The minor bile acids of the toad, *Bufo vulgaris formosus*1

M. Une, T. Kuramoto, and T. Hoshita
Institute of Pharmaceutical Sciences, Hiroshima University School of Medicine, Hiroshima, Japan

**Abstract** Bile from the toad, *Bufo vulgaris formosus*, was found to contain a number of minor bile acids along with two major bile acids, 3α,7α,12α-trihydroxy-5β-cholest-22-ene-24-carboxylic acid and 3α,7α,12α-trihydroxy-5β-cholest-23-en-26-oic acid. The following minor bile acids 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-5α-cholestan-26-oic acid, 3α,7α,12α-trihydroxy-5α-cholestan-26-oic acid, 3α,7α,12α-trihydroxy-5α-cholestan-26-oic acid, varanic acid, and 3α,7α,12α,24-tetrahydroxy-24-methyl-5β-cholestan-26-oic acid. The fact that the toad bile contains not only cholic acid but also 3α,7α,12α-trihydroxy- and 3α,7α,12α,24-tetrahydroxy-5α-cholestan-26-oic acids, which have been recognized as biosynthetic intermediates of cholic acid in mammals, suggests that the toad is capable of synthesizing cholic acid by the same pathway as that for the biosynthesis of the C24 bile acid in mammals. Une, M., T. Kuramoto, and T. Hoshita. The minor bile acids of the toad, *Bufo vulgaris formosus*. J. Lipid Res. 1983. 24: 1468–1474.

**Supplementary key words** gas-liquid chromatography • mass spectrometry

The bile of the toad, *Bufo vulgaris formosus*, has been known to contain two unique higher bile acids, 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid and 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid (1, 2). The former is the only unsaturated C28 steroid to be included as a bile acid. Another special chemical feature of this bile acid is that the location of the carboxylic group of the C28 bile acid differs from that of the same group of all other bile acids so far identified as natural products. The latter of the two unique bile acids possesses a double bond in an unusual position. Intraperitonal injection of the toad with [4-14C]cholesterol as well as [2-14C]mevalonate led to the formation of a number of radioactive bile acids and bile alcohols, but the two unsaturated higher bile acids did not become labeled (3, 4).

In order to obtain at least partial information about the biogenesis of bile acids in the toad, a more complete knowledge of the biliary components of the toad must be secured. We have now examined the minor bile acids in the bile of this species by means of a combination of gas-liquid chromatography and mass spectrometry.

**MATERIALS AND METHODS**

Gas-liquid chromatography–mass spectrometry

GLC–MS was carried out on a Shimadzu GC-MS-7000 gas chromatograph–mass spectrometer. The following operation conditions were employed: column, 3% OV-17 (1m × 3mm); column temperature, 280°C; ionizing current, 60 μA; ionizing voltage, 70 eV.

**Reference bile acids**

Cholic acid (3α,7α,12α-trihydroxy-5β-cholan-24-oic acid) was a commercial product. Allocholic acid (3α,7α,12α-trihydroxy-5α-cholan-24-oic acid) (5), 3α,7α,12α-trihydroxy-5α-cholestan-26-oic acid (6), 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid (7), 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid (2), and 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid (1), were isolated from natural sources. Varanic acid (3α,7α,12α,24-tetrahydroxy-5α-cholestan-26-oic acid) (8) and 3α,7α,12α,24-tetrahydroxy-24-methyl-5β-cholestan-26-oic acid (9) were prepared in this laboratory according to the methods described previously.

**Extraction and fractionation of unconjugated bile acid mixture from the toad bile**

Bile was collected by putting the gallbladders of the toad into ethanol. Evaporation of the filtered solution left crude bile salts. The crude bile salts (8.5 g) were

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dissolved in 200 ml of water and the solution was acidified with 2 N HCl and then extracted with three 150-ml portions of ether. The ethereal extracts were combined and washed with three 100-ml portions of 5% Na₂CO₃ solution to extract acidic materials. The Na₂CO₃ washings were combined, acidified with 2 N HCl, and re-extracted with three 150-ml portions of ether. The pooled ethereal extracts were washed with water, dried over anhydrous Na₂SO₄, and the solvent was evaporated to dryness, leaving a residue (1.4 g) consisting of unconjugated bile acids. The bile acid mixture was methylated by the usual manner with diazomethane and then chromatographed on a silica gel column (100 g) eluted with acetone-ethyl acetate mixtures. The column effluents were monitored by thin-layer chromatography (silica gel G plate; solvent system, ethyl acetate-acetone 7:3).

