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Bile acids and bile alcohols of bullfrog[1]

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Abstract Gallbladder bile of the bullfrog, *Rana catesbeiana* was found to contain a number of minor bile acids and bile alcohols in unconjugated forms. The following bile acids and bile alcohols were identified by combined gas–liquid chromatography–mass spectrometry: cholic acid, allocholic acid, 3α,7α,12α-trihydroxy-5α-cholestan-26-oic acid, 3α,7α,12α-trihydroxy-24-methyl-5β-cholestan-26-oic acid, 3α,7α,12α,24-tetrahydroxy-5α-cholestan-26-oic acid, 3α,7α,12α,24-tetrahydroxy-5β-cholestan-26-oic acid, 3α,7α,12α,26-tetrahydroxy-5α-cholestan-27-oic acid, 3α,7α,12α,26-tetrahydroxy-5β-cholestan-27-oic acid, 3α,7α,12α-trihydroxy-27-nor-5α-cholestan-24-one, and 3α,7α,12α-trihydroxy-27-nor-5β-cholestan-24-one. These minor constituents of the bile may represent intermediates in the biosynthetic pathway of bile acids and bile alcohols in the bullfrog.—**Noma, Y., M. Une, K. Kihira, M. Yasuda, T. Kuramoto, and T. Hoshita.** Bile acids and bile alcohols of bullfrog. *J. Lipid Res.* 1980. **21:** 339–346.

Supplementary key words gas–liquid chromatography · mass spectrometry

The bile of the bullfrog, *Rana catesbeiana*, has been known to contain a number of biogenetically interesting bile acid and bile alcohols. Up to now, the following major bile acid and bile alcohols have been isolated and characterized: trihydroxycoprostanic acid (3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid) (1), 5α-cyprinol (5α-cholestane-3α,7α,12α,26,27-pentol) (2), 5α- and 5β-ranols (27-nor-5α- and 5β-cholestane-3α,7α,12α,24,26-pentols) (2, 3), and the four isomers at C-5 and C-24 of 26-deoxyranol (2). The major bile acid, trihydroxycoprostanic acid has been shown to be an important intermediate in the course of cholic acid (3α,7α,12α-trihydroxy-5β-cholan-24-oic acid) biosynthesis. According to present knowledge (4) the biosynthetic pathway for cholic acid involves the transformation of cholesterol to trihydroxycoprostanic acid via 5β-cholestane-3α,7α,12α-triol. The C_{27} bile acid then undergoes β-oxidation forming cholic acid with the loss of the three-carbon fragment, propionic acid. The occurrence of ranols and 26-deoxyranols as the major bile alcohols presents a metabolic question. The biosynthesis of the 27-norcholestanepolyols clearly indicates a departure from the pathway for the cholic acid production, since at some stage a single carbon atom must be lost from the cholesterol side chain.

In order to better understand the formation of the C_{26} bile alcohols in the bullfrog, we have studied the minor constituents of bullfrog bile.

MATERIALS AND METHODS

General

Melting points, infrared spectra, NMR spectra, and high resolution mass spectra were as described in the preceding paper (5) of this series.

TLC was performed on glass plates coated with silica gel G (0.25 mm) (E. Merck A. G.) using a 10% solution of phosphomolybdic acid in ethanol as the detection reagent.

GLC was run on a Shimadzu GC-6A gas chromatograph using glass column (2 m × 3 mm) packed with 2% OV-1, 3% OV-17, 3% QF-1, or 2% Poly I-110 on 80/100 mesh Gas-Chrom Q. All retention times are given relative to the TMS ether of methyl cholate. Measurements of peak areas were accomplished with a Shimadzu E1A automatic integrator. Preparation of methyl esters and TMS ethers was carried out as described previously (5).

GLC–mass spectrometry was carried out on a JEOL D-300 gas chromatograph–mass spectrometer. The following operating conditions were employed: column, 3% OV-17 (1 m × 3 mm); column temperature, 280°C; ionization current, 300 μA; ionization voltage, 70 eV.

Extraction of unconjugated bile acids and bile alcohols from bullfrog bile

Bile was collected by putting the gallbladders of bullfrog into ethanol. Evaporation of the filtered

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Abbreviations: GLC, gas–liquid chromatography; TLC, thin-layer chromatography; NMR, nuclear magnetic resonance; TMS, trimethylsilyl.

