Inhibitors of sterol synthesis. Tritium-labeled 26-hydroxycholesterol of high specific activity from a byproduct of the Clemmensen reduction of diosgenin

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Abstract (25R)-26-Hydroxycholesterol (I) was synthesized in six steps from (22Z,25R)-cholesta-5,22-diene-3β,26-diol (II) in 31% overall yield. The 26-tert-butyldiphenylsilyl ether of II was converted via its 3β-tosylate to (22Z,25R)-6β-methoxy-26-(tert-butyldiphenylsilyloxy)-3α,5-cyclo-5α-cholest-22-ene (V). Removal of the 26-silyl group of V gave (22Z,25R)-6β-methoxy-3α,5-cyclo-5α-cholest-22-en-26-ol, which was hydrogenated over platinum oxide and then hydrolyzed to I. Catalytic reduction in the presence of deuterium or tritium gas gave [2H]-I or [3H]-I, respectively. Analysis of the [2H]-I by mass spectrometry showed that all the deuterium was located in the sterol side chain, mainly as d2, d3, and d4 species. The 1H and 13C nuclear magnetic resonance (NMR) spectra of [2H]-I indicated that most of the deuterium was located at C-22 and C-23, with lesser amounts at C-24 and minor amounts at C-20, C-21, C-25, and C-27. NMR spectra of [2H]-I and its α-methoxy-α-(trifluoromethyl)phenylacetate diester showed no detectable 20S epimer and ~2% of the 25S epimer. The [3H]-I was prepared analogously to [2H]-I using carrier-free tritium and showed a specific activity of 16.9 Ci/mmol. All synthetic intermediates were characterized fully by 1H and 13C NMR, and representative 1H-1H coupling constants are given for the ring A protons of steroids. - Ni, Y., A. Kisic, W. K. Wilson, and G. J. Schroepfer, Jr. Inhibitors of sterol synthesis. Tritium-labeled 26-hydroxycholesterol of high specific activity from a byproduct of the Clemmensen reduction of diosgenin. J. Lipid Res. 1994, 35: 546-559.

Supplementary key words 1H NMR • 13C NMR • mass spectrometry • i-sterol • conformational analysis

A number of oxygenated sterols have been shown to be highly active as inhibitors of sterol biosynthesis and in the regulation of the levels of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase activity in cultured mammalian cells (1, 2). (25R)-26-Hydroxycholesterol (Ia) is especially potent in this regard (3, 4) and shows synergism with another oxysterol in the lowering of HMG-CoA reductase activity in Chinese hamster ovary cells (5). Other (25R)-26-oxygenated steroids also display high activity in the regulation of the levels of reductase activity (6-8). Mitochondrial 26-hydroxylation of sterols is an obligatory reaction in major pathways of bile acid formation (9-11) and is an initial reaction in the metabolism of 3β-hydroxy-5α-cholestan-8(14)-ene-3β,26-diol, a potent hypcholesterolemic agent (12-14). The 26-hydroxylase has been purified to homogeneity (15), and the gene for the 26-hydroxylase has been cloned and its expression has been detected in several tissues (15). 26-Hydroxycholesterol is present in blood plasma of normal human subjects at concentrations that have been shown to lower the levels of HMG-CoA reductase activity in cultured mammalian cells (17 and references cited therein). Very recently, the immunosuppressant cyclosporin A has been shown to be a potent inhibitor of the mitochondrial 26-hydroxylation of cholesterol (18). These and other observations have provided the impetus for our interest in the chemical synthesis of 26-oxygenated sterols and their isotopically labeled analogs.

We have recently reported the isolation of (22Z,25R)-cholesta-5,22-diene-3β,16β,26-triol as a byproduct of the Clemmensen reduction of diosgenin and its conversion to the corresponding 3β,26-diol II (19). The availability of II suggested a synthetic route to tritium-labeled (25R)-26-hydroxycholesterol of high specific activity by...
Described herein is the preparation of deuterium- and tritium-labeled (25R)-26-hydroxycholesterol from II. A preliminary account of a portion of these results has been presented (19).

EXPERIMENTAL PROCEDURES AND RESULTS

Materials and methods

Melting points (mp) were measured with a Thomas-Hoover apparatus in sealed, evacuated capillary tubes. Optical rotations were measured in CHCl₃ solution at room temperature (−23°C) on a Jasco DIP-4+ digital polarimeter. Infrared spectra (IR) were measured with KBr pellets on a Mattson Galax 6020 Fourier-transform infrared spectrometer; all spectra showed C-H bands at 2980–2800, −1460, and −1370 cm⁻¹. Direct-inlet mass spectra of underivatized sterols were recorded on a Shimadzu QP-1000 quadrupole mass spectrometer by electron impact at 70 eV and are reported as m/z (relative intensity, suggested assignment).

Gas chromatography–mass spectrometry (GC–MS) was carried out with falling needle injection and direct introduction of the helium carrier gas into the ion source of the mass spectrometer (Extrel ELQ-400, quadrupole, electron impact at 70 eV). Bis-trimethylsilyl (TMS) ethers were prepared using bis(trimethylsilyl)trifluoroacetamide (14) and injected in hexane solution onto a DB-5 column (15 m × 0.25 mm, 0.1 μm film thickness, bonded phase of 5% diphenyl 95% dimethyl polysiloxane; J & W Scientific, Folsom, CA) that was held at 200°C for 2 min and increased to 290°C at 10°C per min. Capillary GC was done on a Shimadzu GC-9A unit using splitless injection with flame- ionization detection and nitrogen (1.3 kg per min) as the carrier gas; the DB-5 column (30 m) was held at 200°C for 3 min and increased to 280°C at 20°C per min.

