Identification of (22R)-3α,7α,12α,22- and (23R)-3α,7α,12α,23-tetrahydroxy-5β-cholestanoic acids in urine from a patient with Zellweger's syndrome

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Abstract The nature of two novel C_{27} bile acids present as the taurine conjugates in urine from a patient with Zellweger's syndrome was studied. Bile acids conjugated with taurine were isolated from unconjugated and glycine-conjugated bile acids by means of ion-exchange chromatography. After alkaline hydrolysis of the taurine conjugates, the hydrolysate was acidified and extracted with ether; the extract was again subjected to ion-exchange chromatography to separate neutral from acidic compounds. The neutral fraction, which consisted mainly of two steroidal lactones, was treated with lithium aluminum hydride, and the reduction products were identified as (22R)-5β-cholestane-3α,7α,12α,22,26-pentol and (23R)-5β-cholestane-3α,7α,12α,23,26-pentol by direct comparison of their gas–liquid chromatographic behaviors and mass spectral data with those of chemically synthesized authentic samples. Thus, the chemical structure of two native bile acids present in urine from a patient with Zellweger's syndrome should be formulated as (22R)-3α,7α,12α,22-tetrahydroxy-5β-cholestanoic acid and (23R)-3α,7α,12α,23-tetrahydroxy-5β-cholestanoic acid, respectively. **—Une, M., K. Tsujimura, K. Kihira, and T. Hoshita.** Identification of (22R)-3α,7α,12α,22- and (23R)-3α,7α,12α,23-tetrahydroxy-5β-cholestanoic acids in urine from a patient with Zellweger's syndrome. *J. Lipid Res.* 1989. **30:** 541–547.

Supplementary key words steroidal lactones • gas–liquid chromatography–mass spectrometry • chemical synthesis

Patients with the cerebrohepatorenal syndrome of Zellweger accumulate 3α,7α,12α-trihydroxy-5β-cholestanoic acid (THCA) and other C_{27} bile acids in their bile, serum, and urine as unusual metabolites of cholesterol (1–11). In a previous paper, we reported the occurrence of two novel taurine-conjugated C_{27} bile acids in urine from a patient with Zellweger's syndrome (12). Basesd on their easy conversion to the corresponding lactones during the procedure for the extraction after hydrolysis, these bile acids accounting for 20% of total bile acids were tentatively identified as 22- or 23-hydroxylated derivatives of THCA.

As an extention of our previous study, the present investigation was performed in order to confirm the chemical structure of these novel C_{27} bile acids. This was achieved by the conversion of the steroidal lactones isolated from the urine of the patient into the corresponding neutral bile alcohols, and by the chemical synthesis of reference bile alcohols.

MATERIALS AND METHODS

General

Melting points (mp) were determined with a hot-stage apparatus and are uncorrected.

Infrared (IR) spectra were taken on a JASCO IRA-1 spectrometer as KBr discs. Absorption frequencies are quoted in reciprocal centimeters.

Proton magnetic resonance (PMR) spectra were measured at 90 MHz on a Hitachi R-40 spectrometer. Chemical shifts (δ) are given in ppm downfield from tetramethylsilane internal standard.

Gas–liquid chromatography (GLC) was carried out on a Shimadzu GC-6A gas chromatograph using a glass column (2 m × 3 mm i.d.) packed with 3% OV-17 or 2% OV-1 on 80–100 mesh Gas Chrom Q or an FS-WCOT capillary column (25 m × 0.25 mm i.d.) chemically bonded with OV-1 (Gasukuro Kogyo Inc., Japan). All re-

Abbreviations: TLC, thin-layer chromatography; GLC, gas–liquid chromatography; GLC–MS, gas–liquid chromatography–mass spectrometry; TMS, trimethylsilyl; RRT, relative retention time; PMR, proton nuclear magnetic resonance; IR, infrared; THCA, 3α,7α,12α-trihydroxy-5β-cholestanoic acid.

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tention times are given relative to the trimethylsilyl (TMS) ether of methyl cholate.

Gas–liquid chromatography–mass spectrometry (GLC–MS) was carried out on a Shimadzu GCMS-1000 gas chromatograph–mass spectrometer equipped with a data-processing system (Shimadzu GCMSPAC-90) and Van den Berg's solventless injector. The following conditions were used: column OV-1 (12 m × 0.23 mm i.d.); injection port temperature, 270°C; column oven temperature, 240–270°C, 2°C/min; separator temperature, 270°C; ionizing source temperature, 290°C; flow rate of helium carrier gas, 1.5 ml/min; ionizing energy, 70 eV; tap current, 60 μA.

