Synthesis of 3α,7α,14α-Trihydroxy-5β-cholan-24-oic Acid: A Potential Primary Bile Acid in Vertebrates[1)]

Genta KAKIYAMA,[a] Takashi IIDA,*,[a] Takaaki GOTO,[b] Nariyasu MANO,[b] Junichi GOTO,[b] and Toshio NAMBARA[b]

[a] *Department of Chemistry, College of Humanities and Sciences, Nihon University; Sakurajousui, Setagaya-ku, Tokyo 156–8550, Japan: and* [b] *Graduate School of Pharmaceutical Sciences, Tohoku University; Aobayama, Sendai 980–8578, Japan.* Received November 25, 2003; accepted December 18, 2003

A method for the synthesis of 3α,7α,14α-trihydroxy-5β-cholan-24-oic acid which is a possible candidate of bile acid metabolite in vertebrates was developed. The principal reactions involved were 1) stereoselective remote-hydroxylation of methyl ursodeoxycholate diacetate with dimethyldioxirane, 2) site-selective protection at C-3 by *tert*-butyldimethylsilylation of the resulting 3α,7α,14α-trihydroxy ester, 3) oxidation of the diol with pyridinium dichromate adsorbed on activated alumina, 4) stereoselective reduction of the 7-ketone with zinc borohydride, and 5) cleavage of the protecting group at C-3 with *p*-toluenesulfonic acid. A facile elimination of the 14α-hydroxy group under an acidic or neutral condition is also described. The synthetic reference compound is now available for comparison with unidentified biliary bile acids detected in vertebrate bile.

Key words 3α,7α,14α-trihydroxy-5β-cholan-24-oic acid; dimethyldioxirane; remote-hydroxylation

Chenodeoxycholic acid (CDCA, 3α,7α-dihydroxy-5β-cholan-24-oic acid) and cholic acid (CA, 3α,7α,12α-trihydroxy-5β-cholan-24-oic acid) are formed from cholesterol in the liver and are the dominant primary bile acids in many vertebrate species. CDCA may be considered the building block of all trihydroxy-bile acids and in many vertebrates one additional hydroxy group is added, either to the steroid nucleus or to the isopentanoic acid side chain.[2)] Such additional hydroxylation may occur either at any stage of an intermediate during bile acid biosynthesis or after the mature molecule has been synthesized.

6α-Hydroxylation of CDCA in the pig to give a 3α,6α,7α-trihydroxy acid (hyocholic acid) was reported by Haslewood and Sjövall.[3)] In the rat and mouse, 6β-hydroxylation results in the formation of 3α,6β,7α-trihydroxy bile acid (α-muricholic acid).[4)] In more recent work, 1α-hydroxylation (vulpecholic acid) of CDCA was identified in the Australian opossum.[5)] The 1β-epimer[6)] of this bile acid has been reported to be present in the bile of fruit pigeons and doves (*Columbiformes*) and in the human biological fluids from newborns and from adult patients with cholestatic liver diseases.[7)] The 4β-hydroxy derivative[8)] of CDCA occurs in the biliary bile acids of patients with hepatobiliary diseases and in neonates and newborn infants. Hydroxylation at the C-5 (β-OH) position has been reported to occur in the biliary bile acids of the pheasant,[1)] and 5β-hydroxylation of analogous nor-CDCA was demonstrated in hamster liver.[9)] The presence of the 3α,7α,15α-trihydroxy acid noted in the biliary bile acids of the marsupial and of the swan.[1)] In the most recent work, Hagey *et al.*[10)] have reported the existence of the 3α,7α,16α-trihydroxy acid as a primary bile acid in many species of birds such as herons (*Ardeidae*), pelicans (*Pelecanidae*) and owls (*Tytonidae*).

Hydroxylation on the side-chain of CDCA has also been reported. Haemulcholic [(22*S*)-3α,7α,22-trihydroxy] acid occurs in the bile of bony fish[11)] whereas β-phocacholic [(23*R*)-3α,7α-23-trihydroxy] acid is present in the biliary bile acid of marine mammals,[12)] ducks[13)] and flamingos.[14)] The above findings of the species differences in the bile acid metabolism of vertebrates are, therefore, of interest from the viewpoint of their metabolism and physiological functions, as well as phylogenetic significance. For these reasons, new sites of "third" hydroxylation of CDCA are of considerable interest.

