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 (25*R*)-26-Hydroxycholesterol (**I**) was synthesized in six steps from (22Z,25*R*)-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,25*R*)-6β-methoxy-26-(*tert*-butyldiphenylsilyloxy)-3α,5-cyclo-5α-cholest-22-ene (**V**). Removal of the 26-silyl group of **V** gave (22Z,25*R*)-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 [^{2}H]-**I** or [^{3}H]-**I**, respectively. Analysis of the [^{2}H]-**I** by mass spectrometry showed that all the deuterium was located in the sterol side chain, mainly as d_2, d_3, and d_4 species. The ^{2}H and ^{13}C nuclear magnetic resonance (NMR) spectra of [^{2}H]-**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 [^{2}H]-**I** and its α-methoxy-α-(trifluoromethyl)phenylacetate diester showed no detectable 20S epimer and ~2% of the 25S epimer. The [^{3}H]-**I** was prepared analogously to [^{2}H]-**I** using carrier-free tritium and showed a specific activity of 16.9 Ci/mmol. All synthetic intermediates were characterized fully by ^{1}H and ^{13}C NMR, and representative ^{1}H-^{1}H coupling constants are given for the ring A protons of i-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.

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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). (25*R*)-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 (25*R*)-26-oxygenated sterols 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-cholest-8(14)-en-15-one, a potent hypocholesterolemic 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 (16). 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,25*R*)-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 (25*R*)-26-hydroxycholesterol of high specific activity by

Abbreviations: DEPT, distortionless enhancement by polarization transfer; COSYDEC, ω_1-decoupled ^{1}H-^{1}H correlation spectroscopy; GC, gas chromatography; HETCOR, ^{1}H-^{13}C heteronuclear shift-correlated spectroscopy; HMBC, heteronuclear multiple bond correlation; HMQC, heteronuclear multiple quantum coherence; HPLC, high performance liquid chromatography; IR, infrared; mp, melting point; MS, mass spectrometry or mass spectrum; MPLC, medium pressure liquid chromatography; MTPA, α-methoxy-α-(trifluoromethyl)phenylacetyl; NMR, nuclear magnetic resonance; SC, side chain; TBDPS, *tert*-butyldiphenylsilyl; TLC, thin-layer chromatography; TMS, trimethylsilyl.

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reduction of the Δ^{22} bond with carrier-free tritium gas. Described herein is the preparation of deuterium- and tritium-labeled (25*R*)-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 $CHCl_3$ solution at room temperature (~23°C) on a JASCO DIP-4 digital polarimeter. Infrared spectra (IR) were measured with KBr pellets on a Mattson Galaxy 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 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 cm^2) 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 ^{13}C, ~22°C), a Bruker AMX500 instrument (500.1 MHz for ^1H, 125.8 MHz for ^{13}C, 76.8 MHz for ^2H, 27°C), and a Bruker AC250 instrument (235.4 MHz for ^{19}F, ~22°C) in $CDCl_3$ solution and referenced to internal tetramethylsilane (^1H), $CDCl_3$ at 7.27 ppm (^2H), $CDCl_3$ at 77.0 ppm (^{13}C), or $CFCl_3$ (^{19}F). All ^{13}C 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 (ω_1-decoupled ^1H-^1H correlation spectroscopy; 0.2-s fixed evolution period τ_e, δ ~0.6–2.4, 256 increments), HETCOR (^1H-^{13}C shift correlated spectroscopy; ~50 increments, δ ~0.6–2.6 in f_1), HMBC (heteronuclear multiple bond correlation, 120 increments, δ 10–75 in f_1), and HMQC (heteronuclear multiple-quantum coherence) spectra. An HMQC spectrum of **VIIa** was acquired using the pulse sequence of Bax et al. (20) without ^{13}C decoupling (21): 1.1-s acquisition time, 0.3-s relaxation delay, 0.35-s delay after BIRD pulse, spectral window δ 9–51 in f_1 and δ 0.35–2.15 in f_2, 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 ^{13}C 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 diacetate 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-$AgNO_3$ for MPLC (22, 25) was prepared by adding a solution of $AgNO_3$ (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 injector, 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, tetrabutylammo-

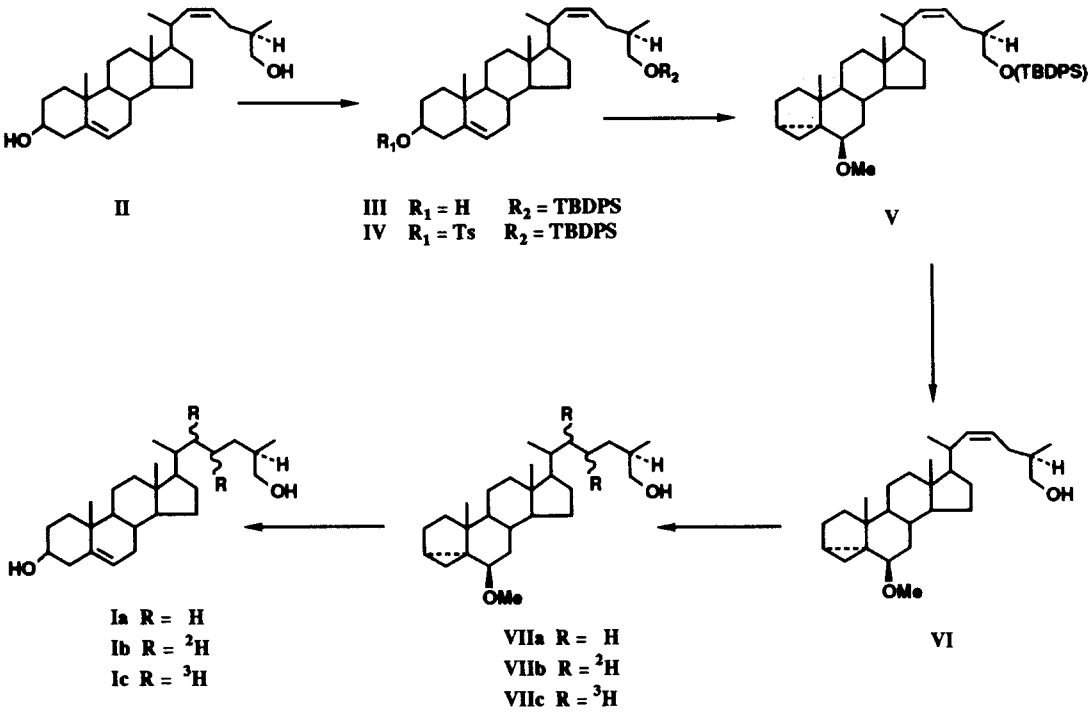

Fig. 1. Synthesis of deuterium- and tritium-labeled (25*R*)-26-hydroxycholesterol from (22Z,25*R*)-cholesta-5,22-diene-3β,26-diol (**II**).

nium fluoride solution, *tert*-butyldiphenylsilyl chloride, bis(trimethylsilyl)trifluoroacetamide, and (S)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetyl (MTPA) chloride (98% ee) were purchased from Aldrich. Radioactivity of sterol solutions was determined on a Packard 4600 liquid scintillation spectrometer using 0.4% 2,5-diphenyloxazole in toluene–ethanol 2:1 as the scintillation fluid. Tritiated sterols **Ic** and **VIIc** were stored in ethyl acetate, hexane, or ethanol solution at −17°C. (22Z,25*R*)-Cholesta-5,22-diene-3β,26-diol (**II**) was prepared as described previously (19) from (22Z,25*R*)-cholesta-5,22-diene-3β,16β,26-triol, a byproduct of the Clemmensen reduction of diosgenin. Chemical structures in the synthesis of **Ia** from **II** are shown in **Fig. 1.**

