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Synthesis of the 1β-Hydroxylated Bile Acids, Unusual Bile Acids in Human Biological Fluids[1)]

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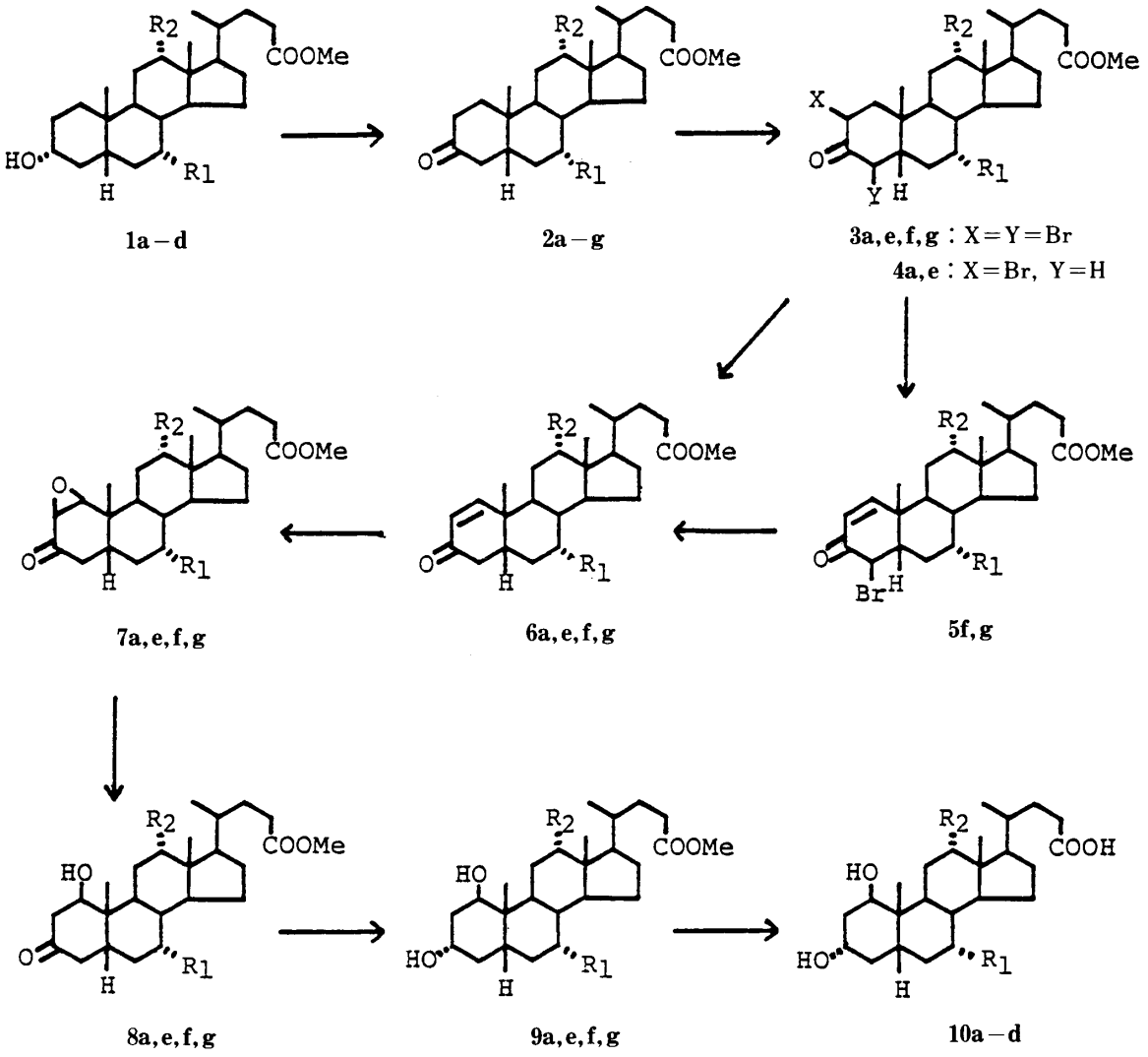
1β-Hydroxylated lithocholic (**10a**), deoxycholic (**10b**), chenodeoxycholic (**10c**) and cholic (**10d**) acids were synthesized from the corresponding bile acid methyl esters (**1a—d**) as starting materials. The Δ^1-unsaturated ketones (**6a**, **e—g**) were prepared from the 3-oxo bile acid esters (**2a—d**) *via* reductive debromination of their 2,4-dibromides (**3a**, **e—g**) with chromous acetate and dehydrobromination with lithium carbonate–lithium bromide, and oxidized with alkaline hydrogen peroxide to give the 1β,2β-epoxyketones (**7a**, **e—g**). Reductive cleavage of the epoxides (**7a**, **e—g**) and subsequent reduction with sodium borohydride afforded the 1β,3α-dihydroxy compounds (**9a**, **e—g**), which were hydrolyzed to the 1β-hydroxylated bile acids (**10a—d**). 1β,3α,12α-Trihydroxy-5β-cholan-24-oic acid (**10b**) was identified in the urine of healthy men and patients with kidney disease by gas chromatography-mass spectrometric analysis. Novel 1β,3α,7α-trihydroxy- (**10c**) and 1β,3α,-7α,12α-tetrahydroxy-5β-cholan-24-oic acids (**10d**) were detected in human meconium.

Keywords——1β-hydroxylated bile acid synthesis; 1β,3α-dihydroxy-5β-cholan-24-oic acid; 1β,3α,7α-trihydroxy-5β-cholan-24-oic acid; 1β,3α,12α-trihydroxy-5β-cholan-24-oic acid; 1β,3α,-7α,12α-tetrahydroxy-5β-cholan-24-oic acid; methyl ester–trimethylsilyl ether derivative; GC-MS; urine; human meconium

Since 3β-hydroxy-5-cholenoic acid was found in the urine of infants with biliary atresia by Makino *et al.*,[2)] much interest has been focused on the abnormal metabolism of bile acids and the formation of the unusual bile acid in connection with hepatobiliary diseases.[3)] Almé *et al.*[4)] found bile acids hydroxylated at the C-1 position in the urine of patients with cholestasis, and tentatively identified them as 1,3,12-trihydroxy- and 1,3,7,12-tetrahydroxycholanoic acids from the inspection of spectral data obtained by gas chromatography-mass spectrometric (GC-MS) analysis. These bile acids have been also detected in the urine of women in late pregnancy[5)] and newborn infants,[6)] and in human meconium.[7)]

It is desirable to obtain authentic samples of these bile acids for further chemical characterization and to aid the development of a specific microassay. Carlström *et al.*[8)] reported on the preparation of 1β,3α,12α-trihydroxy-5β-cholanoic acid together with the 15β-hydroxylated bile acid by microbial transformation of deoxycholic acid with the molds *Penicillium* sp. ATCC 12556, and in the case of cholic acid they obtained trace amounts of the 1β-hydroxylated metabolite. On the other hand, Herz and Ocampo[9)] synthesized methyl 1α,3α-dihydroxy-5β-cholan-24-oate *via* the 1α,2α-oxido-4,6-choladien-3-one from methyl 3β-hydroxy-5-cholenoate as a starting material.

As a continuation of our investigation on the quantitative determination of unusual bile acids in liver diseases,[10)] we wish to report here the synthesis of the 1β-hydroxylated lithocholic (**10a**), deoxycholic (**10b**), chenodeoxycholic (**10c**) and cholic (**10d**) acids and the identification of unusual bile acids (**10b—d**) in urine of healthy men and patients with kidney disease and in human meconium.