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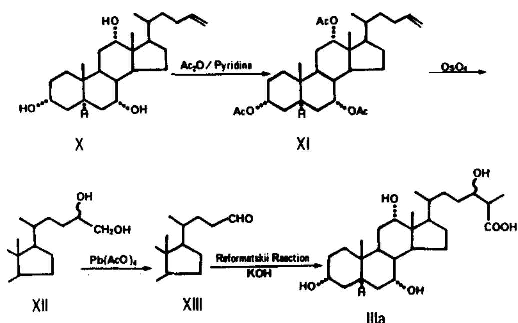

Fig. 1. Partial synthesis of 3α,7α,12α,24-tetrahydroxy-5α-cholestan-26-oic acid. X, 3α,7α,12α-trihydroxy-26,27-dinor-5α-cholest-24-ene; XI, 3α,7α,12α-triacetoxy-26,27-dinor-5α-cholest-24-ene; XII, 3α,7α,12α-trihydroxy-26,27-dinor-5α-cholestane-24,25-diol; XIII, 3α,7α,12α-triacetoxy-5α-cholan-24-al; IIIa, 3α,7α,12α,24-tetrahydroxy-5α-cholestan-26-oic acid.

solution left crude bile salts. The crude bile salts (7.7 g from 52 gallbladders) were dissolved in 200 ml of water and extracted with two 100-ml portions of petroleum ether to remove cholesterol. The aqueous layer was acidified with 2 N HCl and then extracted with three 150-ml portions of ether. The ether extracts were combined and washed with three 100-ml portions of 5% Na_2CO_3 solution to extract acidic materials. The ether layer was washed with water until neutral, dried over anhydrous Na_2SO_4 and the solvent was evaporated to dryness, leaving a residue (28 mg) consisting of unconjugated bile alcohols. The Na_2CO_3 washings were combined, acidified with 2 N HCl, and extracted with three 150-ml portions of ether. Evaporation of the solvent from the washed (H_2O) and dried (Na_2SO_4) extracts left a residue (157 mg) consisting of unconjugated bile acids.

Isolation of bile alcohols

The bile alcohol mixture (25 mg) was dissolved in 1 ml of benzene; this solution was then applied to a column of silica gel (10 g) (E. Merck A. G.) made up in benzene. The column was eluted with benzene–ethyl acetate mixtures. The column fractions were monitored by TLC. Successive elution with a 2:8 mixture gave the less polar bile alcohol, B (18.2 mg), which was recrystallized from ethyl acetate to give crystals with a melting point of 192°C; elution with a 1:9 mixture gave the polar bile alcohol, A (1.7 mg).

Fractionation of bile acids by reversed phase column partition chromatography

The bile acid mixture (150 mg) was subjected to reversed phase column partition chromatography (6) using n-heptane–chloroform 1:9 (v/v) as the stationary phase and 55% aqueous methanol as the moving phase. A glass column was packed with 13.5 g of Hostalene (polyethylene powder, Farbwerke Hoechst, Germany) supporting 9 ml of the stationary phase. The sample was mixed with 1.5 ml of the stationary phase and 3 g of Hostalene, and applied to the column. The column effluents were monitored by TLC. The effluents from 15 ml to 45 ml gave the mixture of more polar bile acids (17.9 mg) such as trihydroxy-C_{24} bile acids and tetrahydroxy-C_{27} bile acids, and the effluents from 45 ml to 150 ml gave the mixture of less polar bile acids (101.1 mg) such as trihydroxy-C_{27} bile acids.

Reference compounds

Cholic acid was a commercial product. Allocholic acid (3α,7α,12α-trihydroxy-5α-cholan-24-oic acid) (7), 3α,7α,12α-trihydroxy-5α-cholestan-26-oic acid (8), and trihydroxycoprostanic acid (1) were isolated from natural sources. 3α,7α,12α-Trihydroxy-24-methyl-5β-cholestan-26-oic acid (9), varanic acid (3α,7α,12α,24-tetrahydroxy-5β-cholestan-26-oic acid) (10), 3α,7α,12α,26-tetrahydroxy-5β-cholestan-27-oic acid (5), 3α,7α,12α-trihydroxy-27-nor-5α-cholestan-24-one (2), and 3α,7α,12α-trihydroxy-27-nor-5β-cholestan-24-one (2) were prepared in this laboratory according to the methods described previously.

Synthesis of 3α,7α,12α,24-tetrahydroxy-5α-cholestan-26-oic acid (Fig. 1)

3α,7α,12α-Trihydroxy-26,27-dinor-5α-cholest-24-ene (X) was obtained from the alkaline hydrolysate of bullfrog bile (11).

3α,7α,12α-Triacetoxy-26,27-dinor-5α-cholest-24-ene (XI). A solution of X (100 mg) in 4 ml of dry pyridine and 5 ml of acetic anhydride was heated on a water bath for 4 hr. The reaction mixture was diluted with water, and 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 provide the triacetate (XI) (107 mg). The infrared spectrum showed no hydroxyl absorption. Infrared spectrum $\nu_{max}^{CHCl_3}$ cm^{-1}: 1735, 1250, 1050 (acetate), 990, 910 (vinyl).

3α,7α,12α-Triacetoxy-26,27-dinor-5α-cholestane-24,25-diol (XII). The triacetate (XI) (100 mg) was dissolved in a mixture of 5 ml of dry ether and 0.4 ml of dry pyridine, then added with 60 mg of osmium tetraoxide and allowed to stand in a closed flask at room temperature for 2 days. The solvent was evaporated under reduced pressure, and the black residue was refluxed with 65 ml of a 12% solution of $NaHSO_3$ in 50% ethanol for 4 hr. A large amount of ethanol was added to the reaction mixture and filtered. The ethanol extract thus obtained was evaporated to dryness to provide XII (95 mg). The infrared spectrum

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showed no vinyl absorption. Infrared spectrum $\nu_{max}^{CHCl_3}$ cm^{-1}: 3440 (hydroxyl), 1735, 1250, 1050 (acetate).

