Inhibitors of sterol synthesis. Characterization of β,γ-unsaturated analogs 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

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Abstract Treatment of 3β-hydroxy-5α-cholest-8(14)-en-15-one (**1**), a potent regulator of cholesterol metabolism, with perchloric acid in methanol resulted in its partial isomerization to the β,γ-unsaturated 15-ketosterols, 3β-hydroxy-5α,14β-cholest-8-en-15-one (**2**) and 3β-hydroxy-5α,14β-cholest-7-en-15-one (**3**), which were easily separated from **1** by chromatography. Isomers **1**, **2**, and **3** could be distinguished by their chromatographic retention times as well as by their physical and spectral properties. Reduction of **2** with sodium borohydride gave 5α,14β-cholest-8-ene-3β,15β-diol (**4**), for which the C-15 configuration was established from the lanthanide-induced shifts of its 3β-*tert*-butyldimethylsilyl ether. ^{1}H and ^{13}C NMR chemical shift differences between **2**, **3**, and **4** indicated the involvement of variable populations of conformers that differ in the flexible C–D ring system and in the side chain. Compounds **2**, **3**, and **4** lowered the levels of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in CHO-K1 cells. —**Wilson, W. K., M. E. Wheeler, F. D. Pinkerton, J. St. Pyrek, and G. J. Schroepfer, Jr.** Inhibitors of sterol synthesis. Characterization of β,γ-unsaturated analogs 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. *J. Lipid Res.* 1991. **32:** 1215–1227.

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3β-Hydroxy-5α-cholest-8(14)-en-15-one (**1**; **Fig. 1**) is a potent regulator of the levels of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase in cultured mammalian cells (1–4). As part of our investigations of the chemistry, metabolism, and actions of **1**, we have pursued the preparation of two of its isomers, i.e., 3β-hydroxy-5α,14β-cholest-8-en-15-one (**2**) and 3β-hydroxy-5α,14β-cholest-7-en-15-one (**3**). We now report the isolation and identification of these sterols, along with their chromatographic and spectral properties and their effects on the levels of HMG-CoA reductase activity in Chinese hamster ovary (CHO-K1) cells. In addition, 5α,14β-cholest-8-ene-3β,15β-diol (**4**), prepared by reduction of **2** with sodium borohydride, was similarly studied.

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 (~22°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. Gas chromatography–mass spectrometry (GC–MS) was carried out with direct introduction of the helium carrier gas into the ion source of the mass spectrometer (EXTREL ELQ-400, quadrupole, electron impact, 20 eV). The following GC conditions

Abbreviations: BSTFA, N,O-bis(trimethylsilyl) trifluoroacetamide; CHO, chinese hamster ovary; COSY, ^{1}H-^{1}H shift-correlated spectroscopy; DEPT, distortionless enhancement by polarization transfer; GC, gas chromatography; HETCOR, ^{1}H-^{13}C heteronuclear shift-correlated spectroscopy; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; HPLC, high performance liquid chromatography: MS, mass spectrometry or mass spectrum; LIS, lanthanide-induced shifts; MPLC, medium pressure liquid chromatography; NMR, nuclear magnetic resonance; PBS, phosphate-buffered saline; SC, side chain; TLC, thin-layer chromatography; TBDMS, *tert*-butyldimethylsilyl; TMS, trimethylsilyl; TMSOH, trimethylsilanol; t_R, retention time; Yb(fod)$_3$,tris-(6,6,7,7,8,8,8-heptafluoro-2,2-dimethyl-3,5-octanedionato)ytterbium.

