Bile salts of the toad, *Bufo marinus:* characterization of a new unsaturated higher bile acid, 3α,7α,12α,26-tetrahydroxy-5β-cholest-23-en-27-oic acid

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Abstract The bile salts present in gallbladder bile of the toad, *Bufo marinus,* were found to consist of a mixture of bile alcohol sulfates and unconjugated bile acids. The major bile alcohol was 5β-bufol; 5α- and 5β-cholestane-3α,7α,12α,26-tetrols occurred as the minor bile alcohols. Bile acids of *Bufo marinus* were cholic acid, allocholic acid, 3α,7α,12α-trihydroxy-5α- and 5β-cholestan-26-oic acids, 3α,7α,12α-trihydroxy-5α- and 5β-cholest-23-en-26-oic acids, 3α,7α,12α,26-tetrahydroxy-5β-cholestan-27-oic acid, and a C_{27} bile acid which has not been previously described. By chromatographic behavior, mass spectral data, and identification of the products of catalytic hydrogenation and ozonolysis, the structure of the new higher bile acid was elucidated as 3α,7α,12α,26-tetrahydroxy-5β-cholest-23-en-27-oic acid. The bile salt pattern of *Bufo marinus* closely resembles that of *Bufo vulgaris formosus,* except for the absence of 3α,7α,12α-trihydroxy-5β-cholest-22-ene-24-carboxylic acid, the major bile acid of the latter toad.—**Yoshii, M., M. Une, K. Kihira, T. Kuramoto, T. Akizawa, M. Yoshioka, V. P. Butler, Jr., and T. Hoshita.** Bile salts of the toad, *Bufo marinus:* characterization of a new unsaturated higher bile acid, 3α,7α,12α,26-tetrahydroxy-5β-cholest-23-en-27-oic acid. *J. Lipid Res.* 1994. **35:** 1646–1651.

Supplementary key words bile acids • bile alcohols • biosynthesis • gas–liquid chromatography • *Bufo marinus* • mass spectrometry

As part of a research program aimed at better understanding of the biosynthesis and molecular evolution of bile salts, we have been examining the bile salts of a large number of vertebrates, especially of more primitive vertebrates such as fishes and amphibians (1).

In this study, we report the results of an examination of the biliary bile acids and bile alcohols of the toad, *Bufo marinus,* by means of combined capillary gas–liquid chromatography and mass spectrometry.

MATERIALS AND METHODS

Reference compounds

Cholesterol and cholic acid were commercial products. Allocholic acid (3α,7α,12α-trihydroxy-5α-cholan-24-oic acid), 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid, and 3α,7α,12α-trihydroxy-5α-cholestan-26-oic acid were isolated from the bile of the giant salamander, *Megalobatrachus japonicus* (2), the bile of the bullfrog, *Rana catesbeiana* (3), and the bile of the iguana, *Iguana iguana* (4), respectively. 3α,7α,12α-Trihydroxy-5α- and 5β-cholest-23-en-26-oic acids and 5β-bufol(5β-cholestane-3α,7α,12α,25,26-pentol) were obtained from the bile of the toad, *Bufo vulgaris formosus* (5, 6). Norcholic acid (3α,7α,12α-trihydroxy-24-nor-5β-cholan-23-oic acid) (7), 3α,7α,12α,26-tetrahydroxy-5β-cholestan-27-oic acid (8), 5α-cholestane-3α,7α,12α,26-tetrol (9), and 5β-cholestane-3α,7α,12α,26-tetrol (10) were synthesized as described previously.

Extraction of bile salts

The bile of *Bufo marinus* was collected by extraction of 10 gallbladders with 50 ml ethanol. Evaporation of the solvent from the filtered extract gave crude bile salts as a solid (55.8 mg).

Abbreviations: TLC, thin-layer chromatography; PHP-LH-20, piperidinohydroxypropyl-Sephadex-LH-20; GLC–MS, gas–liquid chromatography–mass spectrometry; TMS, trimethylsilyl; TeHCA, 3α,7α,12α,26-tetrahydroxy-5β-cholestan-27-oic acid.