3α,7α,12α-Triacetoxy-5α-cholan-24-al (XIII). To a solution of 75 mg of lead tetraacetate in 5 ml of dry benzene, a solution of XII (90 mg) in dry benzene (5 ml) was added at 60°C. After 2 hr at this temperature, 30 ml of water was added to the reaction mixture. The product was extracted with three 50-ml portions of ether. The combined ether extracts were washed with water, dried over anhydrous Na_2SO_4, and evaporated to dryness to yield the aldehyde (XIII) (86 mg). The infrared spectrum showed no hydroxyl absorption. Infrared spectrum $\nu_{max}^{CHCl_3}$ cm^{-1}: 2720, 1725 (carbonyl), 1735, 1250, 1050 (acetate).

3α,7α,12α,24 - Tetrahydroxy - 5α - cholestan - 26 - oic acid (IIIa). Eighty mg of XIII and 6 ml of ethyl α-bromopropionate were added to a mixture of dry toluene (30 ml) and dry benzene (10 ml) with 10 g of granulated zinc and a few crystals of iodine. When the mixture was refluxed on a sand bath a vigorous reaction set in and the solution became cloudy. Gentle refluxing was continued for 1.5 hr with rapid stirring, and then the reaction mixture was cooled. The condensation product was decomposed with 10 ml of 10% H_2SO_4 and the separated benzene–toluene layer was extracted with ether. The extract was washed with water, dried over anhydrous Na_2SO_4, and then the solvents were evaporated to dryness. The brownish oily residue was refluxed with 20 ml of 10% methanolic KOH for 2 hr. After evaporation of methanol from the hydrolysate after addition of certain ml of water, the alkaline solution was extracted with ethyl acetate after acidification with 2 N HCl. The extract was washed with water, dried, and then evaporated to dryness to yield IIIa (39 mg). Infrared spectrum ν_{max}^{KBr} cm^{-1}: 3340 (hydroxyl), 1710 (carboxyl).

Synthesis of 3α,7α,12α,26-tetrahydroxy-5α-cholestan-27-oic acid (IXa) (Fig. 2)

5α-Cyprinol pentaacetate (XV). A solution of 5α-cyprinol (XIV, 3.6 g) in 20 ml of pyridine and 25 ml of acetic anhydride was heated on a water bath for 4 hr. The reaction mixture was diluted with water, and extracted with three 200-ml portions of ether. The combined extracts were washed with water, dried over anhydrous Na_2SO_4, and the solvent was evaporated to dryness. The resulting residue was chromatographed on a column of silica gel (100 g, Merck). The column was eluted with benzene–ethyl acetate mixtures. Elution with a 8:2 mixture gave the pentaacetate (XV, 2.42 g) as a pale yellow gum. The acetate (XV) gave a single spot when examined by TLC using the system benzene–ethyl acetate, 7:3 (R_f = 0.50) and showed in its infrared spectrum no hydroxyl absorption.

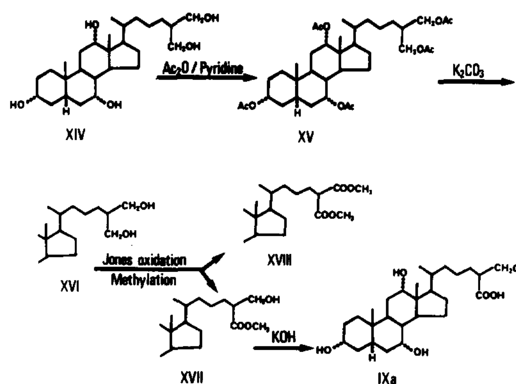

Fig. 2. Partial synthesis of 3α,7α,12α,26-tetrahydroxy-5α-cholestan-27-oic acid. XIV, 5α-cyprinol; XV, 5α-cyprinol pentaacetate; XVI, 3α,7α,12α-triacetoxy-5α-cholestane-26,27-diol; XVII, methyl 3α,7α,12α-triacetoxy-26-hydroxy-5α-cholestan-27-oate; XVIII, dimethyl 3α,7α,12α-triacetoxy-5α-cholestane-26,27-dioate; IXa, 3α,7α,12α,26-tetrahydroxy-5α-cholestan-27-oic acid.