Nuclear magnetic resonance (NMR) spectra were measured on an IBM AF300 spectrometer (75.5 MHz for ¹³C, ~22°C), a Bruker AMX500 instrument (500.1 MHz for ¹H, 125.8 MHz for ¹³C, 76.8 MHz for ²H, 27°C), and a Bruker AC250 instrument (235.4 MHz for ¹⁹F, ~22°C) in CDCl₃ solution and referenced to internal tetramethylsilane (0 ppm), CDCl₃ at 7.27 ppm (¹H), CDCl₃ at 77.0 ppm (¹³C), or CFCl₃ (¹⁹F). All ¹³C shieldings reported here are from 22°C spectra or adjusted accordingly. Standard Bruker software was used to acquire DEPT (distortionless enhancement by polarization transfer), COSYDEC (ω₁-decoupled ¹H–¹H correlation spectroscopy; 0.2-s fixed evolution period τₑ, δ = −0.6–2.4, 256 increments), HET-COR (¹H–¹³C shift correlated spectroscopy; ~50 increments, δ = −0.6–2.6 in f₁), HMBC (heteronuclear multiple bond correlation, 120 increments, δ = 10–75 in f₁), and HMQC (heteronuclear multiple-quantum coherence) spectra. An HMQC spectrum of VIIa was acquired using the pulse sequence of Bax et al. (20) without ¹³C decoupling (21): 1.1-s acquisition time, 0.3-s relaxation delay, 0.35-s delay after BIRD pulse, spectral window δ = 5–51 in f₁ and δ = 0.35–2.15 in f₂, 160 increments, 16-step phase cycling. Coupling constants were derived from line spacings of resolution-enhanced 1D spectra and confirmed in some cases by homodecoupling experiments or spin simulation with NMR’’ (Calleo Scientific Software; Ft. Collins, CO). PC MODEL (Macintosh version 4.4; Serena Software, Bloomington, IN) was used to model sterol structures by molecular mechanics and predict vicinal ¹H NMR coupling constants. ¹H and ¹³C NMR assignments were made from a combination of DEPT, HETCOR, and COSYDEC spectra in conjunction with comparisons of chemical shifts reported for Ia (6) and its diacete ester (22). ¹H NMR stereochemical assignments were made by chemical shift and coupling constant comparisons (22, 23) except in the case of ring A of i-steroids (see below).

Analytical thin-layer chromatography (TLC) was performed using precoated 0.25-mm silica gel G plates ( Analtech, Newark, DE) or aluminum-backed silica gel 60 plates (EM Separations, Gibbstown, NJ). Solvent systems for TLC and column chromatography were: SS-1, 2% ethyl acetate in hexane; SS-2, 2.5% ethyl acetate in hexane; SS-3, 4% ethyl acetate in hexane; SS-4, 5% ethyl acetate in hexane; SS-5, 10% ethyl acetate in hexane; SS-6, 15% ethyl acetate in hexane; SS-7, 25% ethyl acetate in hexane; SS-8, 50% ethyl acetate in hexane; SS-9, 5% water in methanol; SS-10, 40% 2-propanol in methanol. TLC plates were charred by spraying with 5% ammonium molybdate(VI) in 10% sulfuric acid followed by heating. Radio-TLC analyses were carried out on 1-cm sections, as described previously (24). Silica gel (70–230 mesh or 230–400 mesh) was purchased from Aldrich Chemical Co. (Milwaukee, WI), and Unisil was obtained from Clarkson Chemical Co. (Williamsport, PA). Samples for medium pressure liquid chromatography (MPLC) or flash chromatography were dissolved in a solvent no stronger than the initial eluting solvent. Silica gel-AgNO₃ for MPLC (22, 25) was prepared by adding a solution of AgNO₃ (10 g) in water (10–15 ml) to a free-flowing slurry of silica gel (90 g, 70–230 mesh) in acetone (200 ml). The resulting mixture was protected from light, swirled for 5 min, and spun on a rotary evaporator without vacuum for 30 min. Acetone was removed by rotary evaporation, and water was removed under high vacuum (< 0.1 torr). High performance liquid chromatography (HPLC) was performed isocratically at 1 ml/min with a Waters liquid chromatograph (U6K inyector, model 510 pump, and model 481 variable wavelength detector set at 210 nm) on a 5-μm ODS-II Spherisorb column (250 mm × 4.6 mm i.d.).

Deuterium gas was obtained from Cambridge Isotope Labs (Woburn, MA). Platinum oxide, tetrabutyrammo-
Fig. 1. Synthesis of deuterium- and tritium-labeled (25R)-26-hydroxycholesterol from (22Z,25R)-cholesta-5,22-diene-3β,26-diol (II).

To a stirred solution of A5,22Z-3β,26-diol II (630 mg; 1.58 mmol) in dry N,N-dimethylformamide (14 ml) and dichloromethane (14 ml) was added imidazole (262 mg) and tert-butyldiphenylsilyl chloride (0.5 ml). This mixture was stirred at room temperature overnight under nitrogen, diluted with water (20 ml), and extracted with ethyl acetate. The combined extracts were washed with water and brine, dried over sodium sulfate, and evaporated to a residue (1.42 g). The crude product was purified by flash chromatography (75 g silica gel, 230–400 mesh; elution with SS-1 (400 ml), then SS-5; 25-ml fraction volumes). Evaporation of fractions 40–59 furnished III as an oil (0.7 g; 70% yield): > 99% purity by HPLC (SS-10; 8.6 min); [α]D
-34.1° (c 0.6, CHCl3); IR, v
3380, 3071, 3048, 1111, 1055, 824, 700 cm
-1; high resolution MS, calcld. for C39H53O2Si (M-C4H9), 581.3815, found 581.3826; MS,
 m/z 581 (4, M-C4H9), 503 (2, M-C5H5-C3H3), 381 (1), 365 (3), 309 (31, SC-C6H4), 255 (14, M-SC-H2O), 239 (18), 203 (14), 199 (100, Ph2SiOH).

1H and 13C NMR, Table 1 and Table 2. Evaporation of fractions 15–18 gave (22Z,25R)-3β,26-bis(tert-butyldiphenylsilyloxy)cholesta-5,22-diene (0.12 g), identified by its 1H and 13C NMR spectra. Evaporation of fractions 27–30 gave (22Z,25R)-3β-(tert-butyldiphenylsilyloxy)cholesta-5,22-dien-26-ol (0.02 g; 96% pure by HPLC, SS-10), which was identified by 1H and 13C NMR spectra.5

The following notation is used in suggested mass spectral assignments: SC, side chain; pSC, part of side chain excluding C-20 and C-21; TsOH, p-toluenesulfonic acid; Ph, phenyl.

