Synthesis of 3α,6β,7α,12β- and 3α,6β,7β,12β-tetrahydroxy-5β-cholanoic acids

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Chemical synthesis of 3α,6β,7α,12β- and 3α,6β,7β,12β-tetrahydroxy-5β-cholan-24-oic acids is described. 3α,12β-Dihydroxy-5β-chol-6-en-24-oic acid used as the starting material in the synthesis was prepared via oxidation of 3α,12α-dihydroxy-5β-chol-6-en-24-oic acid 3-hemisuccinate at C-12 followed by reduction with potassium/tertiary amyl alcohol. α-Epoxidation of the ester diacetate of 3α,12β-dihydroxy-5β-chol-6-en-24-oic acid with m-chloroperbenzoic acid followed by cleavage of the epoxide with acetic acid and alkaline hydrolysis yielded 3α,6β,7α,12β-tetrahydroxy-5β-cholan-24-oic acid (overall yield 25%). N-Methylmorpholine-N-oxide–catalyzed osmium tetroxide oxidation of the ester diacetate of 3α,12β-dihydroxy-5β-chol-6-en-24-oic acid followed by alkaline hydrolysis yielded 3α,6β,7β,12β-tetrahydroxy-5β-cholan-24-oic acid (overall yield 33%). The structures of the synthesized bile acids were confirmed from their proto nuclear magnetic resonance and mass spectral fragmentation patterns. (*Steroids* **57**:107–111, 1992)

Keywords: steroids; tetrahydroxy bile acids; nuclear magnetic resonance spectroscopy; mass spectrometry; sterols

Introduction

Bile acids are effectively absorbed from the intestine and taken up by the liver with only a small portion of these bile acids excreted in the urine. However, in cholestasis, where serum bile acid concentrations are high, relatively large amounts of bile acids are excreted in the urine, and urinary excretion becomes an important mechanism for bile acid elimination. In addition, in this disease, several highly polar bile acids are excreted in the urine.[1] Bile acids with additional hydroxyl group at C-1 and/or C-6 have already been reported in the urine of patients with cholestatic liver disease.[1–5] 1β-Hydroxy derivatives of cholic acid (3α,7α,12α-trihydroxy-5β-cholan-24-oic acid), chenodeoxycholic acid (3α,7α-dihydroxy-5β-cholan-24-oic acid), and deoxycholic acid (3α,12α-dihydroxyl-5β-cholan-24-oic acid) have all been isolated from the urine of cholestatic patients.[1–3] Of the 6-hydroxylated bile acids, 3α,6α,7α,12α-tetrahydroxy-, 3α,6α,7α-trihydroxy-, 3α,6α,12α-trihydroxy-, and 3α,6α-dihydroxy-5β-cholanoic acids have been reported.[1–6] In addition, several authors have identified 3,6,7,12-tetrahydroxy-5β-cholanoic acids[4–8] but due to unavailability of reference standards, stereochemistry of the hydroxyl groups was not assigned. Recently, synthesis of four isomeric 3α,6,7,12α-tetrahydroxy-5β-cholanoic acids has been reported.[9–11] We report the synthesis and physical characteristics of two tetrahydroxy bile acids, namely, 3α,6β,7α,12β- and 3α,6β,7β,12β-tetrahydroxy-5β-cholanoic acids.

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Experimental

Materials

Cholic acid was purchased from Sigma Chemical Co. (St. Louis, MO, USA) and chenodeoxycholic acid was purchased from Canada Packers, Inc. (Toronto, Ontario, Canada). 3α,12α-Dihydroxy-5β-chol-6-en-24-oic acid was prepared as described previously.[12] All reagents and solvents used were reagent grade and were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Deuterated chloroform and deuterated dimethylsulfoxide were purchased from Aldrich Chemical Co. and were above 99% pure. The elemental analysis of the synthesized compounds was performed at the Spang Microanalytical Laboratory (Eagle Harbor, MI, USA).

