Bile salts of frogs: a new higher bile acid, 3α,7α,12α,26-tetrahydroxy-5β-cholestanoic acid from the bile of *Rana plancyi*[1,2]

M. Une, N. Matsumoto, K. Kihira, M. Yasuhara, T. Kuramoto, and T. Hoshita

Institute of Pharmaceutical Sciences, Hiroshima University School of Medicine, Hiroshima, Japan

Abstract Bile salts of ten species of frogs of the family Ranidae were examined by means of thin-layer chromatography, gas–liquid chromatography, and gas–liquid chromatography–mass spectrometry. Their major bile alcohols were ranol, cyprinol, or bufol. Four species of frogs contained lesser amounts of higher bile acids, and in two species C_{24} bile acids were present. A higher bile acid that has not been previously described was detected in *Rana plancyi*. On the basis of the spectral data and the direct comparison with a synthetic sample, the chemical structure of the new bile acid was established to be 3α,7α,12α,26-tetrahydroxy-5β-cholestanoic acid.—**Une, M., N. Matsumoto, K. Kihira, M. Yasuhara, T. Kuramoto, and T. Hoshita.** Bile salts of frogs: a new higher bile acid, 3α,7α,12α,26-tetrahydroxy-5β-cholestanoic acid from the bile of *Rana plancyi*. *J. Lipid Res.* 1980. **21:** 269–276.

Supplementary key words bile alcohols · gas–liquid chromatography · mass spectrometry

The bile acids occurring in the bile of mammals (including primates) are all hydroxy derivatives of cholanoic acid. These C_{24} bile acids are biosynthesized in the liver from cholesterol by hydroxylation and hydrogenation of the nucleus, followed by oxidative degradation of the side chain (1). In primitive vertebrates such as certain fish, amphibians, and reptiles, the side chain of cholesterol is oxidized but not degraded, thus giving rise to bile alcohols and higher bile acids (2). The occurrence in the primitive vertebrates of bile alcohols and higher bile acids is especially interesting since they may represent evolutionary precursors of the mammalian C_{24} bile acids and are likely intermediates in the biosynthesis of the C_{24} bile acids from cholesterol.

In order to better understand the chemical evolution and the biosynthesis of bile acids, we have continued our studies on bile alcohols and higher bile acids of primitive vertebrates, and have now examined bile acids of ten species of frogs. A new higher bile acid, 3α,7α,12α,26-tetrahydroxy-5β-cholestanoic acid has been identified as a minor bile constituent of *Rana plancyi*, and this has also been prepared by partial synthesis from cholic acid.

EXPERIMENTAL

General

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

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

NMR spectra were obtained in pyridine-d_5 solution at 100 MHz on a JEOL JNM-PS-100 spectrometer. Chemical shifts are given in the δ ppm scale with tetramethylsilane as internal standard.

High resolution mass spectra were recorded with a JEOL JMS-01SG mass spectrometer with an accelerating potential of 10 kV, an ionization potential of 70 eV, and a source temperature of 130°C.

TLC was carried out on Silica Gel G (Merck) using a 10% solution of phosphomolybdic acid in ethanol as the detection reagent.

GLC was run on a Shimadzu GC-6A gas chromatograph using a glass column (2 m × 3 mm) packed with 1.5% OV-1, 3% OV-17, 3% QF-1, or 3% Poly I-110 on 80/100 mesh Gas Chrom Q. All retention times are given relative to the TMS ether of methyl

Abbreviations: IR, infrared; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography; GLC, gas–liquid chromatography; GLC–MS, gas–liquid chromatography–mass spectrometry; TMS, trimethylsilyl; EDTA, ethylenediaminetetraacetic acid; diglyme, diethyleneglycol dimethyl ether.

[1] This study is Part XIII of a series entitled "Comparative biochemical studies of bile acids and bile alcohols". Part XII. M. Une, K. Kihira, T. Kuramoto, and T. Hoshita. *Tetrahedron Lett.* 2527 (1978).

[2] Systematic names of bile acids and bile alcohols referred to in the text by their trivial names are listed in Table 1.

cholate. Measurements of peak areas were accomplished with a Shimadzu E1A automatic integrator.

Mass spectra were obtained by a combination GLC–MS technique. A JEOL Model D-300 gas chromatograph–mass spectrometer was used. The following operating conditions were employed: column, 3% OV-17 (1 m × 3 mm); column temperature, 280°C; ion source temperature, 270°C; ionizing current, 300 μA; ionizing voltage, 70 eV.

Analysis of bile acids and bile alcohols of frogs

Gallbladders from four to ten frogs were cut under ethanol (10–50 ml), and the extract was filtered. Evaporation of the solvent from the filtered extract left crude bile salts as a solid. The bile salts were then analyzed by TLC.