4The 3β,26-bis-TBDPS and 3β-TBDPS ethers of II showed NMR chemical shifts virtually identical to those of III and II, respectively, except in rings A and B, where the following shieldings were observed: 1H NMR, δ 4.10 (H-5), 3.63 (H-6); 13C NMR, δ 76.84 (C-5), 72.24 (C-6), 24.08 (C-7), 22.48 (C-8), 42.28 (C-9), 141.25 (C-10), 129.42 (C-15).
To a solution of 26-TBDPS ether III (1.93 g; 3 mmol) in dry pyridine (20 ml), was added p-toluenesulfonyl chloride (7.8 g). After stirring at room temperature overnight, the solution was poured over ice water (~200 ml) and extracted with ethyl acetate. The combined organic layers were washed with brine, saturated 
C\textsubscript{6}H\textsubscript{6}SO\textsubscript{4}, and additional brine, followed by drying over sodium sulfate and evaporation of the solvent to give IV as an oil (2.41 g; 80% yield); > 99% purity by HPLC (SS-10, \textit{t} \textsubscript{R} 10.5 min); [\alpha]\textsubscript{D}\textsubscript{23} \text{= -29.5° (c 0.7, CHCl\textsubscript{3})}; \textit{IR}, \nu \text{max} 3069, 3044, 1597, 1175, 1131, 937, 702 cm\textsuperscript{-1}; high resolution

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TABLE 2. \(^{13}C\) NMR chemical shifts for synthetic intermediates in the preparation of 26-hydroxycholesterol\(^{12a}\)

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OCH\(_3\)

\(56.55\) \(55.36\) \(56.49\) \(56.49\)

\(^{1}C\) and \(^{13}C\) NMR, Tables 1 and 2.

(22Z,25R)-6β-Methoxy-26-(tert-butyldiphenylsilyloxy)-3α,5-cyclo-5α-cholesta-22-ene (V)

A solution of tosylate IV (2.4 g; 3.03 mmol) and fused potassium acetate (2.4 g) in methanol (120 ml) was refluxed overnight under nitrogen. After cooling of the reaction and evaporation of the solvent, water was added to the residue, followed by extraction with ethyl acetate. The combined organic layers were washed with water and brine, dried over sodium sulfate, and evaporated to an oil (2.0 g; 75% purity by HPLC, SS-10). The crude product was subjected to flash chromatography (40 g silica gel, 230-400 mesh; elution with 0.25% ethyl acetate in hexane; 23-ml fraction volumes). Evaporation of fractions 39-48 gave an analytical sample of V (437 mg) showing > 99% purity by HPLC (SS-10, \(t_R\) 14.0 min); additional material (1.064 g of 94% purity (HPLC, SS-10) was obtained from evaporation of fractions 34-38 and 49-52. Evaporation of fractions 57-85 gave a crude sample of (22Z,25R)-3β-methoxy-26-(tert-butyldiphenylsilyloxy)cholest-5,22-diene (VIII) (0.32 g), which was identified and characterized by \(^1H\) and \(^{13}C\) NMR (Tables 1 and 2) and HPLC (SS-10, \(t_R\) 20.2 min). Analytical data for V: oil; \([\alpha]_D^{23}+11.6^\circ\) (c 0.9, CHCl\(_3\)); IR \(\nu_{max}\) 3071, 3054, 1150, 1111, 1017, 824, 810 cm\(^{-1}\); high resolution MS, calcld. for C\(_{36}\)H\(_{45}\)O\(_3\)Si (M-C\(_\text{H}_2\)), 593.3971, found 593.3958; MS, \(m/z\) 595 (1, M-C\(_\text{H}_4\)), 563 (17, M-CH\(_2\)-C\(_\text{H}_2\)-C\(_\text{H}_4\)), 485 (5, M-CH\(_3\)-C\(_\text{H}_2\)-C\(_\text{H}_4\)), 364 (4, M-CH\(_2\)-C\(_\text{H}_2\)-C\(_\text{H}_4\)-Ph\(_2\)SiOH), 353 (32), 309 (39, SC-C\(_\text{H}_3\)), 281 (73, pSC-C\(_\text{H}_3\)), 255 (9, M-CH\(_3\)-O-SiC\(_\text{H}_3\)), 253 (12, M-CH\(_2\)-O-SiC\(_\text{H}_3\)), 239 (19), 199 (100, Ph\(_2\)SiOH), 109 (41), 107 (45), 105 (70); \(^1H\) and \(^{13}C\) NMR, Tables 1 and 2.

(22Z,25R)-6β-Methoxy-3α,5-cyclo-5α-cholesta-22-en-26-ol (VI)

To a solution of TBDPS ether V (1.45 g, 2.2 mmol) in tetrahydrofuran (100 ml) was added tetrabutylammonium fluoride (72 ml; 1 M solution in tetrahydrofuran), and the...
resulting mixture was stirred at room temperature for 20 h. After evaporation of the solvent, water was added, followed by extraction with ethyl acetate. The combined organic layers were dried over sodium sulfate and evaporated to a yellow oil (2.5 g), which was purified by column chromatography (125 g silica gel, 230-400 mesh; elution with SS-3; 25-ml fraction volumes). Evaporation of fractions 50-80 provided VI as an oil (887 mg; 96% yield) showing a single component by TLC (SS-7, Rf 0.42) and ~99% purity by 1H NMR; [α]D29 +13.8° (c 1.4, CHCl3); IR, νmax 3470, 3079, 3043, 1086, 1053, 1022 cm⁻¹; high resolution MS, calc'd for C28H46O2 414.3498, found 414.3506; MS, m/z 414 (18, M⁺), 399 (39, M-CH₃), 382 (39, M-CH₂OH), 367 (9, M-CH₂OH-CH₃), 359 (62, M-C₆H₅), 356 (9), 287 (3, M-SC), 282 (5), 255 (28, M-CH₂OH-SC), 253 (11, M-CH₂OH-SC), 109 (100), 107 (45), 105 (49); 1H and 13C NMR, Tables 1 and 2.