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Thin-layer chromatography

Thin-layer chromatography (TLC) was performed on silica gel precoated TLC aluminum sheets (0.2 mm thickness, Merck). For two-dimensional TLC of bile salts (11), the ethanolic extract of the gallbladder bile was applied as a single spot and was developed in n-butanol–acetic acid–water 17:2:1 (v/v). After development, the plate was air-dried and then developed in the other direction in chloroform–methanol–acetic acid–water 13:4:2:1 (v/v). Unconjugated sterols, bile acids, and bile alcohols were analyzed using benzene–isopropanol–acetic acid 30:10:1 (v/v) as the solvent system. Conjugated and unconjugated steroids were detected by spraying with phosphomolybdic acid (10 g/100 ml ethanol) followed by heating at 110°C for 5 min.

Group separation of bile salt classes

The crude bile salts were dissolved in 5 ml 90% ethanol and the ethanolic solution was passed through a column of piperidinohydroxypropyl-Sephadex-LH-20(PHP-LH-20) (15 ml) (12). The column was eluted successively with 45 ml 90% ethanol, 150 ml 0.1 M acetic acid in 90% ethanol, and 150 ml 1% ammonium carbonate in 70% ethanol, to give a neutral sterol fraction, an unconjugated bile acid fraction, and a sulfated bile alcohol fraction, respectively.

Solvolysis of sulfated bile alcohols

The sulfated bile alcohol fraction was concentrated to dryness under a reduced pressure, and the resulting residue was dissolved in 2.5 ml of water and the pH was adjusted to 1.0 by adding 1 N HCl; then NaCl (2 g) and 20 ml ethyl acetate were added and the mixture was shaken at 37°C for 3 days. After addition of 25 ml 5% KOH solution, the mixture was extracted with ethyl acetate (15 × 3 ml). The extracts were combined and washed with water. Evaporation of the solvent from the washed extract left a residue, which was hydrolyzed with 5% ethanolic KOH at 75°C for 1 h. Ethanol was removed under N_2 and replaced with water. The solution was cooled and extracted with n-butanol–ethyl acetate 1:1 (v/v) (10 × 3 ml). The extracts were combined and washed with water, and the solvents were evaporated to dryness under a reduced pressure to give a residue consisting mainly of desulfated bile alcohols.

Gas–liquid chromatography and mass spectrometry

Combined gas–liquid chromatography and mass spectrometry (GLC–MS) was carried out on a Hewlett-Packard 5890 gas chromatograph and a JEOL JMS-SX 102 mass spectrometer under the following conditions: column, a fused silica capillary column (15 m × 0.32 mm i.d.) coated (0.15 μm film) with DB-17HT (J & W Scientific); column oven temperature, 200–280°C at a rate of 2°C/min; injection port temperature, 280°C; ion source temperature, 250°C; flow rate of helium carrier gas, 2.0 ml/min; ionizing energy, 70 eV; ionizing current, 300 μA. Neutral materials to be analyzed were chromatographed as their trimethylsilyl (TMS) ether derivatives that were prepared by treating aliquots with hexamethyldisilazane–trimethylchlorosilane–pyridine 2:1:5 (v/v) at room temperature for 2 h; bile acid samples were treated with freshly prepared ethereal diazomethane solution at room temperature for 2 h, and the resulting methyl esters were analyzed as their TMS ether derivatives.

Silica gel chromatography

The unconjugated bile acid methyl esters were transferred onto a column of silica gel 60 (10 g, Merck). The column was successively eluted with 120 ml ethyl acetate, 90 ml ethyl acetate–acetone 1:1 (v/v), and 30 ml acetone, to give a trihydroxy-C_{27} bile acid methyl ester fraction, a trihydroxy-C_{24} bile acid methyl ester fraction, and a tetrahydroxy-C_{27} bile acid methyl ester fraction, respectively.