(22Z,25*R*)-26-*tert*-Butyldiphenylsilyloxy)cholesta-5,22-dien-3β-ol (III)

To a stirred solution of $\Delta^{5,22Z}$-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); $[\alpha]_D^{23}$ −34.1° (*c* 0.6, $CHCl_3$); IR, ν_{max} 3380, 3071, 3048, 1111, 1055, 824, 700 cm^{-1}; high resolution MS, calcd. for $C_{39}H_{53}O_2Si$ (M−C_4H_9), 581.3815, found 581.3826; MS,[3] *m/z* 581 (4, M−C_4H_9), 503 (2, M−C_4H_9−C_6H_6), 381 (1), 365 (3), 309 (31, SC−C_4H_9), 295 (5), 281 (61, pSC−C_4H_9), 255 (14, M−SC−H_2O), 239 (18), 203 (14), 199 (100, Ph_2SiOH); ^1H and ^{13}C NMR, **Table 1** and **Table 2.** Evaporation of fractions 15–18 gave (22Z,25*R*)-3β,26-bis(*tert*-butyldiphenylsilyloxy)cholesta-5,22-diene (0.12 g), identified by its ^1H and ^{13}C NMR spectra.[4] Evaporation of fractions 27–30 gave (22Z,25*R*)-3β-(*tert*-butyldiphenylsilyloxy)cholesta-5,22-dien-26-ol (0.02 g; 96% pure by HPLC, SS-10), which was identified by ^1H and ^{13}C NMR spectra.[4]

[3]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.

[4]The 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 ~3.52 (H-3α), ~5.13 (H-6), 0.98 (H-19); ^{13}C NMR, δ 37.20 (C-1), 31.84 (C-2, C-7, C-8), 73.24 (C-3), 42.48 (C-4), 141.25 (C-5), 121.11 (C-6), 50.07 (C-9), 36.49 (C-10); signals for 3β-TBDPS group (± 0.02 ppm): δ 134.82 (quaternary), 135.75 (ortho), 127.45 (meta), 129.42 (para), 27.01 (methyl), 19.12 (quaternary).

TABLE 1. ^{1}H NMR chemical shifts for synthetic intermediates in the preparation of 26-hydroxycholesterol[a–e]

C-3 Group[f] / C-26 Group[f]	**II** OH OH	**III** OH TBDPS	**IV** OTs TBDPS	**V** 3α,5α-Cyclo TBDPS	**VIII** OMe TBDPS	**VI** 3α,5α-Cyclo OH	**VIIa** 3α,5α-Cyclo OH	**IIa** OH OH
H-1α	1.08	1.08	1.02	1.51	1.04	1.51	1.51	1.08
H-1β	1.85	1.85	1.81	0.86	1.86	0.86	0.86	1.85
H-2α	1.84	1.84	1.81	1.76	1.92	1.76	1.76	1.83
H-2β	1.50	1.50	1.70	1.52	1.43	1.53	1.52	1.50
H-3α	3.52	3.52	4.33	0.89	3.06	0.89	0.89	3.52
H-4α	2.30	2.30	2.27	0.431	2.39	0.432	0.428	2.30
H-4β	2.23	2.23	2.44	0.649	2.16	0.649	0.646	2.23
H-6α	5.35	5.35	5.30	2.77	5.35	2.77	2.77	5.35
H-7α	1.53	1.52	1.48	1.06	1.53	1.06	1.06	1.53
H-7β	1.96	1.97	1.94	1.89	1.97	1.89	1.89	1.97
H-8β	1.46	1.45	1.41	1.72	1.45	1.73	1.73	1.45
H-9α	0.94	0.93	0.88	0.81	0.93	0.82	0.81	0.93
H-11α	1.50†	1.50†	1.46†	1.41	1.49†	1.42	1.40	1.51
H-11β	1.47	1.46	1.42†	1.40	1.46	1.41	1.40	1.46
H-12α	1.19	1.18	1.15	1.15	1.18	1.17	1.13	1.16
H-12β	1.99	1.98	1.96	1.95	1.98	1.97	1.99	2.01
H-14α	1.01†	0.99†	0.96†	1.04†	1.00†	1.04	1.03	0.99
H-15α	1.54	1.52	1.50	1.54	1.52	1.56	1.59	1.58
H-15β	1.04†	1.02†	1.00⅓	1.06††	1.05††	1.11	1.12	1.07
H-16α	1.69	1.66	1.65	1.65	1.65	1.69	1.82	1.82
H-16β	1.16	1.14	1.13	1.14	1.14	1.16	1.26	1.26
H-17α	1.16	1.14	1.13	1.14	1.14	1.16	1.09	1.09
H-18	0.721	0.689	0.665	0.729	0.689	0.756	0.716	0.680
H-19	1.016	1.015	0.970	1.027	1.007	1.028	1.022	1.009
H-20	2.44	2.41	2.40	2.42	2.42	2.44	1.39	1.39
H-21	0.968	0.945	0.932	0.940	0.946	0.962	0.912	0.919
H-22	5.22	5.17	5.15	5.17	5.17	5.22	1.03	1.03
							1.35†	1.35†
H-23	5.20	5.15	5.16	5.15	5.15	5.21	1.24	1.24
							1.34	1.34†
H-24	1.96	1.89	1.88	1.89	1.89	1.97	1.09	1.10
	2.13	2.19	2.18	2.18	2.19	2.12	1.33	1.33
H-25	1.70	1.71	1.70	1.70	1.71	1.70	1.61	1.62
H-26	3.466	3.473	3.468	3.475	3.473	3.468	3.421	3.423
	3.534	3.519	3.512	3.521	3.520	3.535	3.502	3.502
H-27	0.929	0.895	0.889	0.895	0.896	0.929	0.915	0.915
OCH_3				3.328	3.353	3.323	3.322	