**(25R)-6β-Methoxy-3α,5-cyclo-5α-cholestan-26-ol (VIIa)**

To a solution of the Δ24-5α-steroid VII (207 mg; 0.5 mmol) in ethyl acetate (20 ml) was added platinum oxide monohydrate (20 mg). The mixture was stirred under 1 atm of hydrogen for 20 h at room temperature. Filtration and evaporation of the solvent gave a residue (210 mg), which was purified by column chromatography (10 g of AgNO₃-silica gel; 50 cm x 1 cm i.d. column; elution with SS-5; 10-ml fraction volumes). Evaporation of fractions 71-120 gave VIIa as a solid (90 mg; 89% yield): mp 95-96.5°C; [α]D29 +56.9° (c 1.1, CHCl₃); single component by TLC (SS-7). The mass spectrum corresponded to that of VIIa except for deuterium shifts. Analysis of the molecular ion region indicated d₀ (0%), d₁ (8%), d₂ (31%), d₃ (30%), d₄ (19%), d₅ (8%), d₆ (4%), and small amounts of d₇, d₈, and d₉ species (an average of 3.0 deuterium atoms per molecule). A virtually identical distribution of deuterium was observed for ions corresponding to M-CH₃ and M-CH₂OH, whereas the ion corresponding to M-SC-CH₂OH showed no detectable deuterium. The 13C NMR spectrum of VIIb was essentially identical with the spectrum of VIIa except for the absence of signals for C-22 and C-23, attenuation of signals for C-20, C-21, C-24, and C-27, small upfield deuterium isotope shifts (mainly ≤ 0.1 ppm), and minor peaks attributable to deuterium coupling and isotope shifts. No signals were observed for C-22, C-23, or C-24 in the HETCOR spectrum. The 2H NMR spectrum showed the following broad signals (relative intensities estimated by integration): δ 0.89 (7%), D-21, D-27), 1.03 and 1.10 (32%, D-22, D-24), 1.24 (21%, D-23), 1.33 (34%, D-20, D-22, D-24, D-26), 1.60 (5%, D-25), 3.43 and 3.50 (1%, D-26). In the 1H NMR spectrum of VIIb, the side-chain proton signals were broadened and/or diminished in intensity relative to those of the undeuterated steroid VIIa. The IR spectrum of VIIb was essentially identical to that of VIIa except for a broad, weak absorbance at 2150 cm⁻¹ attributed to C-D stretching vibrations.

**(25R)-Cholest-5-ene-3β,26-diol (Ia)**

To a solution of the i-steroid VIIa (46 mg; 0.11 mmol) in dioxane (20 ml) was added water (5 ml) and p-toluenesulfonic acid (46 mg), and the resulting solution was refluxed for 1 h. After addition of water and extraction with ethyl acetate, the combined organic layers were dried over sodium sulfate and evaporated to a solid (44 mg), which was purified by flash chromatography (4 g silica gel, 230-400 mesh; elution with SS-5 and SS-6). Evaporation of fractions 58-60 gave Ia (II mg) of 98% purity, and additional Ia (22 mg) of 96% purity (HPLC analysis) was obtained from adjoining fractions. Recrystallization of the 98% pure sample from methanol furnished an analytical sample of Ia: mp 177-178°C (lit. 177-178°C); [α]D29 -38.6° (c 0.6, CHCl₃); lit (26) [α]D29 -40.6°; single component matching an authentic standard by TLC (SS-8, Rf 0.45) and HPLC (SS-9, tR 4.9 min); the NMR chemical shifts matched reported ones (6) to within ± 0.04 ppm (13C) and ± 0.001 ppm (1H signals).

[3H]-(25R)-6β-Methoxy-3α,5-cyclo-5α-cholestan-26-ol (VIIb)

The Δ24 i-steroid VI (50 mg; 0.12 mmol) was evaporated twice from CH₂OD and then dissolved in ethyl acetate (5 ml) and CH₂COOD (5 ml). Platinum oxide (20 mg) was added, and the mixture was stirred under 1 atm of deuterium gas for 30 min at room temperature. Filtration and evaporation of the solvent gave a residue, which was added to that of a duplicate reaction. The combined residues were adsorbed onto silica gel (200 mg) and subjected to MPLC (14 g of AgNO₃-silica gel; 50 cm x 1 cm i.d. column; elution with SS-4; 5-ml fraction volumes). Evaporation of fractions 71-120 gave VIIb as a solid (90 mg; 89% yield): mp 95-96.5°C; single spot by TLC (SS-7). The mass spectrum corresponded to that of VIIa except for deuterium shifts. Analysis of the molecular ion region indicated d₀ (0%), d₁ (8%), d₂ (31%), d₃ (30%), d₄ (19%), d₅ (8%), d₆ (4%), and small amounts of d₇, d₈, and d₉ species (an average of 3.0 deuterium atoms per molecule). A virtually identical distribution of deuterium was observed for ions corresponding to M-CH₃ and M-CH₂OH, whereas the ion corresponding to M-SC-CH₂OH showed no detectable deuterium. The 13C NMR spectrum of VIIb was essentially identical with the spectrum of VIIa except for the absence of signals for C-22 and C-23, attenuation of signals for C-20, C-21, C-24, and C-27, small upfield deuterium isotope shifts (mainly ≤ 0.1 ppm), and minor peaks attributable to deuterium coupling and isotope shifts. No signals were observed for C-22, C-23, or C-24 in the HETCOR spectrum. The 2H NMR spectrum showed the following broad signals (relative intensities estimated by integration): δ 0.89 (7%), D-21, D-27), 1.03 and 1.10 (32%, D-22, D-24), 1.24 (21%, D-23), 1.33 (34%, D-20, D-22, D-24, D-26), 1.60 (5%, D-25), 3.43 and 3.50 (1%, D-26). In the 1H NMR spectrum of VIIb, the side-chain proton signals were broadened and/or diminished in intensity relative to those of the undeuterated steroid VIIa. The IR spectrum of VIIb was essentially identical to that of VIIa except for a broad, weak absorbance at 2150 cm⁻¹ attributed to C-D stretching vibrations.
the combined organic layers were dried over sodium sulfate and evaporated to a solid (75 mg), which was adsorbed onto silica gel (200 mg) and purified by MPLC (14 g silica gel, 230-400 mesh; elution with SS-6; 8-ml fraction volumes). Evaporation of fractions 80-170 gave Ib (61 mg; 84% yield): single component by TLC (SS-8, $R_f$ 0.40), HPLC (SS-9, $t_R$ 9.5 min; $t_R$ 9.5 min for authentic Ia), and GC ($t_R$ 20.25 min; $t_R$ 20.37 min for authentic Ia); mp 175-177°C. The mass spectrum corresponded to that of Ia except for deuterium shifts. The molecular ion indicated $d_0$ (1%), $d_1$ (8%), $d_2$ (36%), $d_3$ (28%), $d_4$ (18%), $d_5$ (5%), $d_6$ (4%), and small amounts of $d_7$, $d_8$, and $d_9$ species (an average of 2.9 deuterium atoms per molecule).