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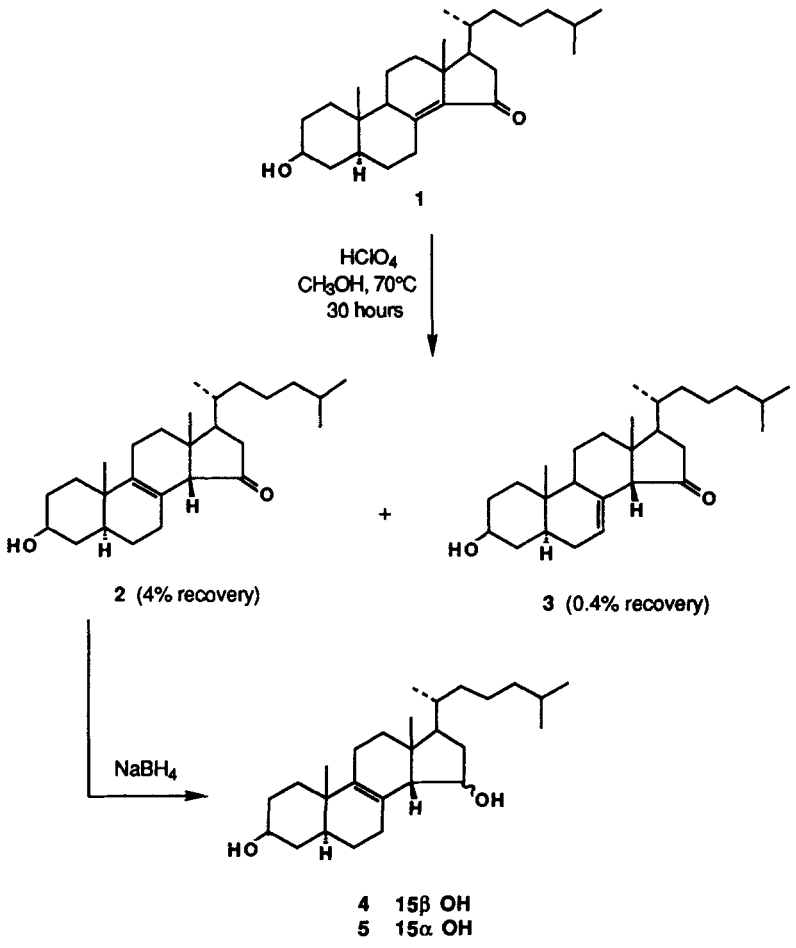

Fig. 1. Formation of β,γ-unsaturated 15-ketosterols **2** and **3** and the reduction of **2** by $NaBH_4$.

were used (falling needle injection): condition A, 15-m Rt_x-20 capillary column (Restek Corp., Bellefonte, PA; 0.25 mm ID, 0.1 μm film thickness, bonded stationary phase of 20% diphenyl–80% dimethyl polysiloxane), 200°C to 280°C at 10°C/min, then held at 280°C; condition B, 30-m DB-1 column (J & W Scientific, Folsom, CA; 0.3 mm ID, 0.1 μm film thickness, bonded stationary phase of 1% diphenyl–99% dimethyl polysiloxane), 200°C for 2 min, then to 290°C at 10°C per min; or condition C, 30-m DB-5 column (0.3 mm ID, 0.1 μm film thickness), 150°C for 1 min, then to 280°C at 40°C per min (or in some cases to 280°C at 10°C per min). Direct-inlet electron-impact mass spectra were recorded on a Shimadzu QP-1000 quadrupole spectrometer at 70 eV. High resolution mass spectra were recorded using a Finnigan MAT90 spectrometer with ionizing energy of 70 eV.

Analytical thin-layer chromatography (TLC) was performed using precoated 0.25 mm silica gel G plates (Analtech; Newark, DE); substances were visualized by spraying with 5% ammonium molybdate(VI) in 10% sulfuric acid followed by heating. Preparative TLC was performed using precoated silica gel G plates (0.5 mm or 1.0 mm plates or 0.3–1.7 mm tapered plates with a preadsorbent layer; Analtech; Newark, DE); substances were visualized by placing the plate under UV light (254 nm), by spraying along the edges of the plate as above, or by spraying with water. TLC solvent systems were: SS-1, ethyl acetate–hexane 1:9; SS-2, ethyl acetate–hexane 2:8, SS-3, ethyl acetate–hexane 3:7; SS-4, ethyl acetate–hexane 4:6; SS-5, ethyl acetate–hexane 1:1; SS-6, ether–benzene 1:1.

High performance liquid chromatography (HPLC) was performed isocratically with a Waters liquid chromatograph (U6K injector, model 510 pump, and model 481 variable wavelength detector set at 210 nm). Analytical HPLC was done at a flow rate of 1.0 ml/min (reversed phase) or 2.0 ml/min (normal phase). A 5 μm ODS-II Spherisorb column (4.6 mm × 250 mm) and a 5 μm Spherisorb silica column (4.6 mm × 250; Custom LC; Houston, TX) were used for analytical HPLC, and an 8 μm Dynamax 60A C_{18} column (21.4 mm × 250 mm; Rainin Instruments; Woburn, MA) was used for preparative HPLC. Medium pressure liquid chromatography (MPLC) was carried out with a Lobar column (37

mm ID × 440 mm; EM Science) containing 40–60 μm silica gel 60. Sterol samples were adsorbed onto silica gel and eluted from a small column onto the main MPLC column.