Analysis of bile acids in urine

A sample of urine (10 ml) obtained from the same patient reported in the previous paper (12) was passed through a Sep-pak C_{18} cartridge (Waters Associates Inc., Midford, MA). After washing the cartridge with 20 ml of water, bile acids were eluted with 20 ml of methanol. The eluate was taken to dryness in vacuo. The residue was dissolved in 1 ml of 90% ethanol and the solution was passed through a column of piperidinohydroxypropyl-Sephadex-LH-20 (PHP-LH-20) (3 ml). After additional washing with 9 ml of 90% ethanol, the column was eluted successively with 24 ml of 0.1 M acetic acid in 90% ethanol, 24 ml of 0.2 M formic acid in 90% ethanol, and 24 ml of 1% ammonium carbonate in 70% ethanol, to give the unconjugated bile acid fraction, the glycine-conjugated bile acid fraction, and the taurine-conjugated bile acid fraction, respectively (12). The taurine-conjugated bile acid fraction was evaporated to dryness. The residue was hydrolyzed at 120°C for 3 hr in 5 ml of 2.5 N KOH. After dilution with water and acidification with dilute HCl, the hydrolysate was extracted with ether. The extract was washed with water and concentrated to dryness to give a residue containing deconjugated bile acids. The residue was dissolved in 90% ethanol (1 ml) and the solution was applied to a column of PHP-LH-20 (3 ml). The column was eluted with 10 ml of 90% ethanol and then with 24 ml of 0.1 M acetic acid in 90% ethanol, to give neutral and acidic fractions, respectively. An aliquot of the neutral fraction was evaporated to dryness and the resulting residue was treated with $LiAlH_4$ (20 mg) in tetrahydrofuran (10 ml). After standing for 3 hr at room temperature, the reaction mixture was diluted with water, acidified with dilute HCl, and extracted with ethyl acetate. The extract was washed with water and concentrated to dryness. A part of the residue was treated with pyridine–hexamethyldisilazane–trimethylchlorosilane 5:2:1 for 2 hr at room temperature. The resulting trimethylsilyl (TMS) ether derivatives were analyzed by GLC and GLC–MS.

Synthesis of (23R)- and (23S)-5β-cholestane-3α,7α,12α,23,26-pentols (Fig. 1)

(23R)- and (23S)-5β-Cholest-25-ene-3α,7α,12α,23-tetrols (I and III) were prepared from norcholic acid according to the method described previously (13).

