Synthesis of 3α,7α-dihydroxy-5β-cholestan-26-oic acid from 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid: configuration in the bile of *Alligator mississippiensis*

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Synthesis of 25R- and 25S-diastereoisomers of 3α,7α-dihydroxy-5β-cholestan-26-oic acid from 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid is described. The 25S-diastereoisomer of 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid was obtained by vigorous hydrolysis of the bile of Alligator mississippiensis *followed by repeated crystallization of the hydrolysate, and the 25R-diastereoisomer was isolated by hydrolysis of the bile salts in bile of* A mississippiensis *with rat feces. Acetylation of the 25R- or 25S-diastereoisomer of methyl 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid under controlled conditions yielded the corresponding 3α,7α-diacetate in approximately 70% yield. The diacetate was quantitatively oxidized to methyl 3α,7α-diacetoxy-12-oxo-5β-cholestan-26-oate, which was converted into the 12-tosylhydrazone in approximately 58% yield. Reduction of the tosylhydrazone with sodium borohydride in acetic acid yielded the 25R- or the 25S-diastereoisomer of 3α,7α-dihydroxy-5β-cholestan-26-oic acid as the major product. Purification via column chromatography yielded the pure diastereoisomers in approximately 25% overall yield. The two diastereoisomers were resolved on thin-layer chromatography and high-performance liquid chromatography. When the bile of* A mississippiensis *was hydrolyzed with rat fecal bacteria, the 3α,7α-dihydroxy-5β-cholestan-26-oic acid isolated via chromatographic purification was shown to be the 25R-diastereoisomer.* (*Steroids* **57**:162–166, 1992)

Keywords: 3α,7α-dihydroxy-5β-cholestan-26-oic acid; 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid; diastereoisomers; alligator bile; thin-layer chromatography; high-performance liquid chromatography; *Alligator mississippiensis*; steroids

Introduction

3α,7α,12α-Trihydroxy-5β-cholestan-26-oic acid (THCA), the major bile acid of the alligator,[1] is present in trace amounts in human bile.[2] However, large amounts of this bile acid are accumulated in bile of patients with extrahepatic bile duct anomalies[3,4] and Zellweger's syndrome.[5] THCA is considered an obligate intermediate in the biosynthesis of cholic acid from cholesterol, formed via a 26-hydroxylation pathway.[6] This bile acid has been shown to be formed from cholesterol[7] and effectively converted into cholic acid both in vitro and in vivo.[8,9] Recently, Salen et al. isolated large quantities of bile alcohols hydroxylated at C-25 in the bile and feces of patients with cerebrotendinous xanthomatosis[10] and they proposed a different mechanism for the biosynthesis of cholic acid that involved 25-hydroxylated intermediates.[11] Because all bile alcohols isolated in cerebrotendinous xanthomatosis were hydroxylated at C-12, they concluded that the biosynthesis of chenodeoxycholic acid involved only the 26-hydroxylation pathway.[12–14] In order to obtain greater insight into the mechanism for the formation of chenodeoxycholic acid in humans, we needed quantities of the proposed obligate intermediate, 3α,7α-dihydroxy-5β-cholestan-26-oic acid (DHCA). The chemical synthesis of this bile acid from chenodeoxycholic acid via Arndt-Eistert synthesis[15] or via electrolytic coupling[16,17] is laborious

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and involves extensive purification steps. Furthermore, the synthesized DHCA is always a mixture of the 25R- and 25S-diastereoisomers. We describe a convenient method for the synthesis of DHCA from THCA, a bile acid that can be isolated in good yield from the bile of *Alligator mississippiensis*.[18,19] Starting with a single diastereoisomer of THCA,[18] the corresponding diastereoisomer of DHCA was prepared in pure form and used to assign the configuration in DHCA in alligator bile.[20,21]

Experimental

All reagents and solvents used were reagent grade and were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Elemental analysis of new compounds was performed at the Spang Microanalytical Laboratory (Eagle Harbor, MI, USA). Melting points were determined on a Thermolyne apparatus, model MP-12600, and are uncorrected.

