Synthesis of 11,12-\(^2\)H\(_2\)- and 11,12-\(^3\)H\(_2\)-labeled chenodeoxycholic and lithocholic acids


Abstract Deuterium- and tritium-labeled chenodeoxycholic acid and lithocholic acid were prepared by catalytic reduction of their respective \(\Delta^{11}\) derivatives. Structures of the intermediates and their isotopic purity were verified by chemical ionization and electron impact mass spectrometry and by nuclear magnetic resonance spectroscopy. Experimental conditions for reductive deuteration were defined which gave complete reduction of the olefin and a product of high isotopic purity. Conditions for optimal tritiation were developed with which little exchange of protons with the solvent occurred; the product had high specific activity.

To test biological stability of the label, the \(^3\)H-labeled chenodeoxycholic acid was administered simultaneously with \(^3\)H-labeled chenodeoxycholic acid to two healthy subjects and the \(^3\)H/\(^\text{H}^\text{\text{2}}\) ratio in bile was determined daily for several days. The ratio remained identical to that administered, suggesting that the 11,12-\(^3\)H label in chenodeoxycholic acid is stable during enterohepatic cycling and can be used for valid estimates of bile acid kinetics in man by the isotope dilution technique.

METHODS

Nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance (NMR) spectra were obtained on a Varian HA-100 NMR spectrometer (Varian Associates, Palo Alto, CA), and chemical shift data are presented in parts per million (ppm) relative to tetramethylsilane as an internal standard.

Mass spectrometry

Electron impact (EI) mass spectra were recorded at Argonne National Laboratory using a Perkin-Elmer model 270 mass spectrometer (Perkin-Elmer Corp., Norwalk, Conn.) with an ionization voltage of 70 eV at a source temperature of 150°C. EI spectra obtained at Syntex Research (Palo Alto, CA) were recorded on a Varian MAT CH-7, also at 70 eV ionization voltage. Chemical ionization (CI) mass spectra were obtained at Argonne National Laboratory using a Biospect (Scientific Research Instruments, Baltimore, MD) CI
mass spectrometer at a source pressure of 0.8 mm of isobutane or methane and a source temperature of 150°C.6

Both CI and EI mass spectrometry were used in the analysis of the deuterium-labeled bile acid products. The ions from the olefin and the deuterated product are separated by four mass units and, if it is assumed that both species will exhibit the same or very similar ionization properties, the ion intensities can be used to determine the mole composition of the saturated product.

Isotope purity

Isotopic composition was calculated by correcting the observed ion intensities for the small proportion of olefin containing two $^{13}$C atoms at natural abundance which will contribute to the apparent content of dideuterated bile acid. Deuterium gas (99.5 atom % $^2$H) was obtained from Matheson Gas Products, Lyndhurst, NJ 07071. Deuterated solvents, $[^3$H]acetone, $[^2$H]ethanol and $[^2$H$_4$]acetic acid (99.5 atom % $^2$H), were obtained from Aldrich Chemical Company, Inc., Milwaukee, WI 53233.

Radiochemical purity

[24-$^{14}$C]Chenodeoxycholic acid (Mallinckrodt, St. Louis, MO), previously shown by zonal scanning to be >98% radiopure (after crystallization four times from ethyl acetate), and [11,12-$^3$H$_2$]chenodeoxycholic acid were recrystallized several times from ethyl acetate, and samples of the crystals were counted to determine $^3$H/$^{14}$C ratios. Recrystallization from methanol–water was performed in a similar manner for [24-$^{14}$C]lithocholic acid (supplied by Dr. Robert H. Palmer; >98% radiopure by zonal scanning after one purification by preparative TLC) and [11,12-$^3$H$_2$]lithocholic acid. Radioactivity was determined by scintillation counting.

Chromatography of products

Resolution of saturated and unsaturated bile acid methyl ester acetates was achieved by argentation thin-layer chromatography (TLC) (10) on glass plates coated to a thickness of 250 $\mu$m with a slurry of silica gel H containing aqueous silver nitrate (12.5%, wt/vol). The TLC plates were developed with benzene–ether 80:20 (v/v). The bile acid methyl esters could not be separated by TLC unless the hydroxyl groups were acetylated.

Gas–liquid chromatography (GLC) of methyl ester acetates gave less satisfactory resolution of the saturated and unsaturated bile acids, but partial resolution could be achieved at 235°C with a 6-ft (2 mm ID) column packed with 1% SP-1000 (Supelco, Inc., Bellefonte, PA).

Melting points were determined on a Fisher–Johns melting point apparatus and are reported uncorrected.