3α,7α,12α-Triacetoxy-5α-cholestane-26,27-diol (XVI). To the stirred solution of pentaacetate (XV, 2.4 g) in methanol (25 ml) at 20°C, a solution of K_2CO_3 (525 mg) in aqueous methanol (5 ml) was added and the stirring was continued. After 5 hr the reaction mixture was diluted with water, and extracted with three 200-ml portions of ether. The combined extracts were washed with water, dried over anhydrous Na_2SO_4, and the solvent was evaporated to dryness under reduced pressure. The residue (1.93 g) was chromatographed on a column of silica gel (100 g, Merck). Elution with a 1:2 mixture of benzene and ethyl acetate gave the triacetoxy-26,27-diol (XVI, 731 mg), as a low melting solid, which could not be recrystallized. Infrared spectrum indicated a band at 3400 cm^{-1} for hydroxyl groups. The purity of XVI was ascertained by TLC (R_f = 0.38 in benzene–ethyl acetate 1:2). NMR (δ ppm): 0.70 (s, 3H, 18-CH_3), 0.76 (s, 3H, 19-CH_3), 0.91 (d, J = 6 Hz, 3H, 21-CH_3), 1.96, 2.00, 2.04 (s, 3H × 3, 3-, 7-, and 12-$OCOCH_3$), 4.06 (d, J = 6 Hz, 4H, 26- and 27-CH_2OH), 5.16 (m, 2H, 3β- and 7β-H), 5.28 (m, 1H, 12β-H).

Methyl 3α,7α,12α-triacetoxy-26-hydroxy-5α-cholestan-27-oate (XVII) and dimethyl 3α,7α,12α-triacetoxy-5α-cholestane-26,27-dioate (XVIII). To an ice-cold solution of the triacetoxy-26,27-diol, XVI (630 mg) in acetone (10 ml) was added to a solution of chromic anhydride (232 mg) in 10% H_2SO_4 (1.8 ml). After 2 hr the reaction mixture was poured in cold water and extracted with three 100-ml portions of ether. The combined extracts were washed with water and dried over anhydrous Na_2SO_4. Evaporation of the solvent yielded

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TABLE 1. R_f values on thin-layer chromatography of bullfrog bile salts and reference compounds

	R_f Value	
	S-8[a]	EA-2[b]
Bile salts of bullfrog	0.90	0.85
	0.74	0.73
	0.48	
	0.35	0.40
	0.22	
	0.00	0.00
Reference compound		
Cholesterol	0.74	0.73
Trihydroxycoprostanic acid	0.48	0.00
Cholic acid	0.36	0.02
3α,7α,12α-Trihydroxy-27-nor-5β-cholestan-24-one	0.35	0.40
3α,7α,12α,26-Tetrahydroxy-5β-cholestan-27-oic acid	0.21	0.00
Taurocholate	0.00	0.00
5α-Cyprinol sulfate	0.00	0.00

[a] S-8, cyclohexane–isopropanol–acetic acid 30:10:1 (v/v).
[b] EA-2, ethyl acetate–acetone 7:3 (v/v).

a mixture (310 mg) of mono- and dicarboxylic acids, which was methylated by the usual manner with diazomethane and then chromatographed on a column of silica gel (12 g). Elution with a 9:1 mixture of benzene–ethyl acetate gave XVIII (169 mg), infrared spectrum $\nu_{max}^{CHCl_3}$ (cm^{-1}): 1710 (ester + acetate); NMR (δ ppm): 0.70 (s, 3H, 18-CH_3), 0.74 (s, 3H, 19-CH_3), 0.89 (d, J = 6 Hz, 3H, 21-CH_3), 1.93, 1.99, 2.02 (s, 3H × 3, 3-, 7-, and 12-$OCOCH_3$), 3.68 (s, 6H, 26- and 27-$COOCH_3$), 5.14 (m, 2H, 3β- and 7β-H), 5.26 (m, 1H, 12β-H). Elution with a 8:2 mixture of benzene–ethyl acetate gave XVII (110 mg), infrared spectrum $\nu_{max}^{CHCl_3}$ (cm^{-1}): 3470 (hydroxyl), 1710 (ester + acetate); NMR (δ ppm): 0.71 (s, 3H, 18-CH_3), 0.76 (s, 3H, 19-CH_3), 0.90 (d, J = 6 Hz, 3H, 21-CH_3), 1.93, 2.00, 2.06 (s, 3H × 3, 3-, 7-, and 12-$OCOCH_3$), 3.66 (s, 3H, 27-$COOCH_3$), 4.06 (d, J = 6 Hz, 2H, 26-CH_2OH), 5.16 (m, 2H, 3β- and 7β-H), 5.26 (m, 1H, 12β-H).

