Bile Acids

XVII. METABOLISM OF \(\alpha\)-MURICHOLIC ACID-24-C\(^{14}\) IN THE RAT\(^*\)

GEORGE D. CHERAYIL,† S. L. HSIA, JOHN T. MATSCHINER,‡ E. A. DOISY, JR., WILLIAM H. ELLIOTT, SIDNEY A. THAYER, AND EDWARD A. DOISY

From the Department of Biochemistry, St. Louis University School of Medicine, St. Louis 4, Missouri

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\(\alpha\)-Muricholic acid (3\(\alpha\),6\(\beta\),7\(\alpha\)-trihydroxy-5\(\beta\)-cholanoic acid)\(^1\) and \(\beta\)-muricholic acid (3\(\alpha\),6\(\beta\),7\(\beta\)-trihydroxy-5\(\beta\)-cholanoic acid), which are constituents of rat bile (2), are metabolites of cheno-
dehydroxycholic acid in both the rat (2-5) and the mouse (6). The \(\alpha\) acid was found to be present in slightly higher concentration than the \(\beta\) acid in rat bile (2). It was also observed that the latter acid is the principal bile acid in the urine of surgically jaundiced rats (4). These and other observations have led to the suggestion that \(\alpha\)-muricholic acid may be an intermediate in the formation of \(\beta\)-muricholic acid and that the inversion of the 7-hydroxy group may proceed through the formation of a 7-keto intermediate (7, 8). The metabolic conversion of the \(\alpha\) acid to the \(\beta\) acid in the rat was reported by Samuelsson (8) and Cherayil et al. (9).

This paper reports our study on the metabolism of \(\alpha\)-muricholic acid in surgically jaundiced rats. During the course of the identification of metabolites of \(\alpha\)-muricholic acid, it became apparent that at least one new radioactive compound was present in the urine. This led to a consideration of the possible reactions of the ketol postulated to be an intermediate between \(\alpha\) and \(\beta\)-muricholic acids. Since it appeared likely that ring B which bears the ketolic structure might be ruptured (10), the seco lactone was prepared. It proved to be identical with the unknown radioactive compound present in the urine.

EXPERIMENTAL PROCEDURE\(^2\)

Synthesis of \(\alpha\)-Muricholic Acid-24-C\(^{14}\)—\(\alpha\)-Muricholic acid was prepared by a procedure previously described (11). After acetylation and chromatographic purification (2) of the triacetate, the norbromide was prepared (12), chromatographed on neutral alumina, and crystallized from aqueous methanol. The properties of this new compound, 23-bromo-24-nor-5\(\beta\)-cholane-3\(\alpha\),6\(\beta\),7\(\alpha\)-triacetate were: m.p. 135-137\(^\circ\); \(\nu_{\text{max}} = 1742\) (acetate), 1239 to 1229 (acetate), 1131, 1036, 931, 893 cm\(^{-1}\).

\[\text{C}_{39}\text{H}_{56}\text{O}_{3}\text{Br} \]

Calculated: C 61.15, H 7.96, Br 14.63

Found: C 61.06, H 8.05, Br 14.30

Carboxyl-labeled \(\alpha\)-muricholic acid was prepared from this bromide through a nitrile synthesis by the method which was utilized previously (3, 13) to label other bile acids. It was purified by chromatography and repeated crystallization from aqueous acetic acid and finally from a mixture of acetone and petroleum ether, m.p. 198-199\(^\circ\); melting point of mixture with authentic \(\alpha\)-muricholic acid showed no depression; specific ac-
tivity, \(1.8 \times 10^4\) d.p.m. per mg.

Preparation of Compounds for Isotopic Dilution

3\(\alpha\)-Hydroxy-6,7-seco-5\(\beta\)-cholane-6,7,24-triolic Acid 3,6-lactone (I) and Dimethyl Ester (II)—The lactone (I) was prepared according to Yamasaki and Chang (14) and purified by partition chromatography (9). It was eluted in Fractions 40-2 to 40-4 and crystallized from a mixture of acetone and hexane, m.p. 232-234\(^\circ\) (reported, 228-229\(^\circ\)) (14). The dimethyl ester (II) was prepared with diazomethane and after chromatography was found in Fractions 0-3 and 0-4. It had the following characteristics: platelets from aqueous methanol, m.p. 158-159\(^\circ\) (reported, 157-158\(^\circ\)) (14); \(\nu_{\text{max}} = 1776, 1761\) (la&one), 1733 (methyl ester), 1166 cm\(^{-1}\) (methyl ester) (Scheme 1).

