Absolute configuration at carbon 23 of 5β-cholestane-3α,7α,12α,23,25-pentol excreted by patients with cerebrotendinous xanthomatosis[1]

Kenji Kihira,[2] Akie Kubota, and Takahiko Hoshita

Institute of Pharmaceutical Sciences, Hiroshima University School of Medicine, Kasumi 1-2-3, Hiroshima, 734, Japan

Abstract Absolute configuration at C-23 of 5β-cholestane-3α,7α,12α,23,25-pentol, one of the bile alcohols isolated from the patients with cerebrotendinous xanthomatosis, was unequivocally determined as 23S by conversion of a key intermediate, (23S)-5β-cholest-25-ene-3α,7α,12α,23-tetrol to either the bile alcohol of known absolute configuration, (23R)-5β-cholestane-3α,7α,12α,23-tetrol, or the naturally occurring 23,25-pentol.—**Kihira, K., A. Kubota, and T. Hoshita.** Absolute configuration at carbon 23 of 5β-cholestane-3α,7α,12α,23,25-pentol excreted by patients with cerebrotendinous xanthomatosis. *J. Lipid Res.* 1984. **25:** 871–875.

Supplementary key words gas–liquid chromatography–mass spectrometry • proton nuclear magnetic resonance spectrometry

Patients with a rare inherited lipid storage disease cerebrotendinous xanthomatosis (CTX) accumulate considerable amounts of six different bile alcohols in their bile and feces (1–3). Two of them have been identified as 5β-cholestane-3α,7α,12α,23-tetrol (3, 4) and 5β-cholestane-3α,7α,12α,23,25-pentol (2, 5) which differ, not only from the other CTX bile alcohols, but also from all other bile alcohols so far identified as natural products in that they possess a 23-hydroxyl group. The absolute configuration at C-23 of the 23-tetrol was established by X-ray crystallography as R (6). However, definite assignment of the configuration at C-23 of the 23,25-pentol remained to be established, though tentative assignments were made on the basis of optical rotation differences (4) or a circular dichroism study (7). We now wish to report the elucidation of the absolute configuration at C-23 of the 5β-cholestane-3α,7α,12α,23,25-pentol isolated from the CTX patients. This was achieved by the conversion of a key intermediate, (23S)-5β-cholest-25-ene-3α,7α,12α,23-tetrol, to both naturally occurring 23-hydroxylated bile alcohols.

MATERIALS AND METHODS

Norcholic acid, 3α,7α,12α-trihydroxy-24-nor-5β-cholan-23-oic acid, was prepared according to the method previously reported (4).

(23R)-5β-Cholestane-3α,7α,12α,23-tetrol (3) (mp 232–233°C) and *5β-cholestane-3α,7α,12α,23ξ,25-pentol* (1, 2) (mp 209–210°C) were isolated from the bile of patients with CTX according to the methods previously reported.

Melting points were determined on a Kofler-hot stage apparatus, and are uncorrected.

Infrared (IR) spectra were obtained on a Shimadzu model IR-408 spectrophotometer as KBr discs. Absorption frequencies are quoted in reciprocal centimeters.

Optical rotations were measured in methanol on a Union Giken model PM-101 automatic polarimeter at 25°C.

Proton nuclear magnetic resonance (PMR) spectra, in δ ppm, were obtained in deuterated pyridine solutions on a Hitachi model R-40 spectrometer (90 MHz) using tetramethylsilane as internal standard.

Thin-layer chromatography (TLC). The samples were separated on silica gel G plates (Merck, 0.25 mm thickness). The spots were detected by spraying with phosphomolybdic acid (10% in ethanol) and heating at 110°C for 5 min.

Gas–liquid chromatography (GLC). The samples, as their trimethylsilyl (TMS) derivatives, were run on a 2 m × 3 mm column packed with 3% OV-17 (column temperature, 270°C), 3% QF-1 (column temperature, 230°C) on 80/100 mesh Gas-Chrom Q, or 25 m × 0.35 mm capillary column coated with SE-30 (column temperature, 270°C). All retention times are described relative to that of TMS ether of methyl cholate (1.00).

Gas–liquid chromatography–mass spectrometry (GLC–MS) was performed on a Shimadzu model GCMS 7000 gas chromatograph–mass spectrometer using the following

Abbreviations: CTX, cerebrotendinous xanthomatosis; IR, infrared; TLC, thin-layer chromatography; GLC, gas–liquid chromatography; RRT, relative retention time; GLC–MS, gas–liquid chromatography–mass spectrometry; PMR, proton nuclear magnetic resonance; TMS, trimethylsilyl.