	R_1	R_2		R_1	R_2
a :	H	H	**e** :	H	OAc
b :	H	OH	**f** :	OAc	H
c :	OH	H	**g** :	OAc	OAc
d :	OH	OH			

Chart 1

The 1β-hydroxylated bile acids (**10a—d**) were synthesized from the corresponding lithocholic (**1a**), deoxycholic (**1b**), chenodeoxycholic (**1c**) and cholic (**1d**) acid methyl esters as starting materials according to the reaction scheme shown in Chart 1. The 3-keto bile acid methyl esters (**2a—d**) were prepared by Jones oxidation of methyl lithocholate (**1a**) and by the selective oxidation of the hydroxyl group at the C-3 position of the other starting materials (**1b—d**) with silver carbonate on Celite in refluxing toluene according to the procedure of Tserng.[11] Prior to the subsequent bromination, the remaining hydroxyl groups of **2b—d** were acetylated with a mixture of acetic anhydride, dimethylaminopyridine and triethylamine to give the corresponding acetates (**2e—g**) in high yields. Then bromination of the 3-ketones (**2a, e—g**) with 2 equivalents of bromine in acetic acid afforded the 2β,4β-dibromoketones (**3a, e—g**) in reasonable yields. The nuclear magnetic resonance (NMR) spectra of these compounds

(**3a**,**e**—**g**) showed the 2α- and 4α-proton signals at around 4.5 ppm as a pair of doublets (J=4.5 and 13 Hz) and at 5.0 ppm as a doublet (J=12 Hz), respectively. The mass spectra (MS) showed the presence of two bromine substituents as three peaks based on the equivalent isotope ratio of bromine.

Selective debromination at the C-4 position of the lithocholic (**3a**) and deoxycholic (**3e**) acid derivatives was performed by treatment with chromous acetate in acetic acid under a nitrogen atmosphere to yield the 2β-monobromides (**4a**, **e**). The structures of **4a** and **4e** were defined on the basis of their NMR spectra, which showed the 2α-proton resonance at 4.68 and 4.44 ppm as doublet of doublets (J=4.5 and 13 Hz), and lacked the 4α-proton resonance at 5.0 ppm in **3**. However, chenodeoxycholic (**3f**) and cholic (**3g**) acid dibromides afforded a mixture of the corresponding 2β- and 4β-monobromides under the above conditions.[12]

This fact is presumably a result of the distorted ring conformation owing to the steric hindrance of the 7α-acetoxy group. Joly and Warnant[13] prepared 17β-acetoxy-4-bromo-3-oxo-1-etiocholene from the corresponding 2,4-dibromide by dehydrobromination with lithium carbonate. According to this procedure, the above dibromides (**3f**, **g**) were converted into the 4β-bromo-Δ^1-3-ketones (**5f**, **g**) by heating with lithium carbonate and lithium bromide in dimethylformamide at 80 °C. The infrared (IR) spectra of **5f** and **5g** exhibited a characteristic absorption of the α,β-unsaturated carbonyl function at 1680 cm^{-1}. The NMR spectra also showed the resonances of two olefinic protons near 6.0 ppm (C_2-H) and 6.8 ppm (C_1-H), and the 4α-proton resonance at around 5.3 ppm as a doublet having a J value of 13 Hz. The 2β-bromides (**4a**,**e**) were dehydrobrominated with calcium carbonate or lithium carbonate–lithium bromide in dimethylformamide to give the Δ^1-3-ketones (**6a**,**e**), the structures of which were determined from their IR spectra showing α,β-unsaturated carbonyl absorptions at 1675 cm^{-1} for **6a** and 1660 cm^{-1} for **6e**. The NMR spectra showed the resonances of the two olefinic protons at the C-1 and C-2 positions at 5.8 and 6.6 ppm, respectively, having a coupling constant of 10 Hz, and the ultraviolet (UV) spectra also suggested the Δ^1-3-oxo structure by the absorption maxima at 230 nm.[14] On the other hand, the 4β-bromides (**5f**,**g**) were readily reduced with zinc dust in acetic acid to yield the Δ^1-3-ketones (**6f**, **g**). The structures were determined from their spectral data in the same way as described above for **6a** and **6e**.

Epoxidation of the enones (**6a**, **e**—**g**) was carried out with hydrogen peroxide in alkaline media, and reesterification of the products with diazomethane gave the desired 1β,2β-epoxyketones (**7a**, **e**—**g**). The NMR spectra of these compounds showed two epoxidic protons as doublets at about 3.2—3.4 ppm with the J value of 4 Hz. Their IR spectra showed saturated carbonyl absorptions around 1700 cm^{-1} instead of the unsaturated ones. The β-orientation of the epoxide was not clear at this stage, but it was presumed by analogy with the result in the epoxidation of the 5β-cholest-1-en-3-one[15] and it was also supported by the NMR spectral data of the following reaction products. Reductive cleavage of the epoxyketones (**7a**, **e**—**g**) was achieved by treatment with chromous acetate in ethanol to give the 1β-hydroxyketones (**8a**, **e**—**g**). The structures of these hydroxyketones were characterized by their NMR spectral data revealed narrow triplet at nearly 4.0 ppm (J=3 Hz), which indicated an equatorial orientation of the C-1 proton.

In general, 5β-steroidal 3-ketones are reduced from the β-side by sodium borohydride to yield 3α-alcohols because of a steric effect of bending of ring A to the α-side.[16] Reduction of the 1β-hydroxyketones (**8a**, **e**—**g**) with sodium borohydride in methanol gave the 1β,3α-hydroxylated bile acid esters (**9a**, **e**—**g**) accompanied with the undesired 3β-isomers in the ratio of *ca.* 2 : 1 as a result of shielding of the β-face by the 1β-hydroxyl group. The structures of these compounds (**9a**, **e**—**g**) were assigned by comparison of their NMR spectral data with those of the starting materials (**1a**—**d**), which showed broad multiplet signals of the axial 3β-protons at 3.7—4.2 ppm. Finally, these esters (**9a**, **e**—**g**) were hydrolyzed with lithium

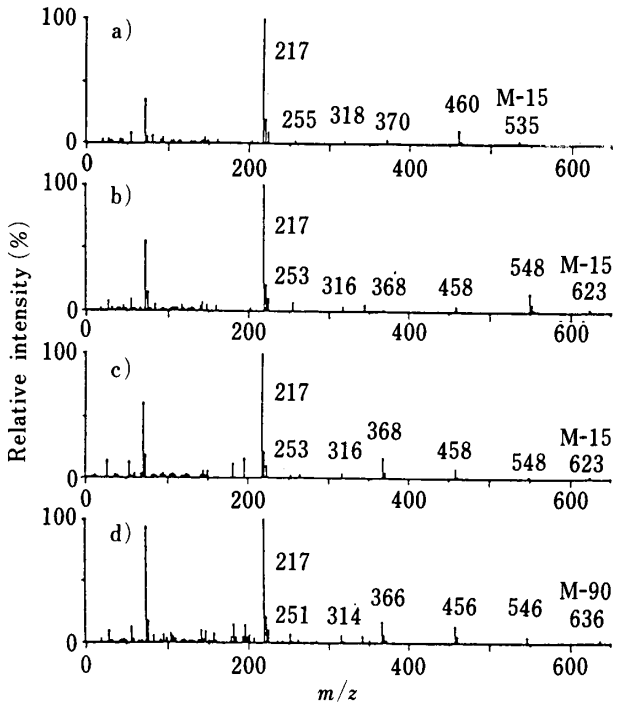


Fig. 1. Mass Spectra of the Methyl Ester–Trimethylsilyl Ether Derivatives of the Synthesized 1β-Hydroxy Bile Acids

a) 1β,3α-Dihydroxy-5β-cholan-24-oic acid (**10a**). b) 1β,3α,12α-Trihydroxy-5β-cholan-24-oic acid (**10b**). c) 1β,3α,7α-Trihydroxy-5β-cholan-24-oic acid (**10c**). d) 1β,3α,7α,12α-Tetrahydroxy-5β-cholan-24-oic acid (**10d**).

