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# Chemical Synthesis of Rare Natural Bile Acids: 11α-Hydroxy Derivatives of Lithocholic and Chenodeoxycholic Acids

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**Abstract** A method for the preparation of 11α-hydroxy derivatives of lithocholic and chenodeoxycholic acids, recently discovered to be natural bile acids, is described. The principal reactions involved were (1) elimination of the 12α-mesyloxy group of the methyl esters of 3α-acetate-12α-mesylate and 3α,7α-diacetate-12α-mesylate derivatives of deoxycholic acid and cholic acid with potassium acetate/hexamethylphosphoramide; (2) simultaneous reduction/hydrolysis of the resulting $\triangle^{11}$-3α-acetoxy and $\triangle^{11}$-3α,7α-diacetoxy methyl esters with lithium aluminum hydride; (3) stereoselective 11α-hydroxylation of the $\triangle^{11}$-3α,24-diol and $\triangle^{11}$-3α,7α,24-triol intermediates with $B_2H_6$/tetrahydrofuran (THF); and (4) selective oxidation at C-24 of the resulting 3α,11α,24-triol and 3α,7α,11α,24-tetrol to the corresponding C-24 carboxylic acids with $NaClO_2$ catalyzed by 2,2,6,6-tetramethylpiperidine 1-oxyl free radical (TEMPO) and NaClO. In summary, 3α,11α-dihydroxy-5β-cholan-24-oic acid and 3α,7α,11α-trihydroxy-5β-cholan-24-oic acid have been synthesized and their nuclear magnetic resonance (NMR) spectra characterized. These compounds are now available as reference standards to be used in biliary bile acid analysis.

**Keywords** 3α,11α-Dihydroxy-5β-cholan-24-oic acid · 11α-Hydroxy-bile acid · 11α-Hydroxylation · NMR · 2,2,6,6-Tetramethylpiperidine 1-oxyl free radical · 3α,7α,11α-Trihydroxy-5β-cholan-24-oic acid

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**Abbreviations**

| | |
|---|---|
| CA | cholic acid |
| CDCA | chenodeoxycholic acid |
| DCA | deoxycholic acid |
| HMPA | hexamethylphosphoramide |
| HR-LC/ESI-MS | high-resolution liquid chromatograph-mass spectrometry with an electrospray ionization |
| LAH | lithium aluminum hydride |
| LCA | lithocholic acid |
| NMR | nuclear magnetic resonance |
| RP-HPLC/RI | reversed-phase high-performance liquid chromatograph with a refractive index detector |
| RP-TLC | reversed-phase thin-layer chromatograph |
| TEMPO | 2,2,6,6-tetramethylpiperidine 1-oxyl free radical |
| TLC | thin-layer chromatograph |

## Introduction

The great variety of bile acids and bile alcohols occurring in vertebrates can be explained by the evolution of differing biochemical pathways that serve to convert cholesterol into amphipathic conjugated bile acids or bile alcohols. Bile acid or bile alcohol composition shows significant variation between orders but not between families, genera, or species. Biliary bile acid composition is a biochemical trait that may provide clues to evolutionary relationships,

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complementing anatomical and genetic analyses (Hofmann & Hagey, 2008, 2014; Hofmann, Hagey, & Krasowski, 2010). Naturally occurring bile acids and bile alcohols differ markedly in their chemical structures, particularly in the number, position, and stereochemistry of hydroxyl groups affixed to the 5β- or 5α-steroid nucleus and in the branched side chain. In inborn errors of bile acid biosynthesis, uncommon bile acids may be formed and excreted into bile or urine. These abnormal bile acids may provide diagnostic information (Sjövall, 2004).

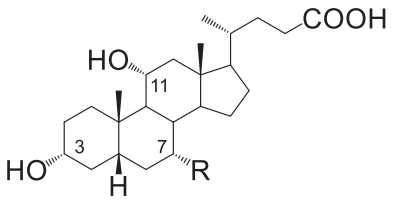
In vertebrates, $C_{24}$ bile acids that are synthesized directly from cholesterol in the hepatocyte *via* $C_{27}$ bile alcohols and $C_{27}$ higher bile acids are termed primary bile acids. The dominant primary bile acid is chenodeoxycholic acid (CDCA, 3α,7α-dihydroxy-5β-cholan-24-oic acid); an additional major site of hydroxylation was at C-12 to yield cholic acid (CA, 3α,7α,12α-trihydroxy-5β-cholan-24-oic acid). In the large intestine, anaerobic modifications of the hydroxyl groups in the primary bile acids occur to form secondary bile acids. The most striking change is 7-dehydroxylation that occurs *via* a 3-oxo-$\triangle^{4,6}$ (Ishida et al., 1998; Sjövall, 2004) nucleotide intermediate. The 7-dehydroxylation product of CA is deoxycholic acid (DCA, 3α,12α-dihydroxy-5β-cholan-24-oic acid); that of CDCA is lithocholic acid (LCA, 3α-hydroxy-5β-cholan-24-oic acid). If one accepts the concept of a default steroid nucleus with hydroxyl groups at C-3, C-7, and/or C-12, then further modifications can be considered as additions to the default structure. The other sites of nuclear hydroxylation in uncommon naturally occurring bile acids are at C-1 (1α-/1β-), C-2 (2α-/2β-), C-4 (4β-), C-5 (5β-), C-6 (6α-/6β-), C-10 (19β-), C-15 (15α-), and C-16 (16α) (Hofmann et al., 2010; Hofmann & Hagey, 2008, 2014).

Recently, bile acids hydroxylated at the rare hydroxylation sites of C-9 (9α-) (Bi, Chai, Song, Lei, & Tu, 2009) and C-11 (11α-) (Ishida et al., 1998) have been identified. A 9α-hydroxy-bile acid (in the form of its taurine conjugate) was isolated from the bile of the Asian black bear (*Selenarctos thibetanus*) (Bi et al., 2009). A bile acid having the structure of 3α,7α,11α-trihydroxy-5β-cholan-24-oic acid (as the taurine conjugate) was isolated from the biliary bile of the sunfish (*Mola mola*) (Ishida et al., 1998).

For some years, we have had an ongoing program of chemical synthesis of rare or potential bile acid metabolites and thus to make them available as reference standards. The conclusive evidence for the assigned structure is based on NMR spectra and some cases on X-ray diffraction. As an example of our work, we have recently reported the preparation of 3α,9α-dihydroxy- and 3α,7α,9α-trihydroxy-5β-cholan-24-oic acids (Iida et al., 2016).