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[2] To whom correspondence should be addressed.

conditions: column, 3% OV-17 (2 m × 3 mm); column temperature, 280°C; ion source temperature, 300°C; ionizing voltage, 70 eV; ionizing current, 150 μA. The samples were analyzed as their TMS derivatives.

EXPERIMENTAL

3α,7α,12α-Triacetoxy-24-nor-5β-cholan-23-oic acid (II)

Two grams of norcholic acid were dissolved in a mixture of acetic acid (10 ml), acetic anhydride (10 ml), and anhydrous sodium acetate (200 mg). The reaction mixture was heated on a steam bath at 100°C for 19 hr, diluted with water (200 ml), and extracted with ether (100 ml × 2). The extract was washed with water and dried over anhydrous Na_2SO_4. After evaporation of the solvent, the residue was crystallized from ethyl acetate to give crystals (2.1 g) of 3α,7α,12α-triacetoxy-24-nor-5β-cholan-23-oic acid (II). Melting point was 112°C; IR, 1750 (carboxyl); PMR, 0.70 (3H, s, 18-C$\underline{H_3}$), 0.85 (3H, s, 19-C$\underline{H_3}$), 0.94 (3H, d, J = 6 Hz, 21-C$\underline{H_3}$), 2.00 (3H × 2, s, C$\underline{H_3}$-COO-), 2.03 (3H, s, C$\underline{H_3}$-COO-), 4.72 (1H, m, 3β-$\underline{H}$), 5.08 (1H, m, 7β-$\underline{H}$), 5.28 (1H, m, 12β-$\underline{H}$).

3α,7α,12α-Triacetoxy-24-nor-5β-cholan-23-ol (III)

To a solution of 3α,7α,12α-triacetoxy-24-nor-5β-cholan-23-oic acid (II, 2 g) dissolved in anhydrous tetrahydrofuran (24 ml) was added triethylamine (0.56 ml) and ethyl chloroformate (0.4 ml) at 0°C. After stirring for 2 hr at 0°C, a solution of $NaBH_4$ (2 g) dissolved in 7.5 ml of water was added dropwise to the reaction mixture. After additional stirring for 2 hr at 0°C, the reaction mixture was diluted with water (200 ml), acidified with 1 N HCl, and extracted with ether (200 ml × 2). The extract was washed with 5% $NaHCO_3$ and water, dried over anhydrous Na_2SO_4, and evaporated to dryness. The residue was chromatographed on silica gel (100 g) using ethyl acetate as eluting solvent. Monitoring on TLC, appropriate fractions were combined and evaporation of the solvent gave an oily residue (1.2 g) of 3α,7α,12α-triacetoxy-24-nor-5β-cholan-23-ol (III). IR, 3400 (hydroxyl) and 1750 (carboxyl); PMR, 0.70 (3H, s, 18-C$\underline{H_3}$), 0.85 (3H, s, 19-C$\underline{H_3}$), 2.00 (3H, d, J = 6 Hz, 21-C$\underline{H_3}$), 2.00 (3H × 2, s, C$\underline{H_3}$-COO-), 2.03 (3H, s, C$\underline{H_3}$-COO-), 3.83 (2H, t, J = 6 Hz, 23-C$\underline{H_2}$-OH), 4.72 (1H, m, 3β-$\underline{H}$), 5.08 (1H, m, 7β-$\underline{H}$), 5.28 (1H, m, 12β-$\underline{H}$).

3α,7α,12α-Trihydroxy-24-nor-5β-cholan-23-al (IV)