3α,7α,12α,26-tetrahydroxy-5α-cholestan-27-oic acid (IXa). XVII (80 mg) was dissolved in 5% methanolic KOH (5 ml) and was refluxed for 2 hr. The solution was diluted with water and extracted with three 100-ml portions of ethyl acetate after acidification with diluted HCl. The extracts were washed with water, dried over anhydrous Na_2SO_4, and evaporated to dryness. The residue was recrystallized from ethyl acetate to give crystals of 3α,7α,12α,26-tetrahydroxy-5α-cholestan-27-oic acid (IXa, 43.7 mg), mp 238–240°. Infrared spectrum ν_{max}^{KBr} (cm^{-1}): 3300 (hydroxyl), 1710 (carboxyl); NMR (δ ppm): 0.80 (s, 3H, 18-CH_3), 0.90 (s, 3H, 19-CH_3), 1.19 (d, J = 6 Hz, 3H, 21-CH_3), 4.09 (m, 1H, 3β-H), 4.16–4.36 (m, 4H, 7β-H, 12β-H, and 26-CH_2OH); High resolution mass spectrum: M^+ 466.3371, (Calcd. for $C_{27}H_{46}O_6$, 466.3295).

RESULTS

TLC analysis of the crude bile salts from bullfrog revealed the presence of a number of minor bile acids and bile alcohols in unconjugated forms (**Table 1**). These unconjugated steroids were obtained from the crude bile salts by ether extraction under acidification, and separated into the bile acid fraction and the bile alcohol fraction as described in the section of Materials and Methods.

GLC analysis revealed that the bile alcohol fraction consisted mainly of two bile alcohols, A and B, in the ratio of 1:9, which were isolated by silica gel adsorption column chromatography.

The less polar bile alcohol, B was identified as 3α,7α,12α-trihydroxy-27-nor-5β-cholestan-24-one (VIb) in the following way. The infrared spectrum of B showed a strong band at 1720 cm^{-1} due to a carbonyl group and a series of prominent bands at 922, 952, 983, 1020, 1042, and 1075 cm^{-1}, which are diagnostically important for bile alcohol carrying the cholic acid-type nucleus (12). The NMR spectrum of B exhibited four methyl resonances: two singlets at δ 0.80 and 1.01, and a doublet at δ 1.18 (J = 6 Hz), ascribable to C-18, C-19, and C-21 methyl groups, respectively; a triplet at δ 0.99 (J = 7 Hz) must be the end of the side chain since the mass spectral evidence described below supports the C_{19} nucleus. The NMR spectrum also showed three multiplets at δ 3.78, 4.10, and 4.23, assigning to C-3, C-7, and C-12 protons,

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TABLE 2. R_f values on thin-layer chromatography and relative retention times on gas–liquid chromatography of bile alcohols obtained from bullfrog bile and reference compounds

Bile Alcohol	R_f Value on TLC		Relative Retention Time[c] on Gas–Liquid Chromatography[d]			
	S-8[a]	EA-2[b]	OV-1	OV-17	QF-1	Poly I-110
Natural bile alcohol A	0.31	0.33	1.12	1.04	1.38	1.10
Synthetic 3α,7α,12α-trihydroxy-27-nor-5α-cholestan-24-one	0.31	0.34	1.12	1.04	1.39	1.10
Natural bile alcohol B	0.35	0.40	1.20	1.18	1.46	1.25
Synthetic 3α,7α,12α-trihydroxy-27-nor-5β-cholestan-24-one	0.35	0.40	1.20	1.18	1.45	1.25

[a] S-8: cyclohexane–isopropanol–acetic acid 30:10:1 (v/v).
[b] EA-2: ethyl acetate–acetone 7:3 (v/v).
[c] Relative to TMS ether of methyl cholate.
[d] Bile alcohols were chromatographed as their TMS ethers.

respectively. The mass spectrum of the TMS ether of B showed a very weak molecular ion, m/e 636, and characteristic fragments at m/e 546 (M-90), 531 [M-(90 + 15)], 456 (M-2 × 90), 441 [M-(2 × 90 + 15)], 366 (M-3 × 90), 343[M-(2 × 90 + side chain)], 294, 281, and 253 [M-(3 × 90 + side chain)] (base peak). The infrared, NMR, and mass spectra of B compared well with those of synthesized authentic 3α,7α,12α-trihydroxy-27-nor-5β-cholestan-24-one (VIb) (2). The natural bile alcohol had the same GLC retention times on four different columns and TLC R_f values in two different solvent systems as the synthesized authentic bile alcohol (**Table 2**).

The more polar bile alcohol, A, was identified as 3α,7α,12α-trihydroxy-27-nor-5α-cholestan-24-one (VIa) in the following way. The infrared spectrum of A showed a carbonyl band at 1720 cm^{-1} and a series of prominent bands at 893, 962, 1010, 1031, and 1087 cm^{-1} which have been described as a characteristic pattern of the allocholic acid-type nucleus (12). The NMR spectrum of A showed the following chemical shifts (δ): 18-CH_3, 0.78 (s); 19-CH_3, 0.88 (s); 21-CH_3, 1.17 (d, J = 6 Hz); 26-CH_3, 0.99 (t, J = 7 Hz); 7β-H, 4.07 (m); 3β-H and 12β-H, 4.20 (m). The mass spectrum of the TMS ether of A exhibited fragment ions at m/e 456 (M-2 × 90), 366 (M-3 × 90), 343 [M-(2 × 90 + side chain)], 259 [M-(90 + A and B rings)], 253 [M-(3 × 90 + side chain)]. The infrared, NMR, and mass spectra and the chromatographic properties (Table 2) of A were completely identical with those of synthesized authentic 3α,7α,12α-trihydroxy-27-nor-5α-cholestan-24-one (VIa) (2).

