Bile salts of the coelacanth, *Latimeria chalumnae*[1]

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Abstract Bile salts of the coelacanth, *Latimeria chalumnae, Smith,* have been analyzed and shown to have three bile alcohols, latimerol, 5α-cyprinol, and 5α-cholestane-3β,7α,-12α,25,26-pentol, two C_{24} bile acids, chenodeoxycholic acid and cholic acid, one C_{26} bile acid, probably 3β,7α,12α-trihydroxy-27-nor-5α-cholestan-26-oic acid, and two C_{27} bile acids, 3α,7α,12α-trihydroxy-5α-cholestan-26-oic acid and 3β,7α,12α-trihydroxy-5α-cholestan-26-oic acid as determined by gas–liquid chromatography and gas–liquid chromatography–mass spectrometry.—**Kihira, K., Y. Akashi, S. Kuroki, J. Yanagisawa, F. Nakayama, and T. Hoshita.** Bile salts of the coelacanth, *Latimeria chalumnae. J. Lipid Res.* 1984. **25:** 1330–1336.

Supplementary key words bile alcohols • sulfate esters • gas–liquid chromatography–mass spectrometry

Bile salts of the coelacanth, *Latimeria chalumnae,* the most primitive of the living bony fishes, have been shown to contain the 26-sulfate of latimerol, 5α-cholestane-3β,7α,12α,26,27-pentol, as the principal bile salt (1, 2). Biochemically latimerol is the most primitive of naturally occurring bile alcohols, having the complete 5α-cholestane carbon skeleton with the β-orientated 3-hydroxyl group as in cholesterol. The coelacanth also contains sulfate esters of 5α-cyprinol, 5α-cholestane-3α,7α,12α,26,27-pentol, and 5α-bufol (3), 5α-cholestane-3α,7α,12α,25,26-pentol, and very small amounts of conjugated bile acids. However, the chemical structures of the bile acids have not yet been elucidated.

This report describes the results of an examination of the biliary bile acids and bile alcohols of the coelacanth by means of glass capillary gas–liquid chromatography and gas–liquid chromatography–mass spectrometry.

MATERIALS AND METHODS

General

Melting points, infrared (IR) spectra, thin-layer chromatography (TLC), and proton nuclear magnetic resonance (PMR) spectra were as described previously (4).

Gas–liquid chromatography (GLC) was performed on a Shimadzu model GC-6A gas chromatograph equipped with a flame ionization detector and Van den Berg's solventless injector. The column used was WCOT, 25 m × 0.35 mm i.d., coated with SE-30 (LKB-Produkter) and the column temperature was 270°C. Bile alcohols were analyzed as their trimethylsilyl (TMS) derivatives and the retention times are given relative to the TMS ether of methyl cholate. Bile acids were analyzed as their methyl ester-dimethylethylsilyl (DMES) ether derivatives and the retention times are given relative to the DMES ether derivative of methyl cholate. Quantitation was accomplished by comparing GLC peak areas of the biological samples with that of external standard, the TMS or DMES ether of methyl cholate.

Gas–liquid chromatography–mass spectrometry (GLC–MS) was carried out on a Shimadzu model GCMS-9000 gas chromatograph–mass spectrometer equipped with a data processing system (Shimadzu GCMSPAC-90) and Van den Berg's solventless injector. The following operating conditions were employed: column: SE-30 (25 m × 0.35 mm i.d.); column oven temperature: 270°C; ionization energy: 22.5 eV; acceleration voltage: 3.5 KV; trap current: 100 μA.

Reference compounds

Cholic acid and chenodeoxycholic acid were commercial products. 5α-cyprinol (5), 5α-bufol (6), 5β-bufol (5β-cholestane-3α,7α,12α,25,26-pentol) (7), 5α-cholestane-3α,7α,12α,26-tetrol (8), 3α,7α,12α-trihydroxy-5α-cholestan-26-oic acid (9), and 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid (10) were isolated from natural sources according to the procedures previously reported.

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

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3α,7α,12α-Trihydroxy-27-nor-5β-cholestan-26-oic acid was prepared according to the method previously reported (11).