RESULTS

Since biliary bile acids of the toad have been known to exist as unconjugated forms (1–4), they were isolated by ethereal extraction of the acidified bile. Preliminary GLC–MS analysis of the ethereal extracts revealed a complex bile acid mixture. Thus, a group separation was undertaken to simplify the bile acid pattern and to purify the sample further. The bile acid mixture was methylated and chromatographed on a silica gel column (100 g) to get five fractions (Table 1). Fig. 1 shows the GLC analysis of bile acid methyl esters as the TMS ether derivatives after the group separation and demonstrates the presence of at least ten different bile acids which are tentatively named as bile acids I–X. Mass fragment ions of these bile acids as the methyl ester-TMS ether derivatives are shown in Table 2 with their RRTs on GLC. The table further shows the relative abundance of the listed bile acids in the mixture as determined by GLC.

Bile acid I was identified as 3α,7α,12α-trihydroxy-5β-cholest-22-ene-24-carboxylic acid. The methyl ester-TMS ether derivative had the same RRT and mass spectrum of the authentic compound. The spectrum shows the molecular ion at m/z 692, and a series of fragment ions at m/z 602, 512, and 422 which are formed by the successive loss of the TMS groups from the molecular ion. The base peak at m/z 253 and the peak at m/z 343 represent loss of the side chain plus three and two nuclear TMS groups, respectively. The fragment at m/z 315 is derived by cleavage of the 6,7- and 9,10-bonds along with the loss of the 7α- and 12α-TMS groups.

Bile acid II was identified as 3α,7α,12α-trihydroxy-5β-cholest-26-oic acid by direct comparison of the RRT and mass spectrum with the authentic compound. The spectrum shows a weak molecular ion at m/z 680, a series of peaks at m/z 590 (M-90), 500 (M-2×90), and 410 (M-3×90), peaks at 665 (M-15), and 345 [M-(side chain + 2×90)], and the base peak at 253 [M-(side chain + 3×90)]. A weak ion was seen at m/z 303. This fragment is formed by cleavage of the 6,7- and 9,10-bonds plus the loss of 7α- and 12α-TMS groups.

Bile acid III was identified as 3α,7α,12α-trihydroxy-5β-cholest-23-en-26-oic acid. The RRT and mass spectrum of the methyl ester-TMS ether derivative of bile acid III were identical with those of the corresponding derivative of the authentic compound. In the mass spectrum, the molecular ion was absent, but the peak due to the loss of a methyl group from the molecular ion was seen at m/z 663. There were two series of fragments, one at m/z 588, 498, and 408, and a second at m/z 461, 371, and 281. The former series results from the successive loss of one, two, and three molecules of TMS-OH from the molecular ion. The latter series results from scission of the bond between C-20 and C-22 followed by the successive loss of TMS-OH molecules. Generally, the base peak of the methyl ester-TMS ether derivatives of bile acids carrying the cholic acid type nucleus appears at m/z 253, [M-(side chain + 3×90)]. Although this spectrum shows a peak at m/z 253, the base peak was seen at m/z 281. These observations suggest a facile rupture of the bond between C-20 and C-22 in bile acid III which has the Δ²3 double bond.

The ratio of the RRTs between bile acid IV and bile acid I was in good agreement with the constant separating factor found between the pairs of bile acids car-

<table>
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<th>Fraction</th>
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<th>Vol.</th>
<th>Wt.</th>
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<td>937</td>
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<td>58</td>
<td>Trihydroxy-5α-higher bile acids (IV, V, VI)</td>
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<td>52</td>
<td>Cholic acid (VII, major) and allocholic acid (VIII)</td>
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<td>8</td>
<td>Cholic acid (VII) and allocholic acid (VIII, major)</td>
</tr>
<tr>
<td>E</td>
<td>25% Acetone-ethyl acetate</td>
<td>200</td>
<td>35</td>
<td>Tetrahydroxy-bile acids (IX, X)</td>
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</table>
Fig. 1. Gas-liquid chromatographic separation of bile acid methyl esters as TMS ether derivatives of each fraction after silica gel column chromatography.