To a solution of pyridinium chlorochromate (1 g) and sodium acetate (70 mg) dissolved in dichloromethane (10 ml), a solution of 3α,7α,12α-triacetoxy-24-nor-5β-cholan-23-ol (III, 1 g) dissolved in dichloromethane (5 ml) was added at 0°C and stirred for 2 hr at room temperature. After dilution with 100 ml of anhydrous ether, the resulting precipitate was filtered off and the filtrate was percolated through a Florisil column (20 g). The column was further eluted with 300 ml of ether. The eluants were combined and evaporated to dryness. The residue (670 mg) was hydrolyzed with 20 ml of 5% methanolic KOH by heating at 60°C for 2 hr. After dilution with 200 ml of water the reaction mixture was extracted with ethyl acetate (200 ml × 2). The extract was washed with water and dried over anhydrous Na_2SO_4. Evaporation of the solvent gave an oily residue (400 mg), which was chromatographed on a silica gel column (40 g) with ethyl acetate–acetone 7:3 as eluting solvent. Monitoring on TLC, appropriate fractions were collected and the solvent was evaporated to dryness to afford a colorless oily residue of 3α,7α,12α-trihydroxy-24-nor-5β-cholan-23-al (IV, 260 mg). IR, 1740 (aldehyde) and 3400 (hydroxyl); PMR, 0.81 (3H, s, 18-C$\underline{H_3}$), 1.00 (3H, s, 19-C$\underline{H_3}$), 1.23 (3H, d, J = 6 Hz, 21-C$\underline{H_3}$), 3.70 (1H, m, 3β-$\underline{H}$), 4.09 (1H, m, 7β-$\underline{H}$), 4.19 (1H, m, 12β-$\underline{H}$), 9.83 (1H, t, J = 3 Hz, 23-C$\underline{H}$O).

(23R)- and (23S)-5β-cholest-25-ene-3α,7α,12α,23-tetrols (Va and Vb)

3α,7α,12α-Trihydroxy-24-nor-5β-cholan-23-al (IV, 250 mg) and β-methallyl chloride (3 ml) were dissolved in anhydrous tetrahydrofuran (10 ml) and this solution was added to a mixture of tetrahydrofuran (25 ml) and sliced Li (250 mg). The reaction mixture was then stirred at 4°C for 24 hr. After the reaction period the reaction mixture was filtered to remove insoluble materials. The filtrate was diluted with water, acidified with 1 N HCl, and extracted with ethyl acetate (100 ml) and ether (100 ml). The extract was washed with water, 5% $NaHCO_3$, and water, successively. After drying over anhydrous Na_2SO_4, the solvent was evaporated to dryness. The residue was chromatographed on a silica gel column (20 g) using a solvent system of acetone graded into ethyl acetate. The fractions eluted with ethyl acetate–acetone 8:2 were collected and evaporation of the solvent gave a colorless oily residue of (23R)-5β-cholest-25-ene-3α,7α,12α,23-tetrol (Va, 90 mg). $[\alpha]_D$ = +40.3° (c = 1.00, methanol); IR, 3400 (hydroxyl) and 890 (end methylene); TLC, R_f 0.49 (ethyl acetate–acetone 7:3); GLC (as TMS ether), RRT 0.91 (3% OV-17); PMR, 0.87 (3H, s, 18-C$\underline{H_3}$), 1.01 (3H, s, 19-C$\underline{H_3}$), 1.37 (3H, d, J = 6 Hz, 21-C$\underline{H_3}$), 1.83 (3H, s, 27-C$\underline{H_3}$), 3.5–4.4 (4H, m, 3β-$\underline{H}$, 7β-$\underline{H}$, 12β-$\underline{H}$, and 23-$\underline{H}$), and 4.98 (2H, s, 26-C$\underline{H_2}$); GLC–MS, m/z (relative intensity), 667 (34%, M − 55), 577 (14, M − 55 − TMSOH), 487 (19, M − 55 − TMSOH × 2), 397 (32, M − 55 − TMSOH × 3), 307 (100, M − 55

− TMSOH × 4), 343 (7, M − side chain − TMSOH × 2), 253 (26, M − side chain − TMSOH × 3).

The fractions eluted with ethyl acetate–acetone 7:3 were collected and evaporation of the solvent gave a colorless oily residue (72 mg). Repeated crystallization from ethyl acetate gave colorless crystals (48 mg) of (23S)-5β-cholest-25-ene-3α,7α,12α,23-tetrol (Vb). Melting point was 194.5–195.0°C; $[\alpha]_D$ = +44.2° (c = 1.00, methanol); IR, 3400 (hydroxyl) and 890 (end methylene); TLC, R_f, 0.21 (the same solvent system for Va); GLC (as TMS ether), RRT 0.94 (3% OV-17); PMR, 0.88 (3H, s, 18-C$\underline{H_3}$), 1.01 (3H, s, 19-C$\underline{H_3}$), 1.41 (3H, d, J = 6 Hz, 21-C$\underline{H_3}$), 1.89 (3H, s, 27-C$\underline{H_3}$), 3.5–4.4 (4H, m, 3β-$\underline{H}$, 7β-$\underline{H}$, 12β-$\underline{H}$, and 23-$\underline{H}$), 4.98 (2H, s, 26-C$\underline{H_2}$); GLC–MS, m/z (relative intensity), 667 (23%, M − 55), 577 (9, M − 55 − TMSOH), 487 (18, M − 55 − TMSOH × 2), 397 (20, M − 55 − TMSOH × 3), 307 (100, M − 55 − TMSOH × 4), 343 (1, M − side chain − TMSOH × 2), 253 (69, M − side chain − TMSOH × 3).