As mentioned above, "third site" hydroxylation at C-1, -4, -5, -6, -12, -15, -16, -22 and -23 in CDCA has now been known and/or characterized in the literature. However, the occurrence of 14α-hydroxylated CDCA has not yet been reported, though it is a logical compound to be formed in vertebrates, as the 3α,5β,7α-trihydroxy compound having a *tert*-hydroxy group at C-5 does occur in some species. In a survey of the biliary bile acids of some 900 vertebrate species, unidentified C_{24} trihydroxy bile acids were often present as major components.[15)] Our labolatory has had a program aimed at synthesizing potential primary bile acids and their metabolites in order to have such compound available. We report here the synthesis of 3α,7α,14α-trihydroxy-5β-cholan-24-oic acid (**1**) and its 7β-epimer (**4**).

Results and Discussion

The synthetic route to 3α,7α,14α-trihydroxy-5β-cholanoic acid (**1**) is shown in Fig. 2. A key intermediate, 3α,7β-diacetoxy-14α-hydroxy-5β-cholanoate (**3a**), was prepared in one-step from methyl ursodeoxycholate 3,7-diacetate (**2a**; methyl 3α,7β-diacetoxy-5β-cholan-24-oate) by remote-oxyfunctionalization of **2a** with a freshly prepared $CHCl_3$ solution of dimethyldioxirane (DMDO).[16,17)] The desired 14α-hydroxy derivative (**3a**) of **2a** was isolated in 10% yield. Alkaline hydrolysis of **3a** with 10% methanolic KOH, followed by acidification with 10% H_2SO_4 yielded the 3α,7β,14α-trihydroxy acid **4**.

Preliminary experiments revealed that the *tert*-14α-hydroxy group of **4** is liable to eliminate under an acidic experimental condition, while it is stable under an alkaline condition. When **4** was treated with conc. H_2SO_4, HCl or *p*-toluenesulfonic acid in methanol, the elimination reaction occurs readily within a few hours to give the Δ^{14}-unsaturated ester (**8a**). The above finding implies that the instability of **4** ham-

* To whom correspondence should be addressed. e-mail: takaiida@chs.nihon-u.ac.jp

pers the chemical synthesis of the desired **1**. Particular caution should therefore be paid for the subsequent reactions as well as for the isolation and purification processes to avoid the elimination of a 14α-hydroxy group in 14α-hydroxylated intermediates.

Esterification of the acid **4** in methanol with (trimethylsilyl)diazomethane in ether solution[18] gave the corresponding methyl ester **4a** quantitatively. Subsequent protection of the equatorial 3α-hydroxy group in **4a** with *tert*-butyldimethylsilyl chloride/imidazole in *N*,*N*-dimethylformamide (DMF)–pyridine solution at $-20\,°C$ for 30 min caused *tert*-butyldimethylsilylation (TBDMSi) site-selectively. The TBDMSi reaction proceeded smoothly and cleanly to give the 3-*tert*-butyldimethylsilyloxy-7β,14α-dihydroxy ester **5a** in an excellent yield (95%). Access of the bulky reagent to the equatorial 7β-hydroxy group in **4a** may be effectively prevented by the axially-oriented 14α-hydroxy group. Attempted direct inversion at C-7 of **5a** by *N*,*N*-dimethylformamide, potassium superoxide/18-crown-6 ether and/or diethyl azodicarboxylate/triphenylphosphine/formic acid,[19] *via* the appropriate 7α-derivatives, was unsuccessful, probably owing to the presence of the 14α-hydroxyl.

When the 3α-*tert*-butyldimethylsilyloxy ester **5a** was subjected to DMDO oxidation for several hours under a neutral condition,[20] the reaction did not proceed at all, probably owing to steric hindrance of the 14α-hydroxy group. Similarly, oxidation of **5a** with pyridinium dichromate (PDC) alone, after 12 h at room temperature, afforded a mixture (ratio, *ca.* 1 : 1) of the 3-*tert*-butylsilyloxy-7-oxo-14α-hydroxy ester **6a** and its dehydrated product of the 14α-hydroxy group. However, by changing the oxidant to PDC absorbed on activated alumina,[21] oxidation reaction proceeded cleanly to give the 7-ketone **6a** in a good isolated yield of 83% without simultaneous formation of the undesirable elimination product at C-14.