(23R)-5β-cholestane-3α,7α,12α,23,26-pentol (II). In a

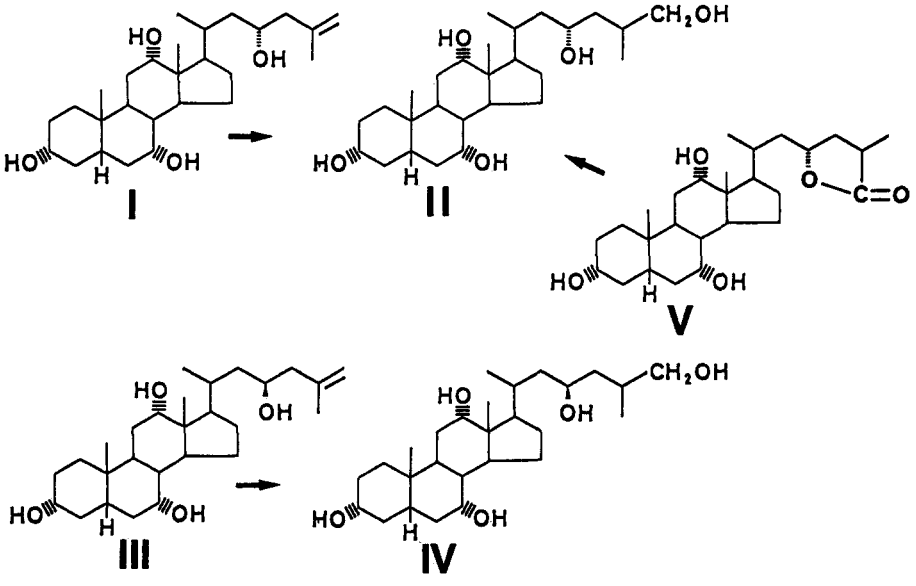

Fig. 1. Synthesis of (23R)- and (23S)-5β-cholestane-3α,7α,12α,23,26-pentols and conversion of natural (23R)-3α,7α,12α-trihydroxy-5β-cholestano-26,23-lactone to (23R)-5β-cholestane-3α,7α,12α,23,26-pentol. I, (23R)-5β-cholest-25-ene-3α,7α,12α,26-tetrol; II, (23R)-5β-cholestane-3α,7α,12α,23,26-pentol; III, (23S)-5β-cholest-25-ene-3α,7α,12α,26-tetrol; IV, (23S)-5β-cholestane-3α,7α,12α,23,26-pentol; V, natural (23R)-3α,7α,12α-trihydroxy-5β-cholestano-26,23-lactone.

dry two-neck flask equipped with a condenser and a pressure-equilibrated dropping funnel were placed diglyme (20 ml), 2-methyl-2-butene (1.5 ml), and sodium borohydride (120 mg). The flask was immersed in an ice bath and boron trifluoride ethereate (1.0 ml) was added dropwise to the well-stirred reaction mixture over a period of 15 min. The reaction mixture was permitted to remain an additional 2 hr at 0–5°C, then (23R)-5β-cholest-25-ene-3α,7α,12α,23-tetrol (50 mg) was added to the solution. After 1 hr, the reaction mixture was treated with 3 N KOH (5 ml) and 30% H_2O_2 (5 ml); it was then allowed to stand for 30 min at room temperature. The reaction mixture was diluted with water and extracted with ethyl acetate after acidification with dilute HCl. The extracts were washed with water, dried over anhydrous Na_2SO_4, and evaporated to dryness. The resulting residue was purified by silica gel column chromatography with increasing amounts of acetone in ethyl acetate to give (23R)-5β-cholestane-3α,7α,12α,23,26-pentol (II) as a noncrystalline material: TLC, R_f 0.38 (ethyl acetate–acetone 3:2); GLC (as TMS ether), RRT 1.44 (3% OV-17) 1.78 (capillary OV-1); IR 3400 (hydroxyl); PMR (δ ppm) 0.87 (s, 3H, 18–CH_3), 1.01 (s, 3H, 19–CH_3), 1.15 (d, J=6 Hz, 3H, 27–CH_3), 1.36 (d, J=6 Hz, 3H, 21–CH_3), 3.69–4.36 (m, 6H, 3β-,7β-,12β-,23-H, and 26-CH_2OH); GLC–MS, *m/z* (relative intensity), 542 (1), 452 (4), 363 (2), 343 (5), 253 (17), 247 (11), 157 (100).

(23S)-5β-Cholestane-3α,7α,12α,23,26-pentol (IV). (23S)-5β-Cholest-25-ene-3α,7α,12α,23-tetrol (III) (50 mg) was treated by the same method described above to give (23S)-5β-cholestane-3α,7α,12α,23,26-pentol (IV) (42 mg): mp 213–215°C (from acetone); TLC, R_f 0.15 (ethyl acetate–acetone 3:2); GLC (as TMS ether), RRT 1.45 (3% OV-17) 1.80 (capillary OV-1); IR 3400 (hydroxyl); PMR (δ ppm) 0.88 (s, 3H, 18–CH_3), 1.02 (s, 3H, 19–CH_3), 1.24 (d, J=6 Hz, 3H, 27–CH_3), 1.43 (d, J=6 Hz, 3H, 21–CH_3), 3.70–4.35 (m, 6H, 3β-,7β-,12β-,23-H and 26-CH_2OH); GLC–MS, *m/z* (relative intensity), 542 (2), 452 (3), 363 (3), 343 (4), 253 (11), 247 (13), 157 (100).

Synthesis of (22R)- and (22S)-5β-cholestane-3α,7α,12α,22,26-pentols (Fig. 2)

(22S)-3α,7α,12α-trihydroxy-5β-cholestano-26,22-lactone (VI) was isolated from the turtle bile as described previously (14).