Catalytic hydrogenation

The material (0.35 mg) eluted in the tetrahydroxy-C_{27} bile acid methyl ester fraction was dissolved in 5 ml of acetic acid and hydrogenated with 10 mg of platinum oxide catalyst at room temperature. After removal of the catalyst by filtration, the filtrate was diluted with 50 ml of water and extracted with ether (20 × 3 ml). The extracts were combined and washed successively with water, 5% Na_2CO_3 solution, and water, dried over anhydrous Na_2SO_4, and concentrated to dryness in vacuo.

Ozonolysis

The material (0.35 mg) eluted in the tetrahydroxy-C_{27} bile acid methyl ester fraction was dissolved in 5 ml of ethanol. Ozone was passed into the solution for 3 min at −15°C, and then 10% sodium hydroxide solution (0.5 ml) and 30% hydrogen peroxide (0.7 ml) were added to the reaction mixture. After stirring for 5 min at room temperature, the reaction mixture was diluted with 50 ml of water, acidified with 1 N HCl, and extracted with ether (20 × 3 ml). The extracts were combined and washed with water, and concentrated to dryness in vacuo.

RESULTS

Group separation of bile salt classes

When the bile salt composition of the toad, *Bufo marinus*, was examined by two-dimensional TLC, spots appeared whose chromatographic behaviors were consistent with those of cholesterol, unconjugated bile acids, and bile alcohol sulfates, but there were no spots corresponding to unconjugated bile alcohols and taurine- or glycine-conjugated bile acids.

The crude bile salts (45 mg) obtained from the ethanolic extract of the toad bile were subjected to ion-exchange column chromatography using PHP-LH-20 to give neutral sterol fraction (dried weight, 6.1 mg), unconjugated bile acid fraction (12.7 mg), and sulfated bile alcohol fraction (19.6 mg). After solvolysis of compounds eluted in the last fraction, the resulting desulfated bile alcohols (15.2 mg) as well as compounds eluted in the first two fractions were derivatized and analyzed by GLC–MS, respectively.

Neutral sterols

On combined GLC–MS analysis of the TMS ether derivatives of the material eluted in the neutral sterol fraction, only one peak was observed whose retention time and mass spectrum were identical with those of the TMS ether of cholesterol.

Bile acids

The result of GLC analysis of the unconjugated bile acid sample as the TMS ether-methyl esters is depicted in **Fig. 1.** At least eight distinct peaks were seen. These peaks were designated provisionally as bile acids 1–8 in order of increasing retention times.

The following bile acids were identified by direct comparison of the retention times and mass spectra with those of authentic compounds: bile acid 1, allocholic acid; bile acid 2, cholic acid; bile acid 3, 3α,7α,12α-trihydroxy-5α-cholestan-26-oic acid; bile acid 4, 3α,7α,12α-trihydroxy-5α-cholest-23-en-26-oic acid; bile acid 5, 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid; bile acid 6, 3α,7α,12α-trihydroxy-5β-cholest-23-en-26-oic acid; bile acid 7, 3α,7α,12α,26-tetrahydroxy-5β-cholestan-27-oic acid (TeHCA).