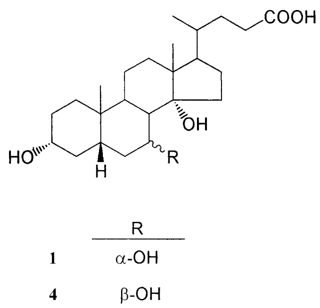

Fig. 1. Structure of Epimeric 3α,7,14α-Trihydroxy-5β-cholanoic Acids

Reduction of oxo- to hydroxy-steroids has been studied extensively.[19] Depending on the reducing agents employed and on the reaction conditions, either α- or β-alcohols, or epimeric mixtures may be obtained. By carrying out the reduction of **6a** using zinc borohydride [$Zn(BH_4)_2$] in ether solution,[22] a reagent that is less basic than $NaBH_4$, the desired 3α-*tert*-butyldimethylsilyloxy-7α,14α-dihydroxy ester **7a** was obtained stereoselectively without concurrent formation of the 7β-epimer **4a**, or partial hydrolysis of the C-24 ester group. Chromatographic purification of the reaction product was unnecessary. Direct recrystallization of the crude **7a**, followed by desilylation afforded the pure ester **1a** nearly quantitatively.

Subsequent cleavage of the *tert*-butyldimethylsilyloxy group at C-3 in **7a**, which is known to be sensitive to an acidic experimental condition, was successfully attained by treating with 0.5% methanolic *p*-toluenesulfonic acid solution for 10 min at room temperature. The deprotection reaction proceeded smoothly to give the desired 3α,7α,14α-trihydroxy ester **1a** quantitatively. Exposure of **7a** to the acid solution for an excess length of time caused degradation of the substrate. Usual alkaline hydrolysis of **1a** with 5% methanolic KOH, followed by acidification with 10% H_2SO_4

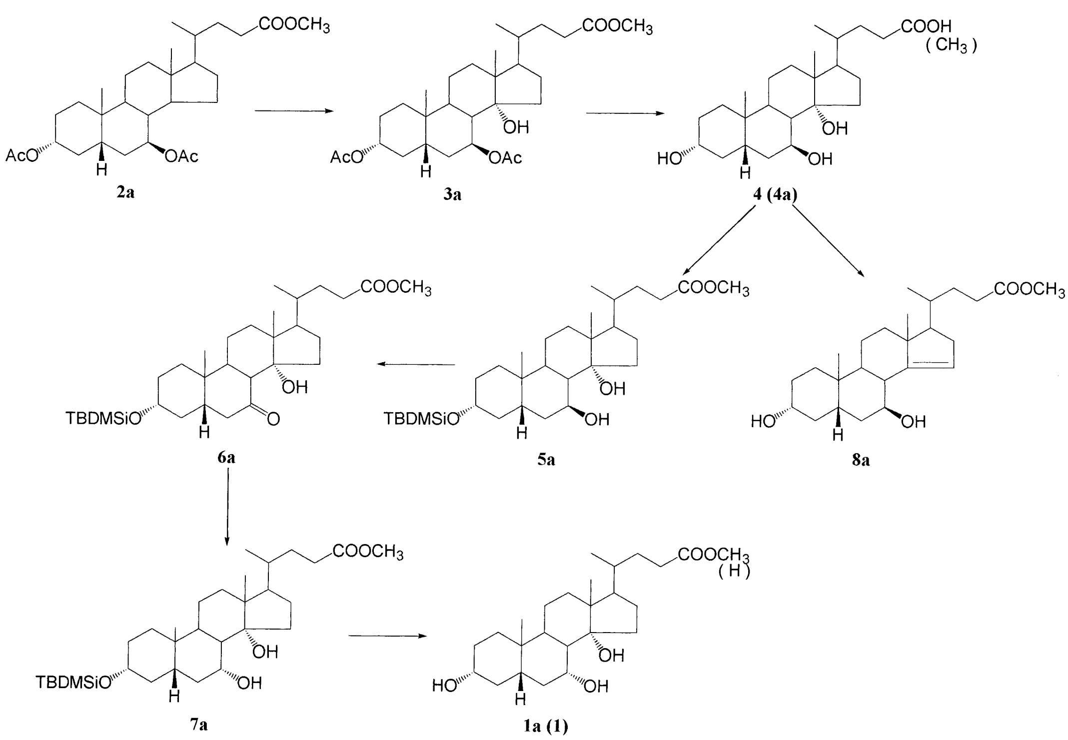

Fig. 2. Synthetic Route to 3α,7α,14α-Trihydroxy-5β-cholanoic Acid

afforded the corresponding 3α,7α,14α-trihydroxy acid **1**.