When an unconjugated bile acid was detected by TLC, the crude bile salts (5.8 mg) were dissolved in 20 ml of water, acidified with diluted HCl, and extracted with ether (20 ml × 3). The ether extracts were combined, washed with water until neutral, dried over anhydrous Na_2SO_4, and evaporated to dryness under reduced pressure, yielding the unconjugated bile acid.

When conjugated bile acids were detected by TLC, the crude bile salts (20 mg) were dissolved in 2.0 ml of 0.1 M acetate buffer (pH 5.6), and the solution was incubated at 37°C for 25 min in the presence of 50 units of cholylglycine hydrolase (E.C. No. 3.5.1.24, Sigma Chemical Co.), 0.2 M disodium salt of EDTA (0.5 ml), and 0.2 M β-mercaptoethanol (0.5 ml). Hydrolysis appeared to be complete, inasmuch as TLC assay of the incubation mixture indicated the disappearance of the spots due to the bile acid conjugates. After the acidification with diluted HCl, the incubation mixture was extracted with ethyl acetate (20 ml × 3). The extracts were combined, washed with water, dried, and evaporated to dryness, yielding a mixture of deconjugated bile acids.

The aqueous phase left from the ether or ethyl acetate extraction was adjusted to pH 7.0 with 1 N NaOH and percolated through an Amberlite XAD-2 resin column (30 cm × 15 mm). The column was washed with water (100 ml) and then with methanol (100 ml). The methanol eluate was evaporated to dryness, leaving a residue consisting of bile alcohol sulfates.

Bile alcohol sulfates were cleaved by the treatment with trichloroacetic acid in dioxane. The sample to be desulfated (1.5–20 mg) was heated with 20 ml of acetic acid–acetic anhydride 1:1 on a water bath for 3 hr, and the solvents were evaporated to dryness under reduced pressure. The residue was dissolved in 10 ml of a 40% solution of trichloroacetic acid in dioxane. After standing at room temperature for 2 weeks, the reaction mixture was diluted with water (80 ml), and extracted with ethyl acetate (60 ml × 3). The ethyl acetate extracts were combined, washed with 2% Na_2CO_3 solution (40 ml × 3) and then with water (40 ml × 3), dried over anhydrous Na_2SO_4, and the solvent was evaporated to dryness. The residue was dissolved in 1 N methanolic KOH (30 ml) and refluxed on a water bath for 2 hr. The hydrolyzate was diluted with water (200 ml) and extracted with n-butanol–ethyl acetate 1:1 (100 ml × 2). The extracts were combined, washed with water, dried, and evaporated, yielding a mixture of desulfated bile alcohols.

The unconjugated bile acid, the deconjugated bile acids, and the desulfated bile alcohols were analyzed by TLC, GLC, and GLC–MS.

For GLC and GLC–MS, the bile acids to be analyzed (1–2 mg) were methylated with diazomethane and silylated with a mixture of hexamethyldisilazane (0.2 ml), trimethylchlorosilane (0.02 ml), and pyridine (0.5 ml) at room temperature for 2 hr. Silylation of bile alcohols was carried out by the same procedure as that of the methylated bile acids. Portions (1–2 μl) of the silylation mixture were analyzed directly by GLC and GLC–MS.

Reference compounds

Cholic acid and chenodeoxycholic acid were commercial products. 3α,7α,12α-Trihydroxy-5α-cholestanoic acid (3), 3α,7α,12α-trihydroxy-5β-cholestanoic acid (4), 27-nor-5α-cholestane-3α,7α,12α,24α-tetrol (5), 27-nor-5β-cholestane-3α,7α,12α,24α-tetrol (5), 5α-ranol (5), 5β-ranol (5), 5α-cyprinol (6), 5β-cyprinol (7), 5α-bufol (8), and 5β-bufol (9) were isolated from natural sources in this laboratory. 5β-Cholestane-3α,7α,12α,26-tetrol was prepared from 3α,7α,12α-trihydroxy-5β-cholestanoic acid by $LiAlH_4$ reduction (10). R_f values on TLC and relative retention times on GLC of reference compounds are listed in **Table 1.**

Synthesis of (25*RS*)-3α,7α,12α,26-tetrahydroxy-5β-cholestanoic acid (VII) (Fig. 3)

Methyl cholate 3-tert-butyl-dimethylsilyl ether (II). To a solution of methyl cholate (I, 10 g) in dimethylformamide (120 ml) were added imidazole (20 g) and tert-butyl-dimethylsilyl chloride (17 g), and the solution was allowed to stand at room temperature for 5 hr. The reaction mixture was diluted with ether, washed with water, dried over anhydrous Na_2SO_4. The solvent and excess reagent were evaporated under reduced pressure. Recrystallization of the residue from isopropyl ether gave crystals (7.0 g) of II, mp 156–157°C; IR ν_{max}^{KBr} cm^{-1} 1725, 3560; NMR (pyridine-d_5) δ ppm 0.07 (s, 6H, 3-OSi$(CH_3)_2$), 0.77 (s, 3H, 18-CH_3), 0.88 (s, 9H, 3-OSiC$(CH_3)_3$), 0.98

