Synthesis of potential C_{27}-intermediates in bile acid biosynthesis and their deuterium-labeled analogs

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In connection with studies of alternative pathways in bile acid biosynthesis, potential intermediates in a pathway starting with 27-hydroxylation of cholesterol have been prepared in natural and deuterated forms. Established methods were used to prepare 27-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid. Clemmensen reduction of kryptogenin in unlabeled and deuterated solvents yielded 27-hydroxycholesterol and 16-oxo-5-cholestene-3β,27-diol, which were separated by adsorption chromatography on Unisil. The labeled 27-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid derived from it consisted of molecules with seven (50%), six (20%), and eight (20%) deuterium atoms, and unlabeled molecules were not detected. The acetates of 27-hydroxycholesterol and methyl 3β-hydroxy-5-cholestenoate were 7α-hydroxylated in a copper-catalyzed reaction with tert-*butylperbenzoate, and the products were purified by chromatography on Unisil. The 7β-epimers were obtained as side products. Labeled 3β,7α-dihydroxy-5-cholenoic acid was prepared in the same way from 3β-hydroxy-5-[2,2,4,4,23-$^2H_5$]-cholenoic acid. The 3-oxo-Δ^4 analogs of the 3β-hydroxy-Δ^5 compounds were prepared by oxidation with cholesterol oxidase. The labeled products had the same isotopic composition as the starting materials. Gas chromatographic retention indices and mass spectral characteristics of the trimethylsilyl ether derivatives of the neutral steroids and the methylated acids are given for all compounds. (Steroids* **58:**119–125, 1993)

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Introduction

Cholic and chenodeoxycholic acids are the major catabolic end products of cholesterol metabolism in humans. They can be formed in the liver via several pathways as demonstrated by administration of isotopically labeled potential intermediates and by studies of enzyme kinetics in vitro.[1] The introduction of a 7α-hydroxyl group has been considered to be the first step in the major pathway of bile acid biosynthesis. However, alternative pathways exist, particularly in the formation of chenodeoxycholic acid, in which the side-chain transformations precede those of the nucleus. Thus, 27-hydroxycholesterol is preferentially converted to chenodeoxycholic acid in humans.[2,3] Three potential intermediates in this pathway, 3β-hydroxy-5-cholestenoic, 3β,7α-dihydroxy-5-cholestenoic, and 7α-hydroxy-3-oxo-4-cholestenoic acids, have recently been identified in human blood.[4] Their levels are characteristically changed in patients with altered bile acid production, possibly reflecting their relative importance as intermediates.[5–8] Based on these findings, a model has been proposed for the biosynthesis of bile acids in humans,[6] in which two major pathways, one "neutral," starting with 7α-hydroxycholesterol, and one "acidic" or "27-oxygenated," starting with 27-hydroxycholesterol, lead to cholic and chenodeoxycholic acids, respectively. When the activity of cholesterol 7α-hydroxylase is stimulated, both bile acids are thought to be formed via 7α-hydroxycholesterol. In order to evaluate the existence and importance of these pathways, unlabeled and labeled versions of potential intermediates were required both for studies in vivo and in vitro, and

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for use as internal standards in gas chromatographic–mass spectrometric analyses. This article describes the preparation and characterization of labeled and unlabeled compounds related to 27-hydroxycholesterol.

Experimental

Chemicals

All solvents were of analytical grade and redistilled; pyridine was redistilled over calcium oxide. Zinc, $HgCl_2$, *tert*-butylperbenzoate, and N-methyl-N-nitroso-p-toluene sulfonamide were from Merck (Darmstadt, Germany) and $CuBr_2$ from BDH Chemicals Ltd. (Poole, England). Imidazole, hexamethyldisilazane, and trimethylsilyl (TMS) chloride were from Fluka (Buchs, Switzerland) and *tert*-butyldimethylsilyl (TBDMS) chloride from Sigma Chemical Co. (St. Louis, MO). Deuteroethanol and deuterium chloride (35% in D_2O) were from MSD Isotopes (Montreal, Canada) and deuterium oxide from Norsk Hydro (Rjukan, Norway). Cholesterol oxidase (from *Nocardia erythropolis*) was from Boehringer (Mannheim, Germany).

Glassware

All glassware except for the round-bottomed flasks were silanized. Cleaning was performed in an ultrasonic bath.