(22S)-5β-cholestane-3α,7α,12α,22,26-pentol (IX). To a solution of (22S)-3α,7α,12α-trihydroxy-5β-cholestano-26,22-lactone (VI) (50 mg) dissolved in tetrahydrofuran (50 ml),

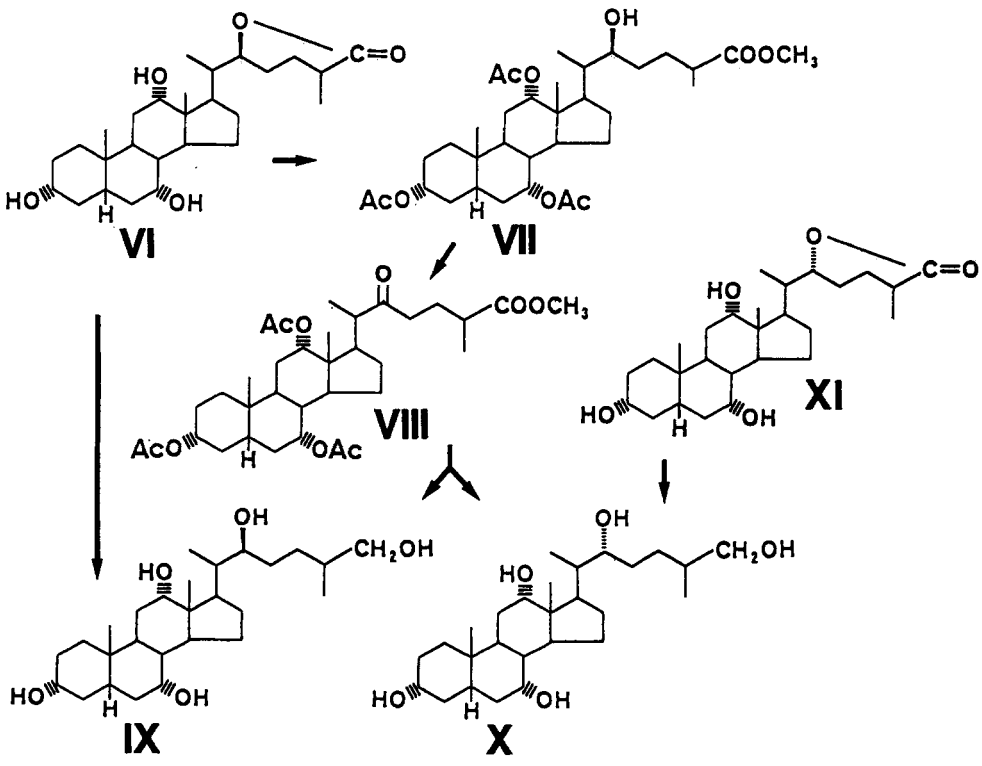

Fig. 2. Synthesis of (22R)- and (22S)-5β-cholestane-3α,7α,12α,22,26-pentols and conversion of natural (22R)-3α,7α,12α-trihydroxy-5β-cholestano-26,22-lactone to (22R)-5β-cholestane-3α,7α,12α,22,26-pentol. VI, (22S)-3α,7α,12α-trihydroxy-5β-cholestano-26,22-lactone; VII, methyl 3α,7α,12α-triacetoxy-22-hydroxy-5β-cholestanoate; VIII, methyl 3α,7α,12α-triacetoxy-22-oxo-5β-cholestanoate; IX, (22S)-5β-cholestane-3α,7α,12α,22,26-pentol; X, (22R)-5β-cholestane-3α,7α,12α,22,26-pentol; XI, (22R)-3α,7α,12α-trihydroxy-5β-cholestano-26,22-lactone.

$LiAlH_4$ (100 mg) was added. The reaction mixture was refluxed for 1 hr, then poured into ice-water, and extracted with ethyl acetate after acidification with diluted HCl. The extract was washed with water and evaporated to dryness. Recrystallization from acetone of the residue (45 mg) afforded colorless prisms of (22S)-5β-cholestane-3α,7α,12α,22,26-pentol (IX): mp 137–139°C, TLC, R_f 0.10 (ethyl acetate–acetone 3:2), GLC (as TMS ether), RRT 1.60 (3% OV-17) 2.05 (capillary OV-1); IR 3400 (hydroxyl); PMR (δ ppm) 0.92 (s, 3H, 18-CH_3), 1.04 (s, 3H, 19-CH_3), 1.15 (d, J=6 Hz, 3H, 27-CH_3), 1.45 (d, J=6 Hz, 3H, 21-CH_3), 3.65–4.43 (m, 6H, 3β-, 7β-,12β-,22-H, and 26-Ch_2OH); GLC–MS, *m/z* (relative intensity), 453 (4), 363 (4), 343 (2), 261 (28), 253 (11), 171 (77), 129 (64), 103 (100).