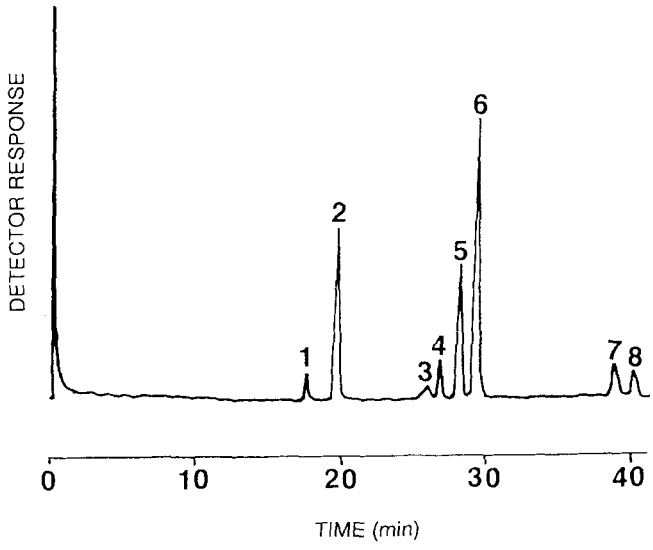

Fig. 1. Gas–liquid chromatogram of the TMS ether-methyl esters of bile acids obtained from the bile of *Bufo marinus*. A DB-17 capillary column (15 m × 0.32 mm) was used; column temperature, 200–280°C at a rate of 2°C/min. The compounds responsible for the peaks 1–8 were identified as the derivatives of allocholic acid, cholic acid, 3α,7α,12α-trihydroxy-5α-cholestan-26-oic acid, 3α,7α,12α-trihydroxy-5α-cholest-23-en-26-oic acid, 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid, 3α,7α,12α-trihydroxy-5β-cholest-23-en-26-oic acid, 3α,7α,12α,26-tetrahydroxy-5β-cholestan-27-oic acid, and 3α,7α,12α,26-tetrahydroxy-5β-cholest-23-en-27-oic acid, respectively.

The GLC retention time of bile acid 8 did not match that of any of known bile acids. The mass spectrum of the TMS ether-methyl ester of bile acid 8 showed the following: *m/z* 766, [M⁺] (18%); 751, [M–15] (48%); 736, (63%); 676, [M–90] (35%); 586, [M–(2 × 90)] (62%); 551, [M–215] (13%); 522, (15%); 496, [M–(3 × 90)] (20%); 461, [M–(90 + 215)] (23%); 431, (23%); 406, [M–(4 × 90)] (10%); 393, (25%); 371, [M–(2 × 90 + 215)] (40%); 355, (19%); 281, [M–(3 × 90 + 215)] (70%); 253, [M–(3 × 90 + side chain)] (38%), 185, (base peak); 103, [CH_2OTMS] (94%). The presence of a series of the peaks at *m/z* 461, 371, and 281 in the spectrum of the TMS ether-methyl ester of bile acid 8 was analogous to the spectrum of the TMS ether-methyl ester of 3α,7α,12α-trihydroxy-5β-cholest-23-en-26-oic acid (bile acid 6), suggesting that bile acid 8 is the Δ^{23}-derivative of a C_{27} bile acid with three nuclear hydroxyl groups and a hydroxyl group in the side chain. The fragment ion at *m/z* 461 results from allylic cleavage at the bond between C-20 and C-22 followed by the loss of a nuclear TMS ether group. The ions at *m/z* 371 and 281 result from the ion at *m/z* 461 by the loss of one and two nuclear TMS ether groups, respectively. The spectral data, together with the biogenetic considerations, suggest that bile acid 8 has the structure 3α,7α,12α,26-tetrahydroxy-5β-cholest-23-en-27-oic acid.

Additional support for this structure was obtained from the comparison of the retention time ratio of the TMS ether-methyl esters with comparable compounds. The retention time ratio of the TMS ether-methyl esters of TeHCA (bile acid 7) and of bile acid 8 was equal to the retention time ratio of the TMS ether-methyl esters of 3α,7α,12α-trihydroxy-5α- and 5β-cholestan-26-oic acids (bile acids 3 and 5) and of their Δ^{23}-derivatives (bile acids 4 and 6), respectively. These values are listed in **Table 1.**

Conclusive evidence for the structure of bile acid 8 was provided by the identification of catalytic hydrogenation and ozonolysis products of bile acid 8 as TeHCA and norcholic acid, respectively.

The unconjugated bile acid fraction was taken to dryness and the residue was methylated with diazomethane in the usual manner. The methyl esters (8.9 mg) were subjected to silica gel column chromatography to give trihydroxy-C_{27} bile acid methyl ester fraction (5.9 mg), trihydroxy-C_{24} bile acid methyl ester fraction (1.6 mg), and tetrahydroxy-C_{27} bile acid methyl ester fraction (0.7 mg). GLC analysis revealed that the tetrahydroxy-C_{27} bile acid methyl ester fraction consisted of the methyl ester of TeHCA (bile acid 7) and the methyl ester of bile acid 8, in the ratio of 3:2.