TABLE 1. R_f values on thin-layer chromatography and relative retention times on gas–liquid chromatography of bile acids and bile alcohols

Bile Acids and Bile Alcohols (Trival Names)	R_f Values on TLC		Relative Retention Times[c] on GLC[d]			
	S-7[a]	EAW-2[b]	OV-1	OV-17	QF-1	Poly I-110
3α,7α,12α-trihydroxy-5β-cholanoic acid (cholic acid)	0.38	0.64	1.00	1.00	1.00	1.00
3α,7α-dihydroxy-5β-cholanoic acid (chenodeoxycholic acid)	0.61	0.84	0.93	1.11	1.03	1.50
3α,7α,12α-trihydroxy-5α-cholestanoic acid	0.41	0.65	1.59	1.45	1.43	1.55
3α,7α,12α-trihydroxy-5β-cholestanoic acid	0.49	0.74	1.67	1.65	1.53	1.78
27-*nor*-5α-cholestane-3α,7α,12α,24α-tetrol	0.22	0.47	1.16	0.77	0.63	0.72
27-*nor*-5β-cholestane-3α,7α,12α,24α-tetrol	0.26	0.53	1.23	0.89	0.67	0.80
27-*nor*-5α-cholestane-3α,7α,12α,24ξ,26-pentol (5α-ranol)	0.07	0.25	2.19	1.42	1.17	1.37
27-*nor*-5β-cholestane-3α,7α,12α,24ξ,26-pentol (5β-ranol)	0.08	0.28	2.31	1.61	1.23	1.53
5β-cholestane-3α,7α,12α,26-tetrol	0.34	0.59	1.73	1.25	0.97	1.30
5α-cholestane-3α,7α,12α,25,26-pentol (5α-bufol)	0.12	0.35	2.55	1.57	1.32	1.50
5β-cholestane-3α,7α,12α,25,26-pentol (5β-bufol)	0.14	0.37	2.69	1.73	1.40	1.70
5α-cholestane-3α,7α,12α,26,27-pentol (5α-cyprinol)	0.11	0.36	2.76	1.84	1.43	1.77
5β-cholestane-3α,7α,12α,26,27-pentol (5β-cyprinol)	0.13	0.39	2.93	2.06	1.53	2.10

[a] S-7: benzene–isopropanol–acetic acid 30:10:1 (v/v/v).
[b] EAW-2: ethyl acetate–acetic acid–water 17:2:1 (v/v/v).
[c] Relative to trimethylsilyl ether of methyl cholate.
[d] Bile acids were chromatographed as their methyl ester–TMS ethers. Bile alcohols were chromatographed as their TMS ethers.

(s, 3H, 19-CH_3), 1.13 (d, J = 6 Hz, 3H, 21-CH_3), 3.56 (s, 3H, $COOCH_3$), 3.60 (m, 1H, 3β-H), 4.03 (m, 1H, 7β-H), 4.17 (m, 1H, 12β-H).

5β-Cholane-3α,7α,12α,24-tetrol 3-tert-butyl-dimethylsilyl ether (III). The silyl ether (II, 5.0 g) was dissolved in dry ether (100 ml). $LiAlH_4$ (2.5 g) was added to the solution in small portions over a 30-min period with cooling in ice-water during each addition. The resulting suspension was heated for 2 hr under reflux, and then poured into ice-cold 10% Rochelle salt solution. The mixture was extracted with three 100-ml portions of ether. The combined extracts were washed with water, dried over anhydrous Na_2SO_4, and then the solvent was evaporated to dryness to yield III as a colorless gum (3.1 g), IR $\nu_{max}^{CHCl_3}$ cm^{-1} 3480; NMR (pyridine-d_5) δ ppm 0.07 (s, 6H, 3-OSi$(CH_3)_2$), 0.78 (s, 3H, 18-CH_3), 0.88 (s, 9H, 3-OSiC$(CH_3)_3$), 1.00 (s, 3H, 19-CH_3), 1.20 (d, J = 6 Hz, 3H, 21-CH_3), 3.56 (m, 1H, 3β-H), 3.75 (t, J = 6 Hz, 2H, 24-CH_2), 4.02 (m, 1H, 7β-H), 4.18 (m, 1H, 12β-H).

3α,7α,12α-Trihydroxy-5β-cholestane-26,27-dioic acid diethyl ester (V). To an ice-cold solution of III (3.0 g) in dry pyridine (20 ml), a solution of p-toluenesulfonyl chloride (1.5 g) in dry pyridine (15 ml) was added dropwise with stirring. Stirring was continued at room temperature for 16 hr, and the reaction mixture was poured into ice-cold, diluted HCl and extracted with ether. Evaporation of the solvent from the washed and dried extract yielded the crude 24-monotosylate of III (3.0 g) in semisolid form, which was used for the next step without further purification or characterization.