Synthesis of 5α-cholestane-3β,7α,12α,25,26-pentol

5α-Cholestane-3α,7α,12α,26-tetrol prepared from 5α-cyprinol sulfate (8) was converted into 5α-cholest-25-ene-3α,7α,12α-triol as described previously (6). The fully acetylated derivative of 5α-cholest-25-ene-3α,7α,12α-triol (580 mg) was partially hydrolyzed with 0.36 N methanolic HCl to yield 7α,12α-diacetoxy-5α-cholest-25-en-3α-ol, which was then oxidized with CrO_3 to give 7α,12α-diacetoxy-5α-cholest-25-en-3-one. The diacetoxycholestenone was reduced with $NaBH_4$ and hydrolyzed with 5% methanolic KOH. The major product was purified on a reversed-phase column (Lober, Lichroprep, RP-8, 2.5 cm × 31 cm, Merck) using 87.5% aqueous methanol as an eluting solvent. Recrystallization from methanol–water gave crystals (78 mg) of 5α-cholest-25-ene-3β,7α,12α-triol with mp of 200.5–201.5°C; TLC (RP-18 plate): R_f 0.55 (solvent system: methanol–water 10:1); IR: 3350 (hydroxyl), 1030, 720 (characteristic bands for 3β,7α,12α-trihydroxy-5α-cholestane nucleus) (2), 1636, 883 (end methylene); PMR (pyridine-d_5): 0.80 (s, 3H, 18-H_3), 0.90 (3H, s, 19-H_3), 1.20 (d, J = 6 Hz, 3H, 21-H_3), 1.70 (s, 3H, 27-H_3), 3.80 (m, 1H, 3α-H), 4.07 (m, 1H, 7β-H), 4.22 (m, 1H, 12β-H), 4.79 (s, 2H, 26-H_2).

5α-Cholest-25-ene-3β,7α,12α-triol (77 mg) was oxidized with OsO_4 according to the procedure described previously (6). Recrystallization from methanol–water gave crystals (60 mg) of 5α-cholestane-3β,7α,12α,25,26-pentol with mp 253°C; IR: 3350 (hydroxyl), 1030, 720; PMR (pyridine-d_5): 0.80 (s, 3H, 18-H_3), 0.90 (s, 3H, 19-H_3), 1.23 (d, J = 6 Hz, 3H, 21-H_3), 1.46 (s, 3H, 27-H_3), 3.80 (m, 1H, 3α-H), 3.83 (s, 2H, 26-H_2), 4.07 (m, 1H, 7β-H), 4.23 (m, IH, 12β-H).

Synthesis of 3β,7α,12α-trihydroxy-5α-cholestan-26-oic acid

The fully acetylated derivative of 5α-cholestane-3α,7α,12α,26-tetrol (400 mg) was partially hydrolyzed with 0.36 N methanolic HCl. The product was purified on a silica gel column chromatograph using benzene–ethyl acetate 3:7 as the eluting solvent to yield a low melting solid (370 mg) of 7α,12α-diacetoxy-5α-cholestane-3α,26-diol: TLC (silica gel G): R_f 0.59 (solvent system: benzene–ethyl acetate 3:7); IR: 3400 (hydroxyl), 1730 (acetate); PMR ($CDCl_3$): 0.71 (3H, s, 19-H_3), 0.76 (3H, s, 18-H_3), 0.80 (3H, d, J = 6 Hz, 21-H_3), 0.90 (3H, d, J = 6 Hz, 27-H_3), 2.07 (6H, s, CH_3-CO-x2), 3.20 (2H, m, 26-H_2OH), 4.30 (1H, m, 3β-H), 4.93 (1H, m, 7β-H), 5.07 (1H, m, 12β-H).

7α,12α-Diacetoxy-5α-cholestane-3α,26-diol (200 mg) was oxidized with Jones reagent. The product was purified on a reversed-phase column (RP-8, 2.5 cm × 31 cm, Merck) using 75% aqueous methanol as an eluting solvent to yield a low melting solid (72 mg) of 3-oxo-7α,12α-diacetoxy-5α-cholestan-26-oic acid: TLC (silica gel G): R_f 0.45 (solvent system: benzene–ethyl acetate 3:7); IR: 1730 (carbonyl, acetate and carboxyl); PMR ($CDCl_3$): 0.75 (3H, s, 18-H_3), 0.80 (3H, d, J = 6 Hz, 21-H_3), 1.05 (3H, s, 19-H_3), 1.23 (3H, d, J = 6 Hz, 27-H_3), 2.07 (6H, s, CH_3-CO-x2), 4.98 (1H, m, 7β-H), 5.09 (1H, m, 12β-H).