[a] Chemical shifts referenced to $Si(CH_3)_4$ signal. Data obtained at 500 MHz in $CDCl_3$ solution at a concentration of 0.02–0.1 M.
[b] Chemical shifts are generally accurate to 0.01 ppm except for values marked by † (± 0.02 ppm) or †† (± 0.05 ppm).
[c] No stereochemical assignments are given for the side-chain protons.
[d] TBDPS ether signals for **III, IV, V,** and **VIII:** δ 7.67 (dd, ortho), 7.41 (m, meta), 7.36 (m, para), 1.060 (s, CH_3). Tosylate signals for **IV:** δ 7.80 (d, 8.3), 7.33 (d, 8.6), 2.44 (s, CH_3).
[e] Selected ^{1}H coupling constants in Hz (average of observed values, deviations generally ≤ 0.2 Hz): H-1α (**V, VI, VIIa**) dd, 13.4, 7.7; H-1β (**V, VI, VIIa**) ddd, 13.4, 11.6, 7.8 (**Ia, VIII**) ddd, 13.3, 3.4, 3.4; H-2α (**V, VI, VIIa**) tdd, 12.1, 7.8, 4.1; H-2β (**V, VI, VIIa**) dd, 12.6, 7.7; H-3α (**Ia, III, VIII**) tt, 11.2, 4.6 (**II**) tdd, 11.1, 5.1, 4.2 (**VI, VIIa**) dt, 8.0, 4.0; H-4α (**Ia, II, III, IV**) ddd, 13.1, 5.1, 2.2 (**V, VI, VIIa**) dd, 8.0, 5.0; H-4β (**V, VI, VIIa**) dd, 5.0, 3.8; H-6 (**Ia, II, III, IV, VIII**) dt, 5.3, 2.0 (**V, VI, VIIa**) t, 2.9; H-7β (**Ia**) dtd, 17.1, 5.0, 2.7 (**VI, VIIa**) dt, 13.5, 3.1; H-12α (**Ia**) td, 12.6, 4.8; H-12β (**Ia**) ddd, 12.9, 4.1, 3.1 (**VI, VIIa**) dt, 12.6, 3.4; H-14α (**Ia**) ddd, 12.6, 10.3, 3.9; H-16α (**VIIa**) dtd, 13.1, 9.5, 5.9; H-20 (**II, V, VI**) tq, ~9, 6.6; H-21 d, 6.6; H-24, upfield signal (**II, VI**) ddd, 14.2, 7.7, 6.3; H-24, downfield signal (**II**) ddd, 14.0, 6.0, 5.0 (**III, IV, V, VI, VIII**) dt, 14.3, 5.2; H-25 (**III**) dqtd, 8.5, 6.7, 6.2, 5.2; H-26, upfield signal (**Ia, II, VI, VIIa**) dd, 10.5, 6.4 (**III, IV, V, VIII**) dd, 9.8, 6.2; H-26, downfield signal (**Ia, VIIa**) dd, 10.5, 5.9 (**II, VI**) dd, 10.5, 6.1 (**III, IV, V, VIII**) dd, 9.8, 6.2; H-27 d, 6.7.
[f] C-3 and C-26 functional groups; TsO, p-$CH_3C_6H_4SO_3$; TBDPS, $tBu(Ph)_2SiO$.

(22Z,25*R*)-3β-Tosyloxy-26-(*tert*-butyldiphenylsilyloxy) cholesta-5,22-diene (IV)

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 $CuSO_4$ solution (to remove pyridine), and additional brine, followed by drying over sodium sulfate and evaporation of the solvent to give **IV** as an oil (2.41 g; 100% yield); > 99% purity by HPLC (SS-10, t_R 10.6 min); $[\alpha]_D^{23}$ −29.5° (*c* 0.7, $CHCl_3$); IR, ν_{max} 3069, 3044, 1597, 1175, 1111, 937, 702 cm^{-1}; high resolution

TABLE 2. ^{13}C NMR chemical shifts for synthetic intermediates in the preparation of 26-hydroxycholesterol[a–c]

C-3 Group[d] / C-26 Group[d]	II OH OH	III OH TBDPS	IV OTs TBDPS	V 3α,5α-Cyclo TBDPS	VIII OMe TBDPS	VI 3α,5α-Cyclo OH	VIIa 3α,5α-Cyclo OH
C-1	37.23	37.22	36.85	33.34	37.16	33.28	33.29
C-2	31.62	31.58	28.59	24.95	27.98	24.89	24.91
C-3	71.77	71.70	82.35	21.45	80.30	21.42	21.43
C-4	42.26	42.23	38.83	13.07	38.66	13.02	13.02
C-5	140.72	140.71	138.81	35.24	140.81	35.17	35.20
C-6	121.67	121.63	123.47	82.39	121.54	82.34	82.37
C-7	31.85	31.85	31.78	35.04	31.89	34.94	34.98
C-8	31.85	31.93	31.69	30.46	31.84	30.40	30.41
C-9	50.13	50.11	49.89	48.08	50.20	48.00	47.94
C-10	36.49	36.46	36.32	43.37	36.87	43.31	43.31
C-11	21.04	21.03	20.93	22.75	21.03	22.70	22.72
C-12	39.68	39.66	39.53	40.21	39.68	40.14	40.22
C-13	42.26	42.18	42.15	42.67	42.20	42.64	42.71
C-14	56.76	56.75	56.63	56.58	56.78	56.49	56.44
C-15	24.24	24.23	24.18	24.14	24.23	24.09	24.11
C-16	28.12	28.09	28.05	28.21	28.10	28.18	28.29
C-17	56.08	56.10	56.06	56.32	56.13	56.21	56.25
C-18	12.17	12.12	12.09	12.53	12.13	12.51	12.21
C-19	19.40	19.40	19.14	19.30	19.37	19.26	19.25
C-20	34.26	34.19	34.17	34.26	34.20	34.29	35.68
C-21	20.60	20.60	20.57	20.60	20.60	20.55	18.60
C-22	138.00	137.52	137.46	137.61	137.53	137.96	36.08
C-23	124.33	124.62	124.67	124.62	124.63	124.31	23.38
C-24	31.38	31.19	31.19	31.22	31.19	31.36	33.48
C-25	36.31	36.38	36.37	36.40	36.39	36.26	35.75
C-26	68.27	68.84	68.84	68.86	68.84	68.16	68.44
C-27	16.52	16.51	16.49	16.51	16.52	16.49	16.47
OCH_3				56.55	55.56	56.49	56.49

[a] Chemical shifts referenced to $CDCl_3$ signal at 77.0 ppm. Data obtained at 75 MHz in $CDCl_3$ solution at a concentration of 0.02–0.2 M.
[b] 26-TBDPS ether signals for **III**, **IV**, **V**, and **VIII** (± 0.02 ppm): δ 134.01 (quaternary), 135.58 (ortho), 127.52 (meta), 129.45 (para), 19.27 (quaternary), 26.85 (methyl).
[c] Signals of 3β-tosylate esters: δ 134.6 (C-SO_3R), 127.59 (ortho), 129.72 (meta), 144.38 (para), 21.61 (para-CH_3); 26-tosylate esters (prepared in the alternate synthetic route) showed: δ 133.02 (C-SO_3R), 127.82 (ortho), 129.73 (meta), 144.55 (para), 21.59 (para-CH_3).
[d] C-3 and C-26 functional groups; TsO, p-$CH_3C_4H_6SO_3$; TBDPS, $tBu(Ph)_2SiO$.

MS, calcd. for $C_{43}H_{60}OSi$ (M-TsOH), 620.4413, found 620.4393; MS, *m/z* 714 (2, M-C_6H_6), 657 (1, M-C_6H_6-C_4H_9), 620 (4, M-TsOH), 563 (17, M-TsOH-C_4H_9), 485 (5, M-TsOH-C_6H_6-C_4H_9), 364 (4, M-TsOH-$C_4H_9Ph_2SiOH$), 353 (32), 309 (39, SC-C_4H_9), 281 (73, pSC-C_4H_9), 255 (9, M-TsOH-SC), 253 (12, M-TsOH-SC-2), 239 (19), 199 (100, Ph_2SiOH), 109 (41), 107 (45), 105 (70); ^1H and ^{13}C NMR, Tables 1 and 2.

(22Z,25*R*)-6β-Methoxy-26-(*tert*-butyldiphenylsilyloxy)-3α,5-cyclo-5α-cholest-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; 25-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,25*R*)-3β-methoxy-26-(*tert*-butyldiphenylsilyloxy)cholesta-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° (*c* 0.9, $CHCl_3$); IR ν_{max} 3071, 3054, 1150, 1111, 1017, 824, 810 cm^{-1}; high resolution MS, calcd. for $C_{40}H_{55}O_2Si$ (M-C_4H_9), 595.3971, found 595.3958; MS, *m/z* 595 (1, M-C_4H_9), 563 (17, M-CH_3OH-C_4H_9), 485 (11), 309 (22, SC-C_4H_9), 281 (56, pSC-C_4H_9), 255 (7, M-CH_3OH-SC), 253 (9, M-CH_3OH-SC-2), 239 (12), 199 (100, Ph_2SiOH), 109 (70), 107 (40), 105 (58); ^1H and ^{13}C NMR, Tables 1 and 2.

