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Determination of the Sulfated Position in 5β-Bufol Sulfate by a Carbon-13 Nuclear Magnetic Resonance Study[1]

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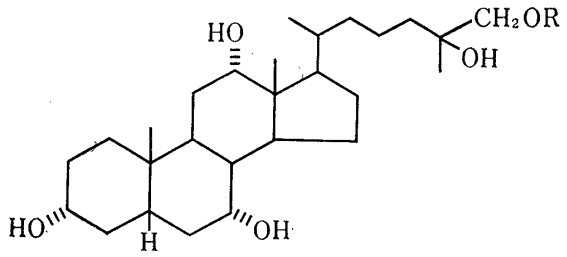
The total structure of 5β-bufol sulfate, which is the major bile salt of the toad, *Bufo vulgaris formosus*, was elucidated as the 26-sulfate ester of 5β-cholestane-3α,7α,12α,25,26-pentol on the basis of ^{13}C-NMR. This method can thus be used for determination of the location of the sulfate ester group in bile alcohol sulfates.

Keywords——bile alcohol sulfate; steroid; ^{13}C-NMR; *Bufo vulgaris formosus*; determination of sulfate ester; sterol

During the course of comparative biochemical studies on bile salts, various bile alcohol sulfates have been found in biles of evolutionally primitive vertebrates as their major bile salts.[2] Although the chemical structures of these bile alcohols have been elucidated after solvolysis of the bile salts to liberate desulfated bile alcohols,[3] the total structures of most of the bile alcohol sulfates have remained obscure, since the determination of the sulfated position is extremely difficult.



Ia : R=H
Ib : R=SO_3^-

Chart 1

In this report we report the usefulness of ^{13}C-NMR spectroscopy for this purpose. By this technique, the location of the sulfate ester group in 5β-bufol sulfate (Ib in Chart 1), a major bile salt of the toad, *Bufo vulgaris formosus*, was determined to be the C-26 position.

5β-Bufol sulfate (Ib) and 5β-bufol (Ia) were obtained from the untreated and solvolyzed biles of the toad, respectively. The chemical structure of 5β-bufol has been elucidated as 5β-cholestane-3α,7α,12α,25,26-pentol (Ia).[4] As model compounds, 5β-cholane-3α,7α,12α,24-tetrol (V) and its 24-sulfate (IV) were prepared by the routes outlined in Chart 2.

Chemical shift date for 5β-bufol, the cholanetetrol, and their sulfates are listed in Table I. The signal assignments of these steroids were carried out by means of single frequency off-resonance decoupling techniques, partially relaxed Fourier transform studies, and comparison with the reported date for steroid derivatives.[5]

On sulfation of bile alcohols, a carbinyl carbon is obviously deshielded, while a β-carbon resonance is displaced somewhat upfield. For example, on going from the cholanetetrol (V) to its 24-sulfate (IV), the signal for C-24 (α-carbon) and C-23 (β-carbon) were displaced by +6.1 ppm and −2.9 ppm, respectively, while signals due to the other carbons (C-1—C-22) remained almost unaffected. Similar chemical shifts due to sulfate substitution have been reported by Kitagawa *et al.*[6] for holothurin B, a triterpene-oligoglycoside sulfate.

Comparison of the ^{13}C-NMR spectrum of 5β-bufol with that of the natural 5β-bufol sulfate indicated that on going from the former to the latter, the C-26 signal was displaced downfield by 4.9 ppm and the C-25 signal was shielded by 1.6 ppm, while the other carbon resonances

of both compounds appeared at essentially the same positions. Thus we concluded that the position of the sulfate ester group in 5β-bufol sulfate is at C-26, and that the bile alcohol sulfate can be formulated as 5β-cholestane-3α,7α,12α,25,26-pentol 26-sulfate (Ib).

TABLE I. ^{13}C Chemical Shifts of 5β-Bufol, 5β-Cholane-3α, 7α, 12α, 24-tetrol and Their Sulfates in CD_3OD Solution

Carbon	5β-Bufol	5β-Bufol sulfate	5β-Cholane-3α,7α,12α,24-tetrol	5β-Cholane-3α,7α,12α,24-tetrol sulfate
1	36.5	36.4	36.5	36.5
2	31.1	31.1	31.2	31.2
3	72.9	72.8	72.8	73.0
4	40.4	40.3	40.5	40.5
5	43.2	43.0	43.2	43.2
6	35.8	35.7	35.9	35.9
7	69.0	68.9	69.0	69.3
8	40.9	40.9	41.0	41.1
9	27.8	27.8	27.9	27.9
10	35.8	35.7	35.9	35.9
11	29.5	29.4	29.6	29.6
12	74.1	74.0	74.1	74.2
13	47.2	47.3	47.4	47.5
14	42.9	42.8	42.9	42.9
15	24.1	24.2	24.2	24.2
16	28.8	28.7	28.8	28.7
17	48.3	48.1	48.1	48.3
18	13.0	13.0	13.0	13.0
19	23.1	23.1	23.2	23.1
20	37.1	37.1	37.1	36.9
21	18.0	18.1	18.0	18.0
22	37.8	37.7	33.2	33.1
23	21.1	21.0	30.4	27.3
24	40.0	40.3	63.6	69.7
25	74.1	72.4		
26	70.3	75.2		
27	23.7	23.9		