Two Monomethyl Esters of Lactone (III) and (IV)—The dimethyl ester (II) (25 mg) was dissolved in 5 ml of methanol and 5 ml of 5% NaOH and heated on a steam bath for 10 min. with rock salt optics. C\(^{14}\) was measured with a Packard Tri-Carb liquid scintillation counter.

1 Yamasaki and Chang (14), who first prepared this compound, named it neodesoxythilobilianic acid. Ziegler (15) subsequently named it 3a-hydroxythilobilianic acid-3,5-lactone after thilobilianic acid of Wieland and Dane (16). Throughout this paper, the systematic name is used to signify the lactone linkage between the hydroxyl group at position 3 and the carboxylic acid formed by oxidation of carbon 6.

2 The designations of the fractions have been abbreviated according to the percentage of benzene in Skellysolve B. For example, 40-2 is the second fraction of the eluent containing 40% benzene in Skellysolve B.
Since carbon 7 is more hindered sterically than carbon 24, this compound was therefore tentatively regarded as the 7-monomethyl ester (IV).

Another monomethyl ester was obtained after the lactone (I) was dissolved in methanolic HCl (6 ml of concentrated hydrochloric acid per 100 ml of methanol) and kept at room temperature overnight. It was crystallized from acetone, m.p. 279-280°C; νmax = 1742 (lactone), 1730 (methyl ester), 1712 (carboxyl), 1171 cm⁻¹ (methyl ester). Neutralization equivalent calculated for a monocarboxylic acid, 435; found, 443.

C₁₈H₂₃O₄
Calculated: C 69.08, H 8.81
Found: C 68.84, H 8.83

This compound was tentatively regarded as the 24-monomethyl ester (III).

Methylation of both monomethyl esters (III and IV) with diazomethane yielded the dimethyl ester (II), which was identified by melting point of mixture (158-159°C) and infrared spectrum.

Methyl 3α,6β-Diacetoxy-7β-hydroxy-5β-cholanoate (VII) Methyl 3α-acetoxy-6α,7α-epoxy-5β-cholanoate (V) was prepared as reported previously (11). Refluxing of V (3.5 g) in glacial acetic acid (75 ml) for 3 ½ hours yielded methyl 3α,6β-diacetoxy-7α-hydroxy-5β-cholanoate (VI) (2.7 g) which was crystallized from aqueous acetic acid as needles, m.p. 90-92°C. Since the crystals were solvated with acetic acid, satisfactory elemental analyses could not be obtained. A solution of 1.160 g of this

These data show that the product is a monomethyl ester.
methyl ester diacetate (VI) in 90% aqueous acetic acid was treated overnight at room temperature with 230 mg of chromic anhydride. The product, methyl 3α,6β-diacetoxy-7-keto-5β-cholanoate (VII) was extracted with ether and crystallized from aqueous methanol to yield 762 mg of needles, m.p. 101-103°.

Further purification was achieved by chromatography on silica gel; a mixture of ether and hexane (1:4) eluted most of the material which was crystallized from aqueous methanol, m.p. 104-106°; \( \lambda_{\text{max}} \) = 1751 (6β-acetoxy-7-keto), 1733 (methyl ester), 1225 (acetoxy), 1172 (methyl ester), 1036 cm\(^{-1}\) (Scheme 2).

\[
\text{C}_{18}\text{H}_{34}\text{O}_{7}
\]
Calculated: C 69.02, H 8.90
Found: C 68.91, H 9.34

**Alkaline Hydrolysis of Methyl 3α,6β-Diacetoxy-7-keto-5β-cholanoate**—Alkaline hydrolysis of the ester (VII) did not provide the desired product, 3α,6β-dihydroxy-7-keto-5β-cholanoic acid. Instead, 3α-hydroxy-6,7-seco-5β-cholane-6,7,24-triolic acid, 3,6-lactone (I) was isolated from the reaction mixture and identified as its dimethyl ester (II), thus allowing rupture of ring B.

A solution of 171 mg of the ester (VII) in 25 ml of 2% methanolic KOH was kept at room temperature overnight. After acidification with dilute HCl, 141 mg of methyl ester was extracted and chromatographed to yield 94 mg in Fractions 20-3 to 40-2. After treatment with diazomethane, the residue in Fraction 40-2 (27.5 mg) crystallized as platelets from aqueous methanol, m.p. 158-159°. The crystalline form and melting point were identical with those of the dimethyl ester of the lactone (II), thus showing rupture of ring B.