(22R)-5β-cholestane-3α,7α,12α,22,26-pentol (X). (22S)-3α,7α,12α-Trihydroxy-5β-cholestano-26,22-lactone (VI) (50 mg) dissolved in pyridine (20 ml) and acetic anhydride (20 ml) was heated on a water bath at 90°C for 8 hr. The reaction mixture was poured into water and extracted with ether. The ethereal extract was washed with water, dried over anhydrous Na_2SO_4, and evaporated to dryness. The resulting triacetate was dissolved in 5% methanolic HCl (20 ml) and the solution was allowed to stand at room temperature for 10 hr. The solution was diluted with water and extracted with ether. The ethereal extract was washed with water, dried over anhydrous Na_2SO_4 and evaporated to dryness. The residue containing methyl 3α,7α,12α-triacetoxy-22-hydroxy-5β-cholestanoate (VII) was then oxidized with CrO_3 (100 mg) in 70% acetic acid (20 ml) at room temperature for 3 hr. The reaction mixture was poured into water and extracted with ether. The ethereal extracts were washed with water and dried over anhydrous Na_2SO_4. The evaporation of the solvent gave a residue (42 mg) containing methyl 3α,7α,12α-triacetoxy-22-oxo-5β-cholestanoate (VIII). The residue was treated with $LiAlH_4$ (50 mg) in tetrahydrofurane (50 ml) under refluxing conditions for 3 hr. The reaction mixture was poured into ice-water and extracted with ethyl acetate after acidification. The extract was washed with water and dried over anhydrous Na_2SO_4. The evaporation of the solvent gave a mixture of two products at a ratio of about 2:1. The separation of these products was achieved by silica gel column chromatography using a solvent system of acetone graded to ethyl acetate. The slower eluted major product was identified as (22S)-5β-cholestane-3α,7α,12α,22,26-pentol (IX) by the comparison of its TLC and GLC properties with those of the authentic sample directly prepared from the turtle lactone, (22S)-3α,7α,12α-trihydroxy-5β-cholestano-26,22-lactone. Thus, the faster eluted minor product was deduced as (22R)-5β-cholestane-3α,7α,12α,22,26-pentol (X). Mass spectral analysis permitted positive identification of the pentol: TLC, R_f 0.35 (ethyl acetate–acetone 3:2), GLC (as TMS ether), RRT 1.36 (3% OV-17) 1.77 (capillary OV-1); GLC–MS, *m/z* (relative intensity), 453 (4), 363 (4), 343 (2), 261 (28), 253 (11), 171 (77), 129 (64), 103 (100).

RESULTS AND DISCUSSION

A urine sample obtained from a patient with Zellweger's syndrome was examined for the two novel C_{27} bile acids that were easily converted to the corresponding steroidal lactones during the extraction procedure after alkaline hydrolysis (12). Since these bile acids have been known to exist as the major components of the taurine-conjugated bile acids (12), the urinary extract was first subjected to ion-exchange chromatography to isolate the taurine-conjugated bile acids, which were then hydrolyzed with potassium hydroxide. After acidification the resulting deconjugated bile acids were extracted with ether and further subjected to ion-exchange chromatography to isolate neutral steroidal lactones from acidic compounds.

Gas–liquid chromatographic analysis of the neutral fraction as the TMS ether derivatives (**Fig. 3**) revealed that the fraction contained two components, the major one giving rise to peak 2 and the minor one giving rise to peak 1. Although neither peak 1 nor peak 2 had an RRT identical to authentic (22S)-3α,7α,12α-trihydroxy-5β-cholestano-26,22-lactone, their mass fragmentation pattern closely resembled that of the turtle lactone except for relative intensities of fragment ions (**Fig. 4**). The molecular ion was not observed, but each spectrum showed a series of fragment ions at *m/z* 574, 484, and 394. There was another series of fragment ions at *m/z* 559, 469, and 379. The former series could arise from the consecutive loss of one, two, and three TMS-OH groups from the molecular ion, and the ions of the latter series represent loss of a methyl group from those of the former series,