A comparison of the ^{1}H-NMR spectra of the epimeric pairs at C-7, 3α,7α,14α- and 3α,7β,14α-trihydroxy esters (**1a**, **4a**), revealed that the axial 7α-H in **4a** resonates at 3.97 ppm as a br m signal, while the corresponding equatorial 7β-H in **1a** occurs at 4.21 ppm as a br s signal. A significant difference between **1a** and **4a** was also observed in the ^{13}C-NMR spectra, in which the C-7 and C-14 signals (69.4 and 87.0 ppm, respectively) in **1a** resonate at lower field than those (66.7 and 84.9 ppm, resectively) in **4a**, probably owing to the 1,3-diaxial interaction between the 7α- and 14α-hydroxy groups. Again, **4a** was gradually decomposed by a prolonged exposure (overnight) with $CDCl_3$ to give the completely dehydrated product, **8a**, even though **4a** was allowed to stand in the neutral solvent. The LR-MS spectra of both the epimers were very similar to each other.

In conclusion, the availability of 3α,7α,14α-trihydroxy acid **1**, as well as the 7β-epimer **4**, should facilitate the identification and characterization of unidentified trihydroxy bile acids present in the biological materials of vertebrates.

Experimental

Melting points (mp) were determined on a micro hot-stage apparatus and are uncorrected. Infrared (IR) spectra were obtained in KBr discs on a Shimadzu FTIR-8300 spectrometer. Proton (^{1}H) and carbon (^{13}C) nuclear magnetic resonance (NMR) spectra were obtained on a JEOL JNM-EX 270 FT instrument at 270 and 68.80 MHz, respectively. Electron ionization (EI) low-resolution mass (LR-MS) spectra were determined on a JEOL JMS-303 mass spectrometer at 70 eV. High-resolution mass (HR-MS) spectra were measured using a JEOL LCmate double-focusing magnetic mass spectrometer equipped with an electrospray ionization (ESI) probe under the positive ion mode (PIM) or the negative ion mode (NIM). HR-MS was also obtained on a JEOL JMS-700 mass spectrometer with an EI probe under the PIM. Thin-layer chromatography (TLC) was performed on precoated silica gel plates (0.25 mm layer thickness; E.Merck, Darmstadt, Germany) using hexane–EtOAc–acetic acid mixtures (30/70/1—80/20/1, v/v/v) or EtOAc–MeOH–acetic acid mixtures (95/5/1, v/v/v) as the developing solvent.

Methyl 3α,7β-Diacetoxy-14α-hydroxy-5β-cholan-24-oate (3a) This compound **3a** was prepared from methyl ursodeoxycholate 3,7-diacetate **2a** (8 g) according to the procedure described in a previous paper[17]; yield, 840 mg, 10%.

3α,7β,14α-Trihydroxy-5β-cholan-24-oic Acid (4) A solution of the 14α-hydroxy ester **3a** (480 mg) in 10% methanolic KOH (10 ml) was refluxed for 30 min. Most of the solvent was evaporated under reduced pressure, and the residue was dissolved in water and then acidified with 10% H_2SO_4 with ice bath cooling. The precipitated solid was filtered, washed with water, and recrystallized from aqueous methanol to give the title compound **4** as a colorless amorphous solid: mp, 200—204 °C; yield, 320 mg, 83%. IR (KBr), ν_{max} cm^{-1}: 1715 (C=O), 3342, 3369, 3435 (OH). ^{1}H-NMR (CD_3OD), δ: 0.82 (3H, s, 18-CH_3), 0.93 (3H, d, J=6.2 Hz, 21-CH_3), 0.97 (3H, s, 19-CH_3), 3.47 (1H, br m, 3β-H), 3.89 (1H, br m, 7α-H). LR-MS (EI-PIM), *m/z*: 408 (M^+, 2%), 390 (M$-H_2O$, 11%), 372 (M$-2H_2O$, 87%), 354 (M$-3H_2O$, 38%), 339 (M$-3H_2O-CH_3$, 17%), 299 (8%), 289 (M$-H_2O-$side chain (S.C.), 41%), 271 (M$-2H_2O-$S.C., 48%), 253 (M$-3H_2O-$S.C., 100%), 211 (M$-3H_2O-$S.C.$-$ring D, 12%), 194 (42%). HR-MS (ESI-NIM), Calcd for $C_{24}H_{39}O_5$ [M+Na]$^+$: 407.2797. Found: *m/z*, 407.2813.