GLC analysis revealed that the bile acid fraction contained at least eight minor bile acids, which were tentatively named 1–8 in order of increasing retention time, along with the major bile acid, trihydroxycoprostanic acid. The bile acid fraction was further separated into the more polar bile acid fraction containing bile acids 1, 2, 5, 6, 7, and 8, and the less polar bile acid fraction containing bile acids 3 and 4 and trihydroxycoprostanic acid. These minor bile acids were in such small amounts that their chemical structures were studied only by a combination of GLC and mass spectrometry. Relative retention times on GLC and the mass spectral fragmentations of the methyl ester-TMS ethers of bile acids 1–8 are shown in **Tables 3** and **4.** These minor bile acids were known compounds that had previously been isolated from natural sources, except bile acids 5 and 7. Thus, bile acids 1, 2, 3, 4, 6, and 8 were identified as allocholic acid (Va), cholic acid (Vb), 3α,7α,12α-trihydroxy-5α-cholestan-26-oic acid (IIa), 3α,7α,12α-trihydroxy-24-methyl-5β-cholestan-26-oic acid, varanic acid (IIIb), and 3α,7α,12α,26-tetrahydroxy-5β-cholestan-27-oic acid (IXb), respectively, by direct comparison of GLC retention times and mass spectra with authentic samples.

The methyl ester-TMS ether of bile acid 5 showed a GLC peak which had a retention time of 0.95, 0.87, 0.94, and 0.92 relative to that of the corresponding

TABLE 3. Relative retention times on gas–liquid chromatography of minor bile acids obtained from bullfrog bile

Bile Acid	Relative Retention Time on Gas–Liquid Chromatography[a]			
	OV-1	OV-17	QF-1	Poly I-110
Bile acid 1[b]	0.94	0.87	0.93	0.82
Bile acid 2	1.00	1.00	1.00	1.00
Bile acid 3	1.59	1.45	1.43	1.55
Bile acid 4	1.90	1.60	1.72	1.88
Bile acid 5	2.29	1.93	1.89	1.74
Bile acid 6	2.42	2.23	2.01	1.89
Bile acid 7	2.91	2.56	2.56	2.58
Bile acid 8	3.07	2.89	2.68	2.77

[a] Relative to TMS ether of methyl cholate. Bile acids were chromatographed as their methyl ester-TMS ethers.
[b] Bile acids 1–8 were identified as follows: 1, allocholic acid; 2, cholic acid; 3, 3α,7α,12α-trihydroxy-5α-cholestan-26-oic acid; 4, 3α,7α,12α-trihydroxy-24-methyl-5β-cholestan-26-oic acid; 5, 3α,7α,12α,24-tetrahydroxy-5α-cholestan-26-oic acid; 6, varanic acid; 7, 3α,7α,12α,26-tetrahydroxy-5α-cholestan-27-oic acid; 8, 3α,7α,12α,26-tetrahydroxy-5β-cholestan-27-oic acid.

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TABLE 4. Mass fragment ions and relative intensities of the methyl ester-TMS derivatives of the minor bile acids[a]

	m/e	Peak 1	Peak 2	m/e	Peak 3	m/e	Peak 4	m/e	Peak 5	Peak 6	Peak 7	Peak 8
$[M]^+$	638			680		694		768				
$[M-15]^+$	623	17	13	665	4	679	3	753	8	7	17	15
$[M-90]^+$	548	8	3	590	3	604	4	678			2	1
$[M-(90 + 15)]^+$	533	2	3	575	1	589		663				
$[M-180]^+$	458	90	40	500	100	514	25	588	42	33	52	25
$[M-(180 + 15)]^+$	443	9	6	485	5	499	4	573				
$[M-270]^+$	368	11	74	410	8	424	56	498	6	67	9	41
$[M-(270 + 15)]^+$	353	9	6	395	3	409	8	483				
$[M-360]^+$								408		10		
$[M-(180 + \text{side chain})]^+$	343	100	31	343	78	343	40	343	100	23	100	41
	281	4	11	303	30	317	15	321		15		
	261	63		281	6	281	24	281		27	6	16
$[M-(270 + \text{side chain})]^+$	253	59	100	253	25	253	100	253	23	100	42	100

[a] These bile acids were identified as follows: peak 1, allocholic acid; peak 2, cholic acid; peak 3, 3α,7α,12α-trihydroxy-5α-cholestan-26-oic acid; peak 4, 3α,7α,12α-trihydroxy-24-methyl-5β-cholestan-26-oic acid; peak 5, 3α,7α,12α,24-tetrahydroxy-5α-cholestan-26-oic acid; peak 6, varanic acid; peak 7, 3α,7α,12α,26-tetrahydroxy-5α-cholestan-27-oic acid; peak 8, 3α,7α,12α,26-tetrahydroxy-5β-cholestan-27-oic acid.