Column-packing materials

Octadecylsilane (ODS)-bonded silica (preparative C_{18}, particle size 55–105 μm) was from Waters Associates Inc. (Milford, MA). The material was washed with 10 ml each of methanol, methanol/chloroform [1 : 1 (v/v)], methanol, and water before use. Activated silicic acid (UNISIL, 200–325 mesh) was obtained from Clarkson Co. Ltd. (Williamsport, PA).

Column beds were prepared in glass columns equipped with gauze-covered valves of polytetrafluoroethylene. All solvents were degassed before use. Suitable flow rates were obtained by application of nitrogen pressure.

Reference steroids

3β-Hydroxy-5-cholenoic acid and cholest-5-ene-3β,7α-diol (7α-hydroxycholesterol) were from Steraloids, Inc. (Wilton, NH). 3β,7α-Dihydroxy-5-cholenoic acid and 3β-hydroxy-5-[2,2,4,4,23-$^2H_5$] cholenoic acid were kindly supplied by Professor M. Tohma (Higashi-Nippon-Gakuen University, Hokkaido, Japan). Kryptogenin (3β,27-dihydroxy-5-cholestene-16,22-dione) was a kind gift from Dr. L. Tökes (Syntex Research, Palo Alto, CA). The purities were checked by gas-liquid chromatography (GLC) after derivatization, and each compound gave a single peak.

Preparation of derivatives

Bile acids were methylated with freshly prepared diazomethane at 0 C, using 2 ml of methanol/diethyl ether (1 : 9 v/v) as solvent. The sample was taken to dryness after 15–30 minutes. TMS derivatives were prepared by addition of about 0.1 ml of pyridine/hexamethyldisilazane/trimethylchlorosilane (3 : 2 : 1 v/v/v) and heating at 60 C for 30 minutes. The reagents were removed under a stream of nitrogen, and the derivatives were dissolved in hexane.

Gas-liquid chromatography (GLC)

GLC was performed using a Carlo Erba (Milan, Italy) HRGC 6000 gas chromatograph connected to a Spectra-Physics (San Jose, CA) SP 4270 integrator. A fused silica column (25 m × 0.32 mm) coated with a 0.25 μm layer of cross-linked methyl silicone (Quadrex Corp., New Haven, CT) was used with an on-column injection system and a flame ionization detector. The temperature of the oven was 60 C during the injection, and was then taken to 290 C at 30 C/min and kept at this temperature. Retention indices (RI) were calculated by comparison of retention times with those of the normal C-30 to C-36 hydrocarbons analyzed under the same conditions.[9] The retention index of a compound is equivalent to 100 times the carbon number of a hypothetical n-alkane with the same adjusted retention time.

Gas-liquid chromatography-mass spectrometry (GC/MS)

GC/MS analyses were performed using a VG 7070E instrument and a methyl silicone column ending in the ion source. A falling-needle injection device was used. The temperatures of the oven and the ion source were 280 C and 290 C, respectively, and the electron energy was 70 eV. Repetitive scanning (2 s/decade) over the m/z range 50–800 was started after a suitable delay. The isotope compositions of ^2H-labeled compounds were calculated from the clusters of the $[M-90]^+$ fragment ions after correction for natural abundance of heavy isotopes and for the occurrence of minor peaks at $[M-91]^+$ as determined from the spectra of the unlabeled compounds.

Synthesis of steroids and their deuterated analogs

Cholest-5-ene-3β,27-diol (27-hydroxycholesterol) was prepared by Clemmensen reduction of kryptogenin.[10,11] Kryptogenin (100 mg) was dissolved in 20 ml 95% aqueous ethanol containing 0.2 ml concentrated hydrochloric acid. Freshly prepared zinc-Hg amalgam (4.0 g) was added and the mixture was refluxed for 3 hours. During the initial 2 hours, 3.3 ml concentrated hydrochloric acid were added dropwise from a separatory funnel. The mixture was cooled, decanted from the zinc-Hg amalgam, and poured into 50 ml of cold water and left overnight in the refrigerator. The precipitate was collected and the remaining solution was neutralized with sodium bicarbonate and extracted with ethyl acetate. The combined extracts were taken to dryness and dissolved together with the dried precipitate in 50 ml of 30% ethyl acetate in trimethylpentane (TMP). Aliquots of 10 ml were subjected to chromatography on Unisil columns (80 mm × 8 mm). After sample application, elution was performed with 100 ml of 30% ethyl acetate in TMP followed by 50 ml each of 40% and 50% ethyl acetate in TMP. Fractions of 10 ml were collected and analyzed by GLC after derivatization. The 27-hydroxycholesterol eluted as a pure compound in 30% ethyl acetate in TMP (fractions 3–10). Small additional amounts were eluted with 40% ethyl acetate in TMP together with 3β,27-dihydroxy-5-cholesten-16-one. Fractions 3–6 were combined and 27-hydroxycholesterol was crystallized from ethyl acetate as long slender needles (36.9 mg). The 16-oxo compound (39.9 mg) was crystallized in the same way.