(22Z,25*R*)-6β-Methoxy-3α,5-cyclo-5α-cholest-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, R_f 0.42) and ~99% purity by ^{1}H NMR; $[\alpha]_D^{23}$ +13.8° (*c* 1.4, $CHCl_3$); IR, ν_{max} 3400, 1098, 1040 cm^{-1}; high resolution MS, calcd. for $C_{28}H_{46}O_2$, 414.3498, found 414.3506; MS, *m/z* 414 (18, M^+), 399 (39, M–CH_3), 382 (39, M–CH_3OH), 367 (9, M–CH_3OH–CH_3), 359 (62, M–C_4H_7), 356 (9), 287 (3, M–SC), 282 (5), 255 (28, M–CH_3OH–SC), 253 (11, M–CH_3OH–SC–2), 109 (100), 107 (45), 105 (49); ^{1}H and ^{13}C NMR, Tables 1 and 2.

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

To a solution of the Δ^{22Z}-i-steroid **VI** (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 $AgNO_3$-silica gel; elution with SS-5; 10-ml fraction volumes). Evaporation of fractions 7–10 gave an analytical sample of **VIIa** (139 mg). The material in fractions 11–22, which consisted of a mixture of **VI** and **VIIa** (46 mg), was subjected to further hydrogenation to afford additional **VIIa** (46 mg; total yield 87%). Characterization of **VIIa:** mp 95–96.5°C; $[\alpha]_D^{23}$ +56.9° (*c* 1.1, $CHCl_3$); single component by TLC (SS-7, developed twice, R_f 0.58); IR ν_{max} 3470, 3079, 3043, 1086, 1053, 1022 cm^{-1}; high resolution MS, calcd. for $C_{28}H_{48}O_2$, 416.3654, found 416.3663; MS, *m/z* 416 (32, M^+), 401 (65, M–CH_3), 384 (53, M–CH_3OH), 369 (15, M–CH_3OH–CH_3), 361 (100, M–C_4H_7), 358 (14), 329 (8), 263 (8), 255 (13, M–SC–CH_3OH), 107 (70), 105 (89); ^{1}H and ^{13}C NMR, Tables 1 and 2. The 125 MHz ^{13}C NMR spectrum showed additional signals of 1–2% intensity at δ 68.28, 33.60, and 16.67 (shieldings adjusted to correspond to 22°C spectra).

(25*R*)-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** (11 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. (6) 177–178°C); $[\alpha]_D^{23}$ −38.6° (*c* 0.6, $CHCl_3$); lit (26) $[\alpha]_D^{23}$ −40.6°; single component matching an authentic standard by TLC (SS-8, R_f 0.45) and HPLC (SS-9, t_R 4.9 min); the NMR chemical shifts matched reported ones (6) to within ± 0.04 ppm (^{13}C) and ± 0.001 ppm (resolved ^{1}H signals).

[^{2}H]-(25*R*)-6β-Methoxy-3α,5-cyclo-5α-cholestan-26-ol (VIIb)

The Δ^{22} i-steroid **VI** (50 mg; 0.12 mmol) was evaporated twice from CH_3OD and then dissolved in ethyl acetate (5 ml) and CH_3COOD (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 $AgNO_3$-silica gel; 50 cm × 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 d_0 (0%), d_1 (8%), d_2 (31%), d_3 (30%), d_4 (19%), d_5 (8%), d_6 (4%), and small amounts of d_7, d_8, and d_9 species (an average of 3.0 deuterium atoms per molecule). A virtually identical distribution of deuterium was observed for ions corresponding to M–CH_3 and M–CH_3OH, whereas the ion corresponding to M–SC–CH_3OH showed no detectable deuterium. The ^{13}C 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 ^{2}H 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-23, D-24), 1.60 (5%, D-25), 3.43 and 3.50 (1%, D-26). In the ^{1}H 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.

[^{2}H]-(25*R*)-Cholest-5-ene-3β,26-diol (Ib)

To a solution of i-steroid **VIIb** (75 mg) in dioxane (32 ml) was added water (7.5 ml) and *p*-toluenesulfonic acid (75 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 (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). 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 δ ~1.34 and an additional doublet-doublet 0.003 ppm upfield of the H-26 resonance at δ 3.50 (possibly due to deuterium isotope effects). The 125-MHz ^{13}C NMR spectrum of **Ib** in $CDCl_3$ containing 5% CD_3OD resembled that of **Ia** ex-

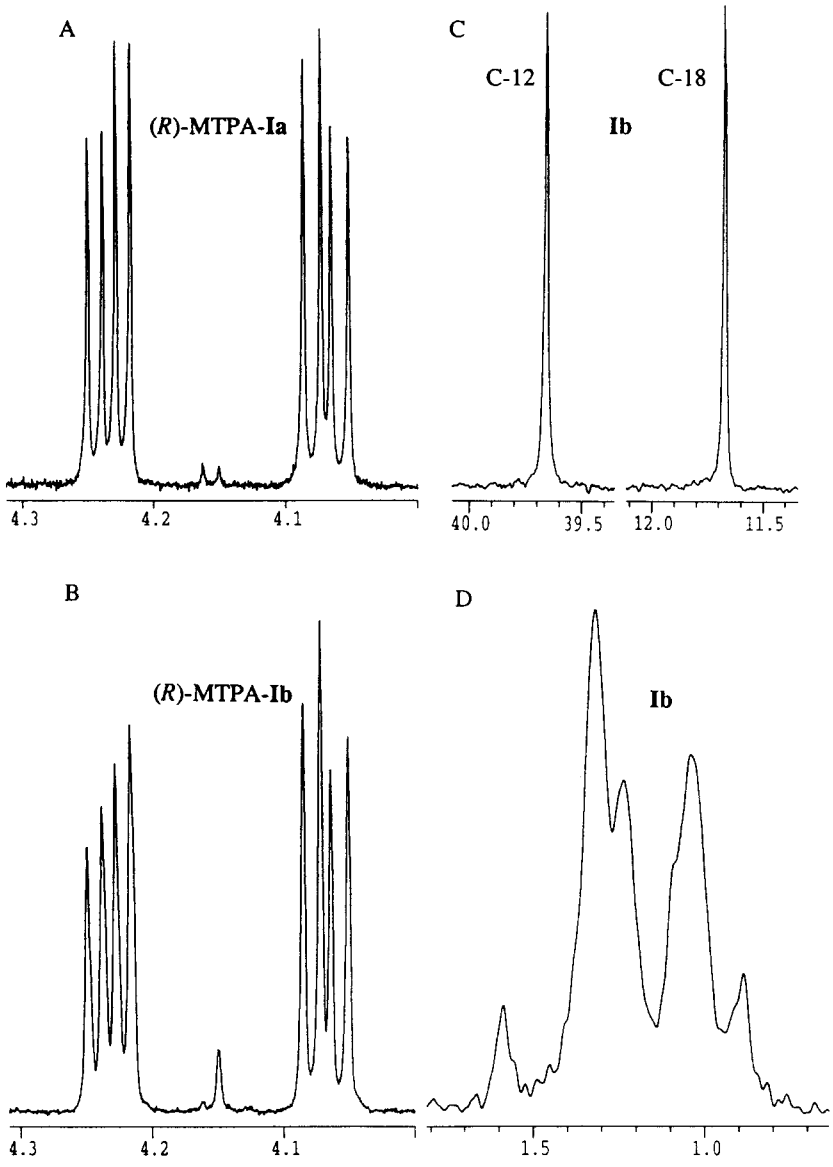

Fig. 2. ^{1}H NMR spectrum of the (*R*)-MTPA diesters of (25*R*)-26-hydroxycholesterol **Ia** (panel A) and the side-chain deuterated analog **Ib** (panel B). ^{13}C NMR signals for C-12 and C-18 of **Ib** (panel C). ^{2}H NMR spectrum of **Ib** (panel D). ^{1}H NMR spectra: 500 MHz, no apodization;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

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 ^2H NMR spectrum (**Fig. 2,** panel D) showed the following broad signals (relative intensities estimated by integration): δ 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).