Fig. 2. $^1$H NMR spectrum of the (R)-MTPA diesters of (25R)-26-hydroxycholesterol Ia (panel A) and the side-chain deuterated analog Ib (panel B). $^{13}$C NMR signals for C-12 and C-18 of Ib (panel C). $^1$H NMR spectrum of Ib (panel D). $^1$H NMR spectra: 500 MHz, no apodization; $^{13}$C spectrum: 125 MHz, inverse gated $^1$H decoupling; 1-Hz line broadening; $^1$H spectrum: 77 MHz, $^1$H decoupled, mild Gaussian apodization; all spectra acquired at 27°C.

Ions corresponding to $M-H_2O-CH_3$, $M-60$, $M-75$, and $M-111$ showed similar amounts of deuterium, whereas ions at $m/z$ 255 and 273 (corresponding to ions involving loss of the side chain) showed complete absence of deuterium. The GC-MS of the TMS ether derivative of Ib showed a similar distribution of deuterium in ions $M^*$, $M-CH_3$, $M-TMSOH$, $M-TMSOH-CH_3$, and $M-129$. The $^1$H NMR spectrum of Ib closely resembled that of Ia except for diminished signal intensities at $\delta \sim 1.34$ and an additional doublet-doublet 0.003 ppm upfield of the H-26 resonance at $\delta$ 3.50 (possibly due to deuterium isotope effects). The 125-MHz $^{13}$C NMR spectrum of Ib in CDCl$_3$ containing 5% CD$_3$OD resembled that of Ia except for diminished signal intensities at $\delta \sim 1.34$ and an additional doublet-doublet 0.003 ppm upfield of the H-26 resonance at $\delta$ 3.50 (possibly due to deuterium isotope effects). The 125-MHz $^{13}$C NMR spectrum of Ib in CDCl$_3$ containing 5% CD$_3$OD resembled that of Ia except for diminished signal intensities at $\delta \sim 1.34$ and an additional doublet-doublet 0.003 ppm upfield of the H-26 resonance at $\delta$ 3.50 (possibly due to deuterium isotope effects). The 125-MHz $^{13}$C NMR spectrum of Ib in CDCl$_3$ containing 5% CD$_3$OD resembled that of Ia except for diminished signal intensities at $\delta \sim 1.34$ and an additional doublet-doublet 0.003 ppm upfield of the H-26 resonance at $\delta$ 3.50 (possibly due to deuterium isotope effects). The 125-MHz $^{13}$C NMR spectrum of Ib in CDCl$_3$ containing 5% CD$_3$OD resembled that of Ia except for diminished signal intensities at $\delta \sim 1.34$ and an additional doublet-doublet 0.003 ppm upfield of the H-26 resonance at $\delta$ 3.50 (possibly due to deuterium isotope effects). The 125-MHz $^{13}$C NMR spectrum of Ib in CDCl$_3$ containing 5% CD$_3$OD resembled that of Ia except for diminished signal intensities at $\delta \sim 1.34$ and an additional doublet-doublet 0.003 ppm upfield of the H-26 resonance at $\delta$ 3.50 (possibly due to deuterium isotope effects). The 125-MHz $^{13}$C NMR spectrum of Ib in CDCl$_3$ containing 5% CD$_3$OD resembled that of Ia except for diminished signal intensities at $\delta \sim 1.34$ and an additional doublet-doublet 0.003 ppm upfield of the H-26 resonance at $\delta$ 3.50 (possibly due to deuterium isotope effects). The 125-MHz $^{13}$C NMR spectrum of Ib in CDCl$_3$ containing 5% CD$_3$OD resembled that of Ia except for diminished signal intensities at $\delta \sim 1.34$ and an additional doublet-doublet 0.003 ppm upfield of the H-26 resonance at $\delta$ 3.50 (possibly due to deuterium isotope effects). The 125-MHz $^{13}$C NMR spectrum of Ib in CDCl$_3$ containing 5% CD$_3$OD resembled that of Ia except for diminished signal intensities at $\delta \sim 1.34$ and an additional doublet-doublet 0.003 ppm upfield of the H-26 resonance at $\delta$ 3.50 (possibly due to deuterium isotope effects).
cept for the following signals: C-22 and C-23 (very weak multiplets), C-24 (weak multiplet), C-17, C-20, C-21, C-25, C-26, and C-27 (diminished in intensity, multiplets upon resolution enhancement). The $^2$H NMR spectrum (Fig. 2, panel D) showed the following broad signals (relative intensities estimated by integration): $\delta$ 0.89 (6%, D-21, D-27), 1.04 and 1.10 (32%, D-22, D-24), 1.24 (19%, D-23), 1.32 (39%, D-22, D-23, D-24), 1.60 (5%, D-25).

$[^2H]-(25R)$-Cholest-5-ene-3$\beta$,26-diol (Ic)

A solution of the $\Delta^{222}$-i-sterol VI (40 mg) in ethyl acetate containing acetic acid and platinum oxide was exposed to tritium gas until an activity of 1 Ci was obtained. After removal of the catalyst and label tritium,$^3$ a portion (420 mCi) of the product (VIIc) was purified by column chromatography on Unisil (35 cm × 1 cm i.d. column; 12-ml fraction volumes; elution with 250 ml of SS-1, 250 ml of SS-4, and 550 ml of SS-5). The contents of fractions 55-67 (299 mCi) had the same $R_f$ (0.5) as VIIa by radio-TLC (SS-7). This material was further chromatographed (AgNO$_3$-silica gel; 40 cm × 1 cm i.d. column; 18-ml fraction volumes; elution with 500 ml of SS-4 and 1250 ml of SS-5). Fractions 36-42 (172 mCi) were combined and evaporated to dryness.

To a solution of the residue in dioxane (10 ml) was added water (2.5 ml) and $p$-toluenesulfonic acid (23 mg). The resulting solution was gently refluxed with stirring under nitrogen for 1 h. After the solution was allowed to cool, water (5 ml) and ethyl acetate (50 ml) were added. The layers were separated, and the aqueous layer was further extracted with ethyl acetate (2 × 50 ml). The combined organic extracts were washed with brine (3 × 10 ml); the aqueous layer and brine washes contained together ~1 mCi. The ethyl acetate solution (~20 ml) was transferred to a round-bottom flask and evaporated to dryness under nitrogen, and the residue was transferred to a Unisil column (35 cm × 1 cm i.d.) in several 10-ml portions of 5% ethyl acetate in hexane. The product was eluted with SS-5 (1000 ml) and SS-6 (1250 ml) and collected in 12.5-ml fractions. Fractions 97-118 were combined and evaporated to dryness.