Preparation of bile acid olefins

Unsaturated bile acids were prepared by a modification of a procedure developed by Nakada and Yamasaki (11,12). Fig. 1 shows the preparation of [11,12-$^2$H$_2$]chenodeoxycholic acid.

3α-Hydroxy-Δ$^{11}$-5β-cholenic acid

Methyl 3α-acetoxy-12α-hydroxy-5β-cholanate (5.0 g, 11.2 mmole), prepared by partial acetylation of methyl deoxycholate (13), was dissolved in 40 ml of pyridine. Fifteen ml of phosphorus oxychloride was added and the solution was maintained at 55°C for 24 hr. The reaction was quenched in ice water, and the product was extracted into ether. The crude product was crystallized three times from aqueous methanol, mp 117–119°C (reported [11] mp 116–118°C). TLC on silver nitrate-impregnated silica gel plates and development with benzene–ether 80:20 (v/v), gave $R_f$ values of 0.78 for methyl 3α-acetoxy-5β-cholanate (under these conditions methyl 3α-acetoxy-Δ$^{11}$-5β-cholenate had an $R_f$ value of 0.44). The NMR spectrum of the product showed two characteristic vinyl proton resonances at 6.10 ppm (1H, dd, $J$ = 11 and 3 Hz) and 5.40 ppm (1H, dd, $J$ = 11 and 1.5 Hz). The CI mass spectrum showed ions at $m/e$ 431 [M$^+$H$^+$] and $m/e$ 371 [M$^+$H$^+$–HOAc]. The EI spectrum showed only a very weak molecular ion at $m/e$ 430 and more intense characteristic ions at $m/e$ 370 [M$^+$–HOAc] and $m/e$ 255 [M$^+$–HOAc–side chain]. The ester was saponified by brief refluxing in 5% alcoholic KOH solution. The solution was

![Fig. 1. Synthesis of [11,12-$^3$H]chenodeoxycholic acid from cholic acid.](image-url)
Fig. 2. Chemical ionization (top) and electron impact (bottom) mass spectra of methyl 3α,7α-diacetoxy-Δ11-5β-cholenate.

Catalytic reduction of bile acid olefins

Optimal conditions for incorporation of deuterium. The goal was to find conditions giving complete reduction to a product with high isotopic purity. For convenience of analysis and purification of the product, we used the 3,7-diacetoxy derivative of chenodeoxycholic acid, but the results obtained should apply equally to the unprotected olefin. Conditions that were tested and the isotopic compositions of the products are summarized in Table 1. A typical deuteration using the selected conditions is outlined below.

Methyl 3α,7α-diacetoxy-5β-Δ11-cholenate, 180.3 mg (0.369 mmole), was dissolved in 5 ml of [O']H ethanol containing 194 ml of platinum oxide and 1 ml of [2H4]-acetic acid as catalyst. The reduction was run at room temperature under deuterium gas (10 lb/min) for 15 min. By CI (Fig. 3), the product contained 98.3% saturated product with high isotopic purity (3% 2H6, 16% 4H1, and 81% 3H2). Protecting groups were removed by saponification induced by addition of KOH (2 g/5 ml water) for 2 days at 25°C. The ethanolic solution was acidified with 1 N HCl and the acid was extracted with ether. TLC analysis (benzene-dioxane-acetic acid 55:40:2) showed a mixture of two products in a 4:1 ratio (Rf 0.43). The major product had the mobility of chenodeoxycholic acid. The minor product was presumably the 7-acetate. The product was purified by column chromatography on silica gel using a gradient of methanol in chloroform. Fractions eluting with 7–15% methanol contained [11,12-2H2]chenodeoxycholic acid.
TABLE 1. Effect of conditions on reduction of the 3,7-diacetoxy-Δ11 olefin and isotopic composition of saturated product

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Olefin</th>
<th>Solvent</th>
<th>PtO2 Catalyst</th>
<th>P atm</th>
<th>Time</th>
<th>Reduction</th>
<th>Saturated Product</th>
<th>3H6</th>
<th>3H4</th>
<th>3H4</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.15</td>
<td>3H2-acetone, 5</td>
<td>100</td>
<td>10</td>
<td>42</td>
<td>71</td>
<td>23</td>
<td>15</td>
<td>83</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.10</td>
<td>3H2-acetone, 5</td>
<td>100</td>
<td>10</td>
<td>20</td>
<td>98</td>
<td>23</td>
<td>46</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.67</td>
<td>O'H-ethanol, 10</td>
<td>126</td>
<td>18</td>
<td>1</td>
<td>67</td>
<td>6</td>
<td>13</td>
<td>81</td>
<td></td>
</tr>
<tr>
<td>3a</td>
<td>0.67</td>
<td>O'H-ethanol, 10</td>
<td>202</td>
<td>18</td>
<td>1</td>
<td>52 (84)</td>
<td>5</td>
<td>14</td>
<td>81</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.37</td>
<td>O'H-ethanol, 5</td>
<td>194</td>
<td>10</td>
<td>0.25</td>
<td>98</td>
<td>4</td>
<td>16</td>
<td>80</td>
<td></td>
</tr>
</tbody>
</table>