To this crude product (3.0 g) dissolved in ethanol (20 ml) was added 15 ml of an ethanol solution which contained 0.5 g of sodium as the sodium ethoxide and 5.0 g of diethyl malonate. The reaction mixture was refluxed for 6 hr. After cooling, the solution was diluted with water, and extracted with three 100-ml portions of ethyl acetate. The extracts were washed with water, dried over Na_2SO_4, and the solvent was evaporated. In order to remove the protecting tert-butyl-dimethylsilyl group (11), the resulting oily residue was dissolved in tetrahydrofuran (60 ml) containing tetra-*n*-butylammonium fluoride (3.0 g). The solution was allowed to stand at room temperature for 3 hr, and extracted with ethyl acetate (100 ml × 3) after dilution with water. The ethyl acetate extracts were washed with water, dried over Na_2SO_4 and evaporated. The crude product obtained was purified by silica gel column chromatography with increasing amounts of acetone in ethyl acetate. The fractions were monitored by TLC. The fraction eluted with 10% acetone in ethyl acetate contained compound V, which was recrystallized from isopropyl ether to yield crystals (1.1 g), mp 154–156°C, IR ν_{max}^{KBr} cm^{-1} 1740, 3440; NMR (pyridine-d_5) δ ppm 0.80 (s, 3H, 18-CH_3), 1.00 (s, 3H, 19-CH_3), 1.14 (t, J = 7 Hz, 6H, 26- and 27-$COOCH_2CH_3$); 1.20 (d, J = 6 Hz, 3H, 21-CH_3), 3.70 (m, 1H, 3β-H), 4.07 (m, 1H, 7β-H), 4.18 (q, J = 7 Hz, 4H, 26- and 27-$COOCH_2CH_3$), 4.22 (m, 1H, 12β-H); High resolution mass spectrum: M^+, 536.3708 (Calcd. for $C_{31}H_{52}O_7$: 536.3711).

3α,7α,12α-Trihydroxy-5β-cholestane-26,27-dioic acid monoethyl ester (VI). A solution of the diethyl ester (V, 1.0 g) in 0.1 N methanolic KOH (40 ml) was allowed to stand at room temperature for 1 hr and extracted with ethyl acetate (200 ml × 3) after dilution with water and acidification with diluted HCl. The ethyl acetate

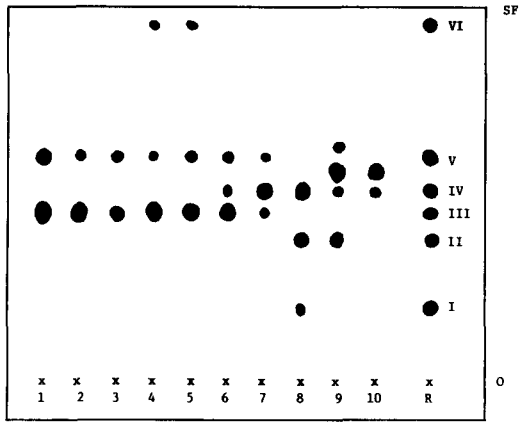

Fig. 1. Thin-layer chromatogram of bile salts from some Ranidae. Solvent system: *n*-butanol–acetic acid–water 17:2:1 (v/v/v). The numbers refer to the following frogs; 1, *Rana amurensis coreana*; 2, *Rana amurensis tsushimensis*; 3, *Rana temporaria dybowskii*; 4, *Rana temporaria coreana*; 5, *Rana temporaria tsushimensis*; 6, *Rana japonica japonica*; 7, *Rana tagoi*; 8, *Rana plancyi*; 9, *Rana tigrina rugulosa*; 10, *Rana erythrea*. R = reference standards, I, taurocholate, II, taurotrihydroxy-5β-cholestanoate, III, 5α-ranol sulfate, IV, 5β-cyprinol sulfate, V, 27-*nor*-5α-cholestanetetrol sulfate, VI, trihydroxy-5α-cholestanoic acid. SF = solvent front. O = origin.

extracts were washed with 2% Na_2CO_3 solution (200 ml × 3), and the washings were acidified with diluted HCl, and extracted with ethyl acetate (200 ml × 3). Evaporation of the solvent from the extract after washing with water and drying over Na_2SO_4 left a residue (960 mg) which was purified by silica gel column chromatography using benzene–isopropanol–acetic acid 30:10:1 as eluting solvent. Recrystallization of the eluate gave crystals (280 mg) of VI, mp 181–183°C; IR ν_{max}^{KBr} cm^{-1} 1740, 1780, 2940, 3400; NMR (pyridine-d_5) δ ppm 0.80 (s, 3H, 18-CH_3), 1.00 (s, 3H, 19-CH_3), 1.20 (t, J = 7 Hz, 3H, 27-$COOCH_2CH_3$), 1.20 (d, J = 6 Hz, 3H, 21-CH_3), 3.74 (m, 1H, 3β-H), 4.07 (m, 1H, 7β-H), 4.22 (m, 1H, 12β-H), 4.25 (q, J = 7 Hz, 2H, 27-$COOCH_2CH_3$).