The resulting solution was gently refluxed with stirring under nitrogen for 36 h. Additional MTPA chloride (10 ml) was added at 16 h, and virtual completion of the reaction after 22 h was indicated by TLC (20% ethyl acetate in hexane, $R_f$ 0.71 for diesters of Ia and Ib, $R_f$ 0.79 for ester of VIIb). The reaction mixture was evaporated to a residue that was triturated with 5% ethyl acetate in hexane and passed through silica gel (50 mm × 4 mm i.d. column); TLC analysis indicated that the MTPA esters were in the first ~5 ml of eluate, which was evaporated to a residue and dissolved in CDCl$_3$ for NMR analysis. The 500 MHz $^1$H NMR spectrum of the MTPA ester of Ia showed the H-26 signals similar to those reported previously (26): $\delta$ 4.233 (dd, 10.7, 5.6 Hz) and 4.069 (dd, 10.7, 6.7 Hz) for the 25R isomer and weak peaks of unequal intensity at $\delta$ 4.233 and 4.069 for the 25S isomer (see Fig. 2). The 19F NMR spectra of the MTPA esters of deuterated sterols Ia and VIIb showed the same doublyt-butyl signals at $\delta$ 4.233 and 4.069 for the 25R isomer and weak peaks of unequal intensity at $\delta$ 4.15 and 4.16 (Fig. 2). Other signals for the esters of Ia and Ib were virtually identical to those reported previously (26).

Preparation of (R)-MTPA esters of Ia, Ib, and VIIb

(S)-(+-)-MTPA chloride (5 µl) was added to a solution of ~1.5 mg of sterol in pyridine-dichloromethane (1:1, 0.6 ml), and the solution was allowed to stand at room temperature for 36 h. Additional MTPA chloride (10 µl) was added at 16 h, and virtual completion of the reaction after 22 h was indicated by TLC (20% ethyl acetate in hexane, $R_f$ 0.71 for diesters of Ia and Ib, $R_f$ 0.79 for ester of VIIb). The reaction mixture was evaporated to a residue that was triturated with 5% ethyl acetate in hexane and passed through silica gel (50 mm × 4 mm i.d. column); TLC analysis indicated that the MTPA esters were in the first ~5 ml of eluate, which was evaporated to a residue and dissolved in CDCl$_3$ for NMR analysis. The 500 MHz $^1$H NMR spectrum of the MTPA ester of Ia showed the H-26 signals similar to those reported previously (26): $\delta$ 4.233 (dd, 10.7, 5.6 Hz) and 4.069 (dd, 10.7, 6.7 Hz) for the 25R epimer and a pair of weak peaks (1% of total H-26 intensity) at $\delta$ 4.15 and 4.16 for the 25S epimer (see Fig. 2).

Attempted preparation of 26-hydroxycholesterol via the 3$\beta,26$-ditosylate of II

The 3$\beta,26$-ditosylate of II (prepared by reaction of II with $p$-toluenesulfonyl chloride-pyridine overnight at room...
temperature) was converted to 6\(\beta\)-methoxy-26-tosyloxy-
3\(\alpha\),5-cyclo-5\(\alpha\)-cholest-22-ene by treatment with potassium
acetate, as described for the preparation of V. After
purification by silica gel chromatography (SS-2), the i-
steroid (50 mg) was hydrogenated (1 atm \(\text{H}_2\), \(\text{PtO}_2\), 23\(^\circ\)C) to
give 6\(\beta\)-methoxy-26-tosyloxy-3\(\alpha\),5-cyclo-5\(\alpha\)-cholestanol
(45 mg). Hydrolysis of the i-steroid (conditions as described
for the preparation of Ia) gave the 26-tosyl derivative of
Ia. However, deprotection of the 26-hydroxyl proved to be
difficult. The best of several attempts to remove the tosyl
group was treatment with sodium acetate in acetic acid at
90\(^\circ\)C overnight to give \(\sim 50\%\) conversion to the 26-acetate of Ia.

DISCUSSION

The goal of this research was to prepare \(3\)H-labeled
(25\(R\))-26-hydroxycholesterol of high specific activity for
use in investigations of its in vitro binding to proteins and
for other studies. Descriptions of the preparation of
26-hydroxycholesterol labeled with isotopes of hydrogen
have been reported previously. In 1968 Wachtel, Emer-
man, and Javitt (28) reported the preparation of \(3\)H-labeled
26-hydroxycholesterol (13.3 \(\mu\)Ci per \(\mu\)mol) by Clemmen-
sen reduction of kryptogenin in the presence of tritiated
water. After further reduction of the crude product with
hydrazine in alkali, the resulting material was diluted with
unlabeled 26-hydroxycholesterol and recrystallized to
constant specific activity. The criterion for radiopurity of
the product was “preparation of the diacetate with no loss
in specific activity.” In 1981 Javitt et al. (29) reported the
preparation of deuterated 26-hydroxycholesterol by Clemmensen reduction of kryptogenin in the presence of
\(\text{D}_2\text{O}\) and \(\text{DCl}\). The product was characterized only by the
MS for its bis-TMS ether derivative, which showed a
major peak at \(m/z 554\) corresponding to the incorporation
of eight atoms of deuterium. In the following year the
same group (30) reported that Clemmensen reduction of
kryptogenin in the presence of CH\(\text{OCH}_2\text{OD}\), \(\text{D}_2\text{O}\), and
\(\text{DCl}\) gave, after column chromatography on alumina,
deuterium-labeled 26-hydroxycholesterol; this material
was further purified by conversion to the \(3\beta,26\)-diacetate
derivative and repeated recrystallization followed by
regeneration of the free sterol, which was recrystallized
from ethyl acetate. Characterization of the labeled
26-hydroxycholesterol obtained in this manner was
limited to negative ion chemical ionization MS and low-
field \(1\)H NMR. The MS studies indicated incorporation of
two to nine deuterium atoms but provided no informa-
tion with respect to isotopic localization. \(1\)H NMR analy-
sis was stated to show the absence of deuterium at C-27,
C-26, and C-25 and the “strongest loss of signals” at C-16
and C-22, with the remainder of the deuterium assumed
to be located on carbon atoms 15, 17, 20, and 23. In 1990
Breuer and Björkhem (31) reported the preparation of [15,15,
16,16,17\(\alpha\)-\(3\)H\(\times\)]-26-hydroxycholesterol “according to previ-
ously published methods.” No characterization of the
product was presented. The pertinent reference cited was
a publication by others (32) involving Clemmensen
reduction of kryptogenin which reportedly gave
26-hydroxycholesterol containing predominantly eight
atoms of deuterium. Another group (33) recently
reported the preparation of deuterium-labeled 26-hydrox-
cholesterol by Clemmensen reduction of kryptogenin in
the presence of \(\text{CH}_3\text{CH}_2\text{OD}, \text{D}_2\text{O},\) and \(\text{DCl}\). The proce-
dure described involved purification of the crude product
by column chromatography on silicic acid followed by
recrystallization of the material corresponding to 26-hydroxy-
cholesterol. The labeled product contained molecules
with five to ten atoms of deuterium with the major species
containing seven atoms of deuterium. Further characteri-
zation of the labeled sterol, including evidence for the
localization of the deuterium, was not presented.