(23R)-5β-Cholestane-3α,7α,12α,23-tetrol (VI) from (23S)-5β-cholest-25-ene-3α,7α,12α,23-tetrol (Vb)

The solution of (23S)-5β-cholest-25-ene-3α,7α,12α,23-tetrol (VIb, 16 mg) dissolved in 10 ml of methanol was hydrogenated with PtO_2 as the catalyst at room temperature. After removal of the platinum catalyst from the reaction mixture the solvent was evaporated to dryness. Repeated crystallization from ethyl acetate of the residue gave 10 mg of colorless needles of (23R)-5β-cholestane-3α,7α,12α,23-tetrol (VI). Melting point was 231.5–232°C; IR, 3400 (hydroxyl); TLC, R_f 0.23 (ethyl acetate–acetone 7:3); GLC (as TMS ether), RRT 0.81 (3% OV-17); GLC–MS, m/z (relative intensity), 667 (1%, M − 57), 544 (5, M − TMSOH × 2), 454 (5, M − TMSOH × 3), 364 (9, M − TMSOH × 4), 343 (6, M − side chain − TMSOH × 2), 253 (58, M − side chain − TMSOH × 3), 159 (100, side chain fragment).

(23S)-5β-cholestane-3α,7α,12α,23,25-pentol (VIIb) from (23S)-5β-cholest-25-ene-3α,7α,12α,23-tetrol (Vb)

(23S)-5β-Cholest-25-ene-3α,7α,12α,23-tetrol (Vb, 15 mg) was dissolved in 5 ml of tetrahydrofuran and this solution was added to a solution of mercuric acetate (15 mg) dissolved in tetrahydrofuran (10 ml) and water (5 ml). While stirring at room temperature for 10 min, 5 ml of a solution of 3 M sodium borohydride was added to the reaction mixture, followed by 5 ml of a solution of 0.5 M sodium borohydride in 3 M NaOH. After dilution with 200 ml of water, the reaction mixture was extracted with ethyl acetate (200 ml × 2). The extract was washed with water, dried over anhydrous Na_2SO_4, and evaporated to dryness. The residue was crystallized from ethyl acetate to give colorless needles (11 mg) of (23S)-5β-cholestane-3α,7α,12α,23,25-pentol (VIIb). Melting point was 211°C; IR, 3400 (hydroxyl); TLC, R_f 0.13 and 0.53 (chloroform–acetone–methanol 5:5:1 and ethyl acetate–water–acetic acid 85:5:10, respectively); GLC (as TMS ether), RRT 1.44 (3% OV-17); GLC–MS, m/z (relative intensity), 722 (1%, M − TMSOH), 667 (3, M − 145), 632 (3, M − TMSOH × 2), 577 (2, M − 145 − TMSOH), 550 (2, M − 262), 542 (4, M − TMSOH × 3), 487 (3, M − 145 − TMSOH × 2), 460 (3, M − 262 − TMSOH), 452 (8, M − TMSOH × 4), 397 (5, M − 145 − TMSOH × 3), 370 (5, M − 262 − TMSOH × 2), 362 (7, M − TMSOH × 5), 344 (8, M − side chain − TMSOH × 2 + H), 307 (15, M − 145 − TMSOH × 4), 253 (17, M − side chain − TMSOH × 3), 143 (35, side chain fragment), and 131 (100, side chain fragment).