A solution of 171 mg of the ester (VII) in 25 ml of 2% methanolic KOH was kept at room temperature overnight. After acidification with dilute HCl, 141 mg of methyl ester was extracted and chromatographed to yield 94 mg in Fractions 20-3 to 40-2. After treatment with diazomethane, the residue in Fraction 40-2 (27.5 mg) crystallized as platelets from aqueous methanol, m.p. 158-159°. The crystalline form and melting point were identical with those of the dimethyl ester of the lactone (II). The structure of this compound was verified by comparison of its infrared absorption spectrum with that of authentic material.

Since the above experiment indicated the instability of the 7-keto compound (VII) in alkali, it was of interest to investigate the behavior of this compound under conditions of processing urine. A solution of 1.182 g of the 7-keto compound (VII) in methanol was treated with 25 ml of 5% aqueous NaOH at 120° in an autoclave for 34 hours. After chromatographic separation and repeated crystallization, 3α,6α-dihydroxy-7-keto-5β-cholanoic acid (VII) (141 mg, m.p. 193-196°) (18), and 3α,7β-dihydroxy-6-keto-5α-cholanoic acid (IX) (68 mg, m.p. 230-240°) (18, 19) were identified. The former acid (VIII) was identified by comparison with a sample prepared as reported previously (18), the latter by comparison with a sample prepared according to Takeda, Komeno, and Igarashi (19). A small amount of 3α-hydroxy-6,7-seco-5β-cholane-6,7,24-triolic acid, 3,6-lactone (I) was identified as its dimethyl ester (II) (3.5 mg) after the residue from the combined mother liquors of VIII, Fractions 20-1 and 20-2 contained 4%; Fractions 60-2 to 60-4, in which cr-muricholic acid is usually eluted, contained 9%; Fractions 40-l to 40-4, which usually contain the dihydroxy-cholanoic acids, contained 9%; Fractions 60-2 to 60-4, in which β-muricholic acid is usually eluted, contained 43%; and Fractions 80-3 to 100-1, in which α-muricholic acid is usually eluted, contained 15%. The column was washed with methanol to yield 8% of the chromatographed C4.

**Identification of Radioactive Metabolites**

**α-Muricholic Acid**—An aliquot (containing 17.3 mg of residue and 5.75 × 10⁵ d.p.m. of C⁴) from Fractions 80-2 to 100-2 (Fig. 1) was mixed with 42.1 mg of authentic α-muricholic acid. The diluted sample was crystallized four times from aqueous acetic acid and four times from acetone and petroleum ether. Specific activities of the crystalline samples were constant (Table I). The radioactive acid was further identified by preparation and chromatography of the methyl ester. No separation of C⁴ occurred. Calculations from the data showed that at least 75% of the C⁴ in Fractions 80-2 to 100-2 was present in α-muricholic acid.

**β-Muricholic Acid**—β-Muricholic acid was isolated from Fraction 60-3, which contained 23.9 mg of residue and 1.60 × 10⁵ d.p.m. of C⁴. The composition of the eluent is given as the percentage of benzene in Skellysolve B, and the volume is given in liters (L). Four fractions were collected for each eluent. The heights of the solid bars from the baseline to the top indicate the percentages of the chromatographed C⁴; the heights of the open bars from the baseline indicate milligrams of eluate.
TABLE I
Isotopic dilution of a-muricholic acid*

<table>
<thead>
<tr>
<th>Solvent</th>
<th>No. of crystallizations</th>
<th>Amount (mg)</th>
<th>Specific activity (d.p.m./mg $\times 10^4$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous acetic acid</td>
<td>4</td>
<td>29.1</td>
<td></td>
</tr>
<tr>
<td>Acetone + petroleum ether</td>
<td>1</td>
<td>23.4</td>
<td>0.7</td>
</tr>
<tr>
<td>Acetone + petroleum ether</td>
<td>2</td>
<td>12.7</td>
<td>10.5</td>
</tr>
<tr>
<td>Acetone + petroleum ether</td>
<td>1</td>
<td>10.2</td>
<td>10.6</td>
</tr>
</tbody>
</table>

* a-Muricholic acid, 42.1 mg, was added to 5.75 $\times 10^4$ d.p.m. (17.3 mg) from Fractions 80-2 to 100-2 to give a calculated specific activity of 1.37 $\times 10^4$ d.p.m. per mg.