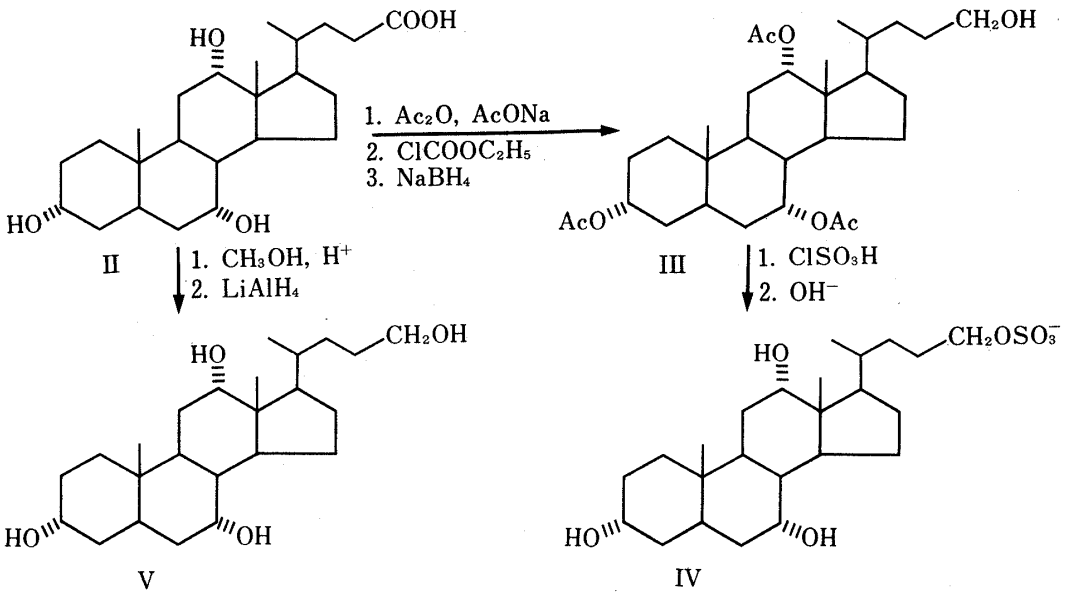


Chart 2

Experimental

General Procedures——Melting points were determined with a Kofler hot-stage apparatus and are uncorrected. Thin-layer chromatography (TLC) was carried out on silica gel G (Merck) with a 10% solution of phosphomolybdic acid in ethanol as the detection reagent. The following solvent systems were employed: CMAW, chloroform–methanol–acetic acid–water (65: 20: 10: 5, by vol.); BE, benzene–ethyl acetate (7:3, by vol.). IR spectra were taken on a JASCO IRA-1 spectrometer as KBr discs. PMR spectra were obtained at 90 MHz on a Hitachi R-40 spectrometer with pyridine-d_5 as the solvent. ^{13}C-NMR spectra were obtained at 100 MHz on a JEOL PFT-100 spectrometer equipped with an EC-6 computer, with CD_3OD as the solvent. All chemical shifts were calculated from internal tetramethylsilane. ^{13}C FT-NMR spectra were recorded at 25.15 MHz at 26° and at a concentration of 0.1—0.2 M in 10 mm tubes. Conditions of FT-NMR measurements were: spectral width, 5 kHz; pulse interval, 1 sec; pulse flipping angle, 45°; acquisition time, 0.4 sec, number of data points, 4096; computer-limited resolution, 0.1 ppm; number of transients, 5000—10000.