[^{3}H]-(25*R*)-Cholest-5-ene-3β,26-diol (Ic)

A solution of the Δ^{22Z} i-steroid **VI** (40 mg) in ethyl acetate containing acetic acid and platinum oxide was exposed to tritium gas until an activity of 1.5 Ci was obtained. After removal of the catalyst and labile tritium,[5] 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 to give **Ic** (140 mCi). As shown in **Fig. 3,** the product showed high purity by radio-TLC on silica gel (SS-8, R_f 0.50) and by radio-HPLC (SS-9, t_R 10.0 min). In both cases, **Ic** comigrated with unlabeled (25R)-26-hydroxycholesterol (**Ia**). A specific activity of ~16.9 Ci per mmol was obtained using a colorimetric assay (27) with calibration curves constructed for authentic 26-hydroxycholesterol.

[5]The catalytic reduction in the presence of tritium gas was done by Amersham International (Buckinghamshire, England).

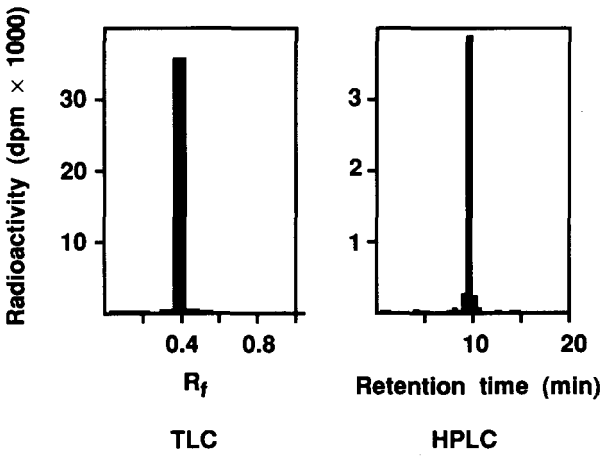

Fig. 3. Chromatographic analysis of ^{3}H-(25*R*)-26-hydroxycholesterol (**Ic**) by radio-TLC on silica gel (elution with 50% ethyl acetate in hexane) and reversed phase radio-HPLC (elution with 5% water in methanol, 0.5-min fractions).

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): δ 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 δ 4.15 and 4.16 for the 25S epimer (see Fig. 2, panel A). The MTPA esters of the deuterated sterols **Ib** and **VIIb** showed the same doublet-doublets at δ 4.233 and 4.069 for the 25R isomer and weak peaks of unequal intensity at δ 4.15 and 4.16 (Fig. 2). Other signals for the esters of **Ia** and **Ib** were virtually identical to those reported previously (26). The ^{19}F NMR spectra of the MTPA diesters of **Ia** and **Ib** showed singlets at δ −71.94 and δ −71.99, and that of the MTPA ester of **VIIb** showed one singlet at δ −72.05; no other signals were observed except ^{13}C satellites (J_{CF} 288.4 Hz) and minor singlets at δ 67.91 and 67.95 (1% of main peaks, in all spectra).

Attempted preparation of 26-hydroxycholesterol via the 3β,26-ditosylate of II

The 3β,26-ditosylate of **II** (prepared by reaction of **II** with *p*-toluenesulfonyl chloride-pyridine overnight at room

temperature) was converted to 6β-methoxy-26-tosyloxy-3α,5-cyclo-5α-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 H_2, PtO_2, 23°C) to give 6β-methoxy-26-tosyloxy-3α,5-cyclo-5α-cholestane (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°C overnight to give ~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, Emerman, and Javitt (28) reported the preparation of ^{3}H-labeled 26-hydroxycholesterol (13.3 μCi per μmol) by Clemmensen 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 D_2O and 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_3CH_2OD, D_2O, and DCl gave, after column chromatography on alumina, deuterium-labeled 26-hydroxycholesterol; this material was further purified by conversion to the 3β,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 five to nine deuterium atoms but provided no information with respect to isotopic localization. ^{1}H NMR analysis 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 [15,15,16,16,17α-$^2H_5$]-26-hydroxycholesterol "according to previously 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-hydroxycholesterol by Clemmensen reduction of kryptogenin in the presence of CH_3CH_2OD, D_2O, and DCl. The procedure described involved purification of the crude product by column chromatography on silicic acid followed by recrystallization of the material corresponding to 26-hydroxycholesterol. The labeled product contained molecules with five to ten atoms of deuterium with the major species containing seven atoms of deuterium. Further characterization 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 3β,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% ND_2ND_2 and KOD. The crude product was acetylated and the resulting material was subjected to chromatography on an alumina column to give 26-hydroxycholesterol 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 diacetate 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 3β-tetrahydropyranyl ether of cholesta-5,25-diene-3β-ol. Using this approach, preparation of (25*RS*)-[25-^{3}H]-3β-tetrahydropyranyloxycholest-5-ene-26-ol (5.97 μCi per μmol) was described.

It should be noted that Kluge, Maddox, and Partridge (36) reported that both Clemmensen reduction of kryptogenin 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 (25*R*)-26-hydroxycholesterol or very extensive characterization of the final 26-hydroxycholesterol.

(22Z,25*R*)-Cholesta-5,22-diene-3β,26-diol (**II**) can be prepared in gram quantities from (22Z,25*R*)-cholesta-5,22-diene-3β,16β,26-triol, a byproduct of the Clemmensen reduction of diosgenin (19). The availability of **II** provided a useful starting material for the preparation of (25*R*)-26-hydroxycholesterol labeled with deuterium or tritium. Selective reduction of the Δ^{22Z} bond of **II** required prior protection of the Δ^5 bond. An effective protecting group for this purpose is the i-steroid, which can be prepared from the Δ^5-3β-tosylate and subsequently hydrolyzed to regenerate the Δ^5-3β-hydroxy system by heating with *p*-toluenesulfonic acid. We initially pursued a synthetic scheme starting with the ditosylate of **II** followed by its conversion to the i-steroid, (22Z,25*R*)-6β-methoxy-26-tosyloxy-3α,5-cyclo-5α-cholest-22-ene.[6] After catalytic hydrogenation (platinum oxide) of the Δ^{22Z} double bond, acid hydrolysis of the i-steroid gave the 26-tosyl derivative of **Ia.** However, difficulties were encountered in the efficient conversion of the 26-tosyl function to the hydroxyl group. To circumvent this problem, the 26-hydroxyl of **II** was first protected as the *tert*-butyldiphenylsilyl (TBDPS) ether **III** under conditions that gave only minor amounts of the 3β-TBDPS and 3β,26-bis-TBDPS ethers. Preparation of the 3β-tosylate **IV** followed by i-steroid formation[6] and removal of the 26-TBDPS group afforded (22Z,25*R*)-6β-methoxy-3α,5-cyclo-5α-cholest-22-en-26-ol (**VI**), which provided the substrate for catalytic reduction with hydrogen, deuterium, or tritium. Catalytic hydrogenation of **VI** over platinum oxide[7] in ethyl acetate gave the saturated i-steroid (**VIIa**), which was hydrolyzed with *p*-toluenesulfonic acid in refluxing dioxane to (25*R*)-26-hydroxycholesterol. The product **Ia** showed spectral and physical properties corresponding to those reported previously (6). This six-step synthesis of (25*R*)-26-hydroxycholesterol from **II** proceeded in an overall yield of 31%.