Methyl 3α,7β,14α-Trihydroxy-5β-cholan-24-oate (4a) To a solution of the 3α,7β,14α-trihydroxy acid **4** (270 mg) in methanol (10 ml) (trimethylsilyl)diazomethane in diethyl ether solution (2 mol/l, 3 ml) was added gradually at 0 °C, and the mixture was left to stand at room temperature for 15 min. The excess reagent and solvents were evaporated and the residue was recrystallized from EtOAc to give the methyl ester **4a** as a colorless amorphous solid: mp, 193—194 °C; yield, 295 mg, *ca.* 100%. IR (KBr), ν_{max} cm^{-1}: 1740 (C=O), 3435 (OH). ^{1}H-NMR ($CDCl_3$), δ: 0.79 (3H, s, 18-CH_3), 0.91 (3H, d, J=5.9 Hz, 21-CH_3), 0.96 (3H, s, 19-CH_3), 3.58 (1H, br m, 3β-H), 3.67 (3H, s, –$COOCH_3$), 3.97 (3H, br m, 7α-H). ^{13}C-NMR ($CDCl_3$), δ: 15.7 (C-18), 18.4 (C-21), 20.0 (C-11), 23.0 (C-19), 27.6 (C-16), 30.2 (C-2), 31.1 and 31.2 (C-22, C-23), 32.2 (C-15), 32.2 (C-20), 34.3 (C-10), 35.1 (C-12), 35.1 (C-9), 35.8 (C-1), 37.3 (C-4), 37.4 (C-6), 42.5 (C-5), 46.5 (C-8), 47.5 (C-13), 49.5 (C-17), 51.5 (–$COOCH_3$), 66.7 (C-7), 71.4 (C-3), 84.9 (C-14), 174.8 (C-24). LR-MS (EI-PIM), *m/z*: 422 (M^+, 5%), 404 (M$-H_2O$, 28%), 386 (M$-2H_2O$, 83%), 368 (M$-3H_2O$, 23%), 353 (M$-3H_2O-CH_3$, 12%), 289 (M$-H_2O-$S.C., 63%), 271 (M$-2H_2O-$S.C., 33%), 265 (51%), 253 (M$-3H_2O-$S.C., 51%), 248 (78%), 211 (M$-3H_2O-$S.C.$-$ring D, 18%), 208 (84%), 195 (100%). HR-MS (EI-PIM), Calcd for $C_{25}H_{42}O_5$ [M]$^+$: 422.3032. Found: *m/z*, 422.3046.

Methyl 3α-*tert*-Butyldimethylsilyloxy-7β,14α-dihydroxy-5β-cholan-24-oate (5a) A solution of the trihydroxy ester **4a** (300 mg) and imidazole (300 mg) dissolved in dry DMF (3 ml) and dry pyridine (150 μl) was cooled at -20 °C with a freezing agent. To the solution was added *tert*-butyldimethylsilyl chloride (370 mg), and the mixture was left stand at -20 °C for 30 min. Water was added to the mixture, and the reaction product was extracted with $CHCl_3$. The combined extract was washed with water, dried with Drierite, and evaporated to give the 3α-*tert*-butyldimethylsilyloxy ester **5a**, which was recrystallized from methanol as colorless needles: mp, 181—183 °C; yield, 360 mg, 95%. IR (KBr), ν_{max} cm^{-1}: 1744 (C=O), 3368, 3435 (OH). ^{1}H-NMR ($CDCl_3$), δ: 0.05 (6H, s, –Si($C\underline{H}_3$)$_2$C(CH_3)$_3$), 0.79 (3H, s, 18-CH_3), 0.88 (9H, s, –Si(CH_3)$_2$C($C\underline{H}_3$)$_3$), 0.90 (3H, d, J=5.9 Hz, 21-CH_3), 0.93 (3H, s, 19-CH_3), 3.53 (1H, br m, 3β-H), 3.67 (3H, s, –$COOCH_3$), 3.97 (1H, br m, 7α-H). LR-MS (EI-PIM), *m/z*: 500 (M$-2H_2O$, 2%), 461 (11%), 369 (M$-2H_2O-$[OSi(CH_3)$_2$C(CH_3)$_3$], 100%), 337 (8%), 253 (M$-2H_2O-$[OSi(CH_3)$_2$C(CH_3)$_3$]$-$S.C., 7%), 207 (5%), 195 (14%). HR-MS (ESI-PIM), Calcd for $C_{31}H_{56}O_5NaSi$ [M+Na]$^+$: 559.3795. Found: *m/z*, 559.3766.