Melting points were determined on a Thermolyne 12,000 apparatus and are uncorrected. Optical rotations were obtained on a Carey model 60 spectropolarimeter with methanol as the

solvent. Infrared spectra were obtained on a Perkin-Elmer model 421 spectrophotometer (The Perkin-Elmer Corp., Norwalk, CO, USA) as KBr discs.

Thin-layer chromatography (TLC) of the bile acids was performed on precoated silica-gel plates (0.25 mm thickness, Analabs, New Haven, CT, USA). Plates were developed in a solvent system of chloroform/methanol, 100:8 (v/v) (solvent system a) or chloroform/methanol/acetic acid, 30:4:2 (v/v/v) (solvent system b). After development, spots were visualized by spraying the plates with phosphomolybdic acid (3.5% in isopropanol), followed by a spray with 10% sulfuric acid and subsequent heating at 110 C for 2 minutes.

Gas–liquid chromatography (GLC) of the methyl ester trimethylsilyl ether derivatives of the bile acids was performed on a Hewlett-Packard model 5890 gas chromatograph (Avondale, PA, USA) equipped with a split/splitless device for capillary columns. A fused silica CP-Sil-19 CB capillary column (25 m; i.d., 0.20–0.22 mm) was used with helium as the carrier gas. The injector and detector temperatures were kept at 260 C and 290 C, respectively. After injection, the oven temperature was kept at 100 C for 2 minutes and then raised at a rate of 35 C/minute to a final temperature of 280 C. Retention times are given relative to that of 5α-cholestane (retention time of 5α-cholestane, 12.15 minutes).

GLC/mass spectrometry (GLC/MS)

The GLC/MS of the synthesized bile acids (as trimethylsilyl ether derivatives of the methyl esters) was performed on a Hewlett-Packard model 5988 capillary GLC/MS operating in the electron impact mode with an ionization energy of 70 electron volts. The GLC operating conditions were identical to those described above.

Nuclear magnetic resonance (NMR) spectroscopy

The high-resolution proton NMR spectra of the synthetic bile acids were obtained in deuterated chloroform/dimethylsulfoxide mixtures (0.5 ml) on a JEOL GSX-400 spectrometer at 400 MHz.

3α,6β,7α,12α-Tetrahydroxy-5β-cholan-24-oic acid (III) and 3α,6β,7β,12α-tetrahydroxy-5β-cholan-24-oic acid (IV). These compounds were synthesized from 3α,12α-dihydroxy-5β-chol-6-en-24-oic acid (Figure 1, **I**) according to the method of Iida et al.[11] Compound **III**, mp 151–153 C (lit. melting point 154–157 C,[11]); $[\alpha]_D^{25}$ + 35.8 (c = 0.65); TLC, R_f, 0.28 (solvent system b); GLC retention time of the trimethylsilyl derivative of its methyl ester relative to 5α-cholestane, 1.56; IR, (cm^{-1}), 3,410, 988 (–OH), 1,718 (>C = O). NMR, δ0.69 (s, 3H, 18-CH_3), 1.00 (d, 3H, J = 5.4 Hz, 21-CH_3), 1.05 (s, 3H, 19-CH_3), 3.41 (brm, 1H, 3β-H), 3.64 (m, 2H, 6α,7β-H), 3.92 (m, 1H, 12β-H). Found: C, 67.77; H, 9.43%. Calculated for $C_{24}H_{40}O_6$: C, 67.89; H, 9.50%. Compound **IV**, mp 225–226 C (lit. mp 234–236 C[11]; $[\alpha]_D^{25}$ +67.8 (c = 0.65); TLC, R_f, 0.40 (solvent system b); GLC retention time of the trimethylsilyl ether derivative of its methyl ester relative to 5α-cholestane, 1.75; IR, (cm^{-1}), 3,410, 1,010 (–OH), 1,718 (>C = O). NMR, δ0.68 (s, 3H, 18-CH_3) 0.98 (d, 3H, J = 5.4 Hz, 21-CH_3), 1.04 (s, 3H, 19-CH_3), 3.39 (brm, 2H, 3β,7α-H), 3.54 (m, 1H, 6α-H), 3.90 (m, 1H, 12β-H). Found: C, 67.83; H, 9.41%. Calculated for $C_{24}H_{40}O_6$: C, 67.89; H, 9.50%. Pertinent ion fragments in the mass spectra of the trimethylsilyl ether derivatives of the methyl esters of both compounds are given in Table 1.