Deuterated 27-hydroxycholesterol was prepared in the same way using deuterium oxide, deuteroethanol, and deuterium chloride in the Clemmensen reduction.

Cholest-5-ene-3β,7α,27-triol (7α,27-dihydroxycholesterol) was prepared from 27-hydroxycholesterol by treatment of the acetate with *tert*-butylperbenzoate.[12] Recrystallized 27-hydroxycholesterol (20 mg) was dissolved in a mixture of 1.5 ml of distilled pyridine and 0.5 ml of acetic acid anhydride and kept at room temperature overnight. After removal of solvent the resulting diacetate of 27-hydroxycholesterol was dissolved in 6

ml of acetic acid, and 5 mg of copper(II) bromide and 150 μl of *tert*-butylperbenzoate were added. The mixture was refluxed at 120 C for 5 minutes, cooled to room temperature, neutralized with 5% (w/v) sodium bicarbonate solution, and extracted with 10 ml of toluene. The toluene phase was washed with water and evaporated to dryness. The residue was dissolved in 2 ml of 10% ethyl acetate in TMP and applied to a column of Unisil (80 mm × 8 mm), which was then eluted with 100 ml of 10% ethyl acetate in TMP. Fractions of 5 ml were collected. Fractions 7–10 containing the acetate of 7α,27-dihydroxycholesterol were combined and taken to dryness. After hydrolysis with 5% potassium hydroxide in methanol at 50 C for 1 hour, acidification with acetic acid and dilution with 1 volume of water, the compound was extracted with a bed of ODS-silica (15 mm × 8 mm) and eluted with methanol. GLC of the TMS ethers showed the presence of 10–15% of the 7β-epimer and the mixture was subjected to chromatography on a Unisil column (65 mm × 8 mm). After application in 2 ml of 30% ethyl acetate in hexane, elution was performed with 10-ml volumes of ethyl acetate/hexane mixtures beginning with 30% ethyl acetate and increasing with 10% each 10 ml. Fractions of 5 ml were collected. Fractions 5–7 (50–60% ethyl acetate) contained the 7β-epimer and fractions 7–9 (60–70% ethyl acetate) the 7α-epimer. The 7α,27-dihydroxycholesterol (4.6 mg) obtained in this way contained about 5% of the 7β-epimer.

Deuterated 7α,27-dihydroxycholesterol was synthesized and purified in the same way from the deuterated 27-hydroxycholesterol.

3β-Hydroxy-5-cholestenoic acid was prepared from 27-hydroxycholesterol essentially as described by Ayaki et al.[13] A mixture of 27-hydroxycholesterol (30 mg) and imidazole (56 mg) was dissolved in 4 ml of anhydrous dimethylformamide, cooled to 0 C, and *tert*-butyldimethylsilyl chloride (44 mg) was added with stirring. After 10 minutes the reaction mixture was diluted with water and extracted twice with 10 ml of diethyl ether. The combined diethyl ether phases were washed with water, dried over anhydrous sodium sulfate, and taken to dryness. The residue was dissolved in 2 ml of hexane and applied to a Unisil column (30 mm × 8 mm). Elution was performed with hexane, followed by 10-ml volumes of dichloromethane/hexane mixtures beginning with 10% dichloromethane and increasing with 10% each 10 ml. Fractions of 5 ml were collected. Fractions 3–5 (20–30% dichloromethane) contained the 3,27-diTBDMS ether (11.3 mg) and fractions 12–14 (60–70% dichloromethane) the 27-monoTBDMS ether (15.8 mg).

The 3-acetate of 27-hydroxycholesterol 27-monoTBDMS ether was obtained by treatment with acetic anhydride/pyridine as described above and extraction into ethyl acetate. The TBDMS ether was hydrolyzed at room temperature overnight in tetrahydrofuran/water/glacial acetic acid, 1.5 : 1 : 3 (v/v/v). The 3-acetate of 27-hydroxycholesterol was dissolved in 5 ml of acetone and oxidized with Jones reagent at room temperature for 15 minutes. Excess reagent was destroyed with ethanol, water was added, and the product was extracted with ethyl acetate. The extract was washed with water and taken to dryness. The acetate of 3β-hydroxy-5-cholestenoic acid was hydrolyzed with 5% (w/v) potassium hydroxide in methanol. After acidification and dilution with 6 volumes of water, the product was extracted with ODS-silica. The yield of free acid was 10.2 mg. The purity, evaluated by GLC of the methyl ester TMS ether derivative, was 89%.