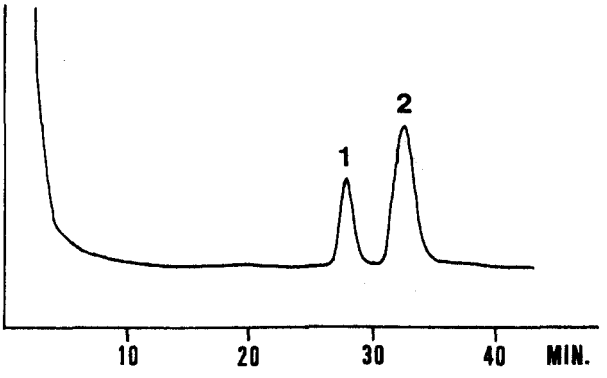

Fig. 3. Gas–liquid chromatogram of TMS ether derivatives of the neutral compounds in the hydrolysate obtained from the taurine-conjugated bile acid fraction. Column, 2% OV-1, column temperature, 260°C. Compounds giving rise to peaks 1 and 2 were identified as (23R)-3α,7α,12α-trihydroxy-5β-cholestano-26,23-lactone and (22R)-3α,7α,12α-trihydroxy-5β-cholestano-26,22-lactone, respectively.

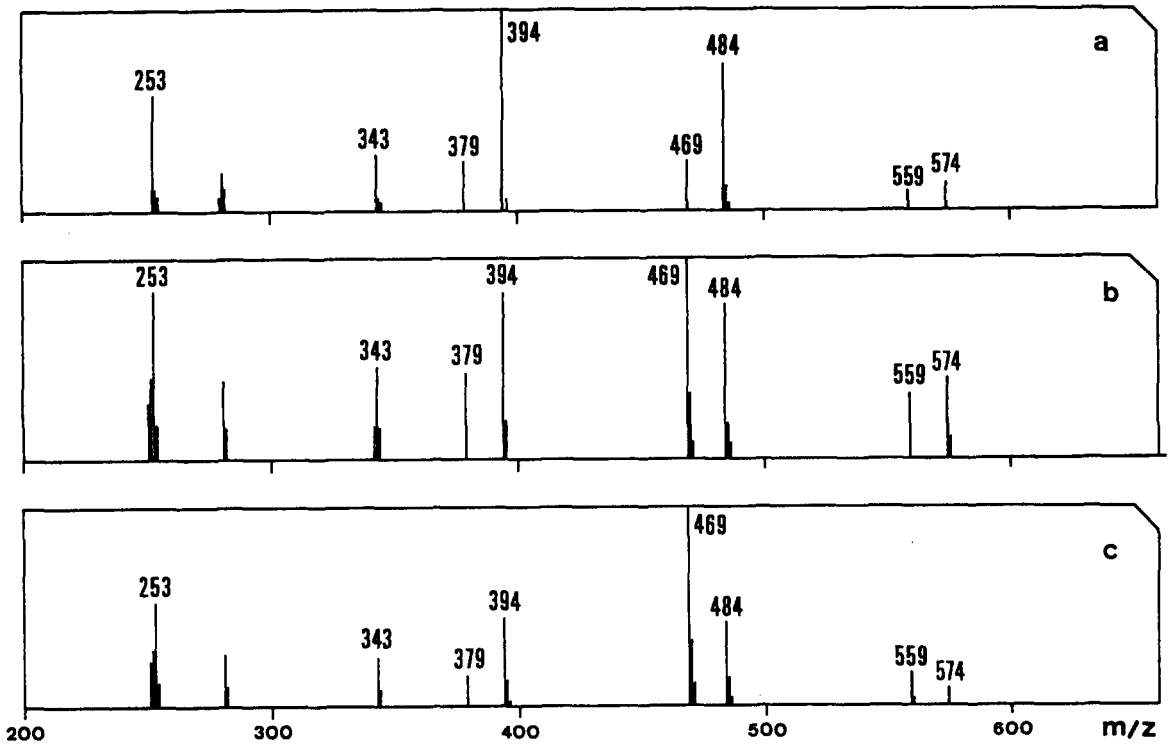

Fig. 4. Mass spectra of TMS ether derivatives of the natural (23R)-3α,7α,12α-trihydroxy-5β-cholestano-26,23-lactone (a), (22R)-3α,7α,12α-trihydroxy-5β-cholestano-26,22-lactone (b), and authentic (22S)-3α,7α,12α-trihydroxy-5β-cholestano-26,22-lactone (c).