(25RS)-3α,7α,12α,26-Tetrahydroxy-5β-cholestanoic acid (VII). To a solution of $NaBH_4$ (200 mg) in diglyme (16 ml) was added LiBr (580 mg), and the resulting solution was stirred at 90°C for 30 min. To this solution was added a solution of the monoethyl ester (VI, 180 mg) in diglyme (8 ml). After stirring for 3 hr at 90°C, the reaction mixture was cooled, diluted with ice-cold diluted HCl, and then extracted with ethyl acetate (100 ml × 3). The extracts were washed with water, dried over anhydrous Na_2SO_4, and the solvent was evaporated to dryness under reduced pressure. The residue was chromatographed on silica gel using the same eluting solvent as for VI. Recrystallization of the eluate form ethyl acetate-isopropyl ether gave crystals (25 mg) of VII, mp 268–269°C; IR ν_{max}^{KBr} cm^{-1} 1720, 2940, 3400; NMR (pyridine-d_5) δ ppm 0.81 (s, 3H, 18-CH_3), 1.01 (s, 3H, 19-CH_3), 1.21 (d, J = 6 Hz, 3H, 21-CH_3), 3.76 (m, 1H, 3β-H), 4.16 (m, 1H, 7β-H), 4.28 (m, 1H, 12β-H), 4.30 (m, 2H, 26-CH_2OH). High resolution mass spectrum: M^+ (methyl ester) 480.3449 (Calcd. for $C_{28}H_{48}O_6$: 480.3391).

RESULTS

Fig. 1 shows a thin-layer chromatogram of bile salts from ten species of frogs of the family Ranidae. All frog gallbladder bile tested contained bile alcohol sulfates as the major components. No free bile alcohols were detected in any samples of bile. Taurine-conjugated bile acids were present in *Rana plancyi* and *Rana tigrina rugulosa*, and an unconjugated bile acid in *Rana temporaria coreana* and *Rana temporaria tsushimensis*.

Thus, the unconjugated bile acid was obtained by ether extraction of the acidified bile salt solution, before the desulfation of the bile alcohol sulfates. Enzymatic hydrolysis of the taurine-conjugated bile acids were carried out according to the method of Nair and Garcia (12). Cleavage of the bile alcohol sulfates was carried out as previously described by Bridgwater, Briggs, and Haslewood (13). Identification of the bile acids and bile alcohols thus obtained was carried out by comparison with known reference compounds listed in Table 1: R_f values on TLC (two different solvent systems), relative retention times on GLC (four different columns), and mass spectrometry. The results of the bile salt determinations are summarized in **Table 2.** Ranol, cyprinol, or bufol was the major bile alcohol in the frogs tested. Lesser amounts of bile acids were found in four species of frogs. A previously unrecognized bile acid was present as the taurine conjugate in *Rana plancyi*. The chemical structure of the new bile acid was deduced as follows.

TLC (Fig. 1) of the bile salts from *Rana plancyi* gave two principal spots with mobilities of cholestanepentol sulfates and taurine-conjugated trihydroxycholestanoates, respectively, and a lesser spot that appeared in the area related to taurine conjugates of trihydroxycholanoates or tetrahydroxycholestanoates. The bile salts (20.0 mg) were treated with cholylglycine hydrolase to give a mixture of deconjugated bile acids (9.2 mg).

A portion of the bile acid mixture was converted to the methyl ester-TMS ethers and examined by GLC (**Fig. 2**). The presence of three bile acids (peaks A, B, and C) were detected, and they were characterized by mass spectrometry. The relative retention times and mass spectra of peaks A and B were the same as those of the TMS ethers of authentic methyl cholate

TABLE 2. Bile salt composition of some Ranidae[a]