Deuterated 3β-hydroxy-5-cholestenoic acid was synthesized and purified in the same way from deuterated 27-hydroxycholesterol.

3β,7α-Dihydroxy-5-cholestenoic acid. The 7α-hydroxyl group was introduced as described for the synthesis of 7α,27-dihydroxycholesterol. 3β-Hydroxy-5-cholestenoic acid (5 mg) was methylated with diazomethane, acetylated, and treated with copper bromide and *tert*-butylperbenzoate in acetic acid. After neutralization, the reaction mixture was extracted with ethyl acetate. The extract was washed with water and taken to dryness. The products were separated on a Unisil column (80 mm × 8 mm) in hexane. After a wash with hexane, elution was performed with 20-ml volumes of ethyl acetate/TMP beginning with 10% ethyl acetate and increasing with 5% every 20 ml. Fractions of 5 ml were collected. Fractions 6–9, containing the methyl ester acetate of 3β,7α-dihydroxy-5-cholestenoic acid, were combined. The ester was hydrolyzed with 5% potassium hydroxide in methanol. After acidification and dilution with 6 volumes of water, the acid was sorbed on a bed of ODS-silica and eluted with methanol. It was then methylated with diazomethane and further purified on a Unisil column (65 mm × 8 mm) to remove the 7β-epimer. After washing with hexane, elution was performed with 10-ml volumes of ethyl acetate/hexane beginning with 30% ethyl acetate and increasing with 10% every 10 ml. Fractions of 5 ml were collected. Fractions 9 and 10 (70% ethyl acetate), containing 1.2 mg of the 7α-epimer, were combined. The purity, evaluated by GLC of the methyl ester TMS ether derivative, was 87%. The free acid was obtained by hydrolysis with 5% potassium hydroxide in methanol.

Deuterated 3β,7α-dihydroxy-5-cholestenoic acid was synthesized and purified in the same way from deuterated 3β-hydroxy-5-cholestenoic acid.

3β,7α-Dihydroxy-5-cholenoic acid. The 7-hydroxylation was performed as described for the synthesis of 3β,7α-dihydroxy-5-cholestenoic acid. The acetate of methyl 3β-hydroxy-5-cholenoate (7 mg) was treated with copper bromide and *tert*-butylperbenzoate in acetic acid. The mixture was neutralized and the products were extracted with ethyl acetate and purified on Unisil. Fractions 8–10, containing the methyl ester acetate of 3β,7α-dihydroxy-5-cholenoic acid, were combined. After hydrolysis, extraction with ODS-silica, and methylation with diazomethane, the compound was further purified on a Unisil column in the same way as the C-27 compound. Fractions containing the 7α-epimer were combined. The yield was 2.8 mg and the purity, evaluated by GLC, was 92%. The free acid was obtained by hydrolysis with 5% potassium hydroxide in methanol.

Deuterated 3β,7α-dihydroxy-5-cholenoic acid was synthesized in the same way from 3β-hydroxy-5-[2,2,4,4,23-$^2H_5$]cholenoic acid.

Preparation of 3-oxo-Δ⁴ sterols and bile acids

The 3β-hydroxy-Δ⁵ steroids and bile acids were oxidized with cholesterol oxidase (EC 1.1.3.6) as described by Brooks et al.[14] The compounds (0.5–1 mg) were dissolved in 500 μl of isopropanol and 6 ml of 0.05 M phosphate buffer (pH 7.0); 5 U of cholesterol oxidase was added, and the mixture was incubated at 37 C for 3 hours. The reaction was stopped by addition of 9 ml methanol and the solution was passed through a bed of ODS-silica (15 mm × 8 mm) prepared in water. The effluent was then diluted with water to 40% methanol for extraction of neutral steroids, or concentrated to a water solution for extraction of bile acids. These solutions were again passed through the same bed of ODS-silica. After washing with water, the 3-oxo-Δ⁴ steroids were eluted with 8 ml of methanol.