Thin-layer chromatography (TLC)

TLC of the various intermediates of THCA was performed on silica gel plates (Analabs, New Haven, CT, USA) in a solvent system of chloroform/methanol/acetic acid, 80 : 1 : 0.5 (v/v) (solvent system A), whereas that of THCA and DHCA was performed in a solvent system of chloroform/methanol/acetic acid, 40 : 2 : 1 (v/v) (solvent system B). The spots were visualized by spraying the plate with phosphomolybdic acid (3.5% in isopropanol) and sulfuric acid (20%) and heating at 110 C for 2 minutes. The two diastereoisomers of DHCA were resolved when the TLC plate was developed three times in a solvent system of chloroform/acetone/methanol, 70 : 20 : 3 (v/v) (solvent system C).

High-performance liquid chromatography (HPLC)

HPLC of 25R- and 25S-diastereoisomers of DHCA was performed on a Waters Associates (Milford, MA, USA) model M-6000 reciprocating pump and a model UK6 septumless loop injector. A Waters Associates model 401 differential refractometer was used and the detector response was recorded with a Spectra-Physics (San Jose, CA, USA) Model SP 4290 integrator. A Waters Associates Radial-Pak μ Bondapak C_{18} reversed-phase column (100 × 8 mm internal diameter, 5 μm particle size) was used for the separations. A 10- to 20-μg sample of the diastereoisomeric DHCA dissolved in 10 μl of methanol was injected into the HPLC column and the compounds were eluted with a solvent system of acetonitrile/water/methanol/acetic acid, 75 : 70 : 20 : 1 (v/v).[22] The flow rate was maintained at 3 ml/minute (operating pressure, ~ 13.8 × 10^3 KPa). The retention volume of 25S-DHCA was 87.8 ml and that of 25R-DHCA was 97.0 ml.[22]

Gas-liquid chromatography (GLC)

The bile acid methyl esters were silylated with Sil-Prep (Alltech Associates, Inc., Deerfield, IL, USA) at 55 C for 30 minutes and the trimethylsilyl ethers were dissolved in 100 μl hexane. The hexane solution (1 μl) was injected into a Hewlett-Packard 5880A gas chromatograph (Downers Grove, IL, USA) equipped with a split-splitless device for capillary columns. A fused silica CP-Sil-5 CB capillary column (25 m), internal diameter 0.20–0.22 mm, was used and helium was used as the carrier gas. The GLC operating conditions were as follows. Injector and detector temperatures were 260 and 290 C, respectively. After injection, the oven temperature was kept at 100 C for 2 minutes, then programmed at a rate of 35 C/minute to a final temperature of 265 C.[23] The 25R- and 25S-diastereoisomers of DHCA were not resolved (retention time, 25.56 minutes; retention time of 5α-cholestane, 12.78 minutes).

Nuclear magnetic resonance (NMR)

The high-resolution proton NMR spectra of the synthesized intermediates were obtained in deuterated chloroform on a JEOL GSX-400 spectrometer at 300 MHz.

GLC/mass spectrometry (GLC/MS)

The GLC/MS of the synthesized products (as their methyl ester trimethylsilyl ether derivatives) was performed on a Hewlett-Packard model 5988 capillary gas–liquid chromatograph–mass spectrometer (Paramus, NJ, USA) operating in the electron impact mode with an ionization energy of 70 eV. The GLC operating conditions were identical to those already described.

Isolation of 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid from A mississippiensis

(25RS)-3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid. Bile of *A mississippiensis* (50 ml) was deproteinized with 300 ml methanol and the supernatant solution was decanted and evaporated. The residue obtained was hydrolyzed with 20 ml diethylene glycol containing 5 g sodium hydroxide exactly as described before.[19] The hydrolysate containing the free bile acids was dissolved in 50 ml ethanol and boiled with 2 g activated charcoal and filtered. The resulting filtrate was evaporated and the residue obtained was crystallized from ethyl acetate to yield colorless needles (2 g), mp 190–192 C; GLC retention time relative to 5α-cholestane, 2.03. TLC of the crystalline product in a solvent system of chloroform/acetone/methanol, 70 : 50 : 10 (v/v, two developments) showed two spots of approximately equal intensity (R_f 0.41 and 0.44) that were shown to be due to the 25R- and 25S-diastereoisomers of THCA by direct comparison with reference standards.[18]