Fifty mg of 3-oxo-7α,12α-diacetoxy-5α-cholestan-26-oic acid in methanol was treated with $NaBH_4$. The reaction mixture was then hydrolyzed with 10% methanolic KOH. Repeated recrystallization from methanol gave colorless needles of 3β,7α,12α-trihydroxy-5α-cholestan-26-oic acid with mp 228–229°C; TLC (silica gel G): R_f 0.38 (solvent system: isooctane–ethyl acetate–acetic acid 5:5:1); IR: 3400 (hydroxyl), 1730 (carboxyl), 1030, 720; PMR (pyridine-d_5): 0.80 (3H, s, 18-H_3), 0.90 (3H, s, 19-H_3), 1.23 (3H, d, J = 6 Hz, 21-H_3), 1.32 (3H, d, J = 6 Hz, 27-H_3), 3.80 (1H, m, 3α-H), 4.09 (1H, m, 7β-H), 4.23 (1H, m, 12β-H).

Analysis of bile alcohols and bile acids in the bile of the coelacanth

A frozen sample (1 ml) of gallbladder bile of the coelacanth was supplied by Japanese Scientific Investigation Party on Coelacanth, Committee for Anatomy and Analysis. The bile sample was extracted with 10 volumes of ethanol at room temperature. The ethanolic extract was evaporated under a reduced pressure to leave crude bile salts as a deep brown solid (about 60 mg). Solvolysis of one-third of the crude bile salts was carried out according to the procedure described previously (12) and extraction of the solvolyzed bile salts with ethyl acetate–n-butanol 1:1 gave a mixture (11 mg) of desulfated bile alcohols. Two mg of the bile alcohol mixture was converted to TMS ether derivatives and analyzed by GLC and GLC–MS. The remaining part (9 mg) of the bile alcohol mixture was crystallized from acetone to give crystals of the principal bile alcohol of the coelacanth, latimerol, with mp 235–236°C, [reported 236°C (2)].

The aqueous phase left from the n-butanol-ethyl acetate extraction was percolated through a Sep-Pak C_{18} cartridge (Waters Associates) and after washing with 15 ml of water the cartridge was eluted with 10 ml of methanol. After removal of the solvent, the residue containing conjugated bile acids was hydrolyzed with 2.5 N KOH solution at 130°C for 3 hr. After dilution with water and acidification with diluted HCl, the hydrolyzate was extracted with ether. After washing with

water, the extract was evaporated to dryness to give a residue (less than 1 mg) containing deconjugated bile acids, which was converted to methyl ester-DMES ether derivatives and analyzed by GLC and GLC–MS.

RESULTS

Preliminary TLC analysis revealed that the coelacanth bile contained bile alcohol sulfates as the major constituents and lesser amounts of taurine-conjugated bile acids. Solvolysis of the crude bile salts gave a mixture of desulfated bile alcohols. The principle bile alcohol of the coelacanth was easily isolated from the bile alcohol mixture by repeated recrystallization, and identified as latimerol by comparison of its melting point and IR spectrum with the reported data. As shown in **Fig. 1**, GLC analysis revealed that the bile alcohol mixture contained two minor bile alcohols corresponding to GLC peaks 1 (2% of the total bile alcohols) and 2 (5%) at RRTs 2.54 and 2.63, respectively, in addition to the major bile alcohol, latimerol, which was chromatographed as the peak 3 (93%) at RRT of 2.81. The concentration of the bile alcohols estimated by GLC was 20.0 mg/ml. The mass spectra 1–3 of the TMS ether derivatives of the bile alcohols corresponding to GLC peaks 1–3, respectively, are shown in **Fig. 2**.