	Free Bile Acid	Taurine-conjugated Bile Acids				Sulfated Bile Alcohols							
	3α,7α,12α-trihydroxy-5α-cholestanoic acid	cholic acid	chenodeoxycholic acid	3α,7α,12α-trihydroxy-5β-cholestanoic acid	3α,7α,12α,26-tetrahydroxy-5β-cholestanoic acid	27-nor-5α-cholestane-3α,7α,12α,24α-tetrol	27-nor-5β-cholestane-3α,7α,12α,24α-tetrol	5α-ranol	5β-ranol	5β-cholestane-3α,7α,12α,26-tetrol	5α-bufol	5β-bufol	5α-cyprinol	5β-cyprinol
	% of total													
Rana amurensis coreana						44		56						
Rana amurensis tsushimensis						16		84						
Rana temporaria dybowskii						39		61						
Rana temporaria coreana	14					15		71						
Rana temporaria tsushimensis	10					25		65						
Rana japonica japonica						20	3	40	26				11	
Rana tagoi						19		25					56	
Rana plancyi		4		37	7									52
Rana tigrina rugulosa		11	8	10						13		46		12
Rana erythrea											68		32	

[a] Quantitation was accomplished by gas–liquid chromatography (OV-17 column) of the trimethylsilyl ether derivatives.

and methyl 3α,7α,12α-trihydroxy-5β-cholestanoate, respectively. The retention time of peak C differed from that of any hitherto known bile acids. In the mass spectrum (Fig. 4) of the compound eluted in peak C, a molecular ion was not seen but a fragment ion at m/e 753 (M-15) was observed. There was a series of fragment ions at m/e 678 (M-90), 588 (M-90 × 2), 498 (M-90 × 3). The spectrum also exhibited ions at m/e 253 and 343, corresponding to the loss of three and two molecules of trimethylsilanol and the side chain. These data suggest that the new bile acid, C, is a tetrahydroxycholestanoic acid with three hydroxyl groups on the nucleus and one in the side chain.

A part of the bile acid mixture was treated with ethereal diazomethane solution and the resulting methyl esters were treated with lithium aluminum hydride. The reduction product was converted to the corresponding TMS ether which was analyzed by gas–liquid chromatography–mass spectrometry. Three major reduction products were identified as 5β-cholane-3α,7α,12α,24-tetrol, 5β-cholestane-3α,7α,12α,26-tetrol, and 5β-cyprinol. Because the C_{24} tetrol and the C_{27} tetrol were formed from cholic acid and 3α,7α,12α-trihydroxy-5β-cholestanoic acid, respectively, the 5β-cyprinol must be the reduction product of the bile acid C. These results suggest that bile acid C should be formulated as 3α,7α,12α,26-tetrahydroxy-5β-cholestanoic acid. Confirmation of this structural assignment was attempted by partial synthesis (**Fig. 3**). Methyl cholate (I) was converted to the 3-tert-butyl-dimethylsilyl ether (II). This compound (II) was reduced with lithium aluminum hydride to produce the 3-tert-butyl-dimethylsilyl ether of 5β-cholane-3α,7α,12α,24-tetrol (III), which via the 24-tosylate (IV) was treated with sodio diethylmalonate in refluxing ethanol. The product was treated with tetra-*n*-butylammonium fluoride in tetrahydrofuran, in order to remove the protecting tert-butyl-dimethylsilyl group, to yield the malonic ester (V). Partial hydrolysis of the malonic ester (V) afforded the half ester (VI). Reduction with lithium

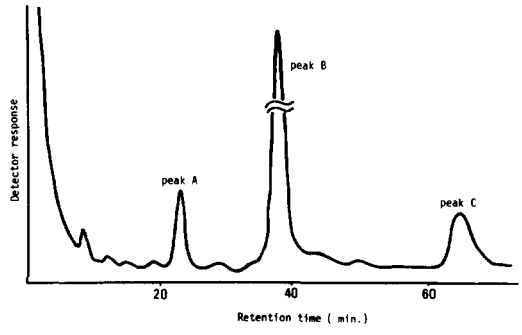

Fig. 2. Gas–liquid chromatographic pattern of bile acids from *Rana plancyi* as the methyl ester–TMS ethers. Column, 3% OV-17; column temperature, 270°C. Peak identifications; A, cholic acid; B, 3α,7α,12α-trihydroxy-5β-cholestanoic acid; C, 3α,7α,12α,26-tetrahydroxy-5β-cholestanoic acid.