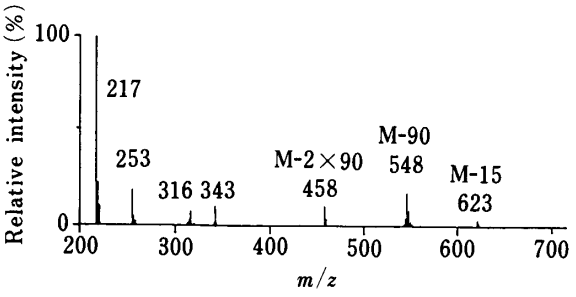


Fig. 2. Mass Spectrum of the Methyl Ester–Trimethylsilyl Ether of 1β,3α,12α-Trihydroxy-5β-cholan-24-oic Acid in Urine from a Healthy Man

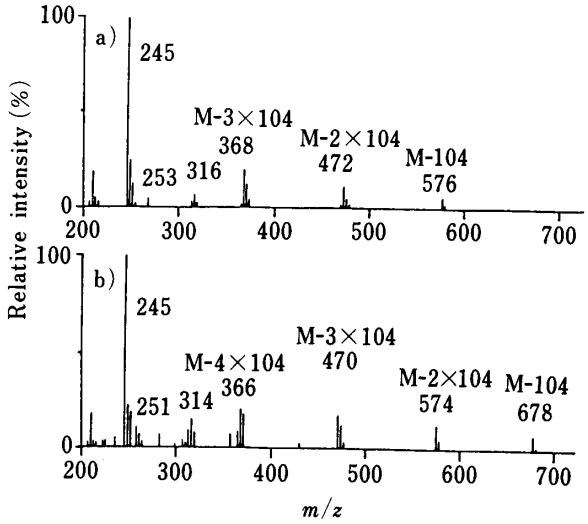


Fig. 3. Mass Spectra of the Methyl Ester–Dimethylethylsilyl Ethers of 1β,3α,7α-Trihydroxy- (a) and 1β,3α,7α,12α-Tetrahydroxy-5β-cholan-24-oic Acids (b) from Human Meconium

hydroxide in methanol to give the desired 1β-hydroxy bile acids (**10a—d**). The spectral data and other physical constants supported these structures.

The MS of the methyl ester trimethylsilyl ether derivatives of the synthesized 1β-hydroxy bile acids (**10a—d**) are shown in Fig. 1. They do not exhibit the molecular ion peak, but an additional hydroxyl group as compared with the starting bile acids is clearly apparent from the peaks of the M − 15 ions and those corresponding to loss of trimethylsilanol molecules (M − n × 90). Moreover, a characteristic base peak at m/z 217 indicated the presence of a 1,3-bistrimethylsiloxy structure,[17] as did the corresponding fragment ions at m/z 314—318 formed by loss of the A-ring and its substituents, and the ABCD ring fragment ion peaks at m/z 251—255.[18] The MS of the 1β,3α,12α-triol (**10b**) and the 1β,3α,7α,12α-tetrol (**10d**)

derivatives are very similar to those of the 1,3,12-trihydroxy and 1,3,7,12-tetrahydroxy bile acids tentatively assigned by Almé, *et al.*[4]

Extraction of the bile acids from urine samples obtained from healthy men and patients with kidney disease was performed by using Bond Elut cartridges, with good recovery. After solvolysis with hydrochloric acid in ethanol–acetone (1 : 9) at pH 1 and alkaline hydrolysis in 4 N sodium hydroxide–methanol (1 : 1), the free bile acids were methylated with diazomethane–ether and converted into the trimethylsilyl ethers with *N*-trimethylsilylimidazole. GC-MS analyses of these derivatives were carried out by the procedure described in the experimental section, and an unusual 1β,3α,12α-trihydroxy-5β-cholan-24-oic acid was identified from both urines by comparison with the retention time (7.2 min) and the MS with those of the synthesized standard (**10b**), as shown in Figs. 1b and 2. These results suggest that 1β-hydroxylated deoxycholic acid was excreted in the urine from both healthy and diseased subjects. Further GC-MS analysis of the bile acids in human meconium indicated the presence of the novel bile acid 1β,3α,7α-trihydroxy-5β-cholan-24-oic acid (**10c**) together with 1β,3α,7α,12α-tetrahydroxy-5β-cholan-24-oic acid (**10d**), which were confirmed as the dimethylethylsilyl ethers as shown in Fig. 3, because the trimethylsilyl ether of **10c** was not separated from the derivatives of cholesterol and chenodeoxycholic acid on the gas chromatogram. 1β,3α-Dihydroxy-5β-cholan-24-oic acid (**10a**) has not yet been detected in any human biological fluids. Detailed studies on the quantitative determination of the 1β-hydroxylated bile acids by GC-MS and its application to the measurement of these unusual bile acids in human biological fluids will be presented in another paper.

Experimental

Melting points were determined on a Mitamura micro hot-stage apparatus and are uncorrected. Optical rotations were taken with a Union Giken PM-201 polarimeter and IR spectra were recorded on a JASCO IR A-102 spectrometer. The UV spectra were measured on a Shimadzu UV-200 spectrophotometer. The NMR spectra were obtained at 90 MHz on a Hitachi R-40 spectrometer, and chemical shifts are given in ppm relative to tetramethylsilane as an internal standard. Abbreviations used: s = singlet, d = dublet, dd = doublet of doublets, t = tirplet, q = quartet, m = multiplet. For column chromatography, silica gel (Merck Kiesel gel 60, 70—230 mesh) was used.

Methyl 12α-Hydroxy-3-oxo-5β-cholan-24-oate (2b), Methyl 7α-Hydroxy-3-oxo-5β-cholan-24-oate (2c) and Methyl 7α,12α-Dihydroxy-3-oxo-5β-cholan-24-oate (2d)——The esters **1b**—**d** in toluene were selectively oxidized with Ag_2CO_3–Celite to give **2b**—**d**, respectively, according to the procedure of Tserng.[11] **2b**: mp 148—149 °C (lit. mp 148—149 °C). IR (Nujol): 3290, 1720 cm^{-1}. NMR ($CDCl_3$) δ: 0.72 (3H, s, 18-CH_3), 1.00 (3H, s, 19-CH_3), 3.63 (3H, s, $COOCH_3$), 3.94—4.08 (1H, m, 12β-H). MS *m/z*: 404 (M^+), 386 ($M^+ - H_2O$). **2c**: mp 131—132 °C (lit. mp 128—129 °C). IR (Nujol): 3460, 1740, 1705 cm^{-1}. NMR ($CDCl_3$) δ: 0.68 (3H, s, 18-CH_3), 0.98 (3H, s, 19-CH_3), 3.61 (3H, s, $COOCH_3$), 3.79—3.97 (1H, m, 7β-H). **2d**: mp 181—182 °C (lit. mp 181—183 °C). IR (Nujol): 3450, 1730, 1700 cm^{-1}. NMR ($CDCl_3$) δ: 0.70 (3H, s, 18-CH_3), 0.98 (3H, s, 19-CH_3), 3.63 (3H, s, $COOCH_3$), 3.80—4.06 (2H, m, 7β, 12β-H).

Methyl 12α-Acetoxy-3-oxo-5β-cholan-24-oate (2e)——*N*,*N*-Dimethylaminopyridine (0.6 g), Et_3N (2.2 g) and Ac_2O (2.2 g) were added to a solution of **2b** (8.24 g) in CH_2Cl_2 (25 ml) under cooling. After being stirred for 30 min, the reaction mixture was diluted with H_2O and extracted with CH_2Cl_2. The organic layer was washed with 2 N HCl, 5% $NaHCO_3$ and saturated NaCl, dried over anhydrous Na_2SO_4 and evaporated *in vacuo*. The residue was recrystallized from MeOH to give **2e** (9.00 g) as colorless prisms, mp 118—121 °C (lit.[19] mp 122 °C). IR (Nujol): 1730, 1710 cm^{-1}. NMR ($CDCl_3$) δ: 0.76 (3H, s, 18-CH_3), 1.00 (3H, s, 19-CH_3), 2.05 (3H, s, $OCOCH_3$), 3.62 (3H, s, $COOCH_3$), 5.02—5.16 (1H, m, 12β-H).