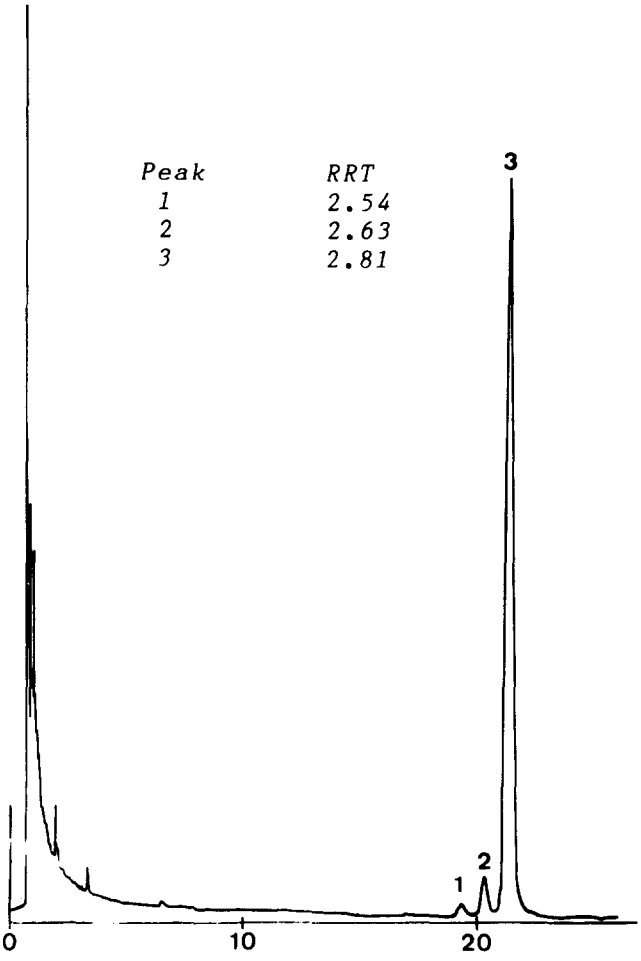

Fig. 1. Gas chromatogram of TMS ether derivative of the bile alcohol mixture of the coelacanth. The peak number corresponds to the mass spectrum number in Fig. 2. Peak 1: 5α-cholestane-3β,7α,12α,25,26-pentol; peak 2: 5α-cyprinol; peak 3: latimerol.

One of the minor bile alcohols, which was chromatographed as the peak 2 on GLC, was identified as 5α-cyprinol. The mass spectrum and the RRT of its TMS ether derivative were completely identical with those of the TMS ether derivative of authentic 5α-cyprinol.

The mass spectrum 1 was almost identical with that obtained from the TMS ether derivative of authentic 5α-bufol, suggesting that the bile alcohol chromatographed as the peak 1 on GLC is a stereoisomer of 5α-bufol. In both spectra, a series of fragment ions at m/z 797, 707, 617, 527, and 437 was seen. This series results from the scission of the bond between C-25 and C-26 followed by the subsequent loss of TMS ether groups (13). The peak at m/z 219 is a side chain fragment resulting from the scission of the bond between C-24 and C-25. The ions at m/z 343 and 253 represent loss of the entire side chain plus two and three nuclear TMS ether groups, respectively. The intensity of the ion at m/z 343 is greater than that of the ion at m/z 253 in both spectra, indicating that the bile alcohol corresponding to the peak 1 as well as 5α-bufol is a 5α-bile alcohol (14). On the GLC, the ratio of the RRTs between the peak 1 (2.54) and 5α-bufol (2.38) was equal to that of the RRTs of latimerol (peak 3) and 5α-cyprinol (peak 2). These results suggest that the peak 1 is of the 3β-epimer of 5α-bufol. In order to confirm this structural assignment, a partial synthesis of 3-epi-5α-bufol, 5α-cholestane-3β,7α,12α,25,26-pentol, was performed. The synthetic 3-epi-5α-bufol (probably a mixture of C-25 isomers, which could not be resolved on the GLC system employed in this study) showed the identical behavior on GLC and mass spectrum to the natural bile alcohol corresponding to the peak 1 on GLC.

Alkaline hydrolysis of the bile salts of the coelacanth afforded a mixture of deconjugated bile acids, of which the GLC chromatogram as the methyl ester-DMES ether derivative is shown in **Fig. 3**. There were at least five bile acid peaks 4–8 at RRTs of 0.76 (8% of the total bile acids), 1.00 (32%), 1.55 (3%), 1.59 (5%), and 1.64 (51%), respectively. The concentration of the total bile acid estimated by GLC was 0.6 mg/ml of bile. The mass spectra 4–8 corresponding to the peaks 4–8, respectively, are shown in **Fig. 4**.

The bile acids corresponding to the peaks 4, 5, and 7 were identified with certainty as chenodeoxycholic acid, cholic acid, and 3α,7α,12α-trihydroxy-5α-cholestan-