All reductions were carried out at room temperature. In experiment 1, pre-reduced catalyst was used, whereas in all other experiments the catalyst was reduced in situ. In experiment 3a, the material used was the product of experiment 3, i.e., it contained only 33% olefin, and at the completion of the reduction, 84% of the material has been reduced. The product of reaction 3a contained three products (resulting from loss of acetyl groups) when examined by thin-layer chromatography.

Percentage reduction was estimated by CI mass spectrometry. See text.

Percentage of deuterated species was calculated from the ion intensities of the saturated products by mass spectrometry. The data are corrected for the 13C fragments originating from the natural abundance of 13C in adjacent masses.

Earlier fractions contained the impurity. Fractions containing chenodeoxycholic acid were combined and evaporated to dryness. The product was dissolved in a small volume of ethyl acetate and treated with hexane just to the point of turbidity, at which point crystallization began. The yield was 51.6 mg (0.131 mmole, 36%) of the free acid, mp 133–138°C (reported [14] mp 138°C).

Optimal conditions for incorporation of tritium. The goal was to find conditions yielding a product of high specific activity. Exchange of protons with solvent had to be minimized; complete reduction of the olefin was deemed less important. Optimal conditions for incorporation of tritium were defined using deuterium for convenience. We elected to use the unprotected olefin to facilitate purification of the final, highly radioactive product which would be obtained after tritiation. Results are given for representative reductions with deuterium gas in Table 2. A typical tritiation using the selected conditions is described here. 3α-Hydroxy-Δ11,5β-cholenic acid (50 mg) or 3α,7α-dihydroxy-Δ11-cholenic acid (50 mg) was dissolved in 2 ml of dioxane containing 50 mg of platinum oxide. The double bond was reduced with tritium gas for 30 min. 2 Hydrogen gas was added to complete

**Fig. 3.** Chemical ionization (top) and electron impact (bottom) mass spectra of methyl 3α,7α-diacetoxy-5β-[11,12-3H]chololate.

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3 Tritiation of olefinic bile acids was carried out by New England Nuclear, Boston, Massachusetts, using carrier free tritium gas and conditions specified in the text.
jected intravenously, and the 3H/14C ratio determined. Data are corrected for the natural abundance of 13C contributed.

| Solvent   | Catalyst | % Reduction | % 3H | % 4H | % 2H | % 3H
<table>
<thead>
<tr>
<th></th>
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<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid</td>
<td>Pd-C</td>
<td>68</td>
<td>60</td>
<td>18</td>
<td>22</td>
<td>18</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>Pd-C</td>
<td>83</td>
<td>76</td>
<td>11</td>
<td>13</td>
<td>11</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>Pd-C</td>
<td>94</td>
<td>16</td>
<td>35</td>
<td>49</td>
<td>35</td>
</tr>
<tr>
<td>Dioxane</td>
<td>Pd-C</td>
<td>62</td>
<td>20</td>
<td>20</td>
<td>60</td>
<td>20</td>
</tr>
</tbody>
</table>

* Reduction conditions as in experiment 3, Table 1.
* Percentage reduction was estimated by CI mass spectrometry. See text for discussion.
* Percentage of deuterated species was calculated from the ion intensities of the saturated products by mass spectrometry. The data are corrected for the natural abundance of 13C contributed by ions at adjacent masses.

Biologic Stability

$[11,12-3\text{H}_2]$Chenodeoxycholic acid and $[11,12-3\text{H}_2]$lithocholic acid were incubated at pH 7.5 with fresh human feces. Anaerobic conditions were maintained in the growth media (thioglycollate, BioQuest) by bubbling nitrogen through the solution. Samples of the reaction mixture were taken at 4, 24, and 48 hr and distilled in a Packard oxidizer (Packard Instruments, Downers Grove, Ill.) for determination of tritium present as $^3\text{H}_2\text{O}$ (15).