(23R)-5β-cholestane-3α,7α,12α,23,25-pentol (VIIa) from (23R)-5β-cholest-25-ene-3α,7α,12α,23-tetrol (Va)

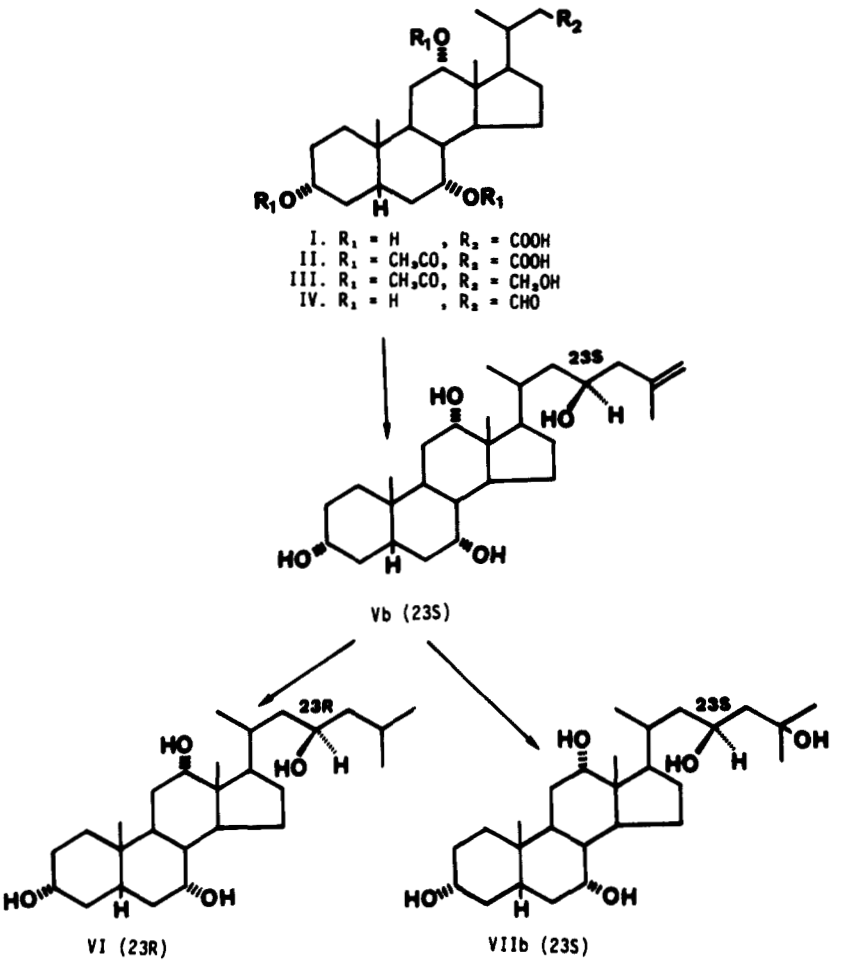
(23R)-5β-Cholest-25-ene-3α,7α,12α,23-tetrol (Vb, 45 mg) was dissolved in 15 ml of tetrahydrofuran and this solution was added to a solution of mercuric acetate (50 mg) dissolved in tetrahydrofuran (10 ml) and water (5 ml). While stirring at room temperature for 2 hr, 15 ml of a solution of 3 M sodium borohydride was added to the reaction mixture, followed by 15 ml of a solution of 0.5 M sodium borohydride in 3 M NaOH. After dilution with 200 ml of water, the reaction mixture was extracted with ethyl acetate (200 ml × 2). The extract was washed with water, dried over anhydrous Na_2SO_4, and evaporated to dryness. The residue was chromatographed on a silica gel column (10 g) and eluted with a solvent system of acetone graded in ethyl acetate. Monitoring on TLC, appropriate fractions were combined and the solvent was removed to give (23R)-5β-cholestane-3α,7α,12α,23,25-pentol (VIIa, 30 mg) as a colorless oily residue. IR, 3400 (hydroxyl); TLC, R_f 0.23 and 0.70 (chloroform–acetone–methanol 5:5:1 and ethyl acetate–water–acetic acid 85:5:10, respectively); GLC (as TMS ether), RRT 1.38 (3% OV-17); GLC–MS, m/z (relative intensity), 722 (1%, M − TMSOH), 667 (3, M − 145), 632 (2, M − TMSOH × 2), 577 (2, M − 145 − TMSOH), 550 (2, M − 262), 542 (3, M − TMSOH × 3), 487 (3, M − 145 − TMSOH × 2), 460 (1, M − 262 − TMSOH), 452 (5, M − TMSOH × 4), 397 (10, M − 145 − TMSOH × 3), 370 (2, M − 262 − TMSOH × 2), 362 (2, M − TMSOH × 5), 344 (3, M − side chain − TMSOH × 2 + H), 307 (5, M − 145 − TMSOH × 4), 253 (12, M − side chain − TMSOH × 3), 143 (32, side chain fragment), and 131 (100, side chain fragment).