(25R)- and (25S)-3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid. To obtain the 25S-diastereoisomer of THCA, (25RS)-THCA (1 g) was crystallized five times from ethyl acetate when the crystals (100 mg) showed a single spot on TLC, R_f, 0.44, in the solvent system already described. The pure compound melted at 200–201 C, $[\alpha]_D^{25}$ = +43.6, and was found to be identical with the 25S-diastereoisomer of THCA by direct comparison with reference standard (melting point, TLC and GLC-MS).[18] In another experiment, 6 ml of the bile of *A mississippiensis* was deproteinized, dissolved in 20 ml chopped meat broth and incubated anaerobically at 37 C for 18 hours with 300 mg of fresh rat feces homogenized in 5 ml chopped meat broth.[19] The reaction was terminated by addition of 1 ml of 10% aqueous NaOH. After centrifugation, the clear supernatant was decanted and the fecal residue was washed with water (2 × 2 ml). The combined aqueous solution was acidified to pH 1 with 50% HCl and extracted with ethyl acetate (3 × 20 ml). The ethyl acetate extract was washed with water to neutrality, dried over anhydrous sodium sulfate, and evaporated to dryness. The dark brown residue obtained was treated with activated charcoal (1 g) and crystallized from ethyl acetate to a light grey colored solid. Two more crystallizations of this solid from ethyl acetate yielded 32 mg of a compound, mp 181–183 C, $[\alpha]_D^{25}$ = +26.8, that was identical with 25R-THCA (melting point, TLC, GLC and mass spectrometry).[18]

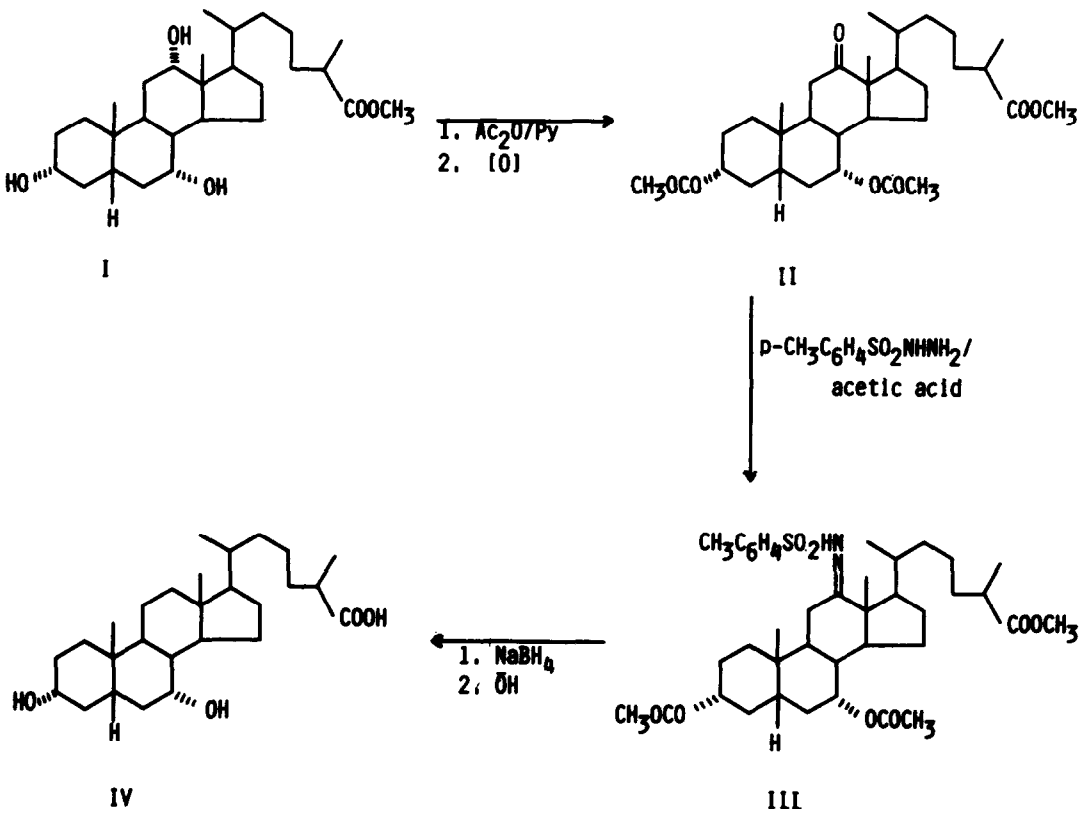

Figure 1 Synthesis of 3α,7α-dihydroxy-5β-cholestan-26-oic acid.