^1H and ^{13}C spectra were recorded in $CDCl_3$ solution in 5 mm tubes on an IBM AF300 spectrometer. ^1H NMR spectra (300.1 MHz) were referenced to tetramethylsilane, and ^{13}C NMR spectra (75.5 MHz) were referenced to $CDCl_3$ at 77.0 ppm. DEPT (distortionless enhancement by polarization transfer), COSY (^1H–^1H shift-correlated spectroscopy), HETCOR (^1H–^{13}C shift-correlated spectroscopy; ~50 increments, δ 0.6–2.6 window in the ^1H dimension) experiments were done with standard Bruker Aspect 3000 software. Lanthanide-induced shifts (LIS) were determined by adding 1–32 μl of a ~0.10 mM solution of $Yb(fod)_3$ in $CDCl_3$ to a solution of 30 mg of **9** in 0.4 ml of $CDCl_3$. ^1H and ^{13}C spectra were collected at 22°C for five molar ratios (0.001 to 0.05 of $Yb(fod)_3$ to sterol. The observed and calculated relative LIS values were determined and compared as described previously (5). Extrapolated to a 1:1 molar ratio of $Yb(fod)_3$: substrate, the LIS for C-15 of **9** was 152 ppm. Molecular mechanics calculations were carried out with either Sybyl (Tripos Associates, St. Louis, MO) on an Iris computer or PC Model 2.0 (Serena Software, Bloomington, IN) on a Macintosh computer. PC Model was used for the LIS calculations, and both Sybyl and PC Model were used to estimate the relative stabilities of conformers.

3β-Hydroxy-5α-cholest-8(14)-en-15-one (**1**), prepared as described previously (6), showed no impurities by TLC (SS-1). 3β-Benzoyloxy-5α,14β-cholest-7-en-15-one (**7**) was prepared as described previously (7, 8).

Supplies were obtained from the following sources: borane–tetrahydrofuran, *tert*-butyldimethylsilyl (TBDMS) chloride, N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA), and tris(6,6,7,7,8,8,8-heptafluoro-2,2-dimethyl-3,5-octanedionato) ytterbium ($Yb(fod)_3$), Aldrich Chemical Co. (Milwaukee, WI); Chinese hamster ovary (CHO-K1) cells, American Type Culture Collection (Rockville, MD); (3*RS*)-[3-^{14}C]HMG-CoA (56 mCi per mmol) and (3*RS*)-[2-^3H]mevalonolactone (176 mCi per mmol), Amersham Corporation (Arlington Heights, IL); trypsin, Gibco Laboratories (Grand Island, NY); Lux tissue culture plasticware, Miles Scientific (Elkhart, IN); powdered Ham's F12 medium (9) and phosphate-buffered saline (PBS: KCl, 2.7 mM; KH_2PO_4, 1.2 mM; NaCl, 137 mM, and Na_2HPO_4, 8.1 mM), Irvine Scientific (Irvine, CA); fetal calf serum, Whittaker M.A. Bioproducts (Elkhart, IN). For cell cultures studies, the sterols were added as ethanolic solutions to Ham's F12 medium supplemented with 5% delipidated (10) fetal calf serum (lipid-deficient medium) and allowed to equilibrate for at least 6 h at room temperature prior to storage at 4°C. Radioactivity was assayed in Packard 4640 and Beckman 9800 liquid scintillation counters using 2,5-diphenyloxazole (0.4%) in toluene or in toluene–ethanol 2:1 as the scintillation fluid. Protein in detergent-solubilized extracts of cultured cells was assayed by the method of Lowry et al. (11) after precipitation with trichloroacetic acid.

Studies of the effects of the 15-oxygenated sterols on HMG-CoA reductase activity were made in CHO-K1 cells. The cells were maintained in Ham's F12 medium supplemented with 5% fetal calf serum (lipid-rich medium) in a humidified atmosphere of 5% CO_2–95% air at 37°C. Each experiment was initiated by inoculating 3.75×10^5 cells into 100-mm dishes containing lipid-rich medium (10 ml), followed by incubation for 48 h. The medium was aspirated and, after rinsing of the plates with PBS (10 ml), lipid-deficient medium (10 ml) was added to one set of plates. A separate set of plates received lipid-deficient medium containing the 15-oxygenated sterols. The cells were incubated for 18 h. The cells incubated in the absence of sterols then received fresh lipid-deficient medium containing the 15-oxygenated sterols, and incubation continued for 4 h.[3] Cells were harvested by scraping, and detergent-solubilized cell preparations were obtained for the assay of HMG-CoA reducase activity using the method of Brown, Dana, and Goldstein (12). Replicate assays (n = 3) were carried out as described by Pinkerton et al. (3), except that the specific activity of the (3*RS*)-[3-^{14}C]HMG-CoA was 20,000 dpm per nmol.