RESULTS AND DISCUSSION

A key intermediate, (23S)-5β-cholest-25-ene-3α,7α,12α,23-tetrol (Vb), which can be converted to ei-

ther (23R)-5β-cholestane-3α,7α,12α,23-tetrol (VI) or (23S)-5β-cholestane-3α,7α,12α,23,25-pentol (VIIb) (**Scheme 1**), was prepared from norcholic acid as follows. Acetylation of norcholic acid (I) followed by reduction with sodium borohydride after treatment with ethyl chloroformate in the presence of triethylamine afforded the triacetoxynorcholanol (III). Oxidation with pyridinium chlorochromate of the primary alcohol (III) followed by hydrolysis gave the trihydroxynorcholanal (IV). By a modified Grignard reaction with methallyl lithium, the aldehyde (IV) was converted to a mixture of 23-epimeric 5β-cholest-25-ene-3α,7α,12α,23-tetrols (Va and Vb). Separation of these epimers was achieved by silica gel column chromatography. Catalytic hydrogenation of the more polar epimer (Vb) gave a 5β-cholestane-3α,7α,12α,23-tetrol (VI), which was identical with the bile alcohol isolated from the CTX patients that has been assigned as the 23R-epimer by X-ray crystallography (6). Both the synthetic and biological tetrols showed superimposable mass and IR spectra, and also identical chromatographic properties on TLC and GLC (**Table 1**). From these results the more polar unsaturated bile alcohol (Vb) was determined to have the 23S configuration. The (23S)-5β-cholest-25-ene-3α,7α,12α,23-tetrol (Vb) was then converted to (23S)-5β-cholestane-3α,7α,12α,23,25-pentol (VIIb) by oxymercuration. By the direct comparison with the synthetic specimen, the bile alcohol isolated from the CTX patients was shown to be (23S)-5β-cholestane-3α,7α,12α,23,25-pentol. The biological pentol had the same mass and IR spectra and the identical chromatographic properties on TLC and GLC (Table 1) as the reference compound (VIIb).

By the chemical conversion of the key intermediate, the 23-hydroxyl groups of the two bile alcohols isolated



Scheme 1. Synthesis of (23R)-5β-cholestane-3α,7α,12α,23-tetrol and (23S)-5β-cholestane-3α,7α,12α,23,25-pentol: I, norcholic acid; II, 3α,7α,12α-triacetoxy-24-nor-5β-cholan-23-oic acid; III, 3α,7α,12α-triacetoxy-24-nor-5β-cholan-23-ol; IV, 3α,7α,12α-trihydroxy-24-nor-5β-cholan-23-al; Vb, (23S)-5β-cholest-25-ene-3α,7α,12α,23-tetrol; VI, (23R)-5β-cholestane-3α,7α,12α,23-tetrol; VIIb, (23S)-5β-cholestane-3α,7α,12α,23,25-pentol.

TABLE 1. Chromatographic properties and melting points of synthetic (VIIa, VIIb, and VI) and biological (CTX) 23-hydroxylated bile alcohols

	23,25-Pentol[a]			23-Tetrol[b]	
	VIIa	VIIb	CTX	VI	CTX
Configuration	23R	23S	23S	23R	23R
Melting points (°C)		211	209–210	231.5–232	232–233
R_f value on TLC[c]					
A	0.70	0.53	0.53	0.63	0.63
B	0.23	0.13	0.13		
RRT on GLC[d]					
OV-17	1.38	1.44	1.44	0.81	0.81
QF-1	1.03	1.07	1.07	0.63	0.63
SE-30	2.20	2.13	2.13		

[a] 5β-Cholestane-3α,7α,12α,23,25-pentol.
[b] 5β-Cholestane-3α,7α,12α,23-tetrol.
[c] Solvent system: ethyl acetate–water–acetic acid 85:5:10 for A, and chloroform–acetone–methanol 5:5:1 for B.
[d] Bile alcohols were analyzed as their TMS derivatives.

from the CTX patients were shown to have the same configuration. These results suggest the biological relationship between 5β-cholestane-3α,7α,12α,23-tetrol and 5β-cholestane-3α,7α,12α,23,25-pentol isolated from the bile and feces of CTX patients. The 23R-tetrol could be a metabolic precursor of the 23S-pentol.

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