derivative of varanic acid (IIIb), on OV-1, OV-17, QF-1, and Poly I-110, respectively. These ratios were in good agreement with constant separating factors found between the pairs of bile acids carrying the allocholic acid-type nucleus and their 5β-counterparts under the employed conditions. The mass spectrum of the methyl ester-TMS ether of bile acid 5 was very similar to that of the corresponding derivative of varanic acid. The only difference was that the relative intensities between the fragment ions at m/e 253 and 343. The intensity of the fragment at m/e 253 was greater than the ion at m/e 343 for varanic acid, while the reverse was true for bile acid 5, suggesting that the latter has the allocholic acid-type nucleus (13). The most probable structure of bile acid 5 is thus 3α,7α,12α,24-tetrahydroxy-5α-cholestan-26-oic acid (IIIa). Confirmation of this structural assignment was attempted by partial synthesis (Fig. 1). 3α,7α,12α-Trihydroxy-26,27-dinor-5α-cholest-24-ene (X), obtained from the alkaline hydrolysate of bullfrog bile (11), was converted to the triacetate (XI). The acetate (XI) was oxidized with osmium tetraoxide and the product (XII) was treated with lead tetraacetate to produce 3α,7α,12α-triacetoxy-5α-cholan-24-al (XIII). Treatment of the aldehyde (XIII) with an excess of ethyl α-bromopropionate and zinc in benzene and toluene followed by alkaline hydrolysis gave the desired 3α,7α,12α,24-tetrahydroxy-5α-cholestan-26-oic acid (IIIa). The synthetic C_{27} bile acid was completely identical in GLC–mass spectrometry with the natural bile acid 5.

The mass spectrum of the methyl ester-TMS ether of bile acid 7 was very similar to that of the corresponding derivative of 3α,7α,12α,26-tetrahydroxy-5β-cholestan-27-oic acid (IXb), except for a difference of the relative intensities between the fragment ions at m/e 253 and 343. In addition, the ratios of GLC retention times of these bile acids were identical with the separating factors found between 5α-bile acid and their 5β-isomers. The most probable structure of bile acid 7 is thus 3α,7α,12α,26-tetrahydroxy-5α-cholestan-27-oic acid (IXa). Confirmation of this structural assignment was attempted by partial synthesis (Fig. 2). 3α,7α,12α-Triacetoxy-5α-cholestan-26,27-diol (XVI) was prepared from 5α-cyprinol (XIV) in two steps involving acetylation of hydroxyl groups at C-3, C-7, C-12, C-26, and C-27, and partial hydrolysis with methanolic potassium carbonate at room temperature of the C-26 and C-27 acetoxyl groups. Oxidation of the triacetoxy-5α-cholestanediol (XVI) followed by methylation gave a mixture of the monocarboxylic acid monomethyl ester (XVII) and the dicarboxylic acid dimethyl ester (XVIII). Alkaline hydrolysis of the monocarboxylic acid monomethyl ester (XVII) gave the desired 3α,7α,12α,26-tetrahydroxy-5α-cholestan-27-oic acid (IXa). The synthetic C_{27} bile acid was completely identical in GLC-mass spectrometry with the natural bile acid 7.

DISCUSSION

The present studies demonstrate the presence of the following unconjugated bile acids and bile alcohols as the minor constituents of the bullfrog bile: allocholic acid (Va), cholic acid (Vb), 3α,7α,12α-trihydroxy-5α-cholestan-26-oic acid (IIa), 3α,7α,12α-trihydroxy-24-methyl-5β-cholestan-26-oic acid, 3α,7α,12α,24-tetrahydroxy-5α-cholestan-26-oic acid (IIIa), varanic acid (IIIb), 3α,7α,12α,26-tetrahydroxy-5α-cholestan-27-oic acid (IXa), 3α,7α,12α,26-tetrahydroxy-5β-cholestan-27-oic acid (IXb), 3α,7α,12α-

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trihydroxy-27-nor-5α-cholestan-24-one (VIa), and 3α,7α,12α-trihydroxy-27-nor-5β-cholestan-24-one (VIb).

We were not surprised to find that in the bullfrog bile the minor bile acids occurred in unconjugated forms, since the major bile acid, trihydroxycoprostanic acid (IIb) also occurred in free form. The bullfrog liver may lack an enzyme that catalyzes the conjugation of bile acids. However, we were surprised to find that the minor bile alcohols, in contrast to the major bile alcohols which were present in the bile as their sulfate esters, occurred in unconjugated forms. The major bile alcohols, ranols (VIIIa and VIIIb), their 26-deoxy derivatives (VIIa and VIIb), and 5α-cyprinol (XIV), were shown to be sulfated at the C-24 or C-26 position but not at C-3, C-7, or C-12 position (14). If the bile alcohol sulfokinase system in the bullfrog liver was specific for the side chain hydroxyl groups, this could explain why the minor bile alcohols, VIa and VIb, which have no side chain hydroxyl group, were not sulfated.