respectively. Each spectrum also showed the ions at *m/z* 253 and 343, indicating that these three compounds carry the same cholic acid type nuclear structure. These data suggest that the compounds giving rise to peaks 1 and 2 are steroidal lactones related structurally to the turtle lactone, namely the lactones of 22- or 23-hydroxylated derivatives of THCA. In order to elucidate the position and the configuration of the hydroxyl group in the side chain, an aliquot of the neutral fraction was treated with lithium aluminum hydride, and the reduction products were converted to the corresponding TMS ether derivatives which were analyzed by GLC and GLC–MS. As shown in **Fig. 5**, GLC analysis revealed the presence of two peaks, 3 (larger) and 4 (smaller), corresponding to peaks 2 and 1 (Fig. 3), respectively. The mass spectrum of peak 3 showed no significant fragment ions in the high field (**Fig. 6a**). However, there existed fragment ions at *m/z* 363 and 453 which were derived from consecutive loss of five and four TMS groups from the molecular ion, respectively. The base peak at *m/z* 103 is known to be a fragment ion formed from the scission of the bond between C-25 and C-26 of 26-hydroxylated bile alcohols (15). The ions at *m/z* 261 and 171 could be assigned as the side chain fragment ions containing two and one TMS groups, respectively, which were formed from the scission of the bond between C-20 and C-22. Based on these mass spectral data, the compound giving rise to peak 3 appears to be a 5β-cholestane-3α,7α,12α,22,26-pentol.

To confirm this structural assignment, (22R)- and (22S)-5β-cholestane-3α,7α,12α,22,26-pentols were prepared from (22S)-3α,7α,12α-trihydroxy-5β-cholestano-

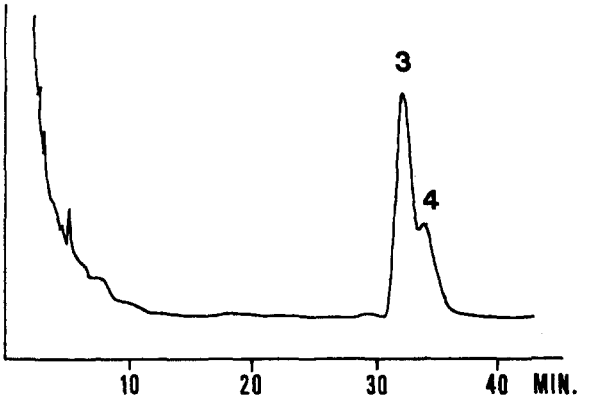

Fig. 5. Gas-liquid chromatogram of TMS ether derivatives of the reduction products with lithium aluminum hydride of the neutral compounds in the hydrolysate obtained from the taurine-conjugated bile acid fraction. Column, 3% OV-17, column temperature, 270°C. Compounds giving rise to peaks 3 and 4 were identified as (22R)-5β-cholestane-3α,7α,12α,22,26-pentol and (23R)-5β-cholestane-3α,7α,12α,23,26-pentol, respectively.