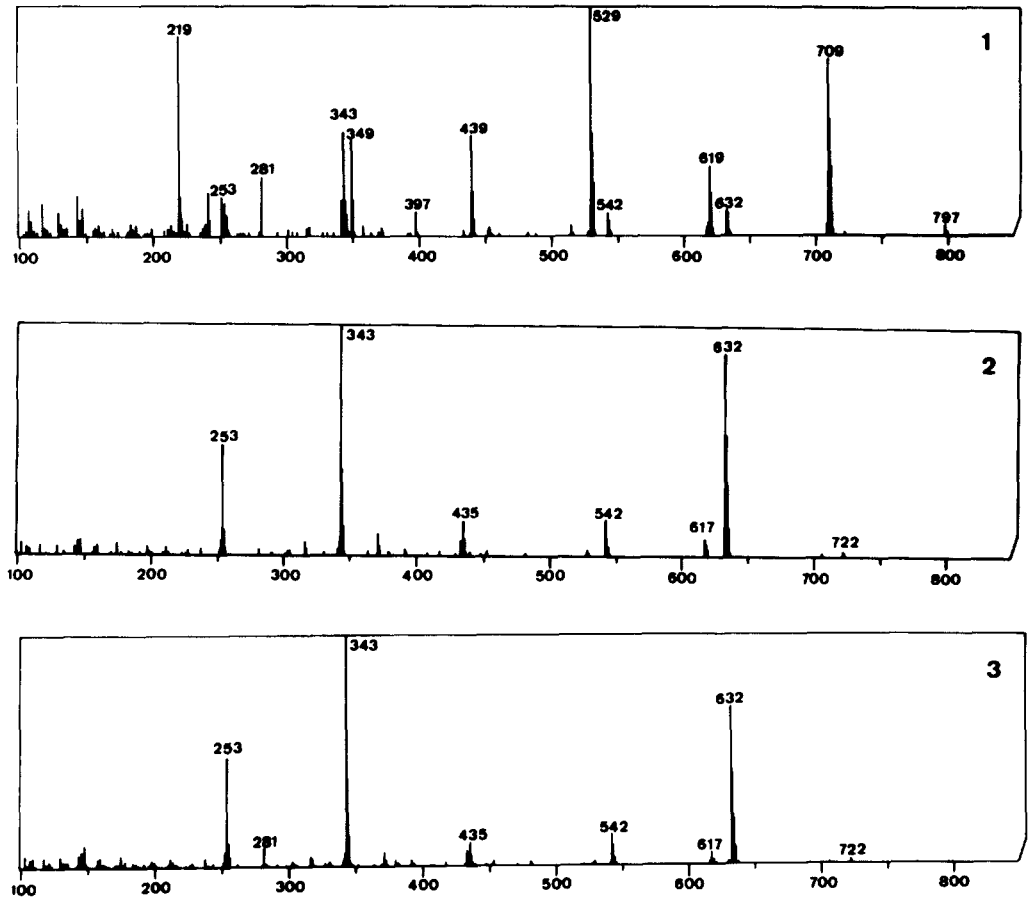

Fig. 2. Mass spectra of the coelacanth bile alcohols (as TMS ether derivatives). The mass spectrum number corresponds to the peak number in Fig. 1. 1: 5α-Cholestane-3β,7α,12α,25,26-pentol; 2: 5α-cyprinol; 3: latimerol.

26-oic acid, respectively, by comparison of their mass spectra and chromatographic properties with those of authentic compounds.

The mass spectrum 8, the major bile acid corresponding to the peak 8 on GLC, was almost identical with the mass spectrum 7 of 3α,7α,12α-trihydroxy-5α-cholestan-26-oic acid. In both spectra, the base peak was seen at 693 which is formed by the loss of ethyl group from the molecular ion. There was a series of fragment ions at m/z 618, 514, and 411, which is formed by the successive loss of the DMES ether groups from the molecular ion. The ions at m/z 357 and 253 represent loss of side chain plus two and three nuclear DMES ether groups, respectively. The ion at m/z 303 is formed by the cleavage of the 6,7- and 9,10-bonds plus loss of two DMES ether groups at C-7 and C-12. These data suggest that the difference between the two is a stereochemical one, most likely at C-3 as in relation between latimerol and 5α-cyprinol. To confirm this structural assignment, a partial synthesis of 3β,7α,12α-trihydroxy-5α-cholestan-26-oic acid was performed. The synthetic 3β-hydroxylated bile acid (probably a mixture of C-25 isomers, which could not be resolved on the GLC system employed in this study) was completely identical, as judged by GLC and GLC–MS, with the natural bile acid corresponding to the peak 8 on GLC.