Results and discussion

The RI values of the synthesized compounds and some side products are summarized in Table 1. The compounds were characterized by their mass spectra (Ta-

Table 1 Retention indices of the TMS or methyl ester TMS ether derivatives of the synthetic and related compounds

Compound[a]	RI[b]	ΔRI[c]	Compound[a]	RI[b]	ΔRI[c]
5-cholestene			4-cholestene		
3β-ol	3,143	—	3-one	3,202	59[d]
3β,7α-diol	3,123	−20[e]	7α-ol-3-one	3,235	33[e]
3β,7β-diol	3,214	71[f]	7β-ol-3-one	3,345	143[f]
3β-ol-7-one	3,389	246[g]	—	—	—
3β,27-diol	3,470 (3,468)	343[h]	27-ol-3-one	3,550	348[h]
3β,7α,27-triol	3,429 (3,427)	−41[e]	7α,27-diol-3-one	3,581 (3,574)	31[e]
3β,7β,27-triol	3,563 (3,560)	93[f]	7β,27-diol-3-one	3,706 (3,698)	156[f]
3β,27-diol-7-one	3,789	319[g]	—	—	—
3β,27-diol-16-one	3,645	175	—	—	—
3β,27-diol-16,22-one	3,795	325	—	—	—
5-cholestenoic acid			4-cholestenoic acid		
3β-ol	3,444 (3,440)	301[i]	3-one	3,528	84[d]
3β,7α-diol	3,418 (3,412)	−26[e]	7α-ol-3-one	3,564 (3,555)	36[e]
3β,7β-diol	3,555 (3,549)	111[f]	7β-ol-3-one	3,695 (3,686)	167[f]
3β-ol-7-one	3,775	331[g]	—	—	—
5-cholenoic acid			4-cholenoic acid		
3β-ol	3,197	−247[k]	3-one	3,265	68[d]
3β-7α-diol	3,185 (3,181)	−12[e]	7α-ol-3-one	3,306	41[e]
3β,7β-diol	3,302 (3,298)	105[f]	7β-ol-3-one	3,421	156[f]
3β-ol-7-one	3,478	—	—	—	—

[a] The position(s), orientation, and nature of functional groups are given below the structure of the steroid skeleton in which they are present.
[b] Retention index on a fused silica capillary column coated with methyl silicone. Values in parentheses refer to the deuterated analog. For definition of retention index, see Experimental, Gas liquid chromatography.
[c] Change of RI upon oxidation or introduction of functional groups.
[d] Conversion of 3β-hydroxy-Δ^5 to the 3-oxo-Δ^4 structure.
[e] Introduction of the 7α-hydroxyl group.
[f] Introduction of the 7β-hydroxyl group.
[g] Introduction of the 7-oxo group.
[h] Introduction of the 27-hydroxyl group.
[i] Oxidation of C-27 methyl to the carboxyl group.
[k] Shortening of C-27 acid to C-24 acid.

ble 2). The configuration of the 7-hydroxy group was deduced from the RI values, which showed the expected characteristic changes upon introduction of functional groups (ΔRI, Table 1). As expected, the labeled compounds had slightly lower RI values than the corresponding unlabeled ones.

27-Hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid

In agreement with the original report[10] the Clemmensen reduction of kryptogenin yielded approximately equal amounts of 27-hydroxycholesterol and 27-hydroxy-5-cholesten-16-one. Instead of subjecting this mixture to a Wolf-Kishner reduction,[10] which would complicate the labeling with deuterium, the two compounds were separated by chromatography on a column of Unisil using 3 g adsorbent for 20 mg steroids (150 : 1). The yield of 27-hydroxycholesterol was about 40%. The 27-hydroxy-5-cholesten-16-one, obtained in a similar yield, can be used to prepare additional amounts of 27-hydroxycholesterol by Wolf-Kishner reduction and purification on Unisil.

The 3β-hydroxy-5-cholestenoic acid was prepared as described by Ayaki et al.[13] Partial reaction of 27-hydroxycholesterol with TBDMS chloride in the presence of imidazol yielded the 27-mono- and 3,27-diTBDMS derivatives in a ratio of 1.4, with only trace amounts of unreacted sterol. The mono-TBDMS and di-TBDMS derivatives were separated by chromatography on Unisil, and 3β-hydroxy-5-cholestenoic acid was prepared from the former by acetylation, hydrolysis of the TBDMS ether, and Jones oxidation. The yield from the 27-mono-TBDMS ether was about 65%. The di-TBDMS derivative can be hydrolyzed to recover 27-hydroxycholesterol.