Bile acids VI and VII were identified as cholic acid and allocholic acid, respectively. The RRTs and mass spectra of the methyl ester-TMS ether derivatives of bile acids VII and VIII were completely identical with those of the TMS ether derivatives of authentic methyl cholate and methyl allocholate, respectively.

Bile acid IX was identified as varanic acid. The methyl ester-TMS ether derivative of bile acid IX had the same RRT and mass spectrum as the authentic compound. The spectrum shows the base peak at m/z 588, 498, 343, and 281. The peak at m/z 321 is attributed to cleavage between C-24 and C-25 followed by elimination of four TMS-OH molecules.

DISCUSSION

Until the present study, only two major bile acids, 3α,7α,12α-trihydroxy-5β-cholest-22-ene-24-carboxylic acid (I) and 3α,7α,12α-trihydroxy-5β-cholest-23-en-26-0ic acid (III) had been isolated from toad bile. The present
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<th>V</th>
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<th>VIII</th>
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<td>762</td>
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<td>6</td>
<td>7</td>
<td>665</td>
<td>4</td>
<td>3</td>
<td>663</td>
<td>7</td>
<td>21</td>
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<td>678</td>
<td>672</td>
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<td>[M-90]</td>
<td>602</td>
<td>29</td>
<td>49</td>
<td>590</td>
<td>3</td>
<td>4</td>
<td>588</td>
<td>7</td>
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<td>678</td>
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<td>[M-90 × 2]</td>
<td>512</td>
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<td>27</td>
<td>500</td>
<td>100</td>
<td>100</td>
<td>498</td>
<td>12</td>
<td>22</td>
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<td>[M-(C_{22}-C_{27} + 90)]</td>
<td>461</td>
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<td>6</td>
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<td>8</td>
<td>8</td>
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<td>408</td>
<td>13</td>
<td>11</td>
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<td>343</td>
<td>34</td>
<td>77</td>
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<tr>
<td>[M-(side chain + 90 × 3)]</td>
<td>315</td>
<td>303</td>
<td>30</td>
<td>70</td>
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<td>27</td>
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<td>% of total</td>
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<td>32.5</td>
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<td>4.1</td>
<td>0.9</td>
<td>2.0</td>
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<tr>
<td>RRT&lt;sup&gt;d&lt;/sup&gt; on GLC&lt;sup&gt;f&lt;/sup&gt;</td>
<td>1.40</td>
<td>1.24</td>
<td>1.63</td>
<td>1.43</td>
<td>1.72</td>
<td>1.53</td>
<td>1.00</td>
<td>0.88</td>
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<tr>
<td>Ratio of 5α/5β</td>
<td>0.89</td>
<td>0.89</td>
<td>0.89</td>
<td>0.88</td>
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<sup>a</sup> Bile acids 1-10 were identified as follows: 1, 3a,7a,12α-trihydroxy-5β-cholesterol-22-ene-24-carboxylic acid; 2, 5α,7α,12α-trihydroxy-5β-cholesterol-26-oic acid; 3, 3a,7a,12α-trihydroxy-5β-cholesterol-23-ene-24-carboxylic acid; 4, 3a,7a,12α-trihydroxy-5α-cholesterol-22-ene-24-carboxylic acid; 5, 3a,7a,12α-trihydroxy-5α-cholesterol-26-oic acid; 6, 3a,7α,12α-trihydroxy-5α-cholesterol-23-ene-24-carboxylic acid; 7, cholic acid; 8, lithocholic acid; 9, 3a,7α,12α,24-tetrahydroxy-24-methyl-5β-cholesterol-26-oic acid.

<sup>b</sup> Fragment A = side chain

<sup>c</sup> Fragment B = side chain + CH$_2$

<sup>d</sup> Relative to methyl cholate-TMS ether.