The mass spectrum 6 showed a pattern comparable to the mass spectrum 8 of 3β,7α,12α-trihydroxy-5α-cholestan-26-oic acid but with 14 mass units less with respect to the fragments containing the side chain. The mass spectrum 6 showed a weak molecular ion at m/z 708 and the base peak at m/z 679 which is formed by the loss of ethyl group from the molecular ion. The series of fragment ions at m/z 604, 500, and 397 results from the successive loss of one, two, and three DMES ether groups from the molecular ion. The fragment at m/z 575 is formed by the loss of a DMES ether group from the m/z 679 fragment. The fragments at m/z 469 and 365 result from the loss of CH_3O- (31 mass units) moiety from the m/z 500 and 397 fragments, respectively. The fragment ion formed by the cleavage of the 6,7- and 9,10-bonds plus loss of two DMES ether groups was seen at m/z 289. In the mass spectrum 6 as well as in the mass spectrum 8, the intensity of the ion

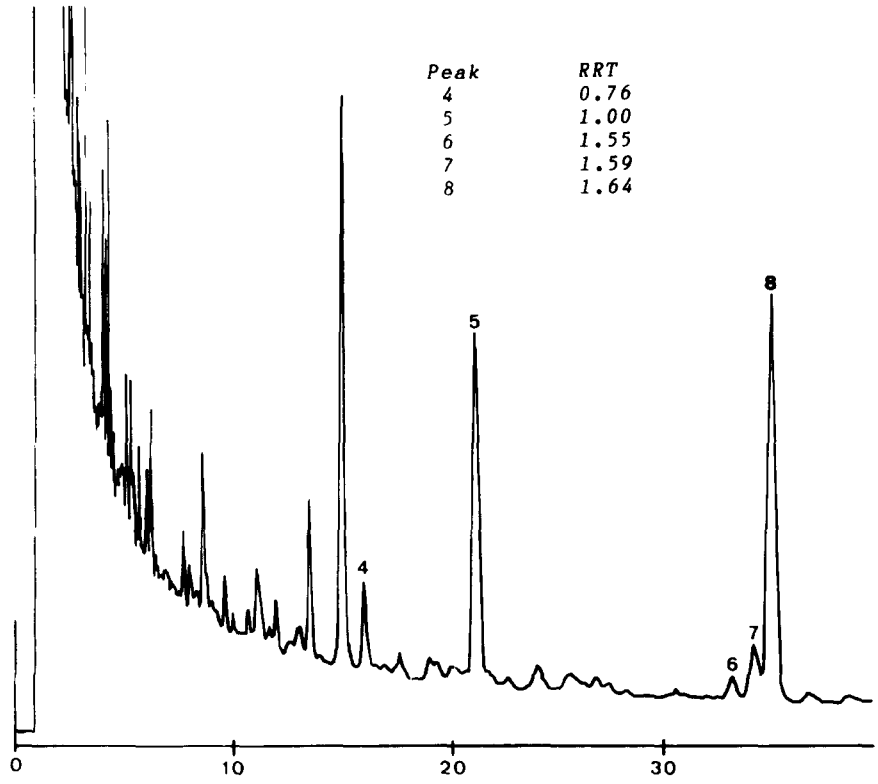

Fig. 3. Gas chromatogram of methyl ester-DMES ether derivative of the bile acid mixture of the coelacanth. The peak number corresponds to the mass spectrum number in Fig. 4. Peak 4: chenodeoxycholic acid; peak 5: cholic acid; peak 6: 3β,7α,12α-trihydroxy-27-nor-5α-cholestan-26-oic acid; peak 7: 3α,7α,12α-trihydroxy-5α-cholestan-26-oic acid; peak 8: 3β,7α,12α-trihydroxy-5α-cholestan-26-oic acid.

at m/z 357 (M-side chain-2 × DMES-OH) was greater than that of the ion at m/z 253 (M-side chain-3 × DMES-OH) suggesting that the bile acid corresponding is a 5α-bile acid (14). These spectral data suggest that the peak 6 is of a trihydroxy-5α-C_{26} bile acid. On GLC, the RRT ratio of the peak 6 and the methyl ester-DMES ether derivative of 3α,7α,12α-trihydroxy-27-nor-5β-cholestan-26-oic acid (RRT = 1.56) was almost equal to that between the peak 8 of 3β,7α,12α-trihydroxy-5α-cholestan-26-oic acid and 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid (RRT = 1.63). These results indicate, but do not prove, that the peak 6 is probably of 3β,7α,12α-trihydroxy-27-nor-5α-cholestan-26-oic acid.