Synthesis of 5β-Cholane-3α,7α,12α,24-tetrol 24-Sulfate (IV)——3α,7α,12α-Triacetoxy-5β-cholan-24-ol (III): Cholic acid triacetate was prepared from cholic acid (II) in the usual manner. Triethylamine (1 ml) and ethyl chlorocarbonate (0.5 ml) were added to a solution of the cholic acid triacetate in freshly distilled tetrahydrofuran (30 ml) with stirring in an ice bath. The mixture was stirred for 2 hr, then a suspension of $NaBH_4$ (0.5 g) in water (20 ml) was added to the solution and the whole was kept at 0° overnight with stirring. The reaction mixture was then poured into ice-water, acidified with dil. HCl and extracted with ether. The ethereal extract was washed with water, 2% $NaHCO_3$, and water successively, dried over anhydrous Na_2SO_4 and evaporated to dryness. The residue (900 mg) was chromatographed on a column of silica gel (100 g, Merck). Elution with a 4: 1 mixture of benzene and ethyl acetate gave the triacetoxy-24-ol (III) (600 mg) as an oily product. The purity of III was ascertained by TLC (Rf=0.45 in BE). The IR spectrum showed a band at 3400 cm^{-1} (hydroxyl group). PMR (δ ppm in pyridine-d_5), 0.69 (s, 3H, 18-CH_3), 0.84 (s, 3H, 19-CH_3), 0.93 (d, J=6 Hz, 3H, 21-CH_3), 1.97, 2.03 (s, 3H×3, 3-, 7-, 12-$OCOCH_3$), 3.82 (t, 2H, 24-CH_2OH), 4.74 (m, 1H, C-3βH), 5.08 (m, 1H, C-7βH), 5.28 (m, 1H, C-12βH).

5β-Cholane-3α,7α,12α,24-tetrel 24-Sulfate (IV): A solution of chlorosulfonic acid (0.2 ml) in anhydrous pyridine (4 ml) was added to a solution of the triacetoxy-24-ol, III, (300 mg) in anhydrous pyridine (10 ml) under ice-cooling. After being heated at 50° for 60 min, the reaction mixture was poured into ice-water, acidified with dil. HCl and extracted with ethyl acetate. The ethyl acetate extract was washed with water, dried over Na_2SO_4 and the solvent was evaporated off. The residue was dissolved in 20 ml of 10% methanolic KOH and the solution was refluxed for 2 hr. The hydrolysate was diluted with water and extracted with ethyl acetate in order to remove nonsulfated material. The aqueous layer was adjusted to pH 10 with dil. HCl and percolated through a column of Amberlite XAD-2 resin (200 ml). After thorough washing with water, the sulfated bile alcohol was eluted with 1% NH_4OH in MeOH (1: 99 v/v). Recrystallization from methanol–ethyl acetate gave crystals (120 mg) of 5β-cholane-3α,7α,12α,24-tetrol 24-sulfate (IV); mp 212—213°; IR KBr cm^{-1}, 3350 (OH), 1520, 1150 (sulfate) 1030, 980, 950, 918 (cholic acid-type nucleus), PMR (δ ppm in CD_3OD), 0.71 (s, 3H, 18-CH_3), 0.91 (s, 3H, 19-CH_3), 1.02 (d, J=6 Hz, 3H, 21-CH_3), 3.32 (m, 1H, C-3βH), 3.80 (m, 1H, C-7βH), 3.96 (t, 2H, 24-$CH_2OSO_3^-$), 3.96 (m, 1H, C-12βH).

Synthesis of 5β-Cholane-3α,7α,12α,24-tetrol (V)——A solution of methyl cholate (1 g) in dry ether (30 ml) was added to a suspension of $LiAlH_4$ (0.5 g) in dry ether (20 ml) with ice-cooling. The reaction was kept at 50° for 2 hr. After usual work-up, the crude product was recrystallized from methanol to give crystals (150 mg) of 5β-cholane-3α,7α,12α,24-tetrol (V); mp 234.5—235.5°, IR KBr cm^{-1}, 3350 (OH), 1030, 980, 950, 918 (cholic acid-type nucleus), PMR (δ ppm in CD_3OD), 0.75 (s, 3H, 18-CH_3), 0.94 (s, 3H, 19-CH_3), 1.17 (d, J=6 Hz, 3H, 21-CH_3), 3.70 (m, 1H, C-3βH), 3.78 (t, 2H, 24-CH_2OH), 4.03 (m, 1H, C-7βH), 4.18 (m, 1H, C-12βH).

Isolation of 5β-Bufol Sulfate (Ib) and 5β-Bufol (Ia)——Gallbladder bile of the toad, *Bufo vulgaris formosus*, was extracted with 20 volumes of ethanol to yield crude bile salts. The ethanol was evaporated off, and the bile salts (350 mg) were dissolved in water, acidified with dil. HCl and extracted with ethyl ether in order to remove unconjugated bile acids.[7] The aqueous layer was adjusted to pH 10 with 2% NaOH and percolated through a column of Amberlite XAD-2 resin (500 g). The column was washed with water until the effluent was salt-free and the bile salts were eluted with 1% NH_4OH–MeOH (1: 99 v/v). 5β-Bufol sulfate was obtained after removal of the solvent. The content of 5β-bufol sulfate in the product was more than 95% on TLC as measured by a dual-wavelength chromatoscanner, as described previously.[8]

5β-Bufol was isolated from the solvolyzed bile of the toad as described previously.[4]

References and Notes

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