Here, we report the successful synthesis of the two hitherto unreported 11α-hydroxy-LCA (3α,11α-dihydroxy-5β-cholan-24-oic acid, **1a**) and 11-hydroxy-CDCA (3α,7α,11α-trihydroxy-5β-cholan-24-oic acid, **1b**) (Fig. 1). We have also re-examined methods of synthesis of C-11 hydroxy-bile acids that were reported in the distant past when DCA was used as starting material for the synthesis of cortisone.

**Fig. 1** Chemical structures of 11α-hydroxy-bile acids




| | R |
|---|---|
| **1a** | H |
| **1b** | OH |

## Experimental

### Materials

DCA (**2a**) and CA (**2b**) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals and solvents were of analytical reagent grade and available from commercial sources. All compounds were dried by azeotropic distillation before use in reactions.

### Instruments

All melting points (mp) were determined on a micro hot stage apparatus and are uncorrected. $^{1}H$ and $^{13}C$ NMR spectra were obtained on a JEOL ECA 500 FT instrument operated at 500 and 125 MHz, respectively, with $CDCl_3$ or $CD_3OD$ containing 0.1% $Me_4Si$ (tetramethylsilane, TMS) as the solvent; chemical shifts were expressed in σ (ppm) relative to TMS (0 ppm). The $^{13}C$ distortionless enhancement by polarization transfer (DEPT; 135 °, 90 °, and 45 °) spectra were measured to determine the exact $^{13}C$ signal multiplicity and to differentiate among $CH_3$, $CH_2$, CH, and C based on their proton environments. In order to further confirm the $^{1}H$ and $^{13}C$ signal assignments for some of the compounds, $^{1}H$–$^{1}H$ correlation spectroscopy (COSY), $^{1}H$ nuclear Overhauser and exchange spectroscopy (NOESY), $^{1}H$ detected heteronuclear multiple quantum coherence (HMQC; $^{1}H$–$^{13}C$ coupling), and $^{1}H$ detected heteronuclear multiple bond connectivity (HMBC; long-range $^{1}H$–$^{13}C$ coupling) experiments were also done. These 2D–NMR spectra were recorded using standard pulse sequences and parameters recommended by the manufacture.

High-resolution liquid chromatograph-mass spectrometry with electrospray ionization (HR-LC/ESI-MS) source was carried out using a AccuTOF JMS-T100LC liquid chromatograph-mass spectrometer (Jeol, Tokyo, Japan) coupled to a Agilent 1200 series binary pump (Agilent Technologies Inc., Santa Clara, CA, USA) operated in the positive or negative ion mode. The ionization conditions were as follows: needle voltage, −2 kV in the positive and negative ion modes; ionization guide peak voltage, 2 kV; ion source temperature, 80 °C; desolvating plate temperature, 250 °C; absolute ring-lens voltage, −15 V; orifice voltage, 75 V, mass range *m/z* 50–1000; and nebulizing gas, $N_2$.

The preparative reversed-phase high-performance liquid chromatograph with refractive index detector (RP-HPLC/RI) consisted of a Hitachi L-7100 pump (Tokyo, Japan), Shodex RI-102 detector (Tokyo, Japan), and Sugai U620 column heater (Wakayama, Japan). Preparative RP-HPLC/RI was carried out by isocratic elution on a Capcell Pak MGII RP-$C_{18}$ column (250 mm × 10 mm internal diameter (I.D.); particle size, 5 μm; Shiseido, Tokyo, Japan) using a mixture of methanol–water–acetic acid (80:20:0.05, v/v/v) as the mobile phase at the flow rate 7.4 mL/min.

Normal-phase thin-layer chromatograph (TLC) was performed on precoated Kieselgel 60$F_{254}$ plates (E. Merck, Darmstad, Germany) using EtOAc-hexane mixtures as the developing solvent. Reversed-phase TLC was performed on precoated RP-18$F_{254s}$ plates (E. Merck, Darmstad, Germany) using methanol–water mixtures as the developing solvent.

### Methyl 3α-Acetoxy-12α-Mesyloxy-5β-Cholan-24-Oate (5a)

To methyl 3α-acetoxy-12α-hydroxy-5β-cholan-24-oate **4a** (2.0 g, 4.5 mmol), prepared from DCA (**2a**) in two steps (methyl esterification and then partial acetylation), dissolved in dry pyridine (10 mL) and magnetically stirred, methanesulfonylchloride (3.5 mL, 45 mmol) was added dropwise. After stirring at room temperature for 16 h, the dark pyridine solution was stirred, diluted with ice chips, and extracted with EtOAc; the reaction was monitored by TLC. The combined EtOAc extract was washed successively with cold, dilute HCl, $NaHCO_3$, and water, decolorized with Norite, dried with anhydrous $MgSO_4$, and evaporated under reduced pressure. The residue was chromatographed on a column of silica gel (150 g) eluting with EtOAc-hexane (35:65, v/v). Recrystallization of the product from EtOAc-hexane gave the title compound **5a** as colorless thin plates (a single spot on TLC): mp, 104–105 °C; 2.15 g (91%). $^1$H NMR (500 MHz, $CDCl_3$) σ: 0.75 (s, 3H, 18-$H_3$), 0.91 (s, 3H, 19-$H_3$), 0.98 (d, 3H, $J$ = 6.3 Hz, 21-$H_3$), 2.03 (s, 3H, 3α-OCO$\underline{CH_3}$), 3.05 (s, 3H, 12α-OSO$_2\underline{CH_3}$), 3.65 (s, 3H, 24-COO$\underline{CH_3}$), 4.69 (brm, 1H, 3β-H), 5.10 (m, 1H, 12β-H). $^{13}$C NMR (125.8 MHz, $CDCl_3$) σ: 12.5 (C-18), 17.8 (C-21), 21.6 (3-OCO$\underline{C}H_3$), 23.3 (C-19), 23.6, 25.9, 26.6, 27.1, 27.2, 27.6, 30.9, 31.1, 32.5, 34.1, 34.3, 34.9, 35.3, 35.9, 39.8 (12-OSO$_2\underline{C}H_3$), 41.9, 46.1, 47.3, 48.8, 51.6 (C-25), 74.2 (C-3), 85.3 (C-12), 170.8 (3-O$\underline{C}$OCH$_3$), 174.6 (C-24). HR-LC/ESI-MS, Calcd for $C_{28}H_{46}O_7SNa$ $[M + Na]^+$, 549.2862; found, *m/z* 549.2862.