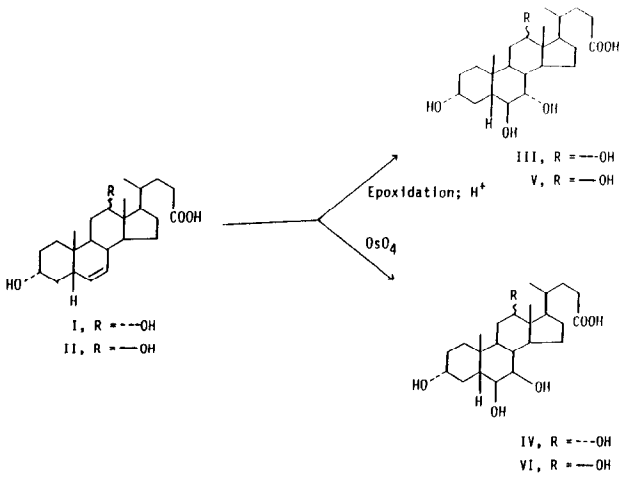

Figure 1 Synthesis of tetrahydroxy bile acids. **I**, 3α,12α-Dihydroxy-5β-chol-6-en-24-oic acid; **II**, 3α,12β-dihydroxy-5β-chol-6-en-24-oic acid; **III**, 3α,6β,7α,12α-tetrahydroxy-5β-cholan-24-oic acid; **IV**, 3α,6β,7β,12α-tetrahydroxy-5β-cholan-24-oic acid; **V**, 3α,6β,7α,12β-tetrahydroxy-5β-cholan-24-oic acid; **VI**, 3α,6β,7β,12β-tetrahydroxy-5β-cholan-24-oic acid.

3α,12β-Dihydroxy-5β-chol-6-en-24-oic acid (II). Succinic anhydride (6 g) was added to **I** (10 g) in carbon tetrachloride (50 ml) and pyridine (10 ml)[13] and the solution was refluxed for 5 hours after which period solvents were evaporated under reduced pressure and the residue was taken in ethyl acetate and washed with water. Evaporation of ethyl acetate yielded 10.7 g of 3α,12α-dihydroxy-5β-chol-6-en-24-oic acid 3-hemisuccinate, which was crystallized from ethyl acetate as colorless needles, mp 195–196 C; TLC, R_f, 0.46 (solvent system a). The hemisuccinate (10 g) was dissolved in acetone (150 ml) and cooled in ice. Jones' reagent (prepared by addition of 2.2 g chromium trioxide to 2.8 ml concentrated sulfuric acid and dilution to 10 ml with water; 20 ml) was slowly added with stirring and the yellow-brown solution was kept at room temperature for 30 minutes. Water (500 ml) was added and the white solid obtained was filtered, washed with water, and dried (9.7 g). The product, 3α-hydroxy-12-oxo-5β-chol-6-en-24-oic acid 3-hemisuccinate, was crystallized from ethyl acetate, mp 227–229 C, TLC, R_f, 0.58 (solvent system a). The 12-oxo acid 3-hemisuccinate (1 g) was hydrolyzed with 5% methanolic KOH and the resulting 3α-hydroxy-12-oxo-5β-chol-6-en-24-oic acid was crystallized from ethyl acetate (0.8 g), single spot on TLC, R_f, 0.50; mp 203–204 C; $[\alpha]_D^{25}$ +80.2 (c = 0.62) (lit. mp 200–201 C; $[\alpha]_D$, 84.51[9]). 3α-Hydroxy-12-oxo-5β-chol-6-en-24-oic acid or its 3-hemisuccinate (7 g) was dissolved in tertiary amyl alcohol (100 ml) and to the refluxing solution, potassium metal (6.2 g) was added over a period of 10 minutes.[14] After refluxing for another 30 minutes, the solution was cooled to room temperature and diluted with water (100 ml). The solution was concentrated to half its volume under reduced pressure at 40 C, cooled in ice, and acidified with 50% HCl. The white solid was filtered, washed with water, and dried. The product (5 g) was methylated and the methyl ester was chromatographed over a column of silica gel (200 g). Elution with hexane/ethyl acetate (60:40) yielded 3.9 g of methyl ester of 3α,12β-dihydroxy-5β-chol-6-en-24-oic acid (Figure 1, **II**) as a colorless semisolid that resisted crystallization, but showed single spot on TLC, R_f, 0.63, solvent system a; $[\alpha]_D^{25}$ +28.1 (c = 0.64); GLC retention time of its trimethylsilyl ether relative to 5α-cholestane, 1.47; IR (cm^{-1}) 3,400, 1,002 (–OH),