Methyl 7α-Acetoxy-3-oxo-5β-cholan-24-oate (2f)——Acetylation of **2c** (3.86 g) in CH_2Cl_2 (14 ml) was carried out with *N*,*N*-dimethylaminopyridine (0.29 g), Et_3N (1.07 g) and Ac_2O (1.07 g) for 1.5 h as described for **2e**. The product was recrystallized from AcOEt–hexane to give **2f** (3.67 g) as colorless needles, mp 117—118 °C. IR (Nujol): 1735, 1725 cm^{-1}. NMR ($CDCl_3$) δ: 0.70 (3H, s, 18-CH_3), 1.03 (3H, s, 19-CH_3), 2.00 (3H, s, $OCOCH_3$), 3.61 (3H, s, $COOCH_3$), 4.85—5.03 (1H, m, 7β-H). *Anal.* Calcd for $C_{27}H_{42}O_5$: C, 72.61; H, 9.48. Found: C, 72.68; H, 9.44.

Methyl 7α,12α-Diacetoxy-3-oxo-5β-cholan-24-oate (2g)——Acetylation of **2d** (1.00 g) in CH_2Cl_2 (3 ml) was carried out with *N*,*N*-dimethylaminopyridine (0.14 g), Et_3N (0.48 g) and Ac_2O (0.48 g) for 1 h as described for **2e**. The product was recrystallized from MeOH to give **2g** (1.32 g) as colorless needles, mp 200—201 °C (lit.[20] 203 °C). IR (Nujol): 1740, 1720, 1710 cm^{-1}. NMR ($CDCl_3$) δ: 0.78 (3H, s, 18-CH_3), 1.02 (3H, s, 19-CH_3), 2.04, 2.08 (2 × 3H, s, $OCOCH_3$), 3.61 (3H, s, $COOCH_3$), 4.88—5.08 (1H, m, 7β-H), 5.01—5.15 (1H, m, 12β-H).

Methyl 2β,4β-Dibromo-3-oxo-5β-cholan-24-oate (3a)——Br_2 (160 mg) in AcOH (12 ml) was added dropwise to a solution of **2a** (780 mg) in AcOH (15 ml) under ice cooling. The reaction mixture was stirred at room temperature for 1 h, and poured into ice-water. The precipitate was separated by filtration and recrystallized from MeOH–Et_2O to give **3a** (821 mg), mp 152—153 °C (lit.[21] mp 145—146 °C). IR (Nujol): 1750, 1715 cm^{-1}. NMR ($CDCl_3$) δ: 0.67 (3H, s, 18-CH_3), 1.09 (3H, s, 19-CH_3), 3.62 (3H, s, $COOCH_3$), 4.77 (1H, dd, $J_1=4.5$ Hz, $J_2=13$ Hz, 2α-H), 5.00 (1H, d, $J=12$ Hz, 4α-H). MS *m/z*: 465 (M^+-Br), 467 (M^++2-Br). *Anal.* Calcd for $C_{25}H_{38}Br_2O_3$: C, 54.95; H, 7.01. Found: C, 54.80; H, 7.01.

Methyl 12α-Acetoxy-2β,4β-dibromo-3-oxo-5β-cholan-24-oate (3e)——Bromination of **2e** (200 mg) was carried out with Br_2 (143 mg) in AcOH as described for **3a**. The product was recrystallized from MeOH–AcOEt to give **3e** (138 mg) as colorless needles, mp 180—183 °C (lit.[22] mp 184—185 °C). IR (Nujol): 1760, 1735 cm^{-1}. NMR ($CDCl_3$) δ: 0.75 (3H, s, 18-CH_3), 1.08 (3H, s, 19-CH_3), 2.07 (3H, s, $OCOCH_3$), 3.63 (3H, s, $COOCH_3$), 4.53 (1H, dd, $J_1=4.5$ Hz, $J_2=13$ Hz, 2α-H), 4.95 (1H, d, $J=12$ Hz, 4α-H), 5.05—5.22 (1H, m, 12β-H). MS *m/z*: 427 ($M^+-115-60$), 429 ($M^++2-115-60$), 431 ($M^++4-115-60$).

Methyl 7α-Acetoxy-2β,4β-dibromo-3-oxo-5β-cholan-24-oate (3f)——Bromination of **2f** (2.23 g) was carried out with Br_2 (1.6 g) in AcOH as described for **3a**. The product was recrystallized from MeOH–$CHCl_3$ to give **3f** (3.05 g) as colorless needles, mp 189—190 °C, $[\alpha]_D^{22}+20.1°$ ($c=0.24$, $CHCl_3$). IR (Nujol): 1730 cm^{-1}. NMR ($CDCl_3$) δ: 0.70 (3H, s, 18-CH_3), 1.12 (3H, s, 19-CH_3), 2.06 (3H, s, $OCOCH_3$), 3.64 (3H, s, $COOCH_3$), 4.73 (1H, dd, $J_1=5$ Hz, $J_2=13$ Hz, 2α-H), 4.91—5.01 (1H, m, 7β-H), 5.38 (1H, d, $J=13$ Hz, 4α-H). MS *m/z*: 427 ($M^+-60-115$), 429 ($M^++2-60-115$), 431 ($M^++4-60-115$). *Anal.* Calcd for $C_{27}H_{40}Br_2O_5$: C, 53.65; H, 6.67; Br, 26.44. Found: C, 53.75; H, 6.66; Br, 26.16.

Methyl 7α,12α-Diacetoxy-2β,4β-dibromo-3-oxo-5β-cholan-24-oate (3g)——Bromination of **2g** (150 mg) was carried out with Br_2 (95 mg) in AcOH as described for **3a**. The white precipitate was recrystallized from benzene–Et_2O to give **3g** (180 mg) as colorless needles, mp 196—198 °C, $[\alpha]_D^{23}+54.7°$ ($c=0.19$, $CHCl_3$). IR (Nujol): 1740 cm^{-1}. NMR ($CDCl_3$) δ: 0.77 (3H, s, 18-CH_3), 1.09 (3H, s, 19-CH_3), 2.10, 2.13 (2×3H, s, $OCOCH_3$), 3.62 (3H, s, $COOCH_3$), 4.63 (1H, dd, $J_1=4$ Hz, $J_2=13$ Hz, 2α-H), 4.93—5.17 (2H, m, 7β, 12β-H), 5.22 (1H, d, $J=13$ Hz, 4α-H). MS *m/z*: 425 ($M^+-2\times60-115$), 427 ($M^++2-2\times60-115$), 429 ($M^++4-2\times60-115$). *Anal.* Calcd for $C_{29}H_{42}Br_2O_7$: C, 52.58; H, 6.39; Br, 24.13. Found: C, 52.67; H, 6.48; Br, 24.35.

Methyl 2β-Bromo-3-oxo-5β-cholan-24-oate (4a)——$Cr(OAc)_2$ (1.5 g) was added portionwise to a solution of **3a** (546 mg) in $CHCl_3$ (4 ml) and AcOH (10 ml) under a stream of N_2. The reaction mixture was stirred at room temperature for 1 h, poured into ice-water and extracted with Et_2O. The organic layer was washed with 2 N NaOH, and saturated NaCl, dried over anhydrous Na_2SO_4 and evaporated *in vacuo*. The product was recrystallized from MeOH to give **4a** (345 mg) as colorless needles, mp 105—107 °C (lit.[23] mp 85 °C), $[\alpha]_D^{22}-10.6°$ ($c=0.47$, $CHCl_3$). IR (Nujol): 1725 cm^{-1}. NMR ($CDCl_3$) δ: 0.68 (3H, s, 18-CH_3), 1.03 (3H, s, 19-CH_3), 3.65 (3H, s, $COOCH_3$), 4.68 (1H, dd, $J_1=4.5$ Hz, $J_2=13$ Hz, 2α-H). *Anal.* Calcd for $C_{25}H_{39}BrO_3$: C, 64.23; H, 8.41; Br, 17.09. Found: C, 64.12; H, 8.36; Br, 17.36.