### Methyl 3α,7α-Diacetoxy-12α-Mesyloxy-5β-Cholan-24-Oate (5b)

The 3α,7α-diacetoxy-12α-hydroxy ester **4b** (2.0 g, 3.9 mmol), prepared in two steps from CA (**2b**), was subjected to mesylation with methanesulfonylchloride (3.2 mL, 41 mmol) and processed as described for the preparation of **5a**. The product was an oily residue. Chromatography of the oily residue on a silica gel column (200 g) and elution with EtOAc-hexane (4:6, v/v) afforded the 12α-mesyloxy derivative **5b**, which was recrystallized from EtOAc-hexane as colorless thin plates (a single spot on TLC): mp, 137–139 °C, yield, 2.14 g (93%). $^1$H NMR (500 MHz, $CDCl_3$) σ: 0.76 (s, 3H, 18-$H_3$), 0.92 (s, 3H, 19-$H_3$), 0.99 (d, 3H, $J$ = 6.3 Hz, 21-$H_3$), 2.02 (s, 3H, 3α-OCO$\underline{CH_3}$), 2.08 (s, 3H, 7α-OCO$\underline{CH_3}$), 3.08 (s, 3H, 12α-OSO$_2\underline{CH_3}$), 3.65 (s, 3H, 24-COO$\underline{CH_3}$), 4.56 (brm, 1H, 3β-H), 4.90 (m, 1H, 7β-H), 5.10 (m, 1H, 12β-H). $^{13}$C NMR (125.8 MHz, $CDCl_3$) σ: 12.4 (C-18), 17.8 (C-21), 21.6 (3-OCO$\underline{C}H_3$), 22.7 (7-OCO$\underline{C}H_3$), 22.7 (C-19), 22.9, 26.8, 27.1, 27.4, 28.6, 30.9, 31.0, 31.3, 34.5, 34.8, 34.8, 35.2, 37.9, 39.8 (12-OSO$_2\underline{C}H_3$), 41.0, 43.1, 46.1, 47.1, 51.6 (C-25), 70.6 (C-7), 74.1 (C-3), 84.6 (C-12), 170.5 (7-O$\underline{C}$OCH$_3$), 170.8 (3-O$\underline{C}$OCH$_3$), 174.5 (C-24). HR-LC/ESI-MS, Calcd for $C_{30}H_{48}O_9SNa$ $[M + Na]^+$, 607.2917; found, *m/z* 607.2910.

### Methyl 3α-Acetoxy-5β-Chol-11-Ene-24-Oate (6a)

To a solution of the 3α-acetoxy-12α-mesyloxy ester **5a** (1.5 g, 2.8 mmol) dissolved in hexamethylphosphoramide (HMPA, 15 mL) was added potassium acetate (6 g). The resulting solution was stirred for 48 h at 100 °C. After cooling at room temperature, the reaction was quenched by adding water gradually and the product was extracted with EtOAc. The combined EtOAc extract was washed with 10% HCl and saturated brine, dried with Drierite, and evaporated to dryness. Chromatography of the crude residue over a column of silica gel (150 g) eluting with EtOAc-hexane (2:8, v/v) resulted in a single component, which was identified as the desired 3α-acetoxy-11-ene **6a**, which was recrystallized from EtOAc-hexane as colorless thin plates (a single spot on TLC): mp, 118–120 °C; yield,

0.96 g (78%). $^{1}$H NMR (500 MHz, $CDCl_3$) σ: 0.72 (s, 3H, 18-$H_3$), 0.88 (s, 3H, 19-$H_3$), 0.99 (d, 3H, $J$ = 6.3 Hz, 21-$H_3$), 2.01 (s, 3H, 3α-OCO$\underline{C}H_3$), 3.66 (s, 3H, 24-COO$\underline{C}H_3$), 4.72 (brm, 1H, 3β-H), 5.39 (dd, 1H, $J$ = 1.7, 10.3 Hz, 11-H), 6.09 (dd, 1H, $J$ = 3.4, 10.3 Hz, 12-H). $^{13}$C NMR (125.8 MHz, $CDCl_3$) σ: 16.7 (C-18), 18.4 (C-21), 21.5 (3-OCO$\underline{C}H_3$), 23.1, 23.7 (C-19), 25.5, 26.7, 28.0, 28.5, 31.0, 31.1, 33.0, 34.4, 34.9, 35.1, 36.0, 41.0, 43.2, 45.1, 51.6 (C-25), 52.0, 53.7, 74.3 (C-3), 125.5 (C-11), 138.9 (C-12), 170.8 (3-O$\underline{C}OCH_3$), 174.6 (C-24). HR-LC/ESI-MS, Calcd for $C_{27}H_{42}O_4Na$ $[M + Na]^+$, 453.2981; found, *m/z* 453.2969.

**Methyl 3α,7α-Diacetoxy-5β-Chol-11-Ene-24-Oate (6b)**

The 3α,7α-diacetoxy-12α-mesyloxy ester **5b** (1.4 g, 2.4 mmol), subjected to the elimination reaction (reaction time, 24 h) with potassium acetate in HMPA and processed as described for the preparation of **6a**, gave a crude product. Chromatography of the product on a column of silica gel (100 g) and elution with EtOAc-hexane (2:8, v/v) afforded the 3α,7α-diactoxy-11-ene **6b**, which was recrystallized from EtOAc-hexane as colorless thin plates (a single spot on TLC): mp, 142–143 °C; yield, 0.95 g (81%). $^{1}$H NMR (500 MHz, $CDCl_3$) σ: 0.73 (s, 3H, 18-$H_3$), 0.89 (s, 3H, 19-$H_3$), 1.00 (d, 3H, $J$ = 6.3 Hz, 21-$H_3$), 2.02 (s, 3H, 3α-OCO$\underline{C}H_3$), 2.05 (s, 3H, 7α-OCO$\underline{C}H_3$), 3.66 (s, 3H, 24-COO$\underline{C}H_3$), 4.60 (brm, 1H, 3β-H), 4.96 (m, 1H, 7β-H), 5.45 (dd, 1H, $J$ = 1.7, 10.3 Hz, 11-H), 6.15 (dd, 1H, $J$ = 2.9, 10.3 Hz, 12-H). $^{13}$C NMR (125.8 MHz, $CDCl_3$) σ: 16.8 (C-18), 18.4 (C-21), 21.5 (3-OCO$\underline{C}H_3$), 22.7 (7-OCO$\underline{C}H_3$), 22.5, 23.1 (C-19), 26.8, 28.5, 30.9, 31.0, 31.9, 34.9, 35.3, 35.3, 36.0, 36.4, 36.7, 40.3, 45.2, 49.3, 51.6 (C-25), 51.6, 70.4 (C-7), 74.0 (C-3), 124.9 (C-11), 139.2 (C-12), 170.5 (7-O$\underline{C}OCH_3$), 170.8 (3-O$\underline{C}OCH_3$), 174.7 (C-24). HR-LC/ESI-MS, Calcd for $C_{29}H_{44}O_6Na$ $[M + Na]^+$, 511.3036; found, *m/z* 511.2989.