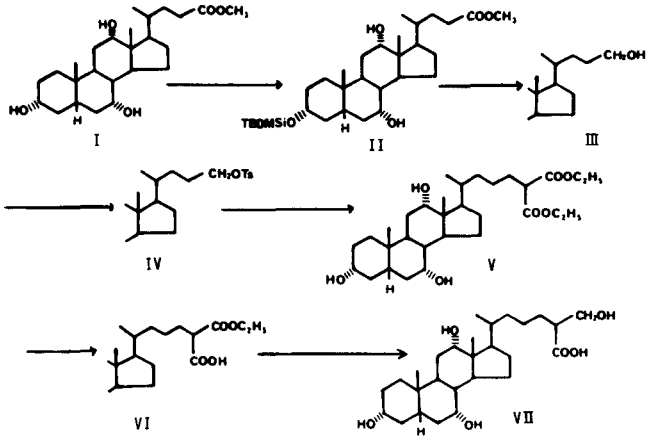

Fig. 3. Synthesis of (25*RS*)-3α,7α,12α,26-tetrahydroxy-5β-cholestanoic acid. TBDMSi = $(CH_3)_3C(CH_3)_2Si$; Ts = $CH_3C_6H_4SO_2$-; I, methyl cholate; II, methyl cholate 3-tert-butyl-dimethylsilyl ether; III, 5β-cholane-3α,7α,12α,24-tetrol 3-tert-butyl-dimethylsilyl ether; IV, 5β-cholane-3α,7α,12α,24-tetrol 3-tert-butyl-dimethylsilyl ether 24-monotosylate; V, 3α,7α,12α-trihydroxy-5β-cholestane-26,27-dioic acid diethyl ester; VI, 3α,7α,12α-trihydroxy-5β-cholestane-26,27-dioic acid monoethyl ester; VII, (25*RS*)-3α,7α,12α,26-tetrahydroxy-5β-cholestanoic acid.

borohydride of the half-ester (VI) gave the desired 3α,7α,12α,26-tetrahydroxy-5β-cholestanoic acid (VII). The synthetic bile acid (VII) was completely identical, as judged by TLC and GLC (**Table 3**) and mass spectrum (**Fig. 4**), with the natural bile acid C.

DISCUSSION

Hydrolysis is an unavoidable step for the analysis of naturally occurring bile acid conjugates. In previous investigations on the bile salts of lower vertebrates, hydrolysis of the C_{27} bile acid conjugates has been done by heating with KOH as with the C_{24} bile acid conjugates. However, the carbon-nitrogen bond in the higher bile acid conjugates is more resistant to saponification than is this bond in the usual bile acid conjugates, presumably because the higher bile acids contain a somewhat hindered carboxyl group, and require prolonged heating in more concentrated alkali at quite high temperature. Under these conditions, significant losses in bile acids are encountered.

We have therefore employed enzymatic hydrolysis for the cleavage of the bile acid conjugates in the bile of frogs. This method has been successfully used for the hydrolysis of the C_{24} bile acid conjugates (12) and has now proved effective for the C_{27} bile acid conjugates.

In a previous paper (14) of this series, we have proposed that the Ranidae may be divided into three groups on the basis of their biochemical differences by the hydroxylation patterns in the side chain of bile alcohols: (a), *R. temporaria, R. pipiens,* and *R. catesbeiana* have mainly 5α- or 5β-ranol; (b), *R. nigromaculata, R. brevipoda,* and *R. rugosa*, 5α- or 5β-cyprinol; and (c), *R. l. limnocharis*, 5β-bufol. The present results support this concept; the main bile alcohol of the Ranidae examined here is ranol, cyprinol, or bufol; thus they can fall into the three bile alcohol categories.

A hitherto undiscovered higher bile acid was detected in *R. plancyi* and identified as 3α,7α,12α,26-tetrahydroxy-5β-cholestanoic acid (VII) on the basis of the spectral data and direct comparison with a synthetic sample. Although there is no experimental proof, 5β-cyprinol (IX) and 3α,7α,12α-trihydroxy-5β-cholestanoic acid (X), which are the major constituents of this frog bile, might be the precursors of this tetrahydroxy-C_{27} acid, since either the oxidation of one of the primary hydroxyl groups in the former or the hydroxylation at the end methyl group of the latter leads to the tetrahydroxycholestanoic acid (**Fig. 5**). It remains to be established whether the tetrahydroxycholestanoic acid (VII) can be further metabolized into cholic acid (XII), which was a minor bile constituent of this frog. According to present knowledge (1), 3α,7α,12α-trihydroxycholestanoic acid (X) is an obligatory intermediate in mammalian cholic acid bio-

TABLE 3. R_f values (thin-layer chromatography) and relative retention times (gas–liquid chromatography) of natural bile acid C of *Rana plancyi* and synthetic 3α,7α,12α,26-tetrahydroxy-5β-cholestanoic acid

Bile Acid	R_f Values on TLC		Relative Retention Times[c] on GLC[d]			
	S-7[a]	EAW-2[b]	OV-1	OV-17	QF-1	Poly I-110
Natural bile acid C	0.26	0.51	3.00	2.80	2.64	3.16
Synthetic 3α,7α,12α,26-tetrahydroxy-5β-cholestanoic acid	0.26	0.51	3.00	2.80	2.64	3.16

[a] S-7: benzene–isopropanol–acetic acid 30:10:1 (v/v/v).
[b] EAW-2: ethyl acetate–acetic acid–water 17:2:1 (v/v/v).
[c] Relative to trimethylsilyl ether of methyl cholate.
[d] Bile acids were chromatographed as their methyl ester-TMS ethers.