Table 1 Mass spectral fragmentation pattern of tetrahydroxy bile acids

Bile acid	Characteristic mass ion fragments (m/z; %)					
	M^+-15	M^+-90	M^+-2 × 90	M^+-3 × 90	M^+-4 × 90	Others
III	711 (2.1)	636 (3.1)	546 (63.3)	456 (75.2)	366 (23.2)	431 (14.8); 341 (100)[a]; 285 (11.1); 251 (71.6); 195 (74.4).
IV	711 (0.4)	636 (2.5)	546 (5.3)	456 (6.4)	366 (3.1)	431 (4.0); 341 (15.6); 285 (98.7); 251 (8.9); 195 (100)[b].
V	711 (3.0)	636 (3.0)	546 (55.8)	456 (86.9)	366 (34.1)	431 (46.2); 341 (100)[c]; 285 (7.4); 251 (81.1); 195 (67.2).
VI	711 (0.4)	636 (1.1)	546 (4.0)	456 (10.9)	366 (5.1)	431 (4.6); 341 (15.8); 285 (74.5); 251 (10.3); 195 (100)[d].

Note. Values in parentheses represent percentages of the various fragment ions relative to the most abundant ion fragment over 100 amu. Base-ion fragment was observed at m/z 73 in all compounds.
[a] Abundance of the ion fragment at m/z 341 relative to base-ion fragment was 24.0%.
[b] Abundance of the ion fragment at m/z 195 relative to base-ion fragment was 85.5%.
[c] Abundance of the ion fragment at m/z 341 relative to base-ion fragment was 21.6%.
[d] Abundance of the ion fragment at m/z 195 relative to base-ion fragment was 59.7%.

1,740 (>CO); NMR, δ0.72 (s, 3H, 18-CH_3), 0.87 (s, 3H, 19-CH_3), 1.00 (d, 3H, J = 5.4 Hz, 21-CH_3), 3.50 (bm, 2H, 3β, 12α-H), 3.67 (s, 3H, $-COOCH_3$), 5.45 (s, 2H, 6- and 7-H); mass spectral fragmentation (m/z) of the methyl ester trimethylsilyl ether: 73 (35.6%), 253 (100), 281 (2.9) 343 (45.0), 368 (8.9), 458 (12.6), and 533 (1.7). Further elution of the column with hexane/ethyl acetate (50:50) yielded 0.9 g of methyl 3α,12α-dihydroxy-5β-chol-6-en-24-oate, R_f, 0.54; GLC retention time of its trimethylsilyl ether relative to 5α-cholestane, 1.59.