Syntheses were next carried out to prepare deuterium-labeled (25*R*)-26-hydroxycholesterol. Catalytic reduction of **VI** over platinum oxide with deuterium gas was much slower than the corresponding reduction with hydrogen and led to incorporation of less than two deuterium atoms per molecule. Addition of acetic acid to the ethyl acetate solution led to a much faster reaction with the incorporation of approximately three deuterium atoms per molecule in the product **VIIb.** The NMR and mass spectra of **VIIb** indicated that all of the deuterium was located in the side chain. The ^{2}H and ^{13}C NMR spectra showed 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. Hydrolysis of the i-steroid **VIIb** gave deuterium-labeled (25*R*)-26-hydroxycholesterol (**Ib**) in 85% yield. Thorough analysis of **Ib** by MS and ^{1}H, ^{2}H, and ^{13}C NMR indicated the same pattern of deuterium incorporation as in **VIIb.**

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 ^{13}C NMR (125 MHz) and by ^{1}H NMR of the MTPA ester (26). Portions of these spectra are shown in Fig. 2. Based on ^{13}C 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 region of the C-12 and C-18 resonances. Examination of the ^{13}C spectra demonstrated the absence of any 20S epimer in either **VIIa** (detection limit 1%) or **Ib** (detection limit 2%). Minor signals in the ^{13}C 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.

In the deuterated sterols **Ib** and **VIIb,** the complexity of the side-chain ^{13}C 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 ^{1}H NMR for the 25S epimer using methodology described previously for 26-hydroxysterols (26). In the resulting ^{1}H NMR spectrum, the H-26 resonance corresponding to one diastereomer (R-MTPA, 25R-sterol and S-MTPA, 25S-sterol) is a pair of well-separated doublet-doublets, between which is the resonance of the nearly isochronous H-26 protons of the other diastereomer (Fig. 2, panel A). Comparison of the integrals of the center and outer resonances of the (R)-MTPA diester of **Ia** indicated 1% of the minor diastereomer. Both the 25S-sterol and the enantiomeric impurity present in the MTPA chloride reagent (stated to be 1%)

[6]Preparation of the i-steroids was accompanied by formation of a significant byproduct that was identified as the Δ^5-3β-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).

[7]Attempts to reduce **VI** with the homogeneous catalyst tris(triphenylphosphine)chlororhodium were unsuccessful; the unreactivity of the Δ^{22E} bond of ergosterol under similar conditions has also been noted (39). Platinum oxide was chosen as the heterogeneous catalyst least prone to promote isotopic scrambling (39, 40).

contribute to this 1% signal. Integration of the outer and inner resonances of the deuterated sterols **Ib** and **VIIb** indicated a ~98:2 ratio of 25R and 25S epimers. The inner resonance consisted of a pair of peaks of unequal intensity corresponding to the H-26 resonance of the minor diastereomer (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 and containing ~1% of the 25S epimer. The deuterated 26-hydroxycholesterol, prepared by reduction of the Δ^{22Z} double bond in the presence of acetic acid, contained no detectable 20S epimer and ~2% of the 25S epimer.

In light of the spectral analyses 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 Clemmensen 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 for any synthesis of tritium-labeled (25R)-26-hydroxycholesterol.

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 Δ^{22} sterols; this ion appears to correspond to the sterol side chain after loss of the C-26 substituent. The Δ^{22Z}-26-TBDPS ethers gave strong ions at *m/z* 281 and *m/z* 199 (Ph_2SiOH), as well as ions from loss of C_4H_9 together with other fragments. The 26-hydroxy-i-steroids **VI** and **VIIa** showed prominent ions for M–CH_3, M–CH_3OH, and M–C_4H_7, but such ions were weak or absent in the 26-TBDPS-i-steroid **V,** whose mass spectrum was dominated by ions characteristic of TBDPS ethers. The MS fragmentation of the i-steroid 6β-methoxy-3α,5-cyclo-5α-cholestane has been thoroughly investigated, and mechanisms have been elucidated for the formation of ions M–CH_3OH and M–C_4H_7 (42).

Full ^{1}H and ^{13}C 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 ^{13}C assignments in Table 2 with those reported for other i-steroids show apparent agreement (43, 44) or minor differences (45).

Analysis of the ^{1}H 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 Δ^5-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 COSYDEC 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 $J_{H1\alpha\text{-}H2\beta}$ 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.[8] Representative ^{1}H 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 $J_{1\alpha\text{-}2\alpha}$, $J_{1\beta\text{-}2\alpha}$, and $J_{1\beta\text{-}2\beta}$

[8]The analysis of the overlapped ring A signals was subsequently confirmed by a ^{1}H-^{13}C correlation spectrum (HMQC) of **VIIa,** which showed the isolated coupling pattern for each ^{1}H signal with good digital resolution. However, in our hands, HMQC spectra show significant distortion from antiphase components and furnish scalar couplings slightly higher than those measured from 1D spectra.

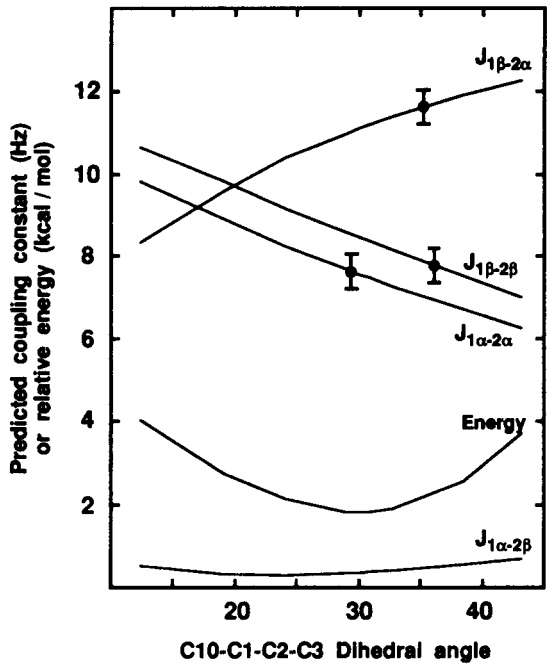

Fig. 4. Calculated energy and predicted coupling constants between C-1 and C-2 protons of 6β-methoxy-i-steroids as a function of the C10-C1-C2-C3 dihedral angle. The three data points (shown with estimated error ranges) are located at the intersection of the curves with the corresponding observed coupling constant.

values to the predicted couplings corresponds to a C10-C1-C2-C3 dihedral angle of ~33°, 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 6β-methoxy-i-steroids were determined from an HMQC spectrum of **VIIa** 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

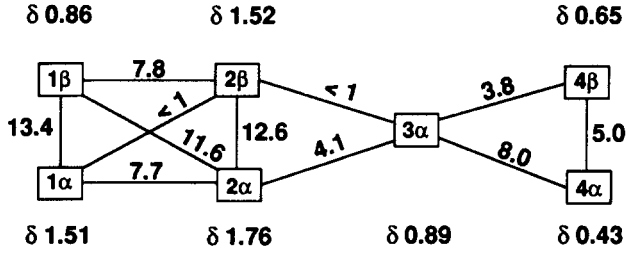

Fig. 5. Representative ^{1}H NMR chemical shifts (δ) and ^{1}H-^{1}H coupling constants in Hz for ring A of i-steroids.

of i-steroids (H-11α, H-11β) and Δ^{22Z} sterols (H-16β, H-17α and H-22, H-23) thwarted further conformational analysis based on ^{1}H NMR couplings.