In 1990 Kok and Javitt (34) reported the preparation of the
\(3\beta,26\)-diacetate derivative of 26-hydroxycholesterol through a series of reactions starting with diosgenin. In
this approach, diosgenin was subjected to Clemmensen
reduction and the crude product was, without further
purification or product characterization, oxidized with
chromium trioxide. The crude oxidation product was,
without further purification or product characterization,
subjected to Wolff-Kishner reduction in the presence of
100\% \(\text{ND}_3\text{ND}_2\) and \(\text{KOD}\). The crude product was
acetylated and the resulting material was subjected to
chromatography on an alumina column to give 26-hydroxy-
cholesterol diacetate, which was recrystallized from
methanol. The product, which was characterized only by
melting point and the presentation of a partial MS, was
reported to consist mostly of \(d_3\) and \(d_4\) species (based
upon analysis of the M-60 region of the MS of the diace-
tate derivative). The published MS data for this region
presented in the upper and insert panels of Figure 1 of
that paper (34) differed significantly in the ion intensities.
No evidence was provided on the localization of the deuterium.

Another approach to the preparation of \(3\)H-labeled
26-hydroxycholesterol was that of Varma, Wickramasinghe,
and Caspi (35) involving hydroboration of the \(3\beta-
tetrahydropyranyl\) ether of cholesta-5,25-diene-3\(\beta\)-ol. Using
this approach, preparation of (25\(SR\))-[25-\(3\)H]-3\(\beta\)-
tetrahydropyranoyloxycholesterol-5-ene-26-ol (5.97 \(\mu\)Ci per
\(\mu\)mol) was described.

It should be noted that Kluge, Maddox, and Partridge
(36) reported that both Clemmensen reduction of kryp-
togenin and Wolff-Kishner reduction of the resulting
22-ketosterol byproduct lead to epimerization at C-20 and
C-25. This report therefore demonstrates the need for
careful attention to the stereochemistry at C-20 and C-25
and the possible requirement of separation of the multiple
compounds resulting from epimerization. The equilibra-
tion of 22-oxo-26-hydroxy and 22-hydroxy-26-oxo species under acidic and basic conditions (36, 37) also raises the possibility of deuterium incorporation at C-25 and C-26. Furthermore, inasmuch as Clemmensen reduction of diosgenin has been shown to lead to a number of significant byproducts (19), attention must be directed towards requirement for the isolation of pure cholest-5-ene-3β,16β, 26-triol for use in its subsequent conversion to (25R)-26-hydroxycholesterol or very extensive characterization of byproducts (19), which was hydrolyzed with p-toluenesulfonic acid. We initially pursued a synthetic scheme starting with the 25s isomer indicated that the sample was almost exclusively (98-99%) the 25R isomer. Both the 25s-sterol and the enantiomeric impurity indicated 1% of the minor diastereomer. The presence of deuterium label in Ib and VIIb at nearly all side-chain positions suggested the possibility of epimerization at C-20 and/or C-25. Consequently, hydrogenation or deuteration products prepared in the presence (Ib, VIIb) or absence (Ia, VIIa) of acetic acid were analyzed for epimerization by 13C NMR (125 MHz) and by 1H NMR of the MTPA ester (26). Portions of these spectra are shown in Fig. 2. Based on 13C NMR shielding differences between C-20 epimers of 26-hydroxycholesterol diacetate (36), the presence of any 20S epimer should be indicated by additional minor signals in the NMR spectrum of VIIb corresponding to those expected (13, 26, 41) for the 25S isomer indicated that the sample was almost exclusively (98-99%) the 25R isomer. In the deuterated sterols Ib and VIIb, the complexity of the side-chain 13C signals (caused by deuterium coupling and deuterium isotope shifts) would obscure any minor signals of the 25S epimer. Consequently, the (R)-MTPA esters of Ia, Ib, and VIIb were prepared and analyzed by 1H NMR for the 25S epimer (detection limit 1%) or Ib (detection limit 2%). Minor signals in the 13C spectrum of VIIa corresponding to those expected (13, 26, 41) for the 25S isomer indicated that the sample was almost exclusively (98-99%) the 25R isomer.

Preparation of the i-steroids was accompanied by formation of a significant byproduct that was identified as the 3β-3β-methoxy ether (compound VIII in the case of the preparation of i-steroid V). A similar byproduct has been reported in the preparation of the i-steroid of stigmasterol (38).

Attempts to reduce VI with the homogeneous catalyst tri(triphenylphosphine)chlororhodium were unsuccessful; the unreactivity of the Δ22 bond of episterol under similar conditions has also been noted (39). Platinum oxide was chosen as the heterogeneous catalyst least prone to promote isotopic scrambling (39, 40).

This six-step synthesis of (25R)-26-hydroxycholesterol from II proceeded in an overall yield of 31%.
Double bond in the presence of acetic acid, contained no stereomer (Fig. 2, panel B). The larger peak at δ 4.15 appears to represent H-26 protons adjacent to deuterium at C-25, an interpretation suggesting that deuterium incorporation at C-25 is accompanied by partial epimerization.