DISCUSSION

The present study has confirmed the results from previous investigations (1, 2) that showed that the coelacanth bile contains latimerol and 5α-cyprinol as the major and the minor bile alcohols, respectively. Biosynthesis of 5α-cyprinol, the 3α-epimer of latimerol, has been studied by Hoshita (15, 16) using carp whose major bile alcohol is this 3α-hydroxylated 5α-bile alcohol. The biosynthetic pathway of 5α-cyprinol might involve the following intermediates: cholesterol → cholest-5-ene-3β,7α-diol → 7α-hydroxycholest-4-en-3-one → 7α,12α-dihydroxycholest-4-en-3-one → 7α,12α-dihydroxy-5α-cholestan-3-one → 5α-cholestane-3α,7α,12α,26-tetrol → 5α-cyprinol. Although there is no experimental evidence, the coexistence of 5α-cyprinol and its 3β-isomer, latimerol, in *Latimeria* bile suggests that latimerol probably arises from cholesterol by the same route as in the biosynthesis of 5α-cyprinol, except for the stereochemistry of the reduction of the 3-oxo group in 7α,12α-dihydroxy-5α-cholestan-3-one, rather than by the route which maintains the 3β-hydroxyl group of cholesterol throughout. In the *Latimeria* liver most of the 3-oxo intermediate would be converted into 5α-cholestane-3β,7α,12α-triol which is then hydroxylated successively at C-26 and C-27 to form latimerol.

Amos et al. (3) have reported the presence of small amounts of 5α-bufol in the coelacanth bile in addition to latimerol and 5α-cyprinol though experimental details had not been given. However, we were unable to find this 3α-hydroxylated bile alcohol. Instead of 5α-bufol, we found a new bile alcohol as the third biliary bile alcohol of the *Latimeria* and elucidated its structure as the 3β-epimer of 5α-bufol, although the configuration