3α,6β,7α,12β-Tetrahydroxy-5β-cholan-24-oic acid (V). The methyl ester of **II** (2.5 g) was acetylated with acetic anhydride/pyridine and the diacetate methyl ester was dissolved in chloroform (20 ml) and *m*-chloroperbenzoic acid (3 g) was added. The resulting solution was kept in the dark for 48 hours and then washed with 1N NaOH solution followed by water washing. The organic layer was evaporated to dryness when a colorless oily residue was obtained. The residue was refluxed with acetic acid (30 ml) for 3 hours, cooled to room temperature and diluted with excess cold water. The resulting solid was filtered and refluxed in 5% methanolic KOH (30 ml) for 2 hours. After addition of water (30 ml), the solution was concentrated to half the volume under reduced pressure at 40 C. The solution was then cooled in ice and acidified with 20% HCl to pH 2. The solid obtained was filtered, washed with water, and dried (1.8 g). This product was chromatographed over a column of silica gel (50 g) and eluted with ethyl acetate followed by increasing proportions of methanol. Elution with 5% methanol in ethyl acetate yielded 0.6 g of a white solid of **V**, crystals from ethyl acetate, mp 168–170 C; $[\alpha]_D^{25}$ +30.2 (c = 0.58). The crystals showed single spot on TLC, R_f, 0.36 (solvent system b): GLC retention time of its methyl ester trimethylsilyl ether relative to 5α-cholestane, 1.51; IR (cm^{-1}), 3,410, 998 (–OH), 1,717 (>C = O). NMR, δ0.76 (s, 3H, 18-CH_3), 1.02 (d, 3H, J = 5.6 Hz, 21-CH_3), 1.08 (s, 3H, 19-CH_3), 3.39 (brm, 2H, 3β, 12α-H), 3.67 (m, 2H, 6α,7β-H). Found: C, 67.98; H, 9.48%. Calculated for $C_{24}H_{40}O_6$: C, 67.89; H, 9.50%. Pertinent ion fragments in the mass spectrum of its methyl ester trimethylsilyl ether are given in Table 1.

3α,6β,7β,12β-Tetrahydroxy-5β-cholan-24-oic acid (VI). The diacetate methyl ester of **II** (2.5 g) was dissolved in tertiary butanol/tetrahydrofuran/water [10:3:1 (v/v/v); 25 ml] and to the solution was added *N*-methylmorpholine *N*-oxide (2.2 g) and osmium tetroxide (50 mg). The mixture was kept at room temperature for 18 hours and the dark brown solution was poured onto water and extracted with ethyl acetate. The organic layer was washed successively with water, 10% HCl, 5% sodium bicarbonate, and finally water and then evaporated to dryness. The oily residue was refluxed in 5% methanolic KOH (30 ml) for 2 hours and worked up exactly as described above for the synthesis of **V**. The product (1.8 g) was chromatographed over a column of silica gel (45 g) and eluted with chloroform/methanol mixtures. Elution with 20% methanol in chloroform yielded 0.75 g of a white residue of **VI**, which was crystallized from ethyl acetate, mp 204–206 C: $[\alpha]_D^{25}$ + 60.3 (c = 0.74). The compound showed single spot on TLC, R_f, 0.59 (solvent system b); GLC retention time of the methyl ester trimethylsilyl ether derivative relative to 5α-cholestane, 1.89; IR (cm^{-1}), 3,410, 1,005 (–OH), 1,718 (>C = O). NMR, δ0.74 (s, 3H, C-18), 1.00 (d, 3H, J = 5.6, C-21), 1.07 (s, 3H, C-19), 3.39 (brm, 3H, 3β,7α,12α-H), 3.55 (m, 1H, 6α-H). Found: C, 67.81; H, 9.46%. Calculated for $C_{24}H_{40}O_6$: C, 67.89; H, 9.50%. Pertinent ion fragments in the mass spectrum of its methyl ester trimethylsilyl ether are given in Table 1.

Results and discussion

For the synthesis of 3α,6β,7α,12β- and 3α,6β,7β,12β-tetrahydroxy-5β-cholan-24-oic acids, 3α,12β-dihydroxy-5β-chol-6-en-24-oic acid (Figure 1, **II**) was used as the starting material. This unsaturated bile acid was prepared in good yield from the corresponding 12α-hydroxy epimer. The 12α-hydroxyl group in **I** was quantitatively oxidized after protection of the 3α-hydroxyl group as the hemisuccinate. Potassium/tertiary amyl alcohol reduction[14] of the 12-oxo compound followed by alkaline hydrolysis yielded **II**. The diaxial trans-6β,7α-diol system was then obtained via treatment of the diacetate methyl ester of **II** with m-chloroperbenzoic acid to form the 6α,7α-epoxide,[15] which on cleavage with acetic acid followed by alkaline hydrolysis yielded 3α,6β,7α,12β-tetrahydroxy-5β-cholan-24-oic acid (**V**) as the major product. To obtain

the axial-equatorial cis-6β,7β-dihydroxy epimer **(VI)**, the fully protected derivative of **II** was treated with *N*-methylmorpholine *N*-oxide in tertiary butanol/tetrahydrofuran/water mixture and catalytic amount of osmium tetroxide[11,15] followed by alkaline hydrolysis and chromatographic purification.