Methyl 3α-*tert*-Butyldimethylsilyloxy-7-oxo-14α-hydroxy-5β-cholan-24-oate (6a) To a magnetically stirred suspension of PDC (800 mg) and activated alumina (activity II, 2 g) in CH_2Cl_2 (10 ml) the 3α-*tert*-butyldimethylsilyloxy ester **5a** (360 mg) in CH_2Cl_2 (10 ml) was added, and the mixture was stirred at room temperature for 5 h. The insoluble matter was filtered and the filtrate was transferred to a column of alumina (activity III, 20 g). Elution with benzene–EtOAc (95 : 5, v/v) yielded the 3α-*tert*-butyldimethylsilyloxy-7-oxo ester **6a**. Recrystallization from methanol gave an analytical pure sample of **6a** as colorless needles: mp, 101—103 °C; yield, 300 mg, 83%. IR (KBr), ν_{max} cm^{-1}: 1699, 1736 (C=O), 3440 (OH). ^{1}H-NMR ($CDCl_3$), δ: 0.03 (6H, s, –Si($C\underline{H}_3$)$_2$C(CH_3)$_3$), 0.74 (3H, s, 18-CH_3), 0.86 (9H, s, –Si(CH_3)$_2$C($C\underline{H}_3$)$_3$), 0.89 (3H, d, J=6.2 Hz, 21-CH_3), 1.18 (3H, s, 19-CH_3), 3.54 (1H, br m, 3β-H), 3.66 (3H, s, –$COOCH_3$). LR-MS (EI-PIM), *m/z*: 477 (M$-$[C(CH_3)$_3$], 100%), 445 (36%), 402 (M$-$[(CH_3)$_3$C(CH_3)$_2$SiOH], 2%), 385 (M$-H_2O-$[OSi(CH_3)$_2$C(CH_3)$_3$], 76%), 367 (M$-2H_2O-$[OSi(CH_3)$_2$C(CH_3)$_3$], 37%), 353 (M$-2H_2O-$[OSi(CH_3)$_2$C(CH_3)$_3$]$-CH_4O$, 87%), 335 (44%), 317 (21%), 207 (38%). HR-MS (ESI-PIM), Calcd for $C_{31}H_{54}ONaSi$ [M+Na]$^+$: 557.3638. Found: *m/z*, 557.3609.

Methyl 3α-*tert*-Butyldimethylsilyloxy-7α,14α-dihydroxy-5β-cholan-24-oate (7a) To a stirred solution of the 3α-*tert*-butyldimethylsilyloxy-7-oxo ester **6a** (230 mg) in benzene (5 ml) a freshly prepared solution of 1.0 M-$Zn(BH_4)_2$ in diethyl ether (5 ml)[22] was added dropwise, and the mixture was stirred for 30 min at room temperature. The organic layer was washed with saturated brine, dried with Drierite, and evaporated to a semi-crystalline product. The residue was recrystallized from methanol to give the title compound **7a** as colorless needles: mp, 150—153 °C; yield, 230 mg, *ca.* 100%. IR (KBr), ν_{max} cm^{-1}: 1747 (C=O), 3417 (OH). ^{1}H-NMR ($CDCl_3$), δ: 0.05 (6H, s, –Si($C\underline{H}_3$)$_2$C(CH_3)$_3$), 0.76 (3H, s, 18-CH_3), 0.88 (9H, s, –Si(CH_3)$_2$C($C\underline{H}_3$)$_3$), 0.89 (3H, d, J=5.9 Hz, 21-CH_3), 0.90 (3H, s, 19-CH_3), 3.42 (1H, br m, 3β-H), 3.67 (3H, s, –$COOCH_3$), 4.15 (1H, br s, 7β-H). LR-MS (EI-PIM), *m/z*: 518 (M$-H_2O$, 1%), 500 (M$-2H_2O$, 1%), 369 (M$-2H_2O-$[(CH_3)$_3$C(CH_3)$_2$SiOH], 100%), 253 (M$-2H_2O-$[(CH_3)$_3$C(CH_3)$_2$SiOH]$-$S.C., 5%). HR-MS (ESI-PIM), Calcd for $C_{31}H_{56}O_5NaSi$ [M+Na]$^+$: 559.3795. Found: *m/z*, 559.3802.