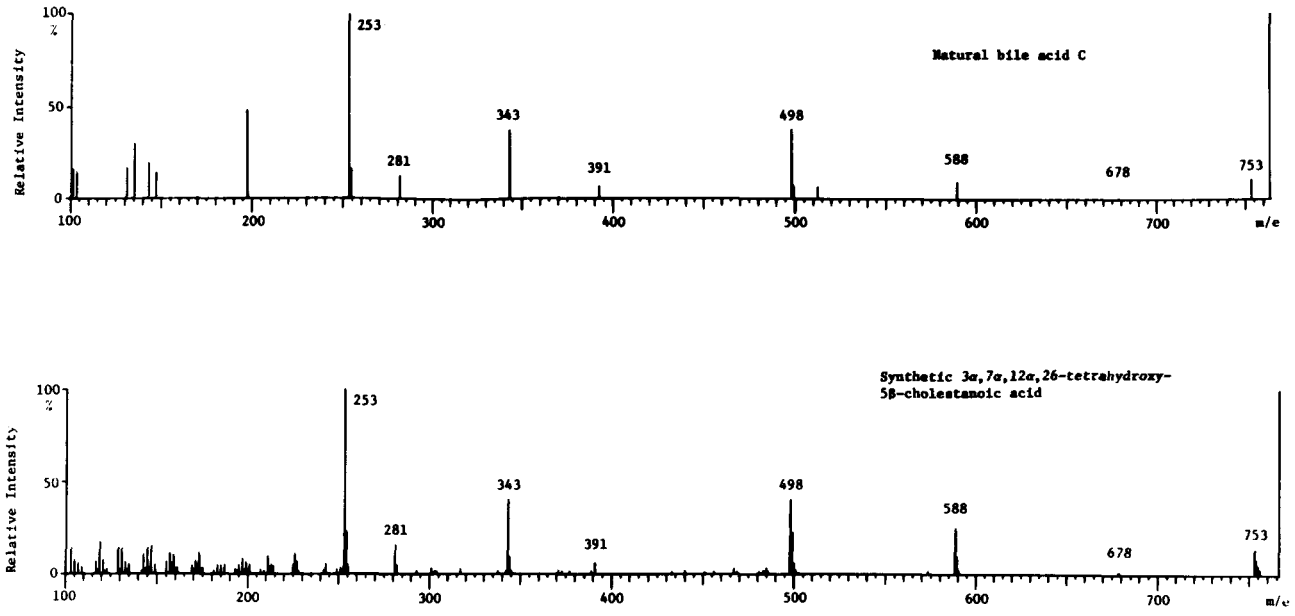

Fig. 4. Mass spectrum of natural bile acid C of *Rana plancyi* and synthetic 3α,7α,12α,26-tetrahydroxy-5β-cholestanoic acid.

synthesis, during which the C_{27} bile acid (X) undergoes β-oxidation to form the C_{24} bile acid (XII) plus propionic acid. If this is the case also in the frog liver, the intermediate during the β-oxidation should be 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid (XI) but not 3α,7α,12α,26-tetrahydroxy-5β-cholestanoic acid (VII). We were unable to find the 24-hydroxylated bile acid (XI) in the bile of *R. plancyi*. Of course, it is possible that the 24-hydroxylated bile acid (XI) is metabolized too quickly to be detected. We defer further discussion for the biosynthetic route of cholic acid in the frog until additional evidence becomes available.

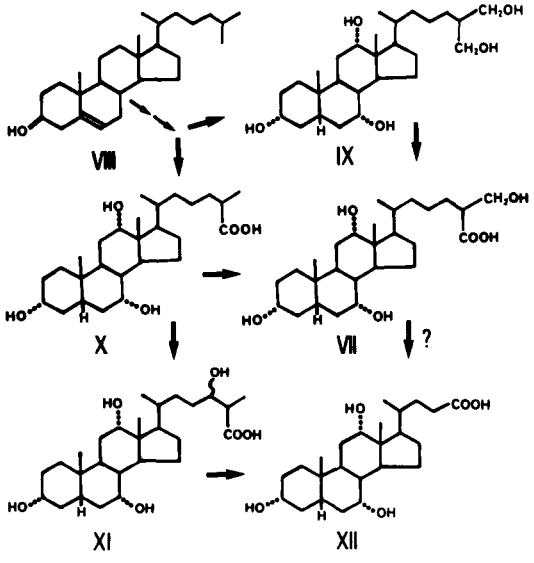

Fig. 5. Possible pathway for the formation of bile acids and bile alcohols in *Rana plancyi*. VII, 3α,7α,12α,26-tetrahydroxy-5β-cholestanoic acid; VIII, cholesterol; IX, 5β-cyprinol; X, 3α,7α,12α-trihydroxy-5β-cholestanoic acid; XI, 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid; XII, cholic acid.

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