The labeled 27-hydroxycholesterol consisted of molecules with 5 to 10 atoms of deuterium (Table 3). No unlabeled molecules were detected. If labeling occurs at carbon atoms 15, 16, 17, 20, 22, and 23, the maximum

Table 2 Mass spectrometric data for TMS or methyl ester TMS derivatives of the unlabeled compounds

Compound[a]	Ions (m/z) and their relative intensities (%)[b]			
	M^+	$[M-15]^+$	$[M-90]^+$	Other ions
C^5-3β,27-ol	546 (9)	531 (4)	456 (27)	417 (30)[c], 255 (13)[d], 129 (81)
C^4-27-ol-3-one	472 (16)	457 (23)	382 (3)	271 (7)[d], 229 (34), 196 (100), 124 (45)
C^5-3β,7α,27-ol	634 (1)	—	544 (33)	129 (6)
C^4-7α,27-ol-3-one	560 (5)	545 (16)	470 (31)	269 (12)[d], 224 (15)
CA^5-3β-ol	502 (15)	487 (10)	412 (51)	446 (7), 373 (87)[c], 291 (26), 255 (24)[d], 129 (100)
CA^4-3-one	428 (25)	413 (6)	—	305 (16), 271 (16)[d], 229 (54), 124 (100)
CA^5-3β,7α-ol	590 (2)	575 (2)	500 (44)	291 (5), 253 (3)[d], 129 (9)
CA^4-7α-ol-3-one	516 (7)	501 (20)	426 (31)	269 (27)[d], 224 (23)
B^5-3β,7α-ol	548 (3)	533 (2)	458 (100)	327 (4)[c], 253 (4)[d], 249 (6), 129 (11)
B^4-7α-ol-3-one	474 (11)	459 (34)	384 (54)	269 (26)[d], 224 (33), 209 (26)

[a] Abbreviations: C, cholestane; CA, cholestanoic acid; B, cholanic acid. Superscript numeral indicates position of double bond, greek letters orientation of hydroxyl groups.
[b] When not among the ions listed, the base peak was at m/z 75.
[c] $[M-129]^+$.
[d] ABCD-ring ion.

Table 3 Isotopic composition of the deuterium-labeled hydroxysterols and bile acids

Compound[a]	Relative abundance (%) of deuterated molecules[b]											
	$^2H_0$	$^2H_1$	$^2H_2$	$^2H_3$	$^2H_4$	$^2H_5$	$^2H_6$	$^2H_7$	$^2H_8$	$^2H_9$	$^2H_{10}$	$^2H_{11}$
C^5-3β,27-ol	—	—	—	—	—	3.9	20.0	48.7	22.1	4.6	0.6	—
C^5-3β,7α,27-ol	—	—	—	—	0.5	2.7	18.4	51.3	21.9	4.7	0.4	—
CA^5-3β-ol	—	—	—	—	1.2	5.0	19.3	46.7	22.2	4.8	0.8	—
CA^5-3β,7α-ol	—	—	—	—	—	3.7	19.6	48.3	22.8	4.8	0.6	—
B^5-3β,7α-ol	—	0.3	3.7	14.6	37.0	28.8	13.7	1.7	0.2	—	—	—

[a] For abbreviations see Table 2.
[b] Calculated from the [M-90]† ions of the TMS or methyl ester TMS ether derivatives.

number of ^2H atoms would be 10. About half of the molecules contained seven ^2H atoms, and species with six and eight ^2H atoms had relative abundances of about 20% each. This isotopic composition differs from that reported by Javitt et al.,[11] who found the $^2H_8$ species to predominate followed by the $^2H_6$ species. The difference may be related to the use in their study[11] of negative ion chemical ionization, where the recorded ions have lost hydrogens. Rigorous exclusion of unlabeled water during the synthesis is also of importance for reproducible results.

The isotopic composition of the labeled 3β-hydroxy-5-cholestenoic acid was not significantly different from that of the precursor 27-hydroxycholesterol (Table 3). This confirms the expected absence of readily exchangeable deuterium atoms in the two compounds.

Cholest-5-ene-3β,7α,27-triol, 3β,7α-dihydroxy-5-cholestenoic acid, and 3β,7α-dihydroxy-5-cholenoic acid

The introduction of a 7α-hydroxyl group into 27-hydroxycholesterol and the two acids was performed using *tert*-butylperbenzoate.[12] The 7β-epimer was formed as one of many side products. The 7α/7β ratio depended on the reaction time. Preliminary experiments with cholesterol indicated that the yields were optimal using a reaction time of 5 minutes at 120 C. The ratio of 7α- to 7β-hydroxylated product decreased with increasing reaction time, as did the total yield of 7-hydroxylated products. This could also be seen by thin-layer chromatographic analysis using the Lifschütz reaction to detect the compounds containing an allylic hydroxyl group.