TABLE II
Isolation of $\beta$-muricholic acid from Fraction 60-8*

<table>
<thead>
<tr>
<th>Solvent</th>
<th>No. of crystallizations</th>
<th>Amount (mg)</th>
<th>Specific activity (d.p.m./mg $\times 10^4$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol + H$_2$O</td>
<td>3</td>
<td>14.0</td>
<td>8.75</td>
</tr>
<tr>
<td>Acetone + petroleum ether</td>
<td>1</td>
<td>10.0</td>
<td>8.73</td>
</tr>
<tr>
<td>Acetone + petroleum ether</td>
<td>2</td>
<td>7.7</td>
<td>8.88</td>
</tr>
</tbody>
</table>

* The residue in this fraction weighed 23.9 mg and contained 1.69 $\times 10^4$ d.p.m.

Fig. 2. Chromatographic analysis of the methylated radioactive material in Fractions 40-2 to 40-4. The composition of the eluent is given as the percentage of benzene in Skellysolve B and the volume is given in milliliters (ML). Four fractions were collected for each eluent. The heights of the solid bars from the baseline to the top indicate percentages of the chromatographed C$^{14}$; the open bar indicates milligrams.

d.p.m. of C$^{14}$. The residue was crystallized three times from aqueous methanol to yield 14.0 mg of crystals with a specific activity of 8.75 $\times 10^4$ d.p.m. per mg. The specific activity remained constant throughout further crystallizations (Table II). The crystals had a melting point of 220–227° and showed no depression on admixture with an authentic sample of $\beta$-muricholic acid. By calculation, at least two-thirds of the radioactivity was present in $\beta$-muricholic acid. In an experiment designed to estimate this value more precisely, an aliquot of Fractions 60-2 to 60-4 from a second animal was diluted with authentic $\beta$-muricholic acid. After crystallization to constant specific activity with a variety of solvents, it was calculated that approximately 95% of the C$^{14}$ was present in $\beta$-muricholic acid.

3a-Hydroxy-6,7-seco-$\alpha$-chole-6,7,24-trioic Acid 3,6-Lactone (I)—The combined Fractions 40-2 to 40-4 (8.69 $\times 10^4$ d.p.m. and 3.8% of the chromatographed C$^{14}$) obtained from a rat which had received 2.82 $\times 10^4$ d.p.m. of a-muricholic-24-C$^{14}$ acid, were methylated with diazomethane and chromatographed (Fig. 2). Two distinct zones of elution of C$^{14}$ were noted; one, comprising Fractions 0-3 and 0-4, contained 21% of the chromatographed C$^{14}$, and the other, with Fractions 20-4 and 40-1, contained 63%.

An aliquot (9.07 $\times 10^4$ d.p.m.) of the combined Fractions 0-3 and 0-4 was diluted with 39.0 mg of the dimethyl ester of the lactone (II), and chromatographed. Most of the ester (II) and 6.47 $\times 10^4$ d.p.m. of C$^{14}$ were eluted in Fractions 0-4 and 20-1. The residues from these two fractions were combined and crystallized to constant specific activity. Partial hydrolysis of this product gave the monomethyl ester (IV) with a specific activity of 1.01 $\times 10^4$ d.p.m. per mg. It was methylated with diazomethane; the regenerated dimethyl ester had a specific activity of 1.03 $\times 10^4$ d.p.m. per mg (Table III). From these data, it was calculated that approximately 45% of the radioactivity in Fractions 0-3 and 0-4 (Fig. 2) was in the dimethyl ester of 3a-hydroxy-6,7-seco-$\alpha$-chole-6,7,24-trioic acid 3,6-lactone (II).

On the basis of the model experiment on alkaline hydrolysis of the 7-cholesteryl methyl ester (VII), it is apparent that in addition to a small amount of the lactone (I), a mixture of other compounds of $\alpha$ and $\beta$ configuration would result from alkaline treatment of possible 6,7-ketol metabolic intermediates in urine. After oxidation with chromic anhydride, these compounds would be identified by gas chromatography.

TABLE III
Identification of 3a-hydroxy-6,7-seco-$\alpha$-chole-6,7,24-trioic acid 3,6-lactone*

<table>
<thead>
<tr>
<th>Solvent</th>
<th>No. of crystallizations</th>
<th>Amount (mg)</th>
<th>Specific activity (d.p.m./mg $\times 10^4$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dimethyl ester</td>
<td></td>
<td>28.9</td>
<td>1.10</td>
</tr>
<tr>
<td>Methanol + water</td>
<td></td>
<td>23.6</td>
<td>1.05</td>
</tr>
<tr>
<td>Ether + acetone + water</td>
<td>2</td>
<td>20.8</td>
<td>1.04</td>
</tr>
<tr>
<td>Ether + petroleum ether</td>
<td>2</td>
<td>16.7</td>
<td>1.09</td>
</tr>
</tbody>
</table>