**5β-Chol-11-Ene-3α,24-Diol (7a)**

To a magnetically stirred solution of 3α-acetoxy-11-ene **6a** (0.96 g, 2.2 mmol) in dry tetrahydrofuran (THF) (20 mL) was added gradually lithium aluminum hydride (LAH, 200 mg, 5.3 mmol) with ice-bath cooling. The mixture was further stirred at room temperature for 30 min; the reaction was monitored by TLC. The reaction was quenched by adding gradually 30% $H_2O_2$ (0.2 mL), 3 N NaOH (0.6 mL), and water (0.6 mL). After evaporation of the solvents, the residue was chromatographed on a column of silica gel (25 g) eluting with EtOAc-hexane (7:3, v/v)~EtOAc-methanol (9:1, v/v). Recrystallization of the major product from methanol–water gave the 3α,24-dihydroxy-11-ene **7a** as colorless thin plates (a single spot on TLC): mp, 184–186 °C; yield, 0.72 g, 90%. $^{1}$H NMR (500 MHz, $CDCl_3$) σ: 0.73 (s, 3H, 18-$H_3$), 0.88 (s, 3H, 19-$H_3$), 1.01 (d, 3H, $J$ = 6.9 Hz, 21-$H_3$), 3.61–3.65 (brm, 3H, 3β-H and 24-$H_2$), 5.41 (dd, 1H, $J$ = 1.2, 10.3 Hz, 11-H), 6.11 (dd, 1H, $J$ = 2.9, 10.3 Hz, 12-H). $^{13}$C NMR (125.8 MHz, $CDCl_3$) σ: 16.8 (C-18), 18.8 (C-21), 23.1, 23.8 (C-19), 25.6, 28.2, 28.6, 29.5, 30.6, 31.8, 34.5, 35.1, 35.3, 36.3, 37.2, 41.2, 43.2, 45.1, 52.2, 53.7, 63.7 (C-24), 71.8 (C-3), 125.5 (C-11), 139.0 (C-12). HR-LC/ESI-MS, Calcd for $C_{24}H_{40}O_2Na$ $[M + Na]^+$, 383.2926; found, *m/z* 383.2897.

**5β-Chol-11-Ene-3α,7α,24-Triol (7b)**

The 3α,7α-diacetoxy-11-ene **6b** (1.96 g, 4.0 mmol), subjected to reductive cleavage with LAH in dry THF at room temperature for 30 min and processed as described for the preparation of **7a**, afforded a crude reaction product. Chromatography of the product on a column of silica gel (50 g) and elution with EtOAc-hexane (9:1, v/v)~EtOAc-methanol (9:1, v/v) afforded the 3α,7α,24-trihydroxy-11-ene **7b**, which was recrystallized from methanol–water as colorless thin plates (a single spot on TLC): mp, 175–177 °C; yield, 1.43 g (95%). $^{1}$H NMR (500 MHz, $CD_3OD$) σ: 0.77 (s, 3H, 18-$H_3$), 0.88 (s, 3H, 19-$H_3$), 1.05 (d, 3H, $J$ = 6.3 Hz, 21-$H_3$), 3.40 (brm, 1H, 3β-H), 3.52 (brm, 2H, 24-$H_2$), 3.91 (m, 1H, 7β-H), 5.50 (dd, 1H, $J$ = 1.7, 10.3 Hz, 11-H), 6.17 (dd, 1H, $J$ = 2.9, 10.3 Hz, 12-H). $^{13}$C NMR (125.8 MHz, $CD_3OD$) σ: 17.0 (C-18), 19.3 (C-21), 23.4, 23.9 (C-19), 29.7, 30.3, 31.4, 33.1, 36.4, 36.5, 36.7, 36.7, 37.6, 39.2, 41.1, 42.4, 46.1, 50.6, 53.3, 63.5 (C-24), 69.4 (C-7), 72.7 (C-3), 126.6 (C-11), 140.1 (C-12). HR-LC/ESI-MS, Calcd for $C_{24}H_{40}O_3Na$ $[M + Na]^+$, 399.2875; found, *m/z* 399.2853.

**5β-Cholane-3α,11α,24-Triol (8a) and 5β-Cholane-3α,12α,24-Triol**

A solution of the 3α,24-dihydroxy-11-ene **7a** (500 mg, 1.4 mmol) in dry THF (20 mL) was cooled to 0 °C. Under a $N_2$ atmosphere, a solution of diborane complex ($B_2H_6$) in THF (10 mL of 1.0 mol/L solution) was injected slowly to the stirred solution of the olefin. The reaction mixture was stirred for 16 h at room temperature; the reaction was monitored by TLC. During ice-bath cooling and stirring, water (1 mL), 3 N NaOH (10 mL), and 30% $H_2O_2$ (10 mL) were added cautiously. After further stirring for 3 h at room temperature, the mixture was neutralized with 10% HCl and extracted with EtOAc. The combined extract was washed with saturated brine, dried with Drierite, and evaporated to dryness. The oily residue, which consisted of two components, was separated by preparative RP-HPLC eluting with a mixture of EtOAc methanol–water (8:2, v/v) as the developing solvent.

The more polar fraction was identified as the desired 3α,11α,24-triol **8a** and recrystallized from methanol–water as colorless needles (a single spot on RP-TLC): mp, 109–112 °C; yield, 226 mg (43%). $^{1}$H NMR and $^{13}$C NMR: see Table 1. HR-LC/ESI-MS, Calcd for $C_{24}H_{42}O_3Na$ $[M + Na]^+$, 401.3032; found, *m/z* 401.3034.