In summary, the combined findings show that hydrogenation in the absence of acetic acid, followed by chromatographic purification and hydrolysis, furnished 26-hydroxycholesterol uncontaminated by the 20s epimer. The corresponding analysis for purity, it is noteworthy that no epimeric or other impurities were detected in Ib by reversed phase HPLC, capillary GC, or TLC on silica gel. We have avoided relying exclusively on chromatographic methods for detecting minor isomeric impurities, which are often marginally resolved from the principal component. The peaks of such impurities present at a level of only a few percent will often be entirely obscured by the tail or front of the nearby principal component peak. For example, the resolution of the 25R and 25S epimers of underivatized 26-hydroxycholesterol by reversed phase HPLC (26) appears to be insufficient to detect a minor impurity of one epimer in the other. Nevertheless, the combination of HPLC and capillary GC is capable of detecting many potential contaminants of 26-hydroxycholesterol, including most byproducts derived from the Clemensen reduction of diosgenin.

Tritium-labeled (25R)-26-hydroxycholesterol (Ic) was prepared under conditions similar to those used in the preparation of Ib. To remove possible epimeric material and other impurities, the i-steroid intermediate was purified on silica gel and on silver nitrate-silica gel, and the diol Ic was further purified on silica gel. The final product showed high purity by radio-TLC and radio-HPLC (Fig. 3). The mass of the final product was estimated by a colorimetric assay, and the specific activity was calculated to be 16.9 Ci per mmol. This specific activity is very considerably higher than reported previously.

All synthetic intermediates were fully characterized by optical rotation, IR, MS, high-resolution MS, and NMR. An abundant m/z 109 ion was characteristic of the mass spectra of the Δ23 sterols; this ion appears to correspond to the sterol side chain after loss of the C-26 substituent. The Δ22,26-TBDDS ethers gave strong ions at m/z 281 and m/z 199 (Ph2SiOH), as well as ions from loss of C2H4 together with other fragments. The 26-hydroxy-i-steroids VI and VIIa showed prominent ions for M-CH3, M-CH2OH, and M-C2H5, but such ions were weak or absent in the 26-TBDDS-i-steroid V, whose mass spectrum was dominated by ions characteristic of TBDDS ethers. The MS fragmentation of the i-steroid 6β-methoxy-3α,5-cyclo-5α-cholestanol has been thoroughly investigated, and mechanisms have been elucidated for the formation of ions M-CH2OH and M-C2H5 (42).

Full 1H and 13C NMR assignments are presented for all synthetic intermediates, including the i-steroids V, VI, and VIIa. The quaternary carbons of i-steroids (C-5, C-10, and C-13) were distinguished in the HMBC spectrum of VIIb, which showed the following pertinent long-range correlations: C-13 (δ 42.71) to H-18, C-5 (δ 35.20) and C-10 (δ 43.31) to H-19, and C-5 to H-7β. Comparison of the 13C assignments in Table 2 with those reported for other i-steroids show apparent agreement (43, 44) or minor differences (45).

Analysis of the 1H NMR resonances in ring A of i-steroids was complicated by the large geometrical differences between the five-membered A ring of i-steroids and the six-membered chair conformation observed for ring A of 5α-sterols and Δ2-sterols. Furthermore, all of the H-1, H-2, and H-3 resonances were overlapped by other signals, and the remoteness of the C-1 and C-2 protons from the C-19 methyl and the C-4 protons made nuclear Overhauser enhancement experiments unattractive. Finally, the near isochronicity of H-1 and H-2 signals at δ ~1.5 portended strong coupling effects that would vastly complicate analysis of the spin system. Nevertheless, precise chemical shift values were derived from COSY/DEC spectra, and predicted coupling constants were obtained by applying a Karplus relation to calculated structures of the i-steroids. Because of the broad energy minima usually encountered in five-membered rings, coupling constants were calculated for a range of conformations by varying the C10-C1-C2-C3 dihedral angle. Similar conformational analyses have been done previously (8, 46). The results shown in Fig. 4 provided the key finding that the predicted value of J1α-1β,1β is <1 Hz and prompted the suggestion that the H-1 and H-2 signals at δ 1.51 and 1.52 might be weakly coupled. This hypothesis proved to be correct and led to the first-order analysis of all the ring A resonances in the 500 MHz spectrum. Representative 1H NMR coupling constants and chemical shifts for ring A protons of i-steroids are presented in Fig. 5. Fig. 4 shows that the best fit of experimental J1α-2α, J1β-2α, and J1β-2β...
values to the predicted couplings corresponds to a C10-C1-C2-C3 dihedral angle of \( \sim 33^\circ \), similar to that from molecular mechanics (30°) or X-ray structures (47) of 6β-methoxy-i-steroids (29° and 31°). All three methods show the five-membered ring to be a 1β-envelope.

Coupling patterns for protons in rings B and C of 6α-methoxy-i-steroids were determined from an HMQC spectrum of 7Ia and by simulation of the spin system containing the nearly isochronous C-11 protons. The coupling constants were similar to those of saturated sterols and produced analogous multiplets except for second-order effects (at 500 MHz) for the H-9α, H-11α, H-11β, and H-12α resonances and simplifications arising from the absence of protons at the 5α and 6β positions. Virtual coupling effects owing to strongly coupled signals of i-steroids (H-11α, H-11β) and \( \Delta^{22\text{Z}} \) sterols (H-16β, H-17α and H-22, H-23) thwarted further conformational analysis based on \(^1\text{H}\) NMR couplings.

In summary, we have presented a chemical synthesis of deuterium- and tritium-labeled (25R)-26-hydroxycholesterol from a byproduct of the Clemmensen reduction of diosgenin. Synthetic (25R)-26-hydroxycholesterol derived from sapogenins is likely to contain epimeric impurities and other contaminants that are detectable only by rigorous analytical techniques. Although most epimeric and other contaminants are separable from (25R)-26-hydroxycholesterol by silica gel chromatography, HPLC, or capillary GC, some of the potential impurities are unlikely to be sufficiently resolved to permit detection at a level of a few percent. We have presented NMR analyses capable of detecting \( \sim 1\% \) of a C-20 or C-25 epimeric impurity in (25R)-26-hydroxycholesterol. We have also demonstrated the use of \(^2\text{H}\) NMR and \(^13\text{C}\) NMR to locate the carbon atoms at which deuterium label is present in (25R)-26-hydroxycholesterol. Although the distribution of the isotopic hydrogen in the side chain of the product described herein may be unsuitable for certain metabolic studies, it does not preclude use in a number of important analytical and biological investigations. Moreover, the synthesis presented herein provides a \(^1\text{H}\)-labeled product of high specific activity (16.9 Ci/mmol) for use in a number of experiments not possible with previously described materials.

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