The 7,27-dihydroxycholesterol obtained under optimal conditions consisted of 88% 7α-epimer and 12% 7β-epimer. The corresponding values for the 7-hydroxylated C_{27} acid were 78% and 22% respectively, and for the 7-hydroxylated C_{24} acid 71% and 29%, respectively. The nature of the other compounds formed was not further studied. The acetates in the crude reaction mixture were partially purified by chromatography on a Unisil column, and the 7α- and 7β-epimers were then separated by a second chromatography after hydrolysis of the acetates. The final yields of 7α-hydroxylated compounds were 20–25% for the C_{27}-compounds and 40% for the C_{24} bile acid. The contamination by 7β-epimer in these products was 4–5%. The yields as well as the purities were considered sufficient for the purposes of in vitro studies and use as internal standards,

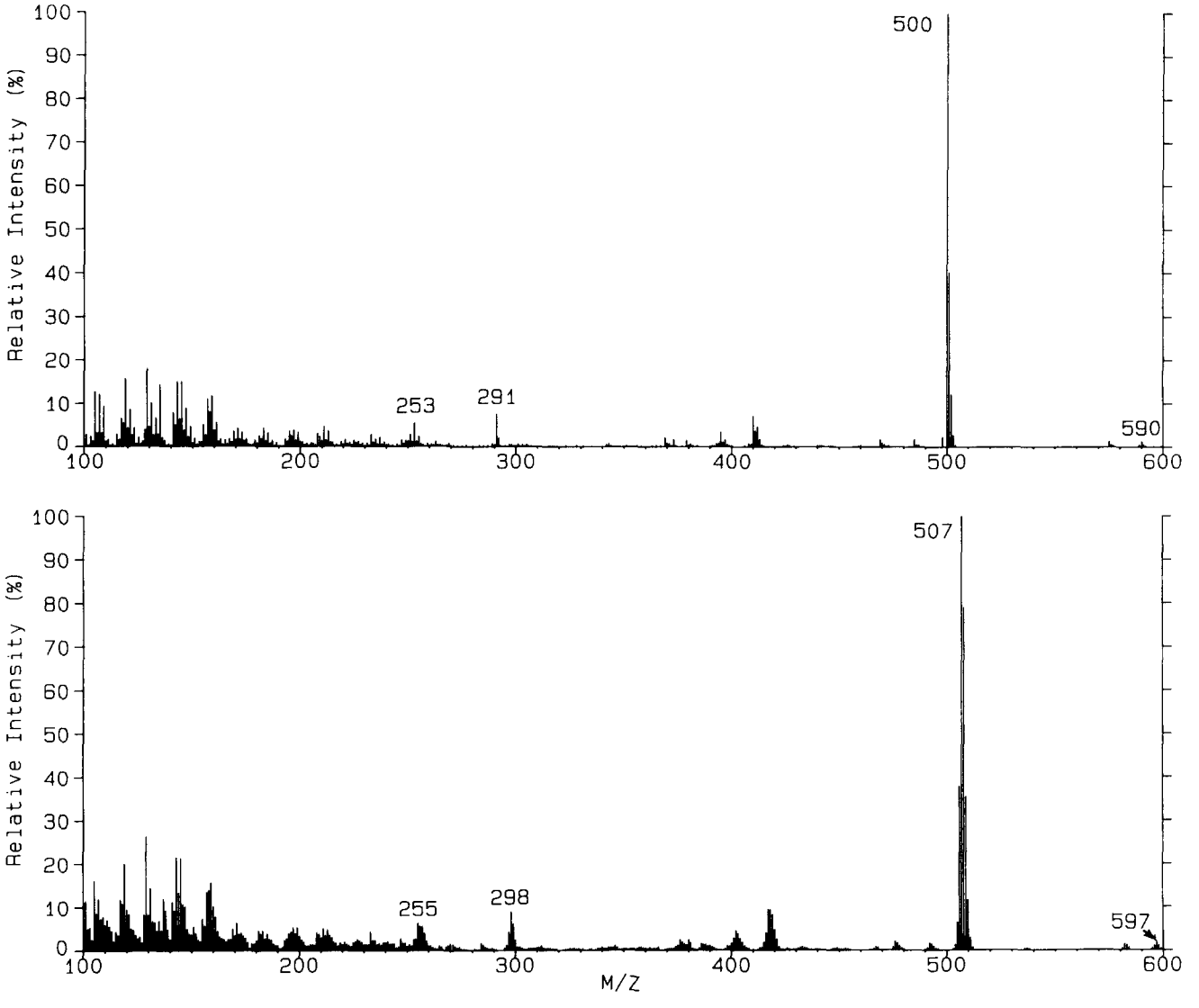

Figure 1 Mass spectra of the methyl ester trimethylsilyl ether derivatives of unlabeled (upper spectrum) and deuterium-labeled (lower spectrum) 3β,7α-dihydroxy-5-cholestenoic acid.

and further attempts were not made to improve the synthetic procedure.