Methyl 12α-Acetoxy-2β-bromo-3-oxo-5β-cholan-24-oate (4e)——The selective debromination of **3e** (100 mg) in $CHCl_3$ (1 ml) and AcOH (5 ml) was performed with $Cr(OAc)_2$ as described for **4a**. The crude product was chromatographed with benzene–Et_2O (30 : 1) as an eluent and recrystallized from MeOH–AcOEt to give **4e** (62 mg) as colorless plates, mp 179—181 °C, $[\alpha]_D^{25}+15.8°$ ($c=0.57$, $CHCl_3$). IR (Nujol): 1740, 1720 cm^{-1}. NMR ($CDCl_3$) δ: 0.75 (3H, s, 18-CH_3), 1.02 (3H, s, 19-CH_3), 2.08 (3H, s, $OCOCH_3$), 3.63 (3H, s, $COOCH_3$), 4.44 (1H, dd, $J_1=4.5$ Hz, $J_2=13$ Hz, 2α-H), 5.06—5.20 (1H, m, 12β-H). *Anal.* Calcd for $C_{27}H_{41}BrO_5\cdot1/2H_2O$: C, 60.67; H, 7.92. Found: C, 60.35; H, 7.74.

Methyl 7α-Acetoxy-4β-bromo-3-oxo-5β-chol-1-en-24-oate (5f)——A solution of **3f** (3 g) in dimethylformamide (DMF 60 ml) was treated with Li_2CO_3 (518 mg) and LiBr (609 mg) under stirring at 80 °C for 4 h in a stream of N_2. The precipitate was filtered off and the filtrate was extracted with AcOEt. The organic layer was washed with 2 N HCl, 5% $NaHCO_3$ and saturated NaCl, dried over anhydrous Na_2SO_4 and evaporated *in vacuo*. The residue was chromatographed with benzene–Et_2O (25 : 1) and the eluate was recrystallized from MeOH to give **5f** (2.13 g) as colorless needles, mp 137—138 °C, $[\alpha]_D^{22}+151.6°$ ($c=0.33$, $CHCl_3$). IR (Nujol): 1740, 1680, 1620 cm^{-1}. NMR ($CDCl_3$) δ: 0.70 (3H, s, 18-CH_3), 1.27 (3H, s, 19-CH_3), 2.08 (3H, s, $OCOCH_3$), 3.63 (3H, s, $COOCH_3$), 4.91—5.09 (1H, m, 7β-H), 5.32 (1H, d, $J=13$ Hz, 4α-H), 6.02 (1H, d, $J=10$ Hz, 2-H), 6.85 (1H, d, $J=10$ Hz, 1-H). *Anal.* Calcd for $C_{27}H_{39}BrO_5$: C, 61.94; H, 7.51; Br, 15.06. Found C, 61.81; H, 7.50; Br, 15.06.

Methyl 7α,12α-Diacetoxy-4β-bromo-3-oxo-5β-chol-1-en-24-oate (5g)——Dehydrobromination of **3g** (180 mg) in DMF (10 ml) with Li_2CO_3 (30 mg) and LiBr (36 mg) was performed as described for **5f**. The product was chromatographed with benzene–Et_2O (20 : 1) and the eluate was recrystallized from MeOH to give **5g** (98 mg) as colorless needles, mp 211—212 °C, $[\alpha]_D^{22}+170.1°$ ($c=0.43$, $CHCl_3$). IR (Nujol): 1730, 1680 cm^{-1}. NMR ($CDCl_3$) δ: 0.78 (3H, s, 18-CH_3), 1.26 (3H, s, 19-CH_3), 2.07, 2.12 (2×3H, s, $OCOCH_3$), 3.63 (3H, s, $COOCH_3$), 4.95—5.11 (2H, m, 7β, 12β-H), 5.30 (1H, d, $J=13$ Hz, 4α-H), 5.99 (1H, d, $J=10$ Hz, 2-H), 6.75 (1H, d, $J=10$ Hz, 1-H). *Anal.* Calcd for $C_{29}H_{41}BrO_7$: C, 59.89; H, 7.11. Found: C, 59.74; H, 7.25.

Methyl 3-Oxo-5β-chol-1-en-24-oate (6a)——$CaCO_3$ (50 mg) was added to a solution of **4a** (50 mg) in DMF (3 ml) and the mixture was refluxed for 1 h under stirring in a stream of N_2. The resulting solution was diluted with

ice-water and extracted with Et_2O. The organic layer was washed with 2 N HCl and saturated NaCl, dried over anhydrous Na_2SO_4 and evaporated *in vacuo*. The crude product was chromatographed on silica gel (7 g). Elution with hexane–AcOEt (9 : 1) and recrystallization of the product from MeOH gave **6a** (29 mg) as colorless plates, mp 144—145 °C (lit.[24] mp 138.5—139.5 °C), $[\alpha]_D^{25}$ +124.5° (c=0.44, $CHCl_3$). IR (Nujol): 1730, 1675 cm^{-1}. UV λ_{max}^{EtOH} nm (ε): 232 (8710). NMR ($CDCl_3$) δ: 0.68 (3H, s, 18-CH_3), 1.17 (3H, s, 19-CH_3), 3.63 (3H, s, $COOCH_3$), 5.87 (1H, d, J=10 Hz, 2-H), 6.79 (1H, d, J=10 Hz, 1-H). *Anal.* Calcd for $C_{25}H_{38}O_3$: C, 77.67; H, 9.91. Found: C. 77.64; H, 9.92.

Methyl 12α-Acetoxy-3-oxo-5β-chol-1-en-24-oate (6e)——Dehydrobromination of **4e** (50 mg) in DMF (3 ml) with Li_2CO_3 (10 mg) and LiBr (10 mg) was performed as described for **5f**. The product was chromatographed with benzene–AcOEt (20 : 1) and recrystallized from MeOH to give **6e** (26 mg) as colorless needles, mp 105—106 °C (lit[22] mp 97—98 °C), $[\alpha]_D^{25}$ +192.5° (c=0.70, $CHCl_3$). IR (Nujol): 1730, 1680, 1610 cm^{-1}. UV λ_{max}^{EtOH} nm (ε): 232 (8220). NMR ($CDCl_3$) δ: 0.77 (3H, s, 18-CH_3), 1.18 (3H, s, 19-CH_3), 2.02 (3H, s, $OCOCH_3$), 3.63 (3H, s, $COOCH_3$), 4.96—5.10 (1H, m, 12β-H), 5.83 (1H, d, J=10 Hz, 2-H), 6.59 (1H, d, J=10 Hz, 1-H). *Anal.* Calcd for $C_{27}H_{40}O_5$: C, 72.94; H, 9.07. Found: C, 72.98; H, 9.25.

Methyl 7α-Acetoxy-3-oxo-5β-chol-1-en-24-oate (6f)——A solution of **5f** (1.5 g) in acetone (50 ml) and AcOH (10 ml) was stirred with Zn dust (500 mg) at room temperature for 1 h. After filtration and evaporation of the reaction mixture, the product was extracted with Et_2O, washed with 5% $NaHCO_3$ and saturated NaCl, and dried over anhydrous Na_2SO_4. The organic layer was evaporated *in vacuo* and the residue was chromatographed with benzene–Et_2O (25 : 1) as an eluent. Recrystallization of the eluate from MeOH gave **6f** (987 mg) as colorless needles, mp 157—158 °C, $[\alpha]_D^{25}$ +99.9° (c=0.32, $CHCl_3$). UV λ_{max}^{EtOH} nm (ε): 230 (8890). IR (Nujol): 1730, 1660 cm^{-1}. NMR ($CDCl_3$) δ: 0.70 (3H, s, 18-CH_3), 1.20 (3H, s, 19-CH_3), 2.03 (3H, s, $OCOCH_3$), 3.63 (3H, s, $COOCH_3$), 4.86—5.04 (1H, m, 7β-H), 5.88 (1H, d, J=10 Hz, 2-H), 6.79 (1H, d, J=10 Hz, 1-H). *Anal.* Calcd for $C_{27}H_{40}O_5$: C, 72.94; H, 9.07. Found: C, 72.66; H, 9.21.