The less polar fraction, which was eluted with the same solvent system was identified as the 3α,12α,24-triol (a single spot on RP-TLC): mp, 96–99 °C (recrystallized from methanol–water); yield, 236 mg (45%). $^{1}$H NMR (500 MHz, $CDCl_3$) σ: 0.67 (s, 3H, 18-$H_3$), 0.89 (s, 3H, 19-$H_3$), 0.97 (d, 3H, $J$ = 6.9 Hz, 21-$H_3$), 3.58–3.62 (brm, 3H, 3β-H and 24-$H_2$), 3.98 (m, 1H, 12β-H). $^{13}$C NMR (125.8 MHz, $CDCl_3$) σ: 12.8 (C-18), 17.8 (C-21), 23.2 (C-19), 23.8, 26.2, 27.2, 27.7, 28.6, 29.6, 30.5, 31.9, 33.7, 34.2, 35.3, 35.5, 36.1, 36.5, 42.2, 46.6, 47.6, 48.3, 63.6 (C-24), 71.9 (C-3), 73.3 (C-12). HR-LC/ESI-MS, Calcd for $C_{24}H_{42}O_3Na$ $[M + Na]^+$, 401.3032; found, *m/z* 401.2984.

### 5β-Cholane-3α,7α,11α,24-Tetrol (8b) and 5β-Cholane-3α,7α,12α,24-Tetrol

The 3α,7α,24-trihydroxy-11-ene **7b** (500 mg, 1.3 mmol) was subjected to the hydroboration/oxidation reaction with $B_2H_6$-NaOH/$H_2O_2$ and processed as described for the preparation of **8a**. The procedure gave a mixture of two components, which were separated by RP-HPLC/RI.

The first, eluted with a mixture of methanol–water (8:2, v/v), consisted of a homogeneous fraction that was recrystallized from methanol–water as colorless needles and characterized as the desired 3α,7α,11α,24-tetrol **8b** (a single spot on RP-TLC): mp, 212–213 °C; yield, 210 mg (40%). $^{1}$H NMR and $^{13}$C NMR: see Table 1. HR-LC/ESI-MS, Calcd for $C_{24}H_{42}O_4Na$ $[M + Na]^+$, 417.2981; found, *m/z* 417.3006.

The second fraction, which was eluted with the same solvent system, was characterized as the 3α,7α,12α,24-tetrol (a single spot on RP-TLC): mp, 232–233 °C (recrystallized from methanol–water); yield, 225 mg (43%). $^{1}$H NMR (500 MHz, $CD_3OD$) σ: 0.72 (s, 3H, 18-$H_3$), 0.92 (s, 3H, 19-$H_3$), 1.02 (d, 3H, $J$ = 6.3 Hz, 21-$H_3$), 3.37 (brm, 1H, 3β-H), 3.51 (brm, 2H, 24-$H_2$), 3.80 (m, 1H, 7β-H), 3.96 (m, 1H, 12β-H). $^{13}$C NMR (125.8 MHz, $CD_3OD$) σ: 13.0 (C-18), 18.0 (C-21), 23.2 (C-19), 24.2, 27.9, 28.8, 29.6, 30.4, 31.2, 33.2, 35.8, 35.9, 37.1, 40.5, 41.0, 43.0, 43.2, 47.4, 48.3, 49.8, 63.6 (C-24), 69.1 (C-7), 72.9 (C-3), 74.1 (C-12). HR-LC/ESI-MS, Calcd for $C_{24}H_{42}O_4Na$ $[M + Na]^+$, 417.2981; found, *m/z* 417.2987.

### 3α,11α-Dihydroxy-5β-Cholan-24-Oic Acid (1a)

To a magnetically stirred solution of the 3α,11α,24-triol **8a** (110 mg, 0.28 mmol) in dry THF (2 mL) and $CH_3CN$ (0.5 mL) was added 2,2,6,6-tetramethylpiperidine 1-oxyl free radical (TEMPO; 5 mg, 30 μmol) and 0.2 M sodium phosphate buffer (pH, 6.7; 0.5 mL). To this solution was very slowly added 70% sodium chlorite ($NaClO_2$, 80 mg, 0.62 mmol) dissolved in water (0.6 mL) and then 5% sodium hypochlorite (bleach, NaClO, 15 μL) diluted in water (0.3 mL). The mixture was further stirred at 35 °C for 16 h, with the course of the reaction being monitored by TLC. The reaction was quenched by adding an ice-cold solution of saturated $Na_2S_2O_3$, and the product was extracted with EtOAc. The combined EtOAc extract was washed with saturated brine, dried with Drierite, and evaporated to dryness. The desired product, 3α,11α-dihydroxy acid **1a**, was purified by passing through preparative RP-HPLC/RI eluting with methanol–water-acetic acid (80:20:0.05, v/v/v) and recrystallized from methanol–water as colorless needles (a single spot on RP-TLC): mp, 103–106 °C [lit. (Long & Gallagher, 1946), mp, 125–145 °C]; yield, 96 mg (84%). $^{1}$H NMR and $^{13}$C NMR: see Table 1. HR-LC/ESI-MS, Calcd for $C_{24}H_{39}O_4$ $[M-H]^-$, 391.2848; found, *m/z* 391.2890.

### 3α,7α,11α-Trihydroxy-5β-Cholan-24-Oic Acid (1b)

The 3α,7α,11α,24-triol **8b** (220 mg, 0.58 mmol) was subjected to selective oxidation with TEMPO/$NaClO_2$/NaClO at 35 °C for 16 h and processed as described for the preparation of **1a** to yield a crude 3α,7α,11α-trihydroxy acid **1b**. Preparative RP-HPLC/RI of the crude **1b** and elution with methanol–water–acetic acid (80:20:0.05) afforded the analytically pure compound, which was recrystallized from methanol–water as colorless needles (a single spot on RP-TLC): mp, 203–206 °C; yield, 188 mg (82%). $^{1}$H NMR and $^{13}$C NMR: see Table 1. HR-LC/ESI-MS, Calcd for $C_{24}H_{39}O_5$ $[M-H]^-$, 407.2798; found, *m/z* 407.2799.

## Results and Discussion

Nuclear 11α-hydroxylation of bile acids in the liver of the sunfish (*Mola mola*) is the result of specific Cytochrome P450 (CYP)-mediated hydroxylation. According to Ishida et al. (1998), the sunfish has gained an enzyme that can introduce an α-hydroxyl group directly at C-11 of the 5β-cholestane skeleton to produce the 11α-hydroxy-bile salts. Another possibility is that the sunfish can catalyze 11β-hydroxylation of the 5β-cholestane skeleton and then convert the configuration of the product from β to α with the help of intestinal flora, as in the case of formation of ursodeoxycholic acid (UDCA) from CDCA.