TABLE 1. Retention times determined by gas-liquid chromatography and relative concentrations of bile acids found in *Bufo marinus* and *Bufo vulgaris formosus*

Bile Acid	Retention Times on GLC[a]	Ratio of Retention Time	*Bufo marinus*	*Bufo vulgaris formosus*[b]
	min		%	%
Allocholic acid	17.56		2.9	0.9
Cholic acid	19.84		19.0	4.1
3α,7α,12α-Trihydroxy-5α-cholest-22-ene-24-carboxylic acid			nd[c]	2.4
3α,7α,12α-Trihydroxy-5β-cholest-22-ene-24-carboxylic acid			nd	42.0
3α,7α,12α-Trihydroxy-5α-cholestan-26-oic acid	25.89	1.03	3.3	0.8
3α,7α,12α-Trihydroxy-5α-cholest-23-en-26-oic acid	26.76		5.5	1.9
3α,7α,12α-Trihydroxy-5β-cholestan-26-oic acid	28.26	1.05	20.0	11.6
3α,7α,12α-Trihydroxy-5β-cholest-23-en-26-oic acid	29.59		39.3	32.5
3α,7α,12α,24-Tetrahydroxy-5β-cholestan-26-oic acid			nd	2.0
3α,7α,12α,24-Tetrahydroxy-24-methyl-5β-cholestan-26-oic acid			nd	1.8
3α,7α,12α,26-Tetrahydroxy-5β-cholestan-27-oic acid	38.96	1.03	5.7	nd
3α,7α,12α,26-Tetrahydroxy-5β-cholest-23-en-27-oic acid	40.32		4.3	nd

[a] A DB-17 capillary column (15 m × 0.32 mm) was used; column temperature, 200–280°C at a rate of 2°C/min.
[b] Reference 6.
[c] Not detected.

A half of the tetrahydroxy-C_{27} bile acid methyl ester fraction was hydrogenated with the presence of platinum oxide catalyst at room temperature. The hydrogenated material was derivatized as the TMS ether-methyl esters and analyzed by GLC–MS. Only one peak was seen and its retention time and mass spectrum were identical with those of the TMS ether-methyl ester of authentic TeHCA. Major ions were: *m/z* 768, [M^+] (6%); 753, [M–15] (39%); 678, [M–90] (9%); 663, [M–(15 + 90)] (7%); 588, [M–(2 × 90)] (99%); 573, [M–(2 × 90 + 15)] (15%); 498, [M–(3 × 90)] (93%); 408, [M–(4 × 90)] (17%); 343, [M–(2 × 90 + side chain)] (55%); 309, (60%); 253 [M–(3 × 90 + side chain)] (base peak).

The remaining half of the tetrahydroxy-C_{27} bile acid methyl ester fraction, which consisted of 57% of the methyl ester of TeHCA and 43% of the methyl ester of bile acid 8, was subjected to ozonolysis and the reaction mixture was treated with alkaline hydrogen peroxide. The reaction products were derivatized as the TMS ether-methyl esters and analyzed by GLC–MS. Two peaks were seen. From their retention times and mass spectral data, one peak (64% of the total recovered bile acid) was identified as that due to the TMS ether derivative of the unchanged TeHCA methyl ester, while another peak (36%) was identified as that due to the TMS ether-methyl ester of norcholic acid, which must be the ozonolysis product of bile acid 8. Major ions in the mass spectrum of the latter were: *m/z* 609, [M–15] (18%); 444, [M–(2 × 90)] (47%); 354, [M–(3 × 90)] (77%); 343, [M–(2 × 90 + side chain)] (12%); 253, [M–(3 × 90 + side chain)] (base peak).

All the evidence establishes that bile acid 8 is 3α,7α,12α,26-tetrahydroxy-5β-cholest-23-en-27-oic acid.