Methyl 7α,12α-Diacetoxy-3-oxo-5β-chol-1-en-24-oate (6g)——Reductive debromination of **5g** (90 mg) in acetone (3 ml) and AcOH (3 ml) was performed with Zn dust (50 mg) at room temperature for 30 min as described for **6f**. The product was chromatographed with benzene–Et_2O (20 : 1) as an eluent and recrystallized from MeOH to give **6g** (63 mg) as colorless needles, mp 180—181 °C, $[\alpha]_D^{22}$ +142.1° (c=0.44, $CHCl_3$). UV λ_{max}^{EtOH} nm (ε): 231 (7270). IR (Nujol): 1740, 1720, 1680 cm^{-1}. NMR ($CDCl_3$) δ: 0.78 (3H, s, 18-CH_3), 1.18 (3H, s, 19-CH_3), 2.05 (6H, s, $OCOCH_3$), 3.63 (3H, s, $COOCH_3$), 4.91—5.11 (2H, m, 7β, 12β-H), 5.86 (1H, d, J=10 Hz, 2-H), 6.68 (1H, d, J=10 Hz, 1-H). *Anal.* Calcd for $C_{29}H_{42}O_7$: C, 69.29; H, 8.42. Found: C, 69.23; H, 8.59.

Methyl 1β,2β-Epoxy-3-oxo-5β-cholan-24-oate (7a)——A 30% H_2O_2 solution (2 ml) was added dropwise to a solution of **6a** (500 mg) in dioxane (20 ml) and 2 N NaOH (1 ml) under ice-cooling and the reaction mixture was stirred at room temperature for 6 h. The resulting solution was diluted with H_2O and extracted with Et_2O. The organic layer was washed with saturated NaCl and dried over anhydrous Na_2SO_4. After evaporation of the solvent, the crude product was methylated with diazomethane–ether and chromatographed on silica gel. Elution with benzene–AcOEt (40 : 1) and recrystallization of the product from MeOH gave **7a** (229 mg) as colorless needles, mp 123—124 °C, $[\alpha]_D^{22}$ −13.6° (c=0.10, $CHCl_3$). IR (Nujol): 1740, 1710 cm^{-1}. NMR ($CDCl_3$) δ: 0.67 (3H, s, 18-CH_3), 1.28 (3H, s, 19-CH_3), 3.25 (1H, d, J=4.5 Hz, 1α-H), 3.40 (1H, d, J=4.5 Hz, 2α-H), 3.63 (3H, s, $COOCH_3$). *Anal.* Calcd for $C_{25}H_{38}O_4$: C, 74.59; H, 9.52. Found: C, 74.51; H, 9.47.

Methyl 12α-Acetoxy-1β,2β-epoxy-3-oxo-5β-cholan-24-oate (7e)——Epoxidation of **6e** (100 mg) in dioxane (6 ml) was carried out with 2 N NaOH (1 ml) and 30% H_2O_2 (0.6 ml) for 24 h as described for **7a**. After methylation of the crude product with diazomethane, chromatography with hexane–AcOEt (6 : 1) as an eluent gave a colorless oily product **7e** (63 mg). IR (Nujol): 1720 cm^{-1}. NMR ($CDCl_3$) δ: 0.75 (3H, s, 18-CH_3), 1.25 (3H, s, 19-CH_3), 2.01 (3H, s, $OCOCH_3$), 3.19 (1H, d, J=4 Hz, 1α-H), 3.28 (1H, d, J=4 Hz, 2α-H), 3.62 (3H, s, $COOCH_3$), 4.96—5.10 (1H, m, 12β-H).

Methyl 7α-Acetoxy-1β,2β-epoxy-3-oxo-5β-cholan-24-oate (7f)——Epoxidation of **6f** (1.5 g) in tetrahydrofuran (THF 15 ml) and EtOH (12 ml) was carried out with 2 N NaOH (3 ml) and 30% H_2O_2 (5 ml) as described for **7a**. Recrystallization of the product from MeOH gave **7f** (1.34 g) as colorless needles, mp 144—145 °C, $[\alpha]_D^{22}$ −30.7° (c=0.23, $CHCl_3$). IR (Nujol): 1720, 1695 cm^{-1}. NMR ($CDCl_3$) δ: 0.70 (3H, s, 18-CH_3), 1.30 (3H, s, 19-CH_3), 2.04 (3H, s, $OCOCH_3$), 3.26 (1H, d, J=4 Hz, 1α-H), 3.44 (1H, d, J=4 Hz, 2α-H), 3.63 (3H, s, $COOCH_3$), 4.81—4.99 (1H, m, 7β-H). *Anal.* Calcd for $C_{27}H_{40}O_6$: C, 70.40; H, 8.75. Found: C, 70.17; H, 8.65.

Methyl 7α,12α-Diacetoxy-1β,2β-epoxy-3-oxo-5β-cholan-24-oate (7g)——Epoxidation of **6g** (150 mg) in EtOH (10 ml) was carried out with 1 N NaOH (2 ml) and 30% H_2O_2 (0.6 ml) for 4 h as described for **7a**. The reaction mixture was treated with 10% $NaHSO_3$ (5 ml) and the precipitate was filtered off. The filtrate was adjusted to pH 4 and evaporated *in vacuo*. The product was extracted with AcOEt, washed with saturated NaCl, dried over anhydrous Na_2SO_4 and evaporated *in vacuo*. After methylation of the residue with diazomethane, chromatography with benzene–Et_2O (20 : 1) as an eluent and recrystallization from iso-Pr_2O gave **7g** (117 mg) as colorless prisms, mp 144—145 °C, $[\alpha]_D^{25}$ +31.8° (c=0.35, $CHCl_3$). IR (Nujol): 1730, 1720 cm^{-1}. NMR ($CDCl_3$) δ: 0.77 (3H, s, 18-CH_3), 1.28 (3H, s, 19-CH_3), 2.06, 2.08 (2 × 3H, s $OCOCH_3$), 3.23 (1H, d, J=4 Hz, 1α-H), 3.33 (1H, d, J=4 Hz, 2α-H), 3.62 (3H, s, $COOCH_3$), 4.86—5.04 (1H, m, 7β-H), 5.00—5.14 (1H, m, 12β-H). *Anal.* Calcd for $C_{29}H_{42}O_8$: C, 67.16; H, 8.16. Found: C, 67.14; H, 8.11.

Methyl 1β-Hydroxy-3-oxo-5β-cholan-24-oate (8a)——$Cr(OAc)_2$ (260 mg) was added portionwise to a solution of **7a** (67.5 mg) in EtOH (5 ml) under ice-cooling in a stream of N_2. The reaction mixture was stirred at room temperature for 30 min, concentrated *in vacuo*, diluted with H_2O, and extracted with Et_2O. The organic layer was washed with saturated NaCl and dried over anhydrous Na_2SO_4, and evaporated *in vacuo*. The residue was chromatographed on silica gel. Elution with benzene–AcOEt (2 : 1) and recrystallization of the product from MeOH gave **8a** (47 mg) as colorless needles, mp 179—181 °C, $[\alpha]_D^{22}$ +26.6° (*c*=0.49, $CHCl_3$). IR (Nujol): 3530, 1710 cm^{-1}. NMR ($CDCl_3$) δ: 0.69 (3H, s, 18-CH_3), 1.14 (3H, s, 19-CH_3), 3.65 (3H, s, $COOCH_3$), 4.14 (1H, t, *J*=3 Hz, 1α-H). *Anal.* Calcd for $C_{25}H_{40}O_4$: C, 74.21; H, 9.97. Found: C, 73.94; H, 9.97.