In summary, we have presented a chemical synthesis of deuterium- and tritium-labeled (25*R*)-26-hydroxycholesterol from a byproduct of the Clemmensen reduction of diosgenin. Synthetic (25*R*)-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 (25*R*)-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 ~1% of a C-20 or C-25 epimeric impurity in (25*R*)-26-hydroxycholesterol. We have also demonstrated the use of ^{2}H NMR and ^{13}C NMR to locate the carbon atoms at which deuterium label is present in (25*R*)-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 ^{3}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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REFERENCES

1. Kandutsch, A. A., H. W. Chen, and H-J. Heiniger. 1978. Biological activity of some oxygenated sterols. *Science.* **201:** 498–501.
2. Schroepfer, G. J., Jr. 1981. Sterol biosynthesis. *Annu. Rev. Biochem.* **50:** 585–621.
3. Esterman, A. L., H. Baum, N. B. Javitt, and G. J. Darlington. 1983. 26-Hydroxycholesterol: regulation of hydroxymethyl glutaryl-CoA reductase activity in Chinese hamster ovary cell culture. *J. Lipid Res.* **24:** 1304–1309.
4. Taylor, F. R., S. E. Saucier, E. P. Shown, E. J. Parish, and A. A. Kandutsch. 1984. Correlation between oxysterol binding to a cytosolic binding protein and potency in the repression of hydroxymethylglutaryl coenzyme A reductase. *J. Biol. Chem.* **259:** 12382–12387.

5. Pinkerton, F. D., R. P. Pelley, and G. J. Schroepfer, Jr. 1992. Synergistic action of two oxysterols in the lowering of HMG-CoA reductase activity in CHO-K1 cells. *Biochem. Biophys. Res. Commun.* **186:** 569–573.
6. Kim, H-S., W. K. Wilson, D. H. Needleman, F. D. Pinkerton, D. K. Wilson, F. A. Quiocho, and G. J. Schroepfer, Jr. 1989. Inhibitors of sterol synthesis. Chemical synthesis, structure, and biological activities of (25*R*)-3β,26-dihydroxy-5α-cholest-8(14)-en-15-one, a metabolite of 3β-hydroxy-5α-cholest-8(14)-en-15-one. *J. Lipid Res.* **30:** 247–261.
7. Siddiqui, A. U., W. K. Wilson, K. E. Ruecker, F. D. Pinkerton, and G. J. Schroepfer, Jr. 1992. Chemical synthesis and spectral properties of 26-oxygenated derivatives of 3β-hydroxy-5α-cholest-8(14)-en-15-one, and their effects on 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in CHO-K1 cells. *Chem. Phys. Lipids.* **63:** 77–90.
8. Swaminathan, S., F. D. Pinkerton, W. K. Wilson, and G. J. Schroepfer, Jr. 1992. Inhibitors of sterol synthesis. Chemical synthesis and spectral properties of (25R)-5α-cholest-8(14)-ene-3β,15β,26-triol, a potential metabolite of 3β-hydroxy-5α-cholest-8(14)-en-15-one and its effects on 3-hydroxy-3-methylglutaryl-coenzyme A reductase in CHO-K1 cells. *Chem. Phys. Lipids.* **61:** 235–242.
9. Björkhem, I. 1985. Mechanism of bile acid biosynthesis in mammalian liver. *In* Sterols and Bile Acids. H. Danielsson and J. Sjövall, editors. Elsevier, Amsterdam. 231–278.
10. Ayaki, Y., E. Kok, and N. B. Javitt. 1989. Cholic acid synthesis from 26-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid in the rabbit. *J. Biol. Chem.* **264:** 3818–3821.
11. Axelson, M., and J. Sjövall. 1990. Potential bile acid precursors in plasma—possible indicators of biosynthetic pathways to cholic and chenodeoxycholic acid in man. *J. Steroid Biochem.* **36:** 631–640.
12. Schroepfer, G. J., Jr., H-S. Kim, J. L. Vermilion, T. W. Stephens, F. D. Pinkerton, D. H. Needleman, W. K., Wilson, and J. S. Pyrek. 1988. Enzymatic formation and chemical synthesis of an active metabolite of 3β-hydroxy-5α-cholest-8(14)-en-15-one, a potent regulator of cholesterol metabolism. *Biochem. Biophys. Res. Commun.* **151:** 130–136.
13. Pyrek, J. S., J. L. Vermilion, T. W. Stephens, W. K. Wilson, and G. J. Schroepfer, Jr. 1989. Inhibitors of sterol synthesis. Characterization of side chain oxygenated derivatives formed upon incubation of 3β-hydroxy-5α-cholest-8(14)-en-15-one with rat liver mitochondria. *J. Biol. Chem.* **264:** 4536–4543.
14. Pyrek, J. S., W. K. Wilson, S. Numazawa, and G. J. Schroepfer, Jr. 1991. Inhibitors of sterol synthesis. Characterization of trimethylsilyl dienol ethers of 3β-hydroxy-5α-cholest-8(14)-en-15-one. Applications in the analysis of mitochondrial metabolites of the 15-ketosterol by gas chromatography–mass spectrometry. *J. Lipid Res.* **32:** 1371–1380.
15. Wikvall, K. 1984. Hydroxylations in biosynthesis of bile acids. Isolation of a cytochrome P-450 from rabbit liver mitochondria catalyzing 26-hydroxylation of C_{27} steroids. *J. Biol. Chem.* **25:** 3800–3804.
16. Andersson, S., D. L. Davis, H. Dahlbäck, H. Jörnvall, and D. W. Russell. 1989. Cloning, structure, and expression of the mitochrondrial cytochrome P-450 sterol 26-hydroxylase, a bile acid biosynthetic enzyme. *J. Biol. Chem.* **264:** 8222–8229.
17. Kudo, K., G. T. Emmons, E. W. Casserley, D. P. Via, L. C. Smith, J. S. Pyrek, and G. J. Schroepfer, Jr. 1989. Inhibitors of sterol synthesis. Chromatography of acetate derivatives of oxygenated sterols. *J. Lipid Res.* **30:** 1097–1111.
18. Princen, H. M. G., P. Meijer, B. G. Wolthers, R. J. Vonk, and F. Kuipers. 1991. Cyclosporin a blocks bile acid synthesis in cultured hepatocytes by specific inhibition of chenodeoxycholic acid synthesis. *Biochem. J.* **275:** 501–505.
19. Ni, Y., H-S. Kim, W. K. Wilson, A. Kisic, and G. J. Schroepfer, Jr. 1993. A revisitation of the Clemmensen reduction of diosgenin. Characterization of byproducts and their use in the preparation of (25R)-26-hydroxysterols. *Tetrahedron Lett.* **34:** 3687–3690.
20. Bax, A., and S. Subramanian. 1986. Sensitivity-enhanced two-dimensional heteronuclear shift correlation NMR spectroscopy. *J. Magn. Reson.* **67:** 565–569.
21. Yang, D., X. Xu, and C. Ye. 1992. Application of HMQC to the measurement of J(H,H) homonuclear coupling constants. *Magn. Reson. Chem.* **30:** 711–715.
22. Siddiqui, A. U., W. K. Wilson, S. Swaminathan, and G. J. Schroepfer, Jr. 1992. Efficient preparation of steroidal 5,7-dienes of high purity. *Chem. Phys. Lipids.* **63:** 115–129.
23. Kirk, D. N., H. C. Toms, C. Douglas, K. A. White, K. E. Smith, S. Latif, and R. W. P. Hubbard. 1990. A survey of the high-field ^{1}H NMR spectra of steroid hormones, their hydroxylated derivatives, and related compounds. *J. Chem. Soc., Perkin. Trans. 2:* 1567–1594.
24. Paliokas, A. M., and G. J. Schroepfer, Jr. 1968. Stereospecificity in the enzymatic conversion of Δ^7-cholesten-3β-ol to 7-dehydrocholesterol. *J. Biol. Chem.* **243:** 453–464.
25. Pascal, R. A., Jr., C. L. Farris, and G. J. Schroepfer, Jr. 1977. Sterol synthesis. Medium pressure chromatography of C_{27} sterol precursors of cholesterol on alumina silver nitrate columns. *Anal. Biochem.* **101:** 15–22.
26. Uomori, A., S. Seo, T. Sato, Y. Yoshimura, and K. Takeda. 1987. Synthesis of (25R)-[26-$^2H_1$] cholesterol and ^{1}H n.m.r. and H. p.l.c. resolution of (25R) and (25S)-26-hydroxycholesterol. *J. Chem. Soc., Perkin Trans. 1:* 713.
27. Abell, L. L., B. B. Levy, B. B. Brodie, and F. F. Kendall. 1952. Simplified method for the estimation of total cholesterol in serum and demonstration of its specificity. *J. Biol. Chem.* **195:** 357–366.
28. Wachtel, N., S. Emerman, and N. B. Javitt. 1968. Metabolism of cholest-5-ene-3β,26-diol in the rat and hamster. *J. Biol. Chem.* **243:** 5207–5212.
29. Javitt, N. B., E. Kok, S. Burstein, B. Cohen, and J. Kutscher. 1981. 26-Hydroxycholesterol. Identification and quantitation in human serum. *J. Biol. Chem.* **256:** 12644–12646.
30. Javitt, N. B., E. Kok, J. Lloyd, A. Benscath, and F. H. Field. 1982. Cholest-5-ene-3β,26-diol: synthesis and biomedical use of a deuterated compound. *Biomed. Mass Spectrom.* **9:** 61–63.
31. Breuer, O., and I. Björkhem. 1990. Simultaneous quantification of several cholesterol autoxidation products by isotope-dilution mass spectrometry. *Steroids.* **55:** 185–192.
32. Javitt, N. B., E. Kok, B. Cohen, and S. Burstein. 1982. Cerebrotendinous xanthomatosis: reduced serum 26-hydroxycholesterol. *J. Lipid Res.* **23:** 627–630.
33. Shoda, J., M. Axelson, and J. Sjövall. 1993. Synthesis of potential C_{27}-intermediates in bile acid biosynthesis and their deuterium-labeled analogs. *Steroids.* **58:** 119–125.
34. Kok, E., and N. B. Javitt. 1990. Cholesterol 26-hydroxylase activity of hamster liver mitochondria: isotope ratio analysis using deuterated 26-hydroxycholesterol. *J. Lipid Res.* **31:** 735–738.
35. Varma, K. R., J. A. F. Wickramasinghe, and E. Caspi. 1969. Biosynthesis of plant sterols. The mode of oxygenation at carbon atom 26 in the formation of sapogenins from