Bile alcohols

GLC (**Fig. 2**) analysis of the TMS ether derivatives revealed that the bile alcohol mixture obtained from the sulfated bile alcohol fraction consisted of three bile alcohols, which were identified as 5α-cholestane-3α,7α,12α,26-tetrol (relative concentrations 5.9% of total bile alcohol), 5β-cholestane-3α,7α,12α,26-tetrol (14.5%), and 5β-bufol (79.6%), respectively, by direct comparison of their GLC retention times and mass spectra with those of authentic compounds.

DISCUSSION

Haslewood (13) has stated in his book that the major bile salt of *Bufo marinus, Bufo b. bufo,* and *Bufo regularis* is 5β-bufol sulfate. However, minor bile salts of these toads had not yet been reported. The toad *Bufo vulgaris formosus* is the only species of the family Bufonidae whose bile salts had received complete chemical analysis. The major bile salt of *Bufo vulgaris formosus* is also 5β-bufol sulfate (5). 5β-Cholestane-3α,7α,12α,25,26,27-hexol and 5α- and 5β-cholestane-3α,7α,12α,26-tetrols were found as the minor constituents of this toad bile (14, 15). The most striking feature of the bile of *Bufo vulgaris formosus* is the presence in it of C_{28} bile acids (6, 16). The chief bile acid of this toad is 3α,7α,12α-trihydroxy-5β-cholest-22-ene-24-carboxylic acid which occurs free (unconjugated) in the bile. Other biliary bile acids were also found as the unconjugates (6). Relative concentrations of the bile acids present in the bile of *Bufo vulgaris formosus* are shown in Table 1.

The present study demonstrates the presence in the bile

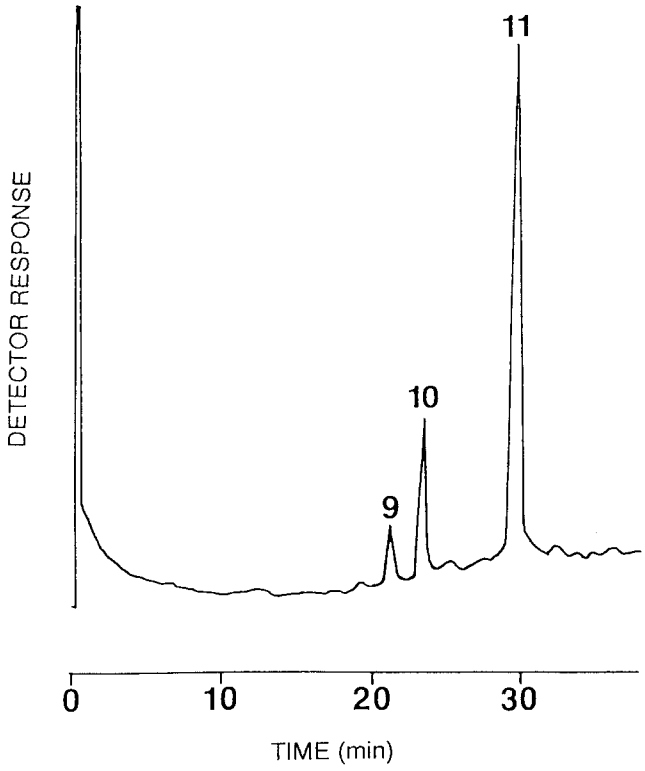

Fig. 2. Gas–liquid chromatogram of the TMS ethers of bile alcohols obtained from the bile of *Bufo marinus* after solvolysis. A DB-17 capillary column (15 m × 0.32 mm) was used; column temperature, 200–280°C at a rate of 2°C/min. The compounds responsible for the peaks 9–11 were identified as the derivatives of 5α-cholestane-3α,7α,12α,26-tetrol, 5β-cholestane-3α,7α,12α,26-tetrol, and 5β-bufol, respectively.

of *Bufo marinus* of ten unconjugated bile acids and sulfate esters of three bile alcohols.