Methyl 3α,7α,14α-Trihydroxy-5β-cholan-24-oate (1a) To a solution of the 3α-*tert*-butyldimethylsilyloxy-7α,14α-dihydroxy ester (170 mg) in methanol (5 ml) was added 1% methanolic *p*-toluenesulfonic acid solution (5 ml). After the mixture was left to stand at room temperature for 10 min, 8% aqueous $NaHCO_3$ solution (1 ml) was added, and most of methanol was removed by evaporation under reduced pressure. The reaction product was extracted with $CHCl_3$, and the combined extract was washed with water, dried with Drierite, and evaporated. The residual oil was recrystallized from EtOAc–hexane to give the desired 3α,7α,14α-trihydroxy ester **1a** as a colorless amorphous solid: mp, 149—152 °C; yield, 135 mg, *ca.* 100%. IR (KBr), ν_{max} cm^{-1}: 1732 (C=O), 3330 (OH). ^{1}H-NMR ($CDCl_3$), δ: 0.79 (3H, s, 18-CH_3), 0.91 (3H, d, J=5.9 Hz, 21-CH_3), 0.93 (3H, s, 19-CH_3), 3.47 (1H, br m, 3β-H), 3.67 (3H, s, –$COOCH_3$), 4.21 (1H, br s, 7β-H). ^{13}C-NMR ($CDCl_3$), δ: 15.7 (C-18), 18.2 (C-21), 19.5 (C-11), 22.6 (C-19), 26.3 (C-16), 27.4 (C-20), 30.3 (C-2), 31.2 and 31.3 (C-22, C-23), 31.8 (C-15), 32.3 (C-6), 34.9

(C-1), 35.2 (C-10), 35.3 (C-12), 35.4 (C-9), 39.4 (C-4), 40.3 (C-5), 41.4 (C-8), 46.8 (C-13), 50.6 (C-17), 51.5 (–COOCH$_3$), 69.4 (C-7), 71.9 (C-3), 87.0 (C-14), 174.7 (C-24). LR-MS (EI-PIM), *m/z*: 404 (M−H$_2$O, 39%), 386 (M−2H$_2$O, 62%), 368 (M−3H$_2$O, 28%), 353 (M−3H$_2$O−CH$_3$, 12%), 289 (M−H$_2$O−S.C., 58%), 271 (M−2H$_2$O−S.C., 48%), 253 (M−3H$_2$O−S.C, 57%), 229 (11%), 212 (100%), 208 (35%), 195 (22%). HR-MS (ESI-PIM), Calcd for $C_{25}H_{42}O_5Na$ [M+Na]$^+$: 445.2930. Found: *m/z*, 445.2921.

3α,7α,14α-Trihydroxy-5β-cholan-24-oic Acid (1) Alkaline hydrolysis of the ester **1a** (70 mg) with 5% methanolic KOH (5 ml), followed by acidification with 10% H_2SO_4 as described in the preparation of **4** afforded the free acid **1**. Recrystallization of **1** from methanol gave an analytical pure sample of **1** as colorless amorphous solid: mp, 166—170 °C; yield, 57 mg, 84%. IR (KBr), ν_{max} cm^{-1}: 1715 (C=O), 3325, 3368, 3435 (OH). ^{1}H-NMR (CD$_3$OD), δ: 0.81 (3H, s, 18-CH$_3$), 0.92 (3H, d, *J*=7.5 Hz, 21-CH$_3$), 0.94 (3H, s, 19-CH$_3$), 3.37 (1H, br m, 3β-H), 4.12 (1H, br s, 7β-H). LR-MS (EI-PIM), *m/z*: 408 (M$^+$+, >1%), 390 (M−H$_2$O, 17%), 372 (M−2H$_2$O, 51%), 354 (M−3H$_2$O, 22%), 339 (M−3H$_2$O−CH$_3$, 17%), 299 (6%), 289 (M−H$_2$O−S.C., 23%), 271 (M−2H$_2$O−S.C., 57%), 253 (M−3H$_2$O−S.C., 100%), 211 (M−3H$_2$O−S.C.−ring D, 6%), 198 (37%). HR-MS (ESI-NIM), Calcd for $C_{24}H_{39}O_5$ [M−H]$^-$: 407.2797. Found: *m/z*, 407.2794.

Methyl 3α,7β-Dihydroxy-5β-chol-14-en-24-oate (8a) A mixture of the 3α,7α,14α-trihydroxy acid **4** (100 mg) in methanol (5 ml) and 5% *p*-toluenesulfonic acid (5 ml) in methanol was left to stand at room temperature for 12 h. Most of the solvent was evaporated, and the residue was extracted with $CHCl_3$. The combined extract was washed with water, dried with Drierite, and evaporated to a residue, which resisted crystallization attempts: yield, 92 mg, 96%. IR (KBr), ν_{max} cm^{-1}: 1736 (C=O), 3435 (OH). ^{1}H-NMR (CDCl$_3$): 0.93 (3H, s, 18-CH$_3$), 0.94 (3H, d, *J*=5.9 Hz, 21-CH$_3$), 0.95 (3H, s, 19-CH$_3$), 3.58 (1H, br m, 3β-H), 3.67 (3H, s, –COOCH$_3$), 3.98 (1H, br m, 7α-H), 5.43 (1H, br s, 15-H). LR-MS (EI-PIM), *m/z*: 386 (M−H$_2$O, 85%), 368 (M−2H$_2$O, 35%), 353 (M−2H$_2$O−CH$_3$, 20%), 271 (M−H$_2$O−S.C., 49%), 253 (M−2H$_2$O−S.C., 100%), 239 (15%), 208 (20%). HR-MS (ESI-PIM), Calcd for $C_{25}H_{40}O_4Na$ [M+Na]$^+$: 427.2842. Found: *m/z*, 427.2856.