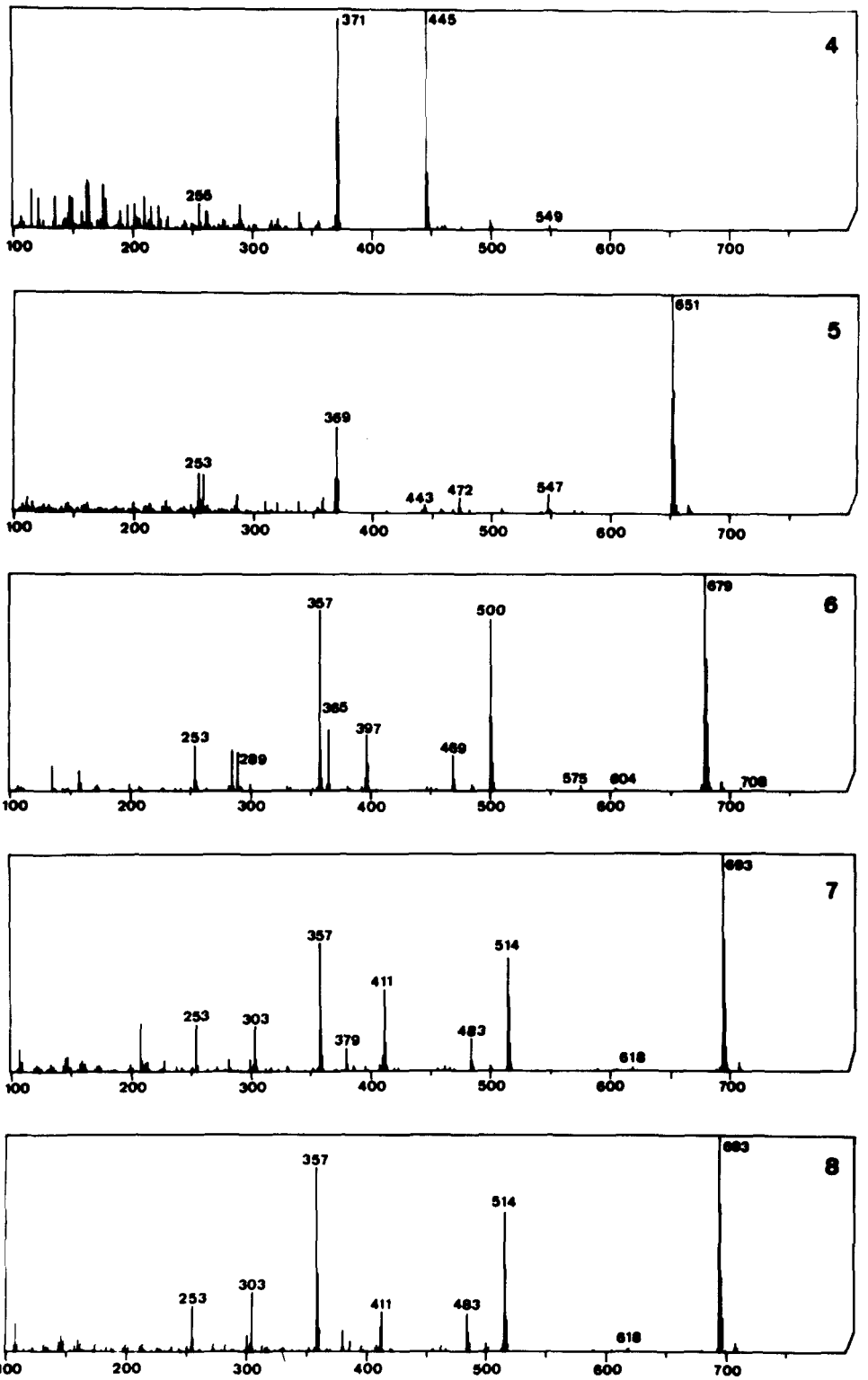

Fig. 4. Mass spectra of the coelacanth bile acids (as methyl ester-DMES ether derivatives). The mass spectrum number corresponds to the peak number in Fig. 3. 4: Chenodeoxycholic acid; 5: cholic acid; 6: 3β,7α,12α-trihydroxy-27-nor-5α-cholestan-26-oic acid; 7: 3α,7α,12α-trihydroxy-5α-cholestan-26-oic acid; 8: 3β,7α,12α-trihydroxy-5α-cholestan-26-oic acid.

at C-25 is not clarified, by the direct comparison with a synthesized sample. It seems more likely that the coelacanth bile contains 3-epi-5α-bufol rather than 5α-bufol in respect to the relative abundance of latimerol and 5α-cyprinol in the bile of this species, and that 3-epi-5α-bufol may possibly represent a subproduct which is formed from 5α-cholestane-3β,7α,12α,26-tetrol, the direct precursor of latimerol, by 25-hydroxylation instead of 27-hydroxylation.

The presence of bile acids in very small amounts in the *Latimeria* bile has previously been reported by Anderson and Haslewood (1, 2). However, their chemical structures have not yet been elucidated. The present study confirmed the presence (0.6 mg/ml bile) of at least five bile acids in the bile of the coelacanth. Three of them were known bile acids, and by direct comparison with authentic samples they were identified as cholic acid, chenodeoxycholic acid, and 3α,7α,12α-trihydroxy-5α-cholestan-26-oic acid, respectively. The remainder were new bile acids. The major one was assumed to be 3β,7α,12α-trihydroxy-5α-cholestan-26-oic acid. Although the configuration at C-25 was not clarified, chemical synthesis permitted the identification of this 3β-hydroxylated higher bile acid. The other one was identified tentatively as 3β,7α,12α-trihydroxy-27-nor-5α-cholestan-26-oic acid by comparison of its properties on GLC and mass spectral data with those of comparable compounds.

It is very interesting that the coelacanth bile contains not only the higher bile acids possessing 27 and presumably 26 carbon atoms but also the most common C_{24} bile acids, cholic acid and chenodeoxycholic acid. Carp and related fishes also contain small amounts of cholic acid in their bile. Hoshita (15, 16) and Kouchi (17, 18) have shown that such fishes could convert labeled cholesterol or cholest-5-ene-3β,7α-diol into radioactive cholic acid as well as 5α-cyprinol. The presence of cholic acid and chenodeoxycholic acid in the coelacanth bile might indicate that the enzyme system capable of degrading the cholesterol side chain was present even in the most primitive bony fish, the coelacanth, as well as in carp and related fishes. It should, however, be noted that the coelacanth is a carnivorous fish, and taurocholate and taurochenodeoxycholate are the major bile salts in almost all species of marine teleosts. The possibility that the 5β-C_{24} bile acids found in the *Latimeria* bile originate from the diet is not fully excluded.

The C_{27} bile acids found in the coelacanth bile seemed to be endogenous since these higher bile acids have not been detected in any other marine fishes. The *Latimeria* C_{27} bile acids may possibly represent subproducts that are formed from 5α-cholestane-3β,7α,12α,26-tetrol and its 3α-epimer, the biosynthetic precursors of the *Latimeria* bile alcohols, by an oxidation of the 26-hydroxyl group to a carboxylic group.

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