Methyl 12α-Acetoxy-1β-hydroxy-3-oxo-5β-cholan-24-oate (8e)——A solution of **7e** (100 mg) in EtOH (5 ml) was treated with $Cr(OAc)_2$ (300 mg) under ice-cooling for 1 h as described for **8a**. The product was chromatographed with benzene–AcOEt (1 : 1) as an eluent and recrystallized from MeOH to give **8e** (58 mg) as colorless needles, mp 210—214 °C, $[\alpha]_D^{24}$ +66.0° (*c*=0.303, $CHCl_3$). IR (Nujol): 3440, 1730, 1700 cm^{-1}. NMR ($CDCl_3$) δ: 0.77 (3H, s, 18-CH_3), 1.12 (3H, s, 19-CH_3), 2.05 (3H, s, $OCOCH_3$), 3.63 (3H, s, $COOCH_3$), 3.95—4.09 (1H, m, 1α-H), 4.99—5.13 (1H, m, 12β-H). *Anal.* Calcd for $C_{27}H_{42}O_6$: C, 70.10; H, 9.15. Found: C, 70.02; H, 9.12.

Methyl 7α-Acetoxy-1β-hydroxy-3-oxo-5β-cholan-24-oate (8f)——A solution of **7f** (500 mg) in EtOH (20 ml) was treated with $Cr(OAc)_2$ (1.5 g) under ice-cooling for 2 h as described for **8a**. The product was chromatographed with benzene–AcOEt (1 : 1) as an eluent and recrystallized from MeOH to give **8f** (336 mg) as colorless needles, mp 155—156 °C, $[\alpha]_D^{25}$ +16.8° (*c*=0.40, $CHCl_3$). IR (Nujol): 3450, 1720, 1700 cm^{-1}. NMR ($CDCl_3$) δ: 0.70 (3H, s, 18-CH_3), 1.14 (3H, s, 19-CH_3), 2.00 (3H, s, $OCOCH_3$), 3.62 (3H, s, $COOCH_3$), 4.05—4.19 (1H, m, 1α-H), 4.86—5.04 (1H, m, 7β-H). *Anal.* Calcd for $C_{27}H_{42}O_6$ C, 69.55; H, 9.17. Found: C , 69.42; H, 9.12.

Methyl 7α,12α-Diacetoxy-1β-hydroxy-3-oxo-5β-cholan-24-oate (8g)——A solution of **7g** (100 mg) in THF (1 ml) and EtOH (5 ml) was treated with $Cr(OAc)_2$ (300 mg) for 2 h as described for **8a**. The product was chromatographed with $CHCl_3$–AcOEt (1 : 1) as an eluent and recrystallized from MeOH to give **8g** (43 mg) as colorless needles, mp 247—249 °C, $[\alpha]_D^{22}$ +66.6° (*c*=0.54, $CHCl_3$). IR (Nujol): 3440, 1730, 1700 cm^{-1}. NMR ($CDCl_3$) δ: 0.73 (3H, s, 18-CH_3), 1.05 (3H, s, 19-CH_3), 1.98 2.02 (2×3H, s, $OCOCH_3$), 3.55 (3H, s, $COOCH_3$), 3.81—3.95 (1H, m, 1α-H), 4.80—5.02 (2H, m, 7β, 12β-H). *Anal.* Calcd for $C_{29}H_{44}O_8$: C, 66.90; H, 8.52. Found: C, 66.81; H, 8.70.

Methyl 1β,3α-Dihydroxy-5β-cholan-24-oate (9a)——Reduction of **8a** (35 mg) was carried out by stirring with $NaBH_4$ (20 mg) in MeOH (2 ml) under ice-cooling for 1 h. The resulting mixture was treated with AcOH (1 drop), evaporated and extracted with AcOEt. The organic layer was washed with 5% $NaHCO_3$ and saturated NaCl, dried over anhydrous Na_2SO_4 and evaporated *in vacuo*. The residue was chromatographed with $CHCl_3$–MeOH (50 : 1) as an eluent. The first eluate afforded the 3β-isomer of **9a** (10 mg) as colorless needles, mp 125—126 °C. NMR ($CDCl_3$) δ: 0.66 (3H, s, 18-CH_3), 1.09 (3H, s, 19-CH_3), 3.63 (3H, s, $COOCH_3$), 3.72—3.89 (1H, m, 1α-H), 4.03—4.20 (1H, m, 3α-H). The second eluate afforded a product which was recrystallized from MeOH to give **9a** (25 mg) as colorless needles, mp 203—205 °C, $[\alpha]_D^{25}$ +47.5° (*c*=0.80, $CHCl_3$). IR (Nujol): 3530, 3440, 1720 cm^{-1}. NMR ($CDCl_3$) δ: 0.66 (3H, s, 18-CH_3), 1.04 (3H, s, 19-CH_3), 3.65 (3H, s, $COOCH_3$), 3.90 (1H, t, *J*=3 Hz, 1α-H), 3.83—4.17 (1H, m, 3β-H). *Anal.* Calcd for $C_{25}H_{42}O_4$: C, 73.85; H, 10.41. Found: C, 73.89; H, 10.62.

Methyl 12α-Acetoxy-1β,3α-dihydroxy-5β-cholan-24-oate (9e)——Reduction of **8e** (25 mg) was carried out with $NaBH_4$ (10 mg) in iso-PrOH (3 ml) under ice-cooling for 30 min. The reaction mixture was treated in the same manner as described for **9a**. The first eluate afforded the 3β-isomer of **9e** (9 mg) as colorless needles, mp 204—205 °C. NMR ($CDCl_3$) δ: 0.74 (3H, s, 18-CH_3), 1.08 (3H, s, 19-CH_3), 2.06 (3H, s $OCOCH_3$), 3.63 (3H, s, $COOCH_3$), 3.83—4.03 (2H, m, 1α, 3α-H), 4.96—5.10 (1H, m, 12β-H). The second eluate was recrystallized from MeOH to give **9e** (15 mg) as colorless needles, mp 203—205 °C, $[\alpha]_D^{22}$ +64.4° (*c*=0.378, $CHCl_3$). IR (Nujol): 3540, 3440, 1740, 1700 cm^{-1}. NMR ($CDCl_3$) δ: 0.72 (3H, s, 18-CH_3), 1.01 (3H, s, 19-CH_3), 2.04 (3H, s, $OCOCH_3$), 3.62 (3H, s, $COOCH_3$), 3.78 (1H, t, *J*=3 Hz, 1α-H) 3.93—4.27 (1H, m, 3β-H), 4.93—5.07 (1H, m, 12β-H). *Anal.* Calcd for $C_{27}H_{44}O_6$: C, 69.79; H, 9.55. Found: C, 69.54; H, 9.50.

Methyl 7α-Acetoxy-1β,3α-dihydroxy-5β-cholan-24-oate (9f)——Reduction of **8f** (364 mg) was carried out with $NaBH_4$ (40 mg) in iso-PrOH (5 ml) under ice-cooling for 30 min. The reaction mixture was treated in the same manner as described for **9a**. The first eluate gave the 3β-isomer of **9f** (110 mg) as an amorphous powder. The second eluate afforded a product which was recrystallized from AcOEt–hexane to give **9f** (241 mg) as colorless needles, mp 93—95 °C, $[\alpha]_D^{22}$ +4.3° (*c*=0.268, $CHCl_3$). IR (Nujol): 3450, 1720 cm^{-1}. NMR ($CDCl_3$) δ: 0.66 (3H, s, 18-CH_3), 1.03 (3H, s, 19-CH_3), 2.05 (3H, s, $OCOCH_3$), 3.62 (3H, s, $COOCH_3$), 3.80—4.16 (2H, m, 1α, 3β-H), 4.75—4.93 (1H, m, 7β-H). *Anal.* Calcd for $C_{27}H_{44}O_6 \cdot 1/2H_2O$: C, 68.47; H, 9.58. Found: C, 68.60; H, 9.72.