There is close resemblance between the bile alcohol patterns of *Bufo vulgaris formosus* and *Bufo marinus.* The principal bile salt of both toads is the sulfate ester of 5β-bufol; 5α- and 5β-cholestane-3α,7α,12α,26-tetrols also occur in the bile of both toads. The biliary bile acid pattern of *Bufo marinus* is similar to that of *Bufo vulgaris formosus.* Both toads have cholic acid, 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid, 3α,7α,12α-trihydroxy-5β-cholest-23-en-26-oic acid, and their 5α-isomers. However, the most obvious difference between the bile acids of the two is the absence of C_{28} bile acids in *Bufo marinus.*

In the toad *Bufo vulgaris formosus* it was shown that radioactive 5α- and 5β-cholestane-3α,7α,12α,26-tetrols (IIIa and IIIb), 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid (IVb), cholic acid (Vb), and 5β-bufol (VI) were found in the bile after injection of [4-^{14}C]cholesterol (I) (15). Radioactive 5β-bufol (VI) and the trihydroxy-5β-cholestanoic acid (IVb) had previously been isolated from the bile of the toad that received the injection of [26,27-^{14}C]5β-cholestane-3α,7α,12α-triol (IIb) (17). These results suggested that, in *Bufo vulgaris formosus,* cholic acid (Vb) is formed from cholesterol (I) via 5β-cholestane-3α,7α,12α-triol (IIb), the 5β-cholestanetetrol (IIIb) and the trihydroxy-5β-cholestanoic acid (IVb) as the biosynthetic intermediates by the same pathway as that in mammals, while 5β-bufol (VI) is formed from the 5β-cholestanetetrol (IIIb) by the catalytic action of a species specific enzyme, 25-hydroxylase (6). In *Bufo vulgaris formosus,* allocholic acid (Va) was thought to be formed from cholesterol (I) by a modification of the biosynthetic pathway to cholic acid (Vb) in which the only difference is the stereospecific formation of the 5α-cholestane-type nuclear structure (6). Thus, 5α-cholestane-3α,7α,12α-triol (IIa), 5α-cholestane-3α,7α,12α,26-tetrol (IIIa), and 3α,7α,12α-trihydroxy-5α-cholestan-26-oic acid (IVa) seem to be the biosynthetic intermediates in the pathway of biosynthesis of allocholic acid (Va) (6). The close resemblance of the bile salt patterns between *Bufo marinus* and *Bufo vulgaris*