Acknowledgements We acknowledge many helpful suggestions of Professor Emeritus Alan F. Hofmann, Department of Medicine, University of California, San Diego. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

References and Notes

1) Part 25 of this series: Iida T., Hikosaka M., Kakiyama G., Shiraishi K., Schteingart C. D., Hagey L. R., Ton-Nu H. T., Hofmann A. F., Mano N., Goto J., Nambara T., *Chem. Pharm. Bull.*, **50**, 1327—1334 (2002).
2) Hofmann A. F., Schteingart C. D., Hagey L. R., “Bile Acids in Liver Diseases,” ed. by Paumgartner G., Beuers U., Kluwer Academic Publishers, Dordrecht, 1995, pp. 3—30.
3) Haslewood G. A. D., Sjövall J., *Biochem. J.*, **57**, 126—130 (1954).
4) Hsia S. L., “The Bile Acids-Chemistry, Physiology, and Metabolism,” Vol. 1, ed. by Nair P. P., Kritchevsky D., Plenum Press, New York, 1971, pp. 95—120.
5) Lee S. P., Lester R., Pyrek J. St., *J. Lipid Res.*, **28**, 19—31 (1987).
6) Hagey L. R., Schteingart C. D., Ton-Nu H.-T., Hofmann A. F., *J. Lipid Res.*, **35**, 2041—2048 (1994).
7) Shoda J., Mahara R., Osuga T., Tohma M., Ohnishi S., Miyazaki H., Tanaka N., Matsuzaki Y., *J. Lipid Res.*, **29**, 847—858 (1988).
8) Dumaswala R., Setchell K. D. R., Zimmer-Nechemias L., Iida T., Goto J., Nambara T., *J. Lipid Res.*, **30**, 847—856 (1989).
9) Schteingart C. D., Hagey L. R., Setchell K. D. R., Hofmann A. F., *J. Biol. Chem.*, **268**, 11239—11246 (1993).
10) Hagey L., Schteingart C. D., Ton-Nu H.-T., Hofmann A. F., *J. Lipid Res.*, **43**, 685—690 (2002).
11) Hoshita T., Hirofugi S., Kazuno T., *J. Biochem.* (Tokyo), **51**, 136—141 (1967).
12) Haslewood G. A. D., *Biochem. J.*, **78**, 352—359 (1961).
13) Klinot J., Jirsa M., Klinotova E., Ubik K., Protiva J., *Collect. Czecho. Chem. Commun.*, **51**, 1722—1730 (1986).
14) Hagey L. R., Schteingart C. G., Ton-Nu H.-T., Odell D., Hofmann A. F., *The Condor*, **92**, 593—597 (1990).
15) Hagey L. R., Thesis Ph. D., “Bile Acid Biodiversity in Vertebrates: Chemistry and Evolutionary Implications,” University of California, San Diego, 1992.
16) Cerré C., Hofmann A. F., Schteingart C. D., Jia W., Maltby D., *Tetrahedron*, **53**, 435—446 (1997).
17) Iida T., Yamaguchi T., Nakamori R., Hikosaka M., Mano N., Goto J., Nambara T., *J. Chem. Soc. Perkin Trans. 1*, **2001**, 2229—2236 (2001).
18) Hashimoto N., Aoyama T., Shioiri T., *Chem. Pharm. Bull.*, **29**, 1475—1478 (1981).
19) Iida T., Nambara T., Chang F. C., “Bile Acids in Gastroenterology,” ed. by Hofmann A. F., Paumgartner G., Stiehl A., Kluwer Academic Publishers, Dordrecht, 1995, pp. 8—26.
20) Sasaki T., Nakamori R., Yamaguchi T., Kasuga Y., Iida T., Nambara T., *Chem. Phys. Lipids*, **109**, 135—143 (2001).
21) Cheng Y.-S., Liu W.-L., Chen S.-H., *Synthesis*, **1980**, 223—224 (1980).
22) Gensler W. J., Johnson F., Sloan A. D. B., *J. Am. Chem. Soc.*, **82**, 6074—6081 (1960).