In order to establish the stereochemistry of the hydroxyl groups at C-6 and C-7, 3α,6β,7α,12α- and 3α,6β,7β,12α-tetrahydroxy-5β-cholan-24-oic acids **(III, IV)** were synthesized according to the recently published method of Iida et al.[11] and their ^{1}H NMR spectra were compared with those of **V** and **VI.** As expected, the 19-methyl signal was deshielded in both **III** (1.05 ppm) and **IV** (1.04 ppm) due to its 1,3-diaxial interaction with the 6β-hydroxyl group.[11] This signal was deshielded by another 0.03 ppm in the spectra of the 12β-hydroxy epimers **V** and **VI** due to the proximity of the 12β-hydroxyl group.[16,17] These resonance frequencies of the 19-methyl group are in accordance with the 6β,7(α and β),12β-trihydroxy sytem in **V** and **VI**. The 12β-H in both the 12α-hydroxy epimers **III** and **IV** resonated at approximately 3.90 ppm, and this signal shifted upfield to 3.39 ppm in the spectra of **V** and **VI** in accordance with their 12β-hydroxy configuration.[17] The 6α- and 7β-hydrogens resonated at approximately 3.65 ppm in the spectra of both **III** and **V,** whereas the 7α-hydrogen signal was observed upfield at approximately 3.39 ppm in the spectrum of the 3α,6β,7β,12β-tetrahydroxy epimer VI, as was observed in the spectrum of the 3α,6β,7β,12α-tetrahydroxy epimer **IV**.

The structures of the isomeric compounds were further established from a comparison of the mass spectral fragmentation pattern of their methyl ester trimethylsilyl ether derivatives with those of α- and β-muricholic acids.[18,19] Thus, all four tetrahydroxy compounds showed mass fragments at m/z 636, 546, 456, and 366 derived by successive losses of trimethylsilanol groups from the molecular ion, suggesting the presence of four hydroxyl groups in the parent compounds and the fragment ion at m/z 251 (derived by loss of side chain and four hydroxyl groups) suggested that all hydroxyl groups were in the ring system. α-Muricholic acid (3α,6β,7α-trihydroxy-5β-cholan-24-oic acid) and β-muricholic acid (3α,6β,7β-trihydroxy-5β-cholan-24-oic acid) have been differentiated from each other by their mass spectral fragmentation pattern.[19] In the mass spectrum of the methyl ester trimethylsilyl ether of α-muricholic acid (with 6β,7α-dihydroxy system), weak ion fragments are observed at m/z 285 and m/z 195 [due to scission between bonds at C-6,7 and C-9,10 (m/z 285) and an additional loss of trimethylsilanol (m/z 195)], whereas the base ion peak is at m/z 253. On the other hand, the steroid ring B cleavage is very facile in the mass spectrum of the methyl ester trimethylsilyl ether of β-muricholic acid (6β,7β-dihydroxy compound) and both ion fragments at m/z 285 and m/z 195 are very prominent in its mass spectrum. As expected for bile acids with 6β,7α-glycol system, both tetrahydroxy compounds **III** and **V** formed via epoxide cleavage showed a weak fragment ion at m/z 195, and the fragment at m/z 285 was barely observed in the mass spectra of their methyl ester trimethylsilyl ether derivatives. On the other hand, in the mass spectra of the methyl ester trimethylsilyl ether derivatives of **IV** and **VI,** formed by osmium tetroxide dihydroxylation, prominent ion fragments at m/z 285 and m/z 195 were observed completely compatible with the 6β,7β-glycal system. Also, the mass spectral fragmentation patterns of the derivatized **III** and **IV** agreed completely with those reported by Kurosawa et al.[10]

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