In order to determine the biologic stability of the label in man during enterohepatic cycling and intestinal transit, preliminary experiments were carried out in healthy subjects, after obtaining informed consent. $[11,12-3\text{H}_2]$Chenodeoxycholic acid (46 $\mu$Ci) and $[24-\text{C}]$chenodeoxycholic acid (10 $\mu$Ci) were injected intravenously, and the $3\text{H}/^{14}\text{C}$ ratio determined in bile as described previously (3). Feces were collected for 3 days, processed as previously described (15), and samples were oxidized using a Harvey biological oxidizer (R. J. Harvey Instrument Company, Hillsdale, NJ). Efficiency was calculated by combusting $^3\text{H}$- and $^{14}\text{C}$-labeled bile acid standards which had been mixed with powdered dried rabbit feces.

RESULTS AND DISCUSSION

Preparation of deuterium-labeled bile acids

For complete reduction of the olefin, acetic acid had to be used together with ethanol. Although reduction occurred nearly as rapidly in ethanol alone, only two-thirds of the olefin was reduced and the addition of fresh catalyst still did not drive the reduction to completion. Further, traces of hydroxide ion normally present in commercial preparations of Adam's catalyst caused considerable hydrolysis of the ester groups during the course of the reaction. Homogeneous catalytic hydrogenation of olefins with Wilkinson's catalyst (tris(triphenylphosphine)chlororhodium) often gives saturated products with high isotopic purity (16), but with the bile acid olefin no reduction occurred, probably because of steric hindrance.

For a product of high isotopic purity, it was necessary to use deuterated solvents and to reduce the catalyst in situ. When reduction was carried out in acetone using prereduced Adam's catalyst (platinum dioxide, prepared by fusion of hexachloroplatinitic acid and sodium acetate), the saturated product contained only 33% $^{13}\text{H}_2$ species. We attribute the low deuterium incorporation to traces of water adsorbed on the catalyst. McKenzie, Mattox, and Kendall (17) also reported that prereduced catalyst was ineffective in reducing the $3\alpha,12\alpha$-diacetoxy-5$\beta$-$\Delta^{b(11)}$-cholenate.

This combination of deuterated ethanol–deuterated acetic acid with in situ catalyst reduction gave excellent reduction (100%) and resulted in a highly labeled product containing 75–85% $^{2}\text{H}$ species. Data presented in Table 3 summarize the isotope composition for a number of preparations of $[11,12-3\text{H}_2]$lithocholic acid and $[11,12-3\text{H}_2]$chenodeoxycholic acid prepared using the ethanol–acetic acid solvent.* The lower yield of $^{2}\text{H}$ species in lithocholic acid can be attributed to traces of protium.

\[ \text{Sample 1: } 7 \quad 27 \quad 66 \]
\[ \text{Sample 2: } 15 \quad 18 \quad 67 \]

\[ \text{Sample 1: } 4 \quad 13 \quad 83 \]
\[ \text{Sample 2: } 9 \quad 16 \quad 75 \]

* An $[11,12-3\text{H}_2]$chenodeoxycholic acid product with still higher isotopic purity (96 to 98%) has been prepared using deuteration conditions developed at Merck, Sharp & Dohme, Canada Ltd., Montreal, Canada (I. Lesk and H. Koch, personal communication); this preparation is now available from the manufacturer.
containing solvent of crystallization occluded in the crystal lattice of the unsaturated starting material. The low yield (36%) of [11,12-3H]chenodeoxycholic acid after deuteration of the di-acetate can be explained by incomplete hydrolysis at the 7 position (c.f. 14), by some loss during silica gel chromatography, and by a considerable loss during the crystallization procedure. When the reaction is carried out with greater mass, the yield should be much greater (>80%).

Preparation and radiochemical purity of tritium-labeled bile acids

The least exchange between solvent and olefin was obtained with platinum oxide in dioxane; these conditions afforded 62% reduction with 60% 2H isotopic purity. Thus, calculated specific activity of the tritiated product may be misleading in that the mass may include some of the olefin. However, the effect is small.

When [11,12-3H2]chenodeoxycholic acid or [11,12-3H2]lithocholic acid was cocrystallized with a 14C-labeled preparation of the same acid, radioactivity ratios remained constant (Table 4), thus confirming radiochemical purity of the tritiated chenodeoxycholic and lithocholic acids prepared by this method. Although this procedure indicates that label is present on the indicated bile acid, it provides no information on the amount of olefin present or the position of the tritium label.

Biologic stability

After incubation with a fecal homogenate, no radioactivity was detected in the distillate, suggesting stability of the label to bacterial enzymes, at least under these experimental conditions. After simultaneous administration of [11,12-3H]chenodeoxycholic acid and [24-14C]chenodeoxycholic acid to two healthy subjects, the 3H/14C ratio of bile was found to remain similar to that administered for 4 days. When the 3H/14C ratio of fecal radioactivity was determined, it was lower than that administered (Table 5), suggesting some loss of the label during distal intestinal passage, presumably mediated by bacterial enzymes.