A need for a moderate supply of **1a** and **1b** as reference specimens prompted us to examine prior literature preparations of the compounds. Microbial biotransformation

**Table 1** Complete $^{1}H$ and $^{13}C$-NMR spectral data for synthetic 11α-hydroxylated compounds (**8a**, **8b**, **1a**, and **1b**)

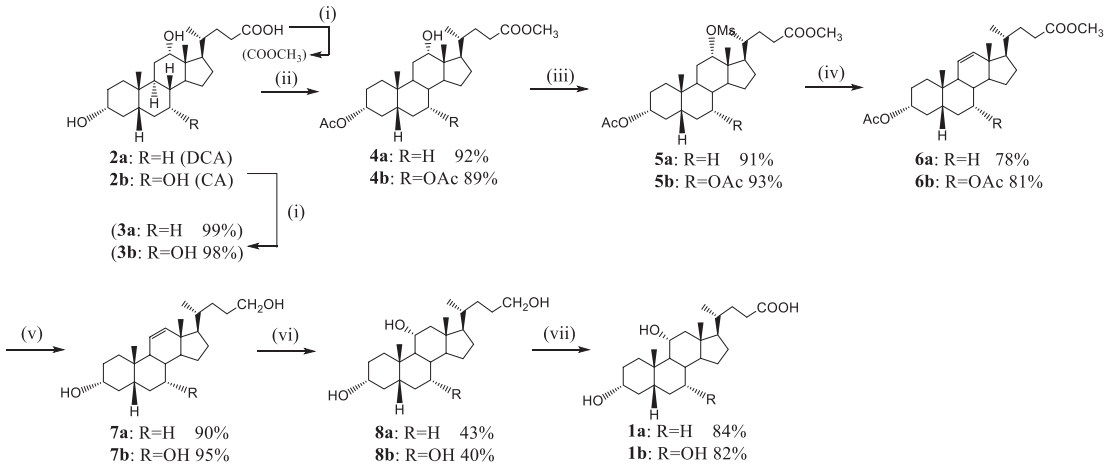
| | **8a**[a] | | | | **8b**[a] | | | | **1a**[b] | | | | **1b**[b] | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Proton | | | | Proton | | | | Proton | | | | Proton | |
| No | Type | Carbon | α | β | Type | Carbon | α | β | Type | Carbon | α | β | Type | Carbon | α | β |
| 1 | $CH_2$ | 38.33 | 2.56 | 1.01 | $CH_2$ | 39.35 | 2.58 | 0.97 | $CH_2$ | 39.21 | 2.64 | 0.95 | $CH_2$ | 39.35 | 2.55 | 0.97 |
| 2 | $CH_2$ | 31.79[c] | 1.50 | 1.72 | $CH_2$ | 32.42 | 1.59 | 1.59 | $CH_2$ | 32.18[c] | 1.59 | 1.59 | $CH_2$ | 32.42 | 1.59 | 1.59 |
| 3 | CH | 72.15 | — | 3.68 | CH | 73.24 | — | 3.41 | CH | 72.83 | — | 3.57 | CH | 73.24 | — | 3.41 |
| 4 | $CH_2$ | 36.96 | 1.74 | 1.52 | $CH_2$ | 41.10 | 2.27 | 1.67 | $CH_2$ | 37.71 | 1.76 | 1.47 | $CH_2$ | 41.11 | 2.25 | 1.65 |
| 5 | CH | 43.74 | — | 1.37 | CH | 44.57 | — | 1.34 | CH | 45.27 | — | 1.34 | CH | 44.57 | — | 1.32 |
| 6 | $CH_2$ | 27.67 | 1.28 | 1.83 | $CH_2$ | 36.25 | 1.52 | 1.98 | $CH_2$ | 28.86 | 1.28 | 1.89 | $CH_2$ | 36.24 | 1.50 | 1.96 |
| 7 | $CH_2$ | 26.31 | 1.43 | 1.15 | CH | 69.01 | — | 3.79 | $CH_2$ | 27.49 | 1.43 | 1.19 | CH | 68.99 | — | 3.77 |
| 8 | CH | 34.85 | — | 1.39 | CH | 40.21 | — | 1.52 | CH | 36.24 | — | 1.43 | CH | 40.21 | — | 1.52 |
| 9 | CH | 47.53 | 1.52 | — | CH | 41.17 | 1.96 | — | CH | 48.28 | 1.56 | — | CH | 41.17 | 1.96 | — |
| 10 | C | 35.81 | — | | C | 37.46 | — | | C | 36.89 | — | | C | 37.46 | — | |
| 11 | CH | 69.55 | — | 3.85 | CH | 69.77 | — | 3.82 | CH | 69.86 | — | 3.79 | CH | 69.74 | — | 3.81 |
| 12 | $CH_2$ | 52.23 | 1.17 | 2.29 | $CH_2$ | 52.25 | 1.26 | 2.29 | $CH_2$ | 52.59 | 1.28 | 2.23 | $CH_2$ | 52.21 | 1.26 | 2.27 |
| 13 | C | 43.16 | — | | C | 43.84 | — | | C | 44.16 | — | | C | 43.87 | — | |
| 14 | CH | 55.67 | 1.15 | — | CH | 51.05 | 1.52 | — | CH | 56.86 | 1.19 | — | CH | 51.04 | 1.52 | — |
| 15 | $CH_2$ | 24.32 | 1.57 | 1.04 | $CH_2$ | 24.63 | 1.72 | 1.03 | $CH_2$ | 25.33 | 1.59 | 1.06 | $CH_2$ | 24.62 | 1.72 | 1.03 |
| 16 | $CH_2$ | 28.32 | 1.87 | 1.26 | $CH_2$ | 29.28 | 1.93 | 1.32 | $CH_2$ | 29.17 | 1.89 | 1.32 | $CH_2$ | 29.15 | 1.92 | 1.34 |
| 17 | CH | 56.12 | 1.15 | — | CH | 57.49 | 1.23 | — | CH | 57.39 | 1.19 | — | CH | 57.29 | 1.21 | — |
| 18 | $CH_3$ | 13.14 | 0.66 | | $CH_3$ | 13.00 | 0.70 | | $CH_3$ | 13.45 | 0.70 | | $CH_3$ | 13.00 | 0.70 | |
| 19 | $CH_3$ | 23.85 | 1.04 | | $CH_3$ | 24.06 | 1.03 | | $CH_3$ | 24.40 | 1.06 | | $CH_3$ | 24.05 | 1.03 | |
| 20 | CH | 35.62 | 1.39 | | CH | 36.99 | 1.43 | | CH | 36.64 | 1.43 | | CH | 36.69 | 1.46 | |
| 21 | $CH_3$ | 18.66 | 0.94 | | $CH_3$ | 19.14 | 0.99 | | $CH_3$ | 18.69 | 0.97 | | $CH_3$ | 18.72 | 0.99 | |
| 22 | $CH_2$ | 31.76[c] | 1.08, 1.45 | | $CH_2$ | 33.13 | 1.08, 1.48 | | $CH_2$ | 32.11[c] | 1.28, 1.80 | | $CH_2$ | 32.22 | 1.30, 1.80 | |
| 23 | $CH_2$ | 29.45 | 1.45, 1.63 | | $CH_2$ | 30.23 | 1.41, 1.61 | | $CH_2$ | 31.97 | 2.20, 2.33 | | $CH_2$ | 31.92 | 2.20, 2.33 | |
| 24 | $CH_2$ | 63.63 | 3.61 | | $CH_2$ | 63.54 | 3.52 | | C | 178.10 | — | | C | 178.07 | — | |