3β-Hydroxy-5α,14β-cholest-8-en-15-one (2) and 3β-hydroxy-5α,14β-cholest-7-en-15-one (3)

To a solution of 3β-hydroxy-5α-cholest-8(14)-en-15-one (**1**, 10.0 g) in methanol (150 ml) was added two drops of perchloric acid. The solution was heated under reflux for 30 h, during which time the reaction mixture was periodically analyzed by HPLC (**Table 1**). The reaction mixture was allowed to cool, water (90 ml) was added, and the resulting precipitate was redissolved by the addition of methanol (150 ml) and heating. Cooling afforded crystals, which were filtered to give a solid (8.43 g) that, on the basis of ^1H and ^{13}C NMR analyses, contained only the $\Delta^{8(14)}$-15-ketosterol **1** and was not studied further. Water (150 ml) was added to the filtrate, and the resulting solution was extracted with CH_2Cl_2 (150 ml, twice) and

[3]Transfer of CHO-K1 cells from lipid-rich medium to lipid-deficient medium results in a marked elevation of HMG-CoA reductase activity, which reaches a maximum at 16–24 h after transfer to the lipid-deficient medium (2, 4). Therefore, all of the experiments reported herein involved incubation of the cells in lipid-deficient media for 18 h. In experiments designed to determine the effects of the oxysterols on this elevation, the compounds were added at the beginning of the 18-h period. In experiments designed to determine the effects of the oxysterols on the elevated levels of enzyme activity, the compounds were added at the end of the 18-h period. The incubations were then continued for 4 h, a time period at which marked suppression of the elevated levels of HMG-CoA reductase activity by **1** occurs (5).

TABLE 1. Formation of β,γ-unsaturated ketone **2** by refluxing **1** in acidic methanol[a,b]

Time	2	1
h	%	
1	0.3	99.7
2.2	0.7	99.3
4.2	1.2	98.8
8.3	2.1	97.9
26	4.8	95.2
30	4.8	95.2

[a]Analysis by reversed phase HPLC (water-methanol 1:9, UV detection at 210 nm). Percentages of **1** (t_R 20 min) and **2** (t_R 14.5 min) were corrected for their HPLC response factors at 210 nm. HPLC analysis of a known mixture (5.3 mg of **1**, 10.0 mg of **2**) gave peak areas of 8.24 for **1** and 6.90 for **2**. Thus, the Δ^8 ketone **2** had a response factor 2.25 times that of **1**.

[b]The amount of the Δ^7 ketone **3**, which was not resolved from **2**, was estimated to be ~ 10% of the amount of **2** based on the masses of **2** and **3** isolated by chromatography.

$CHCl_3$ (150 ml; 50 ml). The combined extracts were dried over anhydrous sodium sulfate. Evaporation of the solvent under reduced pressure gave a solid (1.01 g) that was adsorbed onto silica gel (3 g) and subjected to MPLC (ethyl acetate–hexane 1:5). The fractions (20 ml) were analyzed by TLC (SS-3), and appropriate fractions were combined to give **2** (454 mg; R_f 0.35; fractions 113–138), **1** (395 mg; R_f 0.26; fractions 174–207), and **3** (36 mg; R_f 0.22; fractions 243–272).

The material from fractions 113–138 was subjected to preparative TLC (SS-5) to give an analytical sample of **2**: mp 112–113°C; $[\alpha]_D^{22}$ −61.1° (*c*, 0.6); IR, 3400, 3000–2800, 1730, 1468, 1375, 1152, and 1036 cm^{-1}; TLC, single spots at R_f 0.50 (SS-5) and 0.55 (SS-6); single component on HPLC (methanol–water 92:8) with a retention time of 11.8 min; high resolution MS: ion at *m/z* 400 (M^+), 400.3340 (calc. for $C_{27}H_{44}O_2$: 400.3339), see also

TABLE 2. Ion abundances in the mass spectra of 3β-hydroxy-15-oxygenated sterols and their trimethylsilyl ether derivatives[a,b,c]

	Free Sterols, Direct Inlet				TMS Ethers, GC-MS			
	1	**2**	**3**	**4**				
Suggested Assignment[d]	$\Delta^{8(14)}$ 15-keto	14β-Δ^8 15-keto	14β-Δ^7 15-keto	14β-Δ^8 15β-OH	$\Delta^{8(14)}$ 15-keto	14β-Δ^8 15-keto	14β-Δ^7 15-keto	*e*
M^+	400 (91)	400* (67)	400* (59)	402* (26)	472 (80)	472 (16)	472 (16)	472 (30)
M-CH_3	385 (26)	385* (99)	385* (80)	387* (41)	457 (34)	457