cholesterol. *J. Biol. Chem.* **244:** 3951–3957.
36. Kluge, A. F., M. L. Maddox, and L. G. Partridge. 1985. Synthesis of (20R,25R)-cholest-5-ene-3β,26-diol and the occurrence of base-catalyzed 1,5-hydride shift in a steroidal 1,5-ketol. *J. Org. Chem.* **50:** 2359–2365.
37. Seo, S., A. Uomori, and K. Takeda. 1986. Direct observation of the reverse 1,5-hydride shift: the mechanism of acid-catalyzed isomerization at C-25 of spirostanols. *J. Org. Chem.* **51:** 3823–3827.
38. Steele, J. A., and E. Mosettig. 1963. The solvolysis of stigmasteryl tosylate. *J. Org. Chem.* **28:** 571–572.
39. Tökés, L., and L. J. Throop. 1972. Introduction of deuterium into the steroid system. *In* Organic Reactions in Steroid Chemistry. Vol. 1. J. Fried and J. A. Edwards, editors. Van Nostrand Reinhold Co., New York. 178–221.
40. Rylander, P. N. 1985. Hydrogenation Methods. Academic Press, New York. 29–36.
41. Batta, A. K., T. H. Williams, G. Salen, D. N. Greeley, and S. Shefer. 1980. Differentiation between the 25R- and 25S-isomers of 5β-cholestane-3α,7α,26-triol by ^{13}C NMR spectroscopy. *J. Lipid Res.* **21:** 130–135.
42. Brown, F. J., I. J. Massey, and C. Djerassi. 1980. The mass spectrometric behavior of 3α,5-cyclo-5α-cholestan-6β-yl alkyl ethers. *Can. J. Chem.* **58:** 2592–2599.
43. Smith, W. B., D. L. Deavenport, J. A. Swanzy, and G. A. Pate. 1973. Steroid ^{13}C chemical shifts. Assignments via shift reagents. *J. Magn. Reson.* **12:** 15–19.
44. D'Auria, M. V., F. De Riccardis, L. Minale, and R. Riccio. 1990. Synthesis of 24-methyl-26-hydroxysteroid side-chains: models for stereochemical assignments in poly-hydroxylated marine steroids. *J. Chem. Soc., Perkin Trans. 1:* 2889–2893.
45. González Sierra, M., D. A. Bustos, M. E. Zudenigo, and E. A. Rúveda. 1986. Configurational assignment of epimeric 22,23-epoxides of steroids by carbon-13 NMR spectroscopy. *Tetrahedron.* **42:** 755–758.
46. Schneider, H-J., U. Buchheit, N. Becker, G. Schmidt, and U. Siehl. 1985. ^{1}H NMR analyses, shielding mechanisms, coupling constants, and conformations in steroids bearing halogen, hydroxy, oxo groups, and double bonds. *J. Am. Chem. Soc.* **107:** 7027–7039.
47. Horibe, I., H. Nakai, T. Sato, S. Seo, K. Takeda, and S. Takatsuto. 1989. Stereoselective synthesis of the C-24 and C-25 stereoisomeric pairs of 24-ethyl-26-hydroxy- and 24-ethyl-[26-^{2}H]sterols and their Δ^{22} derivatives: reassignment of ^{13}C N.m.r. signals of the *pro-R* and *pro-S* methyl groups at C-25 of 24-ethylsterols. *J. Chem. Soc., Perkin Trans. 1:* 1957–1967.