Value of labeling technique

The bile acid labeling procedure described here is fairly simple and rapid once the unsaturated precursor has been prepared; it is applicable to most types of bile acids. By dehydration of the 7 or 12 hydroxyl group and reduction with deuterium or tritium, it appears possible to produce labeled deoxycholic and ursodeoxycholic acids in addition to chenodeoxycholic and lithocholic acids. Since the method requires the sacrifice of a hydroxyl group, it cannot be used to produce a labeled cholic acid.

Although our data suggest that the 11,12-3H label in chenodeoxycholic acid is stable in healthy man during enterohepatic circulation, we believe that additional validation of this label is desirable before its extensive use in studies of bile acid metabolism in man. We have recently compared the behavior of the 11,12-3H label with that of the 24-14C label in chenodeoxycholic acid and found good agreement for estimates of bile acid pool size and synthesis rate determined by each nuclide (19). Still, the loss of 3H in the fecal sample suggests that [11,12-3H]chenodeoxycholic acid resembles [2,4-3H]chenodeoxycholic acid in being stable while in the exchangeable bile acid pool, but labile when in the bacteria-rich

<table>
<thead>
<tr>
<th>Crystallization</th>
<th>3H/14C Ratioa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before</td>
<td>1.00</td>
</tr>
<tr>
<td>1</td>
<td>0.97</td>
</tr>
<tr>
<td>2</td>
<td>0.99</td>
</tr>
<tr>
<td>3</td>
<td>0.99</td>
</tr>
<tr>
<td>4</td>
<td>1.01</td>
</tr>
</tbody>
</table>

a Ratio before initial crystallization has been normalized to 1.00.

### TABLE 5. Stability of label in [11,12-3H]chenodeoxycholic acid

<table>
<thead>
<tr>
<th>Subject</th>
<th>Day</th>
<th>3H/14C Ratio</th>
<th>Day</th>
<th>3H/14C Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>0.97</td>
<td>1</td>
<td>0.88</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.96</td>
<td>2</td>
<td>0.88</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.99</td>
<td>3</td>
<td>0.86</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.99</td>
<td>4</td>
<td>0.84</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.95</td>
<td>5</td>
<td>0.83</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>0.94</td>
<td>3</td>
<td>0.81</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.95</td>
<td>4</td>
<td>0.81</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.97</td>
<td>5</td>
<td>0.80</td>
</tr>
</tbody>
</table>

* [11,12-3H]chenodeoxycholic acid (46 μCi) and [24-14C]-chenodeoxycholic acid (10 μCi) were given intravenously and bile was collected from an indwelling tube. The ratio is expressed relative to that administered which has been normalized to 1.0.

b Radioactivity was determined in bile without isolation of the chenodeoxycholic acid since it has been shown previously that virtually all of the radioactivity will be present as conjugates of chenodeoxycholic acid (22).

c Feces were collected and kept refrigerated until homogenization. A 1.0-g aliquot was combusted using a commercial sample oxidizer as described in the text.
mien of the distal intestine. Of course this label cannot be used in species that hydroxylate bile acids in position 12, but this has been observed only in the chicken (20). The [11,12-2H] bile acids would appear to be the best preparation of deuterium-labeled bile acids presently available, and should be useful for tracer studies of bile acid metabolism in pediatric and obstetric patients because radiation hazard is obviated (8,19).

In recent unpublished experiments, we have scrutinized the biologic stability of the [11,12-3H] label in lithocholic acid and found that the label may be lost in some individuals during enterohepatic cycling. If these observations are correct, [11,12-3H] lithocholic acid cannot be used for a valid estimate of bile acid kinetics in man by the isotope dilution technique. However, lithocholic acid has a unique enterohepatic circulation in that it is probably largely absorbed from the colon after deconjugation and desulfation (21), whereas most chenodeoxycholic acid is absorbed from the small intestine without deconjugation (22). Thus lability of the label in [11,12-3H] lithocholic acid may not imply lability of the label of [11,12-3H] chenodeoxycholic acid.

The biological stability of 3H bile acids prepared by the Wilzbach method also remains uncertain, since one preparation exhibited extensive loss of label (4), while another preparation, after extensive purification by partition chromatography, showed little and uniform loss when used in a small group of subjects (7). Thus, as emphasized by Panveliwalla, Pertsemlidis, and Ahrens (4), extensive validation of any preparation of 3H bile acid seems mandatory before its widespread application to the characterizing of bile acid metabolism can be accepted.

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