[a]Measured in $CDCl_3$ at 500.2 MHz in $^{1}H$ NMR and at 125.8 MHz in $^{13}C$ NMR. Chemical shifts were expressed as $\delta$ ppm relative to TMS.

[b]Measured in $CD_3OD$ at 500.2 MHz in $^{1}H$-NMR and at 125.8 MHz in $^{13}C$-NMR. Chemical shifts were expressed as $\delta$ ppm relative to TMS.

[c]Assignments along a vertical column bearing the same superscript may be interchanged.

**Fig. 2** Synthetic route to 11α-hydroxy-bile acids from DCA and CA




*Reagents and conditions*: (i) *p*-Toluenesulfonic acid/ MeOH, at 40 °C for 1 h. (ii) $Ac_2O$/ pyridine/ toluene, at r.t. for 16 h. (iii) Methanesulfonylchloride/ dry-pyridine, at r.t. for 16 h. (iv) Potassium acetate/ HMPA, at 100 °C for 24 h (at 100 °C for 48 h). (v) $LiAlH_4$/ THF, at r.t. for 30 min. (vi) Diborane-THF solution/ THF, at r.t for 16 h ; NaOH/ $H_2O_2$, at r.t. for 3 h. (vii) $NaClO_2$/ TEMPO/ NaClO/ $CH_3CN$/ THF, at 35°C for 16 h .

(Bhatti & Khera, 2012; Bortolini, Medici, & Poli, 1997) has been an important tool for structural modification of such steroids as 11-deoxycortisol and progesterone to the corresponding 11α-hydroxy steroids (Kolet, Niloferjahan, Haldar, Gonnade, & Thulasiram, 2013; Petric, Hakki, Bernhardt, Zigon, & Cresnar, 2010; Swizdor, Panek, & Milecka-Tronina, 2014). Although the simultaneous 11α,15β- and 11β,15β-dihydroxylations of LCA have been reported by a fungus *Cunninghamella blakesleeana* ST-22 (Jodoi, Nihira, Naoki, Yamada, & Taguchi, 1987), such biological transformation is severely restricted for the selective 11α-hydroxylation in bile acids.

Meanwhile, 3α,11α-dihydroxy-5β-cholan-24-oic acid (**1a**), one of the earliest 11α-hydroxy-bile acids known, has been synthesized chemically by several lengthy and low-yield procedures (Gallagher & Long, 1946; Long & Gallagher, 1946; Turner, Mattox, Engel, Mckenzie, & Kendall, 1946). The first synthetic route involved the Wolff-Kishner/isomerization reaction of the 12-hydrazone derivative of methyl 3α,11β-diacetoxy-12-keto-5β-cholan-24-oate (Long & Gallagher, 1946) or its 3α,11β-dihydroxy acid (Gallagher & Long, 1946). The second approach consisted of the catalytic hydrogenation of 3α-hydroxy-11-keto-5β-cholanoic acid, which was obtained from methyl 3α-hydroxy-11-keto-12ξ-bromo-5β-cholanate (Turner et al., 1946). However, the two routes were less straightforward and inelegant than indicated in the literatures; very low yields of **1a** were obtained only after a laborious multistep process. Our simplified method for preparing 11α-hydroxy-bile acids (**1a** and **1b**), which proceeds through easily prepared, well-defined intermediates, affords pure products in good isolated yields.

As outlined in Fig. 2, the key intermediates in our work were the $\triangle^{11}$-3α,24-diol and $\triangle^{11}$-3α,7α,24-triols (**7a** and **7b**), which were prepared in four steps starting from DCA (**2a**) and CA (**2b**). Thus, selective acetylation of the methyl esters (**3a** and **3b**) of **2a** and **2b** under mild conditions resulted in the formation of the partially acetylated compounds at C-3 and C-7 (**4a** and **4b**) (Fieser & Rajagopalan, 1950; Iida, Tamura, Matsumoto, & Chang, 1985), respectively, which in turn were treated with methanesulfonylchloride to yield the corresponding 12α-mesyloxy derivatives (**5a** and **5b**) nearly quantitatively. When **5a** or **5b** was subjected to deoxygenation with potassium acetate in HMPA at 100 °C, a single olefinic product, $\triangle^{11}$-3α-acetate or $\triangle^{11}$-3α,7α-diacetate ester (**6a** or **6b**), was obtained in a good isolated yield of 78 (or 81%). In the $^1$H NMR spectra, the olefinic proton signals occurred at σ 5.39 (dd, 11-H) and 6.09 (dd, 12-H) in **6a** and at σ 5.45 (dd, 11-H) and 6.15 (dd, 12-H) in **6b**, suggesting that an unsaturated bond was introduced between the C-11 and C-12. Additional evidence in support of the presence of the $\triangle^{11}$-bond was also provided by the $^{13}$C NMR signals arising from the C-11 at σ 125.5 and the C-12 at σ 138.9 in **6a** and the C-11 at σ 124.9 and the C-12 at σ 139.2 in **6b**.

Subsequent treatment of **6a** and **6b** with LAH at room temperature resulted in simultaneous reduction/hydrolysis at C-3/C-7 and C-24 to afford the key intermediates, $\triangle^{11}$-3α,24-diol (**7a)** and $\triangle^{11}$-3α,7α,24-triol (**7b**), in high yields (90 and 95%), respectively. In the $^1$H NMR spectrum of

**7b**, the $^{1}H$ signals appearing at σ 5.50 (dd, 11-H) and 6.17 (dd, 12-H), together with at σ 3.40 (brm, 3β-H), 3.91 (m, 7β-H), and 3.52 (brm, 24-$H_2$), supported the evidence of the structure of **7b**. In addition, the $^{13}C$ signals in **7b** occurring at σ 126.6 (C-11) and 140.1 (C-12), along with at σ 72.7 (C-3), 69.4 (C-7), and 63.5 (C-24), provided a confirmatory evidence. Essentially, similar $^{1}H$ and $^{13}C$ NMR characteristics were observed in **7a**.