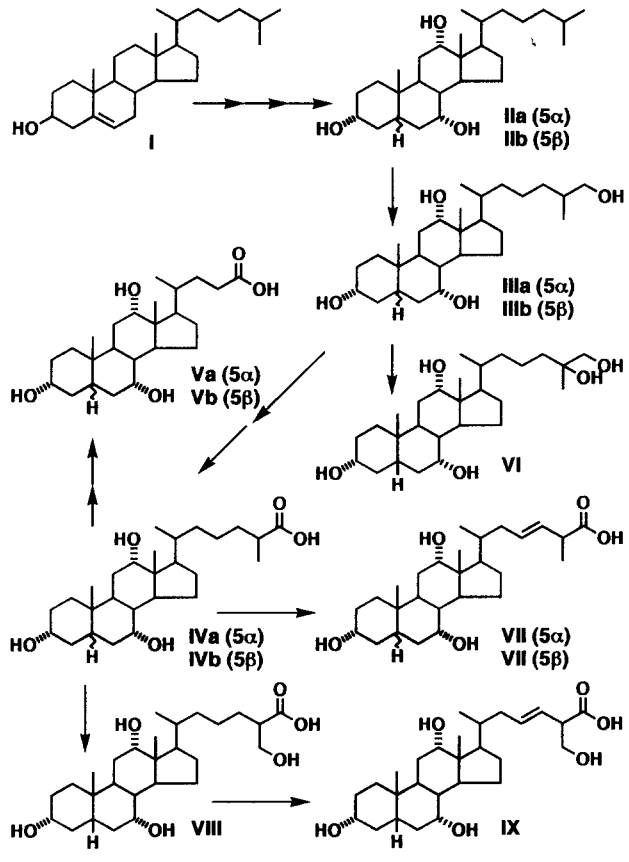

Fig. 3. Postulated pathways for the biosynthesis of bile acids and bile alcohols in the Bufonidae. I, cholesterol; IIa and IIb, 5α- and 5β-cholestane-3α,7α,12α-triols; IIIa and IIIb, 5α- and 5β-cholestane-3α,7α,12α,26-tetrols; IVa and IVb, 3α,7α,12α-trihydroxy-5α- and 5β-cholestan-26-oic acids; Va, allocholic acid; Vb, cholic acid; VI, 5β-bufol; VIIa and VIIb, 3α,7α,12α-trihydroxy-5α- and 5β-cholest-23-en-26-oic acids; VIII, 3α,7α,12α,26-tetrahydroxy-5β-cholestan-27-oic acid; IX, 3α,7α,12α,26-tetrahydroxy-5β-cholest-23-en-27-oic acid.

formosus suggests that the biosynthetic routes for allocholic acid (Va), cholic acid (Vb), and 5β-bufol (VI), including their biosynthetic intermediates, IIa, IIb, IIIa, IIIb, IVa, and IVb in the former toad, are the same as those in the latter toad. The fact that 5α-cholestane-3α,7α,12α,25,26-pentol, the 5α-isomer of 5β-bufol, could not be detected in the bile of both toads suggests that 5α-cholestane-3α,7α,12α,26-tetrol (IIIa) is not the substrate for the 25-hydroxylase that catalyzes the conversion of 5β-cholestane-3α,7α,12α,26-tetrol (IIIb) into 5β-bufol (VI).

In the biosynthetic experiments with [4-^{14}C]cholesterol, the chief bile acid of *Bufo vulgaris formosus*, 3α,7α,12α-trihydroxy-5β-cholest-22-ene-24-carboxylic acid, did not become labeled (15). The absence of incorporation of radioactivity into the unsaturated C_{28} bile acid was also observed in a similar experiment and after the injection of [2-^{14}C]mevalonate into toads (18). Une, Kuramoto, and Hoshita (6) suggested that the bio-origin of 3α,7α,12α-trihydroxy-5β-cholest-22-ene-24-carboxylic acid is a C_{28} sterol, campesterol, which has been found as a minor sterol in the liver of *Bufo vulgaris formosus* (19). In the present study, we could not discover campesterol, the postulated precursor for the biosynthesis of 3α,7α,12α-trihydroxy-5β-cholest-22-ene-24-carboxylic acid, in *Bufo marinus* bile. This fact may be explained by the absence of the unsaturated C_{28} bile acid in this toad.

One (T.H.) of the authors had suggested that in *Bufo vulgaris formosus* 3α,7α,12α-trihydroxy-5β-cholest-23-en-26-oic acid (VIIb) is formed from 3α,7α,12α-trihydroxy-5β-cholest-22-ene-24-carboxylic acid by the pathway including the decarboxylation along with the migration of the Δ^{22}-double bond to the Δ^{23}-position followed by the reoxidation of the terminal methyl group of the side chain (1). However, the present study demonstrates that *Bufo marinus* bile contains 3α,7α,12α-trihydroxy-5β-cholest-23-en-26-oic acid (VIIb) but not 3α,7α,12α-trihydroxy-5β-cholest-22-ene-24-carboxylic acid. It seems, therefore, likely that the biosynthetic precursor of 3α,7α,12α-trihydroxy-5β-cholest-23-en-26-oic acid (VIIb) is not 3α,7α,12α-trihydroxy-5β-cholest-22-ene-24-carboxylic acid. The simultaneous detection of three pairs of the saturated C_{27} bile acids (IVa, IVb, and VIII) and their Δ^{23}-derivatives (VIIa, VIIb, and IX) in the bile of *Bufo marinus* suggests the possibility that the latter, IVa, IVb, and VII, are formed from the former, VIa, VIb, and IX, respectively, by the dehydrogenation at C-23 and C-24 to introduce the Δ^{23}-double bond.

The postulated pathways for the biosynthesis of bile acids and bile alcohols in the Bufonidae are shown in **Fig. 3.**

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