Isolation of (25R)-3α,7α-dihydroxy-5β-cholestan-26-oic acid from A mississippiensis

The mother liquor obtained during the isolation of (25R)-THCA from alligator bile that was treated with rat feces was evaporated to dryness. The residue (approximately 0.6 g) was dissolved in 0.5 ml ethyl acetate and poured over 30 g silica gel and eluted with hexane followed by increasing proportions of ethyl acetate in hexane. Elution with hexane/ethyl acetate (30 : 70) yielded a pale yellow semisolid (15 mg) that showed a major spot on TLC (solvent system B), R_f 0.62, that corresponded to the R_f value for DHCA by direct comparison with a synthetic reference standard.[15] The compound was purified by preparative TLC in solvent system B (7 mg) and was crystallized from aqueous methanol, mp 173–175 C; $[\alpha]_D^{25} = +6.3$; TLC, R_f, 0.44 (solvent system C, three developments); HPLC retention volume, 97.0 ml; GLC retention time relative to 5α-cholestane, 2.00.

Methyl 3α,7α-diacetoxy-12-oxo-5β-cholestan-26-oate (II)

(25RS)-THCA (0.6 g) was kept overnight with 5% methanolic H_2SO_4 and then poured over crushed ice-water. The precipitated methyl ester was collected, washed with water, dried, and crystallized from ethyl acetate/hexane, mp 154–156 C, R_f, 0.08 (solvent system A). The methyl ester (Figure 1, **I**) (0.6 g) was dissolved in pyridine (7 ml) and benzene (20 ml) and to the solution, acetic anhydride (7 ml) was added.[24] The resulting solution was kept at room temperature for 18 hours and then poured over crushed ice. The product was extracted with benzene, washed with water, and dried. Evaporation of the solvent yielded 0.65 g of a semisolid that showed one major spot, R_f, 0.46 (solvent system A), in addition to a spot corresponding with unreacted methyl THCA. The crude product was applied over 10 g solica gel in a column and eluted with 100 ml chloroform and then with 100 ml of 1% methanol in chloroform when 420 mg of methyl 3α,7α-diacetoxy-12α-hydroxy-5β-cholestan-26-oate was obtained. It was crystallized from methanol, mp 144–145 C. Found: C, 70.18; H, 9.54%. Calculated for $C_{32}H_{52}O_7$: C, 70.07; H, 9.49%; IR (KBr), 1,732, 1,255 cm^{-1}; NMR, δ 0.76 (3H, s, 18-CH_3), 1.02 (3H, s, 19-CH_3), 1.04 (3H, d, J = 6.5Hz, 21-CH_3), 1.17 (3H, d, J = 6.9Hz, 27-CH_3), 2.04, 2.09 (6H, 2s, -$OCOCH_3$), 3.70 (3H, s, -$COOCH_3$), 4.02 (1H, bs, 12-H), 4.55–4.64 (1H, bm, 3-H) and 4.93 (1H, m, 7-H).

To a stirred solution of methyl 3α,7α-diacetoxy-12α-hydroxy-5β-cholestan-26-oate (350 mg) in acetone (10 ml) at 0–5 C was added 0.5 ml of a solution of Jones' reagent (prepared by addition of 2.8 ml concentrated sulfuric acid into 2.2 g chromium trioxide and dilution to 10 ml with water). The resulting orange solution was kept at room temperature for one half hour and then poured over crushed ice. The precipitate was collected, washed with water, and dried (340 mg). The product **(II)** showed a single spot on TLC (system A), R_f, 0.68, and was crystallized from methanol, mp 138–140 C. Found: C, 70.39; H, 9.21%. Calculated for $C_{32}H_{50}O_7$: C, 70.33; H, 9.16%; IR (KBr), 1,735, 1,710, 1,250 cm^{-1}; NMR, δ 0.89 (3H, s, 18-CH_3), 1.14, 1.15 (6H, 2s, 19-CH_3 with overlapping 21-CH_3), 1.18 (3H, d, J = 6.9Hz, 27-CH_3), 2.05, 2.06 (6H, 2s, -$OCOCH_3$), 3.71 (3H, s, -$COOCH_3$), 4.57–4.62 (1H, bm, 3-H) and 5.04 (1H, m, 7-H).