The second curious character of the minor bile alcohols is that they have a keto group at C-24. During the course of comparative biochemical studies of bile salts, a large number of bile alcohols have been isolated from natural sources and characterized (2, 5, 14). However, in contrast to bile acids, some of which have one or two keto groups in the molecules, no bile alcohol possessing such a group has so far been obtained from natural sources. To the best of our knowledge this is the first demonstration of the natural occurrence of keto bile alcohols. Although it cannot be excluded that the keto bile alcohols, like keto bile acids (15), are formed secondarily by intestinal microorganisms during the enterohepatic circulation, it seems more logical to assume that these C_{26} keto bile alcohols, VIa and VIb are biosynthetic precursors of the C_{26} major bile alcohols, 26-deoxyranols (VIIa and VIIb) and ranols (VIIIa and VIIIb). It has been reported that, in the bullfrog, 5β-ranol (VIIIb) and 26-deoxy-5β-ranol (VIIb) are formed from 5β-cholestane-3α,7α,12α-triol and varanic acid (IIIb), as well as from cholesterol (I) (16–18). Hence, the route (**Fig. 3**) involving decarboxylation of C_{27} keto bile acids, 3α,7α,12α-trihydroxy-24-oxo-5α- and 5β-cholestan-26-oic acids (IVa and IVb) to the C_{26} keto bile alcohols (VIa and VIb) has been proposed for the biosynthetic pathway of ranols (VIIIa and VIIIb) (14). This proposal receives further support from the present finding of the postulated intermediates, VIa and VIb in the bullfrog bile.

3α,7α,12α,26-Tetrahydroxy-5β-cholestan-27-oic acid (IXb) and its 5α-isomer (IXa) may be formed from trihydroxycoprostanic acid (IIb) and its 5α-isomer (IIa), respectively, by hydroxylation of the C-26 methyl group.

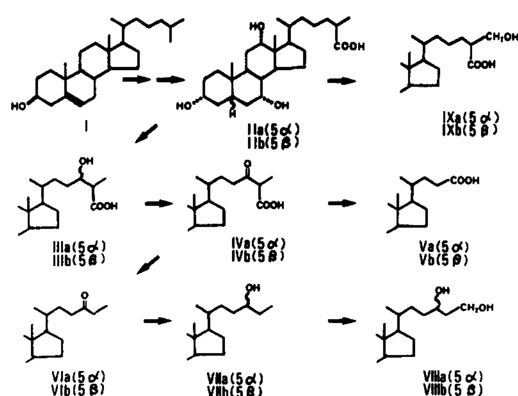

Fig. 3. Possible route for biosynthesis of bile acids and bile alcohols in bullfrog. I, cholesterol; IIa, 3α,7α,12α-trihydroxy-5α-cholestan-26-oic acid; IIb, trihydroxycoprostanic acid; IIIa, 3α,7α,12α,24-tetrahydroxy-5α-cholestan-26-oic acid; IIIb, varanic acid; IVa, 3α,7α,12α-trihydroxy-24-oxo-5α-cholestan-26-oic acid; IVb, 3α,7α,12α-trihydroxy-24-oxo-5β-cholestan-26-oic acid; Va, allocholic acid; Vb cholic acid; VIa, 3α,7α,12α-trihydroxy-27-nor-5α-cholestan-24-one; VIb, 3α,7α-12α-trihydroxy-27-nor-5β-cholestan-24-one; VIIa, 26-deoxy-5α-ranol; VIIb, 26-deoxy-5β-ranol; VIIIa, 5α-ranol; VIIIb, 5β-ranol.

Origin of 3α,7α,12α-trihydroxy-24-methyl-5β-cholestan-26-oic acid is obscure. The Δ^{22} derivative of this C_{28} bile acid and another C_{28} bile acid, 3α,7α,12α-trihydroxy-5β-cholest-22-ene-24-carboxylic acid have been found in the bile of toad *Bufo vulgaris formosus* (19, 20). It has been shown that these unsaturated C_{28} bile acids were not formed from cholesterol or mevalonate in the toad (21, 22). It is possible that in these amphibians the C_{28} bile acids are formed from dietary C_{28} sterols (14, 23).

The presence of C_{24} bile acids in the bullfrog had been postulated since labeled cholic acid was obtained from the bullfrog that received [2-^{14}C]mevalonate (22). The present studies confirmed the presence of cholic acid (Vb) and allocholic acid (Va) in this frog bile, although in lesser concentrations. It is conceivable that in the bullfrog (Fig. 3) most of the 24-keto C_{27} bile acids (IVa and IVb) are transformed into the 24-keto C_{26} bile alcohols (VIa and VIb), and then into deoxyranols (VIIa and VIIb) and ranols (VIIIa and VIIIb), but lesser amounts of the 24-keto C_{27} bile acids (IVa and IVb) escaped from the action of decarboxylase and would be converted to allocholic and cholic acids (Va and Vb).

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