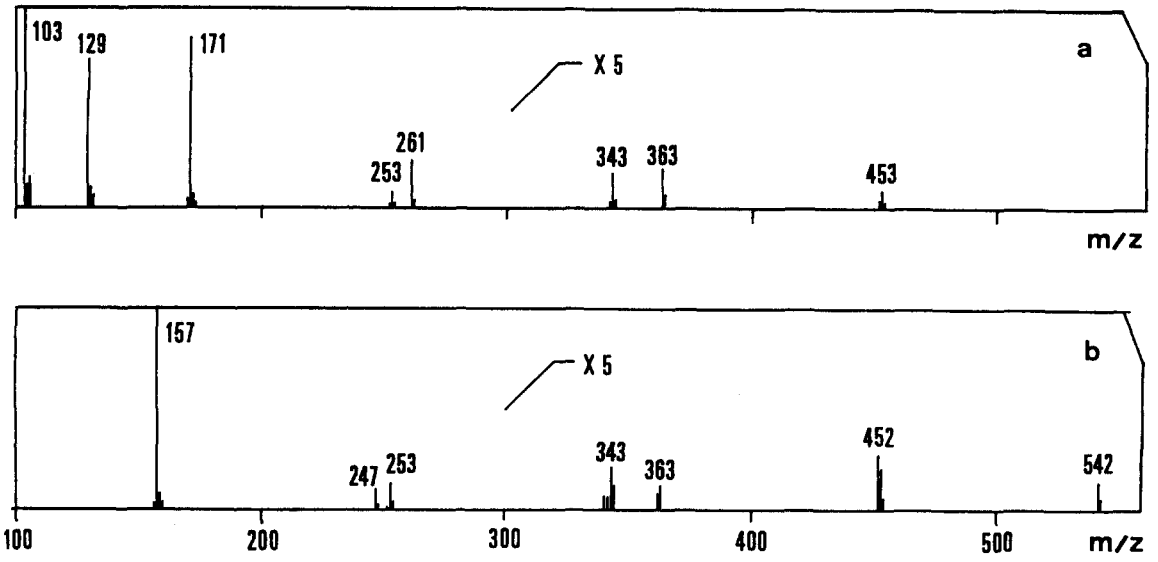

Fig. 6. Mass spectra of TMS ether derivatives of the reduction products with lithium aluminum hydride of the neutral compounds in the hydrolysate obtained from the taurine-conjugated bile acid fraction. Mass spectra a and b were identical with those of TMS ethers of synthetic (22R)-5β-cholestane-3α,7α,12α,22-26-pentol and (23R)-5β-cholestane-3α,7α,12α,23,26-pentol, respectively.

26,22-lactone which was isolated from the bile of the turtle, *Amyda japonica* (14). (22S)-5β-Cholestane-3α,7α,12α,22,26-pentol was directly obtained by the reduction of the turtle lactone with lithium aluminum hydride. (22R)-5β-Cholestane-3α,7α,12α,22,26-pentol was obtained along with the 22S-epimer by the reduction of methyl 3α,7α,12α-triacetoxy-22-oxo-5β-cholestan-26-oate with lithium aluminum hydride, which was prepared by acetylation of the turtle lactone followed by methanolysis and oxidation with chromium trioxide. The RRTs on GLC and mass spectra of peak 3 were identical with those of (22R)-5β-cholestane-3α,7α,12α,22,26-pentol. Accordingly, the compound giving rise to peak 2 should be formulated as (22R)-3α,7α,12α,22-tetrahydroxy-5β-cholestanoic acid.

In the mass spectrum of peak 4 (Fig. 6b), there existed fragment ions at *m/z* 542, 452, and 363 derived from successive loss of three to five TMS groups from the molecular ion. The base peak was observed at *m/z* 157. This ion is observed in the mass spectra of bile alcohols possessing a hydroxyl group at C-23, and known to be a side chain fragment formed by scission of the bond between C-22 and C-23 (13). Thus the compound giving rise to peak 4 appears to be a 5β-cholestane-3α,7α,12α,23,26-pentol.

To confirm this structural assignment, (23R)- and (23S)-5β-cholestane-3α,7α,12α,23,26-pentols were synthesized from each isomer of (23R)- and (23S)-5β-cholest-25-ene-3α,7α,12α,23-tetrols, respectively, by hydroboration. The GLC RRTs and mass spectrum of peak 4 were identical with those of the synthetic (23R)-5β-cholestane-3α,7α,12α,23,26-pentol. Thus the compound giving rise to peak 1 was determined to be (23R)-3α,7α,12α,23-tetrahydroxy-5β-cholestanoic acid.

Patients with Zellweger's syndrome show evidence of impairment of cholesterol metabolism associated with incomplete degradation of the cholesterol side chain. Thus, large amounts of THCA, which in normal humans is degraded quickly to cholic acid, is accumulated in bile, serum, and urine of patients with this syndrome. Other unusual metabolites detected in the biological fluids of patients with Zellweger's syndrome were 1β-, 6α, 24-, and 26-hydroxylated derivatives of THCA (8, 12). The formation of these tetrahydroxycholestanoic acids may reflect the operation of compensating mechanisms to THCA accumulation in patients with Zellweger's syndrome. The presently identified C_{27} bile acids, 3α,7α,12α,22- and 3α,7α,12α,23-tetrahydroxy-5β-cholestanoic acids are also metabolites of THCA under conditions of THCA accumulation. Urinary excretion of the accumulated THCA may be facilitated by 22- and 23-hydroxylations as well as 1β-, 6α-, 24-, and 26-hydroxylations.

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