Methyl 3α,7α-diacetoxy-12-oxo-5β-cholestan-26-oate-12-tosylhydrazone (III)

To a stirred solution of **II** (300 mg) in 7 ml acetic acid was added 300 mg of *p*-toluenesulfonyl hydrazide and the mixture was

allowed to stand at room temperature for 18 hours. The reaction mixture was poured over ice-cold water and the semisolid product obtained was extracted with ethyl acetate. The organic layer was washed with 5% Na_2CO_3 solution and then with water to neutrality and evaporated. The oily residue was applied over 10 g silica gel in a column and eluted with chloroform followed by increasing proportions of methanol. Elution with 50 ml of 0.5% methanol in chloroform gave unreacted **II** (60 mg). Further elution with 100 ml of 1% methanol in chloroform yielded 230 mg of **III** as a colorless semisolid which was crystallized from methanol as microscopic needles, mp 146–150 C; R_f, 0.30, (solvent system A). Found: C, 65.42; H, 8.10%. Calculated for $C_{39}H_{58}O_8N_2S$: C, 65.55; H, 8.12%; IR (KBr), 3,175, 1,730, 1,630, 1,590, 1,330, 1,255, 1,160, 810 cm^{-1}; NMR, δ 0.85 (3H, s, 18-CH_3), 1.01 (3H, s, 19-CH_3), 1.06 (3H, d, J = 3.8Hz, 21-CH_3), 1.17 (3H, d, J = 5.9Hz, 27-CH_3), 2.07, 2.08 (6H, 2s, -$OCOCH_3$), 2.47 (3H, s, Ar-CH_3), 3.71 (3H, s, -$COOCH_3$), 4.58–4.61 (1H, bm, 3-H), 5.02 (1H, m, 7-H) and 7.33, 7.87 (2H each, 2d, J = 8.1Hz, *p*-substituted phenyl).

(25RS)-3α-7α-dihydroxy-5β-cholestan-26-oic acid (IV)

To a stirred solution of **III** (150 mg) in acetic acid (4 ml) at 50 C was gradually added sodium borohydride (150 mg) over a period of 15 minutes. The contents were stirred at 50 C for another hour and then at room temperature for 6 hours. The reaction mixture was then poured over crushed ice and filtered. The solid obtained was dissolved in 5 ml methanol and 5 ml of 10% sodium hydroxide was added. The resulting solution was heated to reflux for 3 hours, cooled to room temperature, and the contents were poured into cold water. After acidification with dilute hydrochloric acid, the precipitate obtained was filtered, washed with water to neutrality, and dried. TLC (system B) showed the presence of three compounds, R_f, 0.62, 0.46, and 0.34, respectively. The crude product was applied over 8 g silica gel and eluted with chloroform followed by increasing proportions of methanol. Elution with 2% methanol in chloroform (30 ml) yielded 80 mg of a semisolid, single spot on TLC, R_f, 0.62, which agreed with that due to DHCA **(IV)**. The compound was crystallized from aqueous methanol, mp 170–173 C [literature mp of (25RS)-DHCA, 173–175 C[16]]. It was found to be identical with DHCA on comparative TLC and GLC and its methyl ester trimethylsilyl ether derivative showed mass spectral fragmentation pattern identical to that from a reference standard.[25] TLC of **IV** in solvent system C (three developments) showed two spots, R_f, 0.44 and 0.46, and on HPLC two peaks were observed with retention volumes of 87.8 and 96.9 ml, respectively. Continued elution of column with the same solvent (30 ml) yielded 20 mg of a mixture containing a more polar compound also (R_f, 0.46), which was not characterized. Finally, elution with 30 ml of 3% methanol in chloroform yielded a white solid (15 mg; R_f, 0.34) that was identical with THCA.