When **7a** and **7b** were subjected to the hydroboration/oxidation with $B_2H_6$ and $H_2O_2$/NaOH, the isolated products were the 3α,11α,24-triol (**8a**, 43%) and 3α,7α11α,24-tetrol (**8b**, 40%), accompanied by the simultaneous formation of the 3α,12α,24-triol (45%) and 3α,7α12α,24-tetrol (43%), respectively. The result implies that anti-Markownikoff's attack of $B_2H_6$ on the disubstituted $\triangle^{11}$-bond occurred from the less sterically hindered α-side and gave roughly equivalent amounts of the two possible 11α- and 12α-hydroxy isomers, which were separated efficiently by RP-HPLC/RI; no more sterically crowded 11β- and 12β-hydroxy compounds were produced. The $^{1}H$ NMR spectra of **8a** exhibited the $^{1}H$ signals arising from the 3β-H at σ 3.68 (brm), the 11β-H at σ 3.85 (brm) and the 24-$H_2$ at σ 3.61 (brm). On the other hand, the $^{13}C$ signals appeared for the methine C-3 at σ 72.2, the methine C-11 at σ 69.6 and the methylene C-24 at σ 62.2 in the DEPT experiment. Essentially, identical $^{1}H$ and $^{13}C$ NMR spectra were observed in **8b**.

Recently, successful use of $NaClO_2$ catalyzed by TEMPO and NaClO for oxidation of alcohols suggested that a selective oxidation of primary hydroxy group to its carboxyl group without oxidizing a secondary hydroxy group might be feasible (Anelli, Biffi, Montanari, & Quici, 1987; de Nooy, Besemer, & van Bekkum, 1996; Khripach et al., 2005; Zhao et al., 1999). Expectedly, mild reaction of **8a** and **8b** with $NaClO_2$ in the presence of the coreagents resulted in selective oxidation of the primary 24-hydroxy group to afford the desired 3α,11α-dihydroxy and 3α,7α,11α-trihydroxy acids (**1a** and **1b**) in excellent isolated yields of 84 and 82%, respectively.

Table 1 shows the complete $^{1}H$ and $^{13}C$ NMR signal assignments for 11α-hydroxylated compounds (**8a**, **8b**, **1a**, and **1b**). The presence and stereochemical configuration of the 11α-hydroxy group in the compounds were determined by a combined use of the DEPT and several $^{1}H/^{1}H$ and $^{1}H/^{13}C$ shift correlated 2D–NMR techniques, which include COSY, NOESY, HMQC, and HMBC. For example, the structure of **1a** was confirmed as follows. Thus, the $^{13}C$ signals appearing at σ 72.8 and 69.9 in the DEPT experiment, together with the $^{1}H$ signals occurring at σ 3.57 (3β-H, brm) and 3.79 (11β-H, brm), were assigned to the C-3 and C-11 methine carbons bearing a hydroxy group. In the HMBC, the appearance of the $^{1}H/^{13}C$ correlated peak arising from the 18-$H_3$ (σ 0.70, s) was assigned to the C-12 (σ 52.6). The $^{1}H/^{13}C$ correlation peaks in the HMQC were observed between the 12α-H (σ 1.28)/C-12 and 12β-H (σ 2.23)/C-12. The $^{1}H/^{1}H$ correlation peaks in the COSY were detected between the 12α-H/11β-H (σ 3.79, brm), 12β-H/11β-H, and 9α-H (σ 1.56)/11β-H, indicating the presence of a hydroxy group at the C-11 position. The stereochemical configuration of an equatorially oriented 11α-hydroxy group in **1a** was confirmed by measuring the NOESY, in which the distinct correlation peaks were detected between the 1,3-diaxially oriented 18-$H_3$ (σ 0.70, s)/11β-H and 19-$H_3$ (σ 1.06, s)/11β-H as well as the spatially closed 12β-H/11β-H. Essentially, similar $^{1}H$ and $^{13}C$ NMR correlations were also observed in **1b**, and their characteristics in the 5β-steroid nucleus were very similar to those reported for naturally occurring 5β-cholestane-3α,7α,11α,26,27-pentol and 3α,7α,11α-trihydroxy-5β-cholanoic acid taurine conjugate (Ishida et al., 1998).

The most promising method for preparing 11α-hydroxy-bile acids would seem to be the application of the so-called *anti-Markownikoff* hydroxylation with $B_2H_6$ and $H_2O_2$/NaOH of appropriate $\triangle^{9(11)}$- or $\triangle^{11}$-5β-cholene derivatives. Nussim, Mazur, and Sondheimer (1964) reported that the hydroxylation of $\triangle^{9(11)}$-5α-steroids with A/B-*trans* junction yielded almost exclusively the corresponding 11α-alcohols; however, the reaction did not proceed at all for $\triangle^{9(11)}$-5β-steroids with A/B-*cis* junction; on the other hand, the reaction of $\triangle^{11}$-5α-steroids yielded the corresponding 11α- and 12α-alcohols in a ratio of 1:1. In preliminary experiments, we found that 11α-hydroxylation of $\triangle^{9(11)}$-5β-cholen-3α,24-diol (Iida et al., 2016) was unsuccessful under various conditions, in analogy with the previous finding of other $\triangle^{9(11)}$-5β-steroids (Nussim et al., 1964). Furthermore, an attempted reductive cleavage with $AlH_2Cl$ (Iida et al., 2016) of methyl 3α-acetoxy-11α,12α-epoxy-5β-cholan-24-oate, which was easily prepared from **6a** intermediate, failed to afford 5β-cholan-3α,12α,24-triol as the major product. Hydroxylation of **6a**, instead of **7a**, was also unsatisfactory, yielding a complex mixture of products, probably because of the simultaneous formation of partially hydrolyzed products at C-3/C-7 and C-24 by the alkaline reagent. Therefore, the use of **7a** and **7b** as substrates is essential for the 11α-hydroxylation with $B_2H_6$ and $NaOH/H_2O_2$.

In conclusion, the availability of 3α,11α-dihydroxy-5β-cholan-24-oic acid (**1a**) and 3α,7α,11α-trihydroxy-5β-cholan-24-oic acid (**1b**) as standards should facilitate their identification when present in vertebrates.

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**Conflict of interest** We declare no conflict of interest.

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