Methyl 7α,12α-Diacetoxy-1β,3α-dihydroxy-5β-cholan-24-oate (9g)——Reduction of **8g** (50 mg) was carried out with $NaBH_4$ (20 mg) in iso-PrOH (3 ml) for 1 h as described for **9a**. The first eluate gave the 3β-isomer of **9g** (12 mg) as colorless needles, mp 114—115 °C. NMR ($CDCl_3$) δ: 0.72 (3H, s, 18-CH_3), 1.06 (3H, s, 19-CH_3), 2.04, 2.06 (2×3H, s, $OCOCH_3$), 3.86—4.00 (1H, m, 1α-H), 4.13—4.28 (1H, m, 3α-H), 4.82—5.10 (2H, m, 7β, 12β-H). The second eluate afforded a product which was recrystallized from iso-Pr_2O to give **9g** (38 mg), mp 207—208 °C, $[\alpha]_D^{25}$ +52.6° (*c*=0.300, $CHCl_3$). IR (Nujol): 3430, 1740, 1710 cm^{-1}. NMR ($CDCl_3$) δ: 0.73 (3H, s, 18-CH_3), 1.02 (3H, s, 19-CH_3), 2.05, 2.09 (2×3H, s, $OCOCH_3$), 3.69 (3H, s, $COOCH_3$), 3.73—3.87 (1H, m, 1α-H), 3.72—4.08 (1H, m, 3β-H), 4.77—4.90 (1H, m, 7β-H), 4.90—5.02 (1H, m, 12β-H). *Anal.* Calcd for $C_{29}H_{46}O_8$: C, 66.38; H, 9.22. Found: C, 66.39; H,

8.95.

1β,3α-Dihydroxy-5β-cholan-24-oic Acid (10a)——A mixture of **9a** (313 mg) in MeOH (10 ml) and 2 N LiOH (2 ml) was refluxed for 1 h. After concentration of the solution *in vacuo*, the residue was diluted with H_2O and adjusted to pH 1 with 2 N HCl. Filtration of the precipitate and crystallization from EtOH–hexane afforded **10a** (240 mg) as colorless needles, mp 274—278 °C. IR (Nujol): 3300, 3200, 1680 cm^{-1}. NMR (pyridine-d_5) δ: 0.62 (3H, s, 18-CH_3), 1.28 (3H, s, 19-CH_3), 4.12—4.28 (1H, m, 1α-H), 4.61—4.99 (1H, m, 3β-H). *Anal.* Calcd for $C_{24}H_{40}O_4 \cdot 1/2H_2O$: C, 71.78; H, 10.29. Found: C, 71.64; H, 10.01.

1β,3α,12α-Trihydroxy-5β-cholan-24-oic Acid (10b)——Hydrolysis of **9e** (218 mg) in MeOH (6 ml) was carried out with 2 N LiOH (2 ml) as described for **10a**. Recrystallization of the product from EtOH–hexane gave **10b** (178 mg) as colorless needles, mp 262—264 °C. IR (Nujol): 3400, 3300, 1705 cm^{-1}. NMR (pyridine-d_5) δ: 0.75 (3H, s, 18-CH_3), 1.34 (3H, s, 19-CH_3), 4.10—4.36 (2H, m, 1α, 12β-H), 4.58—4.96 (1H, m, 3β-H). *Anal.* Calcd for $C_{24}H_{40}O_5$: C, 70.55; H, 9.87. Found: C, 70.16; H, 9.86.

1β,3α,7α-Trihydroxy-5β-cholan-24-oic Acid (10c)——Hydrolysis of **9f** (241 mg) in MeOH (10 ml) was carried out with 2 N LiOH (3 ml) as described for **10a**. Recrystallization of the product from EtOH–hexane gave **10c** (154 mg) as colorless needles, mp 266—270 °C. IR (Nujol): 3380, 3280, 1705 cm^{-1}. NMR (pyridine-d_5) δ: 0.69 (3H, s, 18-CH_3), 1.30 (3H, s, 19-CH_3), 3.92—4.10 (1H, m, 7β-H), 4.12—4.28 (1H, m, 1α-H), 4.40—4.78 (1H, m, 3β-H). *Anal.* Calcd for $C_{24}H_{40}O_5$: C, 70.55; H, 9.87. Found: C, 70.08; H, 9.91.

1β,3α,7α,12α-Tetrahydroxy-5β-cholan-24-oic Acid (10d)——Hydrolysis of **9g** (200 mg) in MeOH (6 ml) was carried out with 2 N LiOH (2 ml) as described for **10a**. Recrystallization of the product from EtOH–hexane gave **10d** (112 mg) as colorless needles, mp 288—290 °C. IR (Nujol): 3400, 3300, 1705 cm^{-1}. NMR (pyridine-d_5) δ: 0.81 (3H, s, 18-CH_3), 1.40 (3H, s, 19-CH_3), 4.03—4.38 (3H, m, 1α, 7β, 12β-H), 4.48—4.86 (1H, m, 3β-H). *Anal.* Calcd for $C_{24}H_{40}O_6$: C, 67.89; H, 9.50. Found: C, 67.39; H, 9.37.

Gas Chromatography-Mass Spectrometry (GC-MS)——GC-MS was performed on a Shimadzu-LKB 9000 system equipped a multiple ion detector and a data processing system (Shimadzu GC-MS PAC-300M). A gas chromatographic column (2 m × 2.5 mm i.d. glass coil) packed with 1.5% Poly I-110 on Gas Chrom Q (100—120 mesh) was used at 255 °C.[10] The flow rate of He carrier gas was 30 ml/min. The temperatures of the flash heater and separator were 280 and 300 °C, respectively. The MS were recorded at 70 eV with an ion source temperature of 290 °C.

Derivatization——Each bile acid or mixture of bile acids was derivatized to the methyl ester with diazomethane–ether at room temperature. After removal of excess reagents, the trimethylsilyl or dimethylethylsilyl ethers were prepared by heating the residue with *N*-trimethylsilyl- or *N*-dimethylethylsilyl-imidazole in acetonitrile at 60 °C for 30 min. Excess reagents were evaporated off in an N_2 stream and the residue was redissolved in *n*-hexane prior to GC-MS analysis. The methyl ester–trimethylsilyl and dimethylethylsilyl ether derivatives showed the following retention times: **10a**, 12.2 and 6.5 min; **10b**, 7.2 and 8.1 min; **10c** 8.3 and 10.2 min; **10d**, 5.5 and 11.4 min, respectively.

Extraction of Bile Acids from Human Biological Fluids——Urine (5 ml) or meconium (1 mg) dissolved in 0.1 N NaOH (2 ml) was acidified with 2 N HCl to pH 4.0. The solution was passed through a Bond Elut cartridge (Analytichem International, CA, U.S.A.). The cartridge was washed with H_2O (5 ml), and the bile acid conjugates were eluated with MeOH–$CHCl_3$ (9 : 1, 5 ml). Solvolysis of the sulfates was carried out at pH 1 with 2 N HCl in EtOH–acetone (1 : 9) at 37 °C for 1 h, and the amino acid conjugates were hydrolyzed with 4 N NaOH–MeOH (1 : 1, 10 ml) at 80 °C for 16 h. The free bile acids were extracted with Bond Elut, purified on Sephadex LH-20 (Pharmacia Fine Chemicals, Uppsala, Sweden) and derivatized to the methyl ester–trimethylsilyl ethers.

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