7-Monomethyl ester

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Amount (mg)</th>
<th>Specific activity (d.p.m./mg $\times 10^4$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ether + petroleum ether</td>
<td>10.9</td>
<td></td>
</tr>
<tr>
<td>Methanol + water</td>
<td>4</td>
<td>1.6</td>
</tr>
</tbody>
</table>

* Unlabeled 3a-hydroxy-6,7-seco-$\alpha$-chole-6,7,24-trioic acid 3,6-lactone (39.0 mg) was added to 9.07 $\times 10^4$ d.p.m. from Fractions 0-3 and 0-4 (Fig. 2) to give a calculated specific activity of 2.33 $\times 10^4$ d.p.m. per mg.

† Calculated on a molar basis to compare with the dimethyl ester.

† Formed by methylation of the 7-monomethyl ester with diazomethane.
yield either 3-keto-6,7-seco-5α-cholane-6,7,24-trioic acid (X) or its 5β isomer (XI). Accordingly, Fractions 40-1 to 40-4 were examined for the presence of radioactive 5α- and 5β-seco acids (X and XI) after oxidation in the presence of appropriate carriers. Only the 5β-seco acid was detected (Table IV).

**Examination for Other Possible Metabolites**—In our preliminary experiments, aliquots from Fractions 40-1 to 40-4 (Fig. 1) were admixed with chenodeoxycholic and 3α,6β-dihydroxy-5β-cholanic acids. After several crystallizations, the specific activities remained constant (9). However, preparation of the methyl esters and methyl ester diacetates and chromatography of diacetoxy-7-keto-5α-cholanoate by the action of dilute alkali in methanol + water. Furthermore, since no radioactive 5α-seco acid (X) could be detected, it is not likely that the intermediate could have been present in urine at the time of alkaline hydrolysis. If the radioactive lactone resulted from the presence of the postulated intermediate in voided urine, it must have been formed by air oxidation prior to hydrolysis. Alternatively, the lactone may be formed during metabolism of α-muricholic acid by rupture of ring B.

The absence of dihydroxy metabolites of α-muricholic acid in Fractions 40-1 to 40-4 indicates that elimination of a hydroxy group from ring B did not occur. Except for the small amount (approximately 0.4%) of radioactivity identified as the lactone (I) and the 3β-seco acid (XI), most of the C14 in Fractions 40-1 to 40-4 was unidentified. If the lactone was formed by metabolic rupture of the steroid nucleus, other similarly complex substances may comprise the unidentified material.

## SUMMARY

α-Muricholic acid-24-C14 was synthesized and administered to rats with ligated bile ducts. Most of the administered C14 was obtained in the urine during a 10-day period. Approximately 11% of the chromatographed C14 was found to be associated with α-muricholic acid, and 41% was associated with β-muricholic acid.

A small amount (0.4%) of the chromatographed C14 was found to be in 3α-hydroxy-6,7-seco-5β-cholane-6,7,24-trioic acid 3,6-lactone. This lactone was also formed from methyl 3α,6β-diacetoxy-7-keto-5β-cholanoate by the action of dilute alkali in the presence of air. The lactone may be a metabolite of α-muricholic acid or an artifact produced from a proposed 6,7-ketol intermediate.

## REFERENCES


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**Table IV**

Identification of 3-keto-6,7-seco-5β-cholane-6,7,24-trioic acid (X) as its trimethyl ester*  

<table>
<thead>
<tr>
<th>Solvent</th>
<th>No. of crystallizations</th>
<th>Amount (mg)</th>
<th>Specific activity (d.p.m./mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol + water</td>
<td>2</td>
<td>12.0</td>
<td>1119</td>
</tr>
<tr>
<td>Acetone + water</td>
<td>2</td>
<td>9.8</td>
<td>1130</td>
</tr>
<tr>
<td>Ether + petroleum ether</td>
<td>2</td>
<td>6.3</td>
<td>1132</td>
</tr>
</tbody>
</table>

* An aliquot containing 1.80 × 10^6 d.p.m. from Fractions 20-4 and 40-1 (Fig. 2) after hydrolysis and chromatography was mixed with 29.9 mg of 3α,5α-dihydroxy-7-keto-5β-cholanic acid (VIII) and oxidized with excess chromic anhydride. The trimethyl ester of the resulting seco acid (XI) was prepared with diazomethane and crystallized to constant specific activity. By calculation, 9% of the C14 in Fractions 20-4 and 40-1 (Fig. 2) was in compounds that formed the 5β-seco acid.


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