal.* Calcd for $C_{27}H_{46}O_5 \cdot H_2O$: C, 69.23; H, 10.26. Found: C, 69.18; H, 9.93. High-resolution MS: 432.3210 (error −2.6 mMU) for $C_{27}H_{44}O_4$($C_{27}H_{46}O_5 - H_2O$). IR ν_{max}^{KBr} cm^{-1}: 3400, 2950, 1480, 1380, 1080, 1050, 1020, 980, 960, 920, 860. ^{1}H-NMR (in CD_3OD) δ: 4.65—4.45 (2H, m), 4.45—4.30 (1H, dd, J=6.3, 6.3 Hz), 3.94 (1H, br), 3.78 (1H,

m), 3.70 (2H, d, $J=6.3$ Hz), 3.45—3.28 (1H, m), 2.40—2.15 (2H, m), 2.10—1.04 (23H, m), 1.02 (3H, d, $J=6.5$ Hz), 0.91 (3H, s), 0.71 (3H, s). ^{13}C-NMR (in D_3OD) δ: 88.3 (d), 74.8 (d), 73.6 (d), 72.4 (t), 69.8 (d), 64.6 (t), 48.8 (d), 48.2 (s), 44.7 (d), 43.9 (d), 43.7 (d), 41.8 (d), 41.2 (t), 37.7 (d), 37.3 (t), 36.7 (s), 36.6 (t), 35.4 (t), 31.9 (t), 30.3 (t), 29.5 (d), 28.6 (d), 25.0 (t), 24.4 (q), 18.9 (q), 13.8 (q). These data indicate that compound **4** is 24,26-epoxy-5β-cholestane-3α.7α,12α,27-tetrol (anhydroscymnol).

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