<sup>f</sup> Column, 3% OV-17; column temperature, 280°C.
study demonstrates the presence of the following minor bile acids in toad bile: 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid (II); 3α,7α,12α-trihydroxy-5α-cholestan-26-oic acid (V); 3α,7α,12α-trihydroxy-5α-cholest-22-ene-24-carboxylic acid (IV); 3α,7α,12α-trihydroxy-5α-cholest-23-en-26-oic acid (VI); cholic acid (VII); allocholic acid (VIII); varanic acid (IX); 3α,7α,12α,24-tetrahydroxy-24-methyl-5β-cholestan-26-oic acid (X). The two unsaturated 5α-bile acids (IV and VI) were not fully identified because of the lack of standards. It is well known that mass spectra of isomeric 5α- and 5β-bile acids are similar, i.e., the process of fragmentation is essentially the same (10). Only occasional differences are discerned in the relative intensities of selected fragment ions. Elliott (11) has reported a more facile elimination of the 3α-TMS group in 5β-bile acids than in their 5α-isomers. The intensity of the fragment at m/z 343 [M-(side chain + 2 × 90)] is greater in the latter than in the former. It has been reported that the intensity of the fragment ion resulting from cleavage of the 6,7- and 9,10-bonds in the spectra of the methyl ester-TMS ether derivatives of 5α-bile acids is greater than in the comparable 5β-bile acids (11). Based on this evidence, tentative identifications of IV and VI were made not only by the comparisons of their gas-liquid chromatographic retention time ratios but also by the comparison of their mass spectra with the two major 5β-bile acids, I and III, respectively. The remainder of the minor acids were identified with certainty by direct comparison of their chromatographic properties and mass spectra to those of authentic compounds.

The presence of cholic acid (VII) in toad bile had been postulated since labeled cholic acid was obtained from the toad that received [2,14C]mevalonate (4). The study described here confirmed the presence of this C24 bile acid (VII). The biosynthetic sequence between cholesterol and cholic acid in mammals has been extensively studied (12). At the present time, it is believed that the major pathway for the mammalian cholic acid biosynthesis involves the following intermediates (Fig. 2): cholesterol (XI) → cholest-5-ene-3β,7α-diol (XII) → 7α-hydroxycholest-4-en-3-one (XIII) → 7α,12α-dihydroxycholest-4-en-3-one (XIV) → 5β-cholestane-3α,7α,12α-triol (XV) → 5β-cholestan-3α,7α,12α,26-tetrol (XVI) → 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid (II) → varanic acid (IX) → cholic acid (VII). The presence and formation from cholesterol of the tetrol (XVI) in the toad has been reported by Hoshita et al. (3). The demonstration of the previous and present studies that the intermediates, 5β-cholestanetetrol (XVI), trihydroxy-5β-cholestanolic acid (II), and varanic acid (IX), occur in toad bile strongly suggest that the pathway for the synthesis of cholic acid (VII) in the toad is the same as that in mammals.

The present study also demonstrates the presence of another C24 bile acid which was identified as the 5α-isomer of cholic acid, allocholic acid (VIII). Current information suggests that allocholic acid is derived from either cholestanol or cholesterol. Cholestanol has been shown to be converted to allocholic acid in rats (13) and gerbils (14) by a pathway similar to that for the biosynthesis of cholic acid from cholesterol. However, it is hardly conceivable that the major source of the 5α-bile acid (VIII) found in toad is cholestanol, since very little...
of this 5α-sterol was found in toad liver (our unpublished observation). Hoshita, Shefer, and Mosbach (15) have shown that liver microsomes from the green iguana, a species in which the major biliary bile acid is the taurine conjugate of allocholic acid, convert 7α,12α-dihydroxycholest-4-en-3-one (XIV) into 5α-cholestan-3α,7α,12α-triol (XVII) rather than the 5β-isomer (XV) involved in cholic acid biosynthesis. This indicates that allocholic acid can be formed from cholesterol by a modification of the biosynthetic pathway to cholic acid in which the only difference is the stereospecific saturation of Δ4-double bond of XIV. Thus, 7α,12α-dihydroxycholest-4-en-3-one (XIV) is the last intermediate common to both cholic acid (VII) and allocholic acid (VIII). 5α-Cholestane-3α,7α,12α,26-tetrol (XVIII) and 3α,7α,12α-trihydroxy-5α-cholestan-26-oic acid (V) seem to be the intermediates in the pathway of biosynthesis of allocholic acid (VIII) from the 5α-cholestanetriol (XVII). Hoshita et al. (3) have reported the occurrence and formation from cholesterol of the 5α-tetrol (XVIII) in the toad, and the present study demonstrates the presence of the trihydroxy-5α-cholestanolic acid (V) in toad bile. These facts suggest that in the toad a lesser amount of 7α,12α-dihydroxycholest-4-en-3-one (XIV), a greater fraction of which is converted to 5β-cholestan-3α,7α,12α-triol (XV), is transformed into 5α-cholestan-3α,7α,12α-triol (XVII) and then into the 5α-C27-tetrol (XVIII), the 5α-C27-bile acid (V), and allocholic acid (VIII).