The isotopic compositions of the labeled 7α,27-dihydroxycholesterol and 3β,7α-dihydroxy-5-cholestenoic acid were not significantly different from that of the labeled 27-hydroxycholesterol used as precursor (Table 3). Figure 1 shows the mass spectrum of the TMS ethers of unlabeled and labeled 3β,7α-dihydroxy-5-cholestenoic acid. The peak at m/z 291 given by the unlabeled compound contains the D ring and side chain[15] and a comparison with the corresponding cluster of peaks around m/z 298 in the spectrum of the labeled compound confirms that all the deuterium atoms are in this part of the molecule. The intensities of the ABCD ring fragment ions at m/z 253 (unlabeled) and m/z 253–259 (labeled) suggest that the major species of labeled molecules have two to three ^2H atoms in the D ring, indicating that about 70% of the ^2H atoms are in the side chain.

Also in the case of 3β,7α-dihydroxy-5-cholenoic acid, the precursor of which was labeled at carbon atoms 2, 4, and 23, there was no loss of deuterium upon introduction of the 7α-hydroxy group.

Compounds with a 3-oxo-Δ^4 structure

When available, enzymatic reactions are preferable to chemical ones for preparation of the small quantities of potential intermediates that are needed for studies in vitro or analytical use. Oxidation with cholesterol oxidase is a well established and simple procedure for conversion of 3β-hydroxy-Δ^5 to 3-oxo-Δ^4 steroids.[14] The recoveries, evaluated by GLC of the TMS or methyl ester TMS ether derivatives, were high (Table 4), indicating that elimination of the 7α-hydroxyl group to yield 3-oxo-4,6-dienes was of minor or no importance as a side reaction. The usefulness of the cholesterol oxidase reaction in structural analyses by GLC[14]

Table 4 Recoveries of 3-oxo-Δ^4 steroids by oxidation of 3β-hydroxy-Δ^5 steroids with cholesterol oxidase

Compound[a]	Recovery (%)
Sterols	
C^4-3-one	112
C^4-7α-ol-3-one	95
C^4-27-ol-3-one	95
C^4-7α,27-ol-3-one	96
Bile acids	
CA^4-3-one	106
CA^4-7α-ol-3-one	98
B^4-7α-ol-3-one	88

[a] For abbreviations see Table 2.

is confirmed by the results shown in Table 1. The ΔRI : s for the derivatives upon conversion of a 3β-hydroxy-Δ^5 to a 3-oxo-Δ^4 structure is 59 for cholesterol and 80 for the corresponding cholestenoic acid, which increases to 112 and 146, respectively, upon introduction of a 7α-hydroxy group. The influence of the side chain structure is also evident from the values in Table 1.

Two deuterated 3-oxo-Δ^4 compounds were prepared for use as internal standards from 7α,27-dihydroxycholesterol and 3β,7α-dihydroxy-5-cholestenoic acid. Their isotopic composition did not differ significantly from those of the substrates and no unlabeled product was detected.

The use of the synthesized compounds to evaluate potential pathways and compartmentation of bile acid biosynthesis will be described separately. Using the unlabeled compounds as substrates and the labeled ones as internal standards in GC/MS analyses, we have demonstrated the presence in pig liver of 7α-hydroxylase(s) different from cholesterol 7α-hydroxylase and active on the side-chain oxygenated 3β-hydroxy-Δ^5 steroids.[16,17] Both the microsomal and mitochondrial fractions had 7α-hydroxylating activity, and mitochondria were found to contain all enzymes required to convert cholesterol via 27-hydroxycholesterol into 7α-hydroxy-3-oxo-4-cholestenoic acid.[16] Similar reactions have also been found in the human liver,[18] indicating that a pathway of bile acid biosynthesis exists that is not regulated by the activity of cholesterol 7α-hydroxylase. The availability of the compounds described in this article was a prerequisite for the detection of this pathway.

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