(25R)- and (25S)-3α,7α-dihydroxy-5β-cholestan-26-oic acid

The above sequence of reactions was repeated using 25 mg of 25R- or 25S-diastereoisomer of THCA when, after purification, 6 mg of pure 25R- or 25S-diastereoisomer of DHCA was obtained (25R-diastereoisomer, crystals from aqueous methanol, mp 172–174 C; $[\alpha]_D^{25}$ = +5.7; HPLC retention volume, 97.0 ml and 25S-diastereoisomer, pure on TLC, but failed to crystallize, $[\alpha]_D^{25}$ = +15.4; HPLC retention volume, 87.8 ml). When subjected to TLC in solvent system C, each diastereoisomer showed single spot, 25R-diastereoisomer, R_f, 0.44 and 25S-diastereoisomer, R_f, 0.46. When the DHCA isolated from alligator bile was cochromatographed with the 25R- and 25S-diastereoisomers of DHCA obtained above, this compound corresponded with the slower moving spot (25R-diastereoisomer).

Results and discussion

Except for the Kolbe electrolytic cross-coupling method used by Bridgwater[16] and modified by Briggs,[17] the reported methods for the conversion of C_{24} bile acids into C_{27} bile acids produce mixtures of the 25R- and 25S-diastereoisomers.[15,26,27] The only method reported for the synthesis of 25R- and 25S-diastereoisomers of DHCA involves electrolytic coupling of chenodeoxycholic acid and the half ester of D- or L-methylsuccinic acid.[17] However, the synthesis of optically pure isomers of methylsuccinic acid is tedious. Furthermore, electrolytic coupling results in a large number of side products[28] and pure DHCA can only be obtained after repeated column chromatography and yield of the final compound is very low.[17,28]

We considered that transformation of THCA into DHCA via elimination of the 12-hydroxyl group would be a better way to obtain pure DHCA. THCA could be converted into a 12-oxo derivative by standard methods[24]; however, the conventional method of reducing the 12-oxo group to a methylene group (Wolf-Kishner or Huang-Minlon reduction) requires refluxing of the hydrazone of the compound with strong alkali solutions at high temperatures. In a previous report, we have shown that THCA tends to isomerize at C-25 under these vigorous conditions[19]; therefore, DHCA obtained in this way would be a mixture of the 25R- and 25S-diastereoisomers. Iida and Chang recently used a mild method for the reduction of the 12-oxo group to a methylene group in their synthesis of chenodeoxycholic acid from cholic acid.[29] The method involves sodium borohydride reduction of the tosylhydrazone of the carbonyl compound.[30] Unlike Wolf-Kishner or Huang-Minlon reduction, the reduction of the tosylhydrazone with sodium borohydride is noninvasive. We preferred this method because we needed to prepare the pure 25R- and 25S-diastereoisomers of DHCA. We have successfully obtained the 25R- and 25S-diastereoisomers of THCA from alligator bile and have unequivocally characterized them by radiographic crystallography.[18] Because the chirality at C-25 is not disturbed during the tosylhydrazone formation or sodium borohydride reduction, the DHCA obtained from THCA must have the same configuration as the starting THCA.

Partial acetylation of methyl THCA was performed according to the method of Fieser and Rajagopalan[24] and the pure 3α,7α-diacetate was obtained by column chromatography. Chromic acid oxidation followed by tosylhydrazone formation yielded the 12-tosylhydrazone that on controlled reduction with sodium borohydride and chromatographic purification gave pure DHCA. Repeating the sequence of reactions starting with a pure diastereoisomer of THCA, the 25R- and 25S-diastereoisomers of DHCA were also prepared. The two diastereoisomers had identical GLC retention time but could be resolved on TLC and HPLC.[22] These characteristics were used to show that the natural

isomer of DHCA in the bile of *A mississippiensis* has 25R-configuration. The isolation of 25R-diastereoisomer of THCA in both alligator[18] and human[31] bile suggests that the hepatic enzymes must be stereo-specific in the ω-hydroxylation of 5β-cholestane-3α,7α,12α-triol, as has already been demonstrated by in vivo studies.[32–34] The availability of both diastereoisomers of DHCA will enable the characterization of its natural isomer in human bile[35] and will help establish if a similar stereospecificity exists for the microsomal and mitochondrial 26-hydroxylation of 5β-cholestane-3α,7α-diol to DHCA in mammalian species.

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