The present study demonstrates the presence in a lesser amount of the saturated 5β-C28 bile acid, 3α,7α,12α,24-tetrahydroxy-24-methyl-5β-cholestan-26-oic acid (X). The structure of this tetrahydroxy-C28 bile acid (X) closely resembles that of varanic acid (IX). The only difference between X and IX is the existence of the C-28 methyl group. A small amount of 24-methylcholesterol, presumably campsterol (XX), has been found in toad liver (16), it is likely that the C28 bile acid (X) is formed from the C28 sterol (XX) by a pathway similar to that for the biosynthesis of the C27 bile acid (IX) from cholesterol. In contrast to IX, a part of which is further oxidized to cholic acid (VII) with the intermediary formation of the β-keto acid, the 24-methylated bile acid (X) would not be further oxidized since the 24-hydroxyl group is tertiary and could not be oxidized to a keto group.

The present finding of 3α,7α,12α,24-tetrahydroxy-24-methyl-5β-cholestan-26-oic acid (X) suggests a biochemical origin of the most abundant toad biliary bile acid, 3α,7α,12α-trihydroxy-5β-cholesterol-22-ene-24-carboxylic acid (I). Although the position of the carboxyl group of the major C28 bile acid (I) differed from that of the same group of the minor C28 bile acid (X), the side chain carbon skeletons of these bile acids are identical to each other and to that of campsterol (XX). Since the oxidation of the C-26 methyl group of campsterol (XX) would produce the carboxyl group of X, whereas the oxidation at C-28 methyl group would lead to the carboxyl group of I, it is not unreasonable to assume that the C28 sterol (XX) is the source common to both the C28 bile acids, I and X (Fig. 3).

3α,7α,12α-Trihydroxy-5α-chol-22-ene-24-carboxylic acid (IV) seems to be formed from campsterol (XX) by a modification of the pathway to the 5β-isomer (I).

Recently, Ali, Stephenson, and Elliott (17) reported the presence of 3α,7α,12α-trihydroxy-5β-cholesterol-23-en-26-oic acid (III) in bile of Varanus monitor, the major bile acid of which is varanic acid (IX). The co-existence and the structural relationship of these C27 bile acids suggest that in this reptile the Δ23-C27 bile acid, III, is a biological dehydration product of varanic acid (IX). However, it seems unlikely that in the toad, the second principal bile acid, III, is a metabolite of varanic acid (IX), because in our previous investigations (3, 4) with labeled cholesterol as well as mevalonate, the label was incorporated into cholic acid and its biosynthetic precursors in the toad, but the two major bile acids, III and I, did not become labeled. Until other evidence...
becomes available, we postulate that 3α,7α,12α-trihydroxy-5β-cholest-23-en-26-oic acid (III) is a metabolite of 3α,7α,12α-trihydroxy-5β-cholest-22-en-24-carboxylic acid (I), because decarboxylation and migration of the double bond followed by oxidation of the terminal methyl group of the latter (I) would lead to the former (III). If this is correct, 3α,7α,12α-trihydroxy-5α-cholest-23-en-26-oic acid (VI) would be formed from the unsaturated 5α-C27 bile acid (IV) by a pathway analogous to that for the formation of the 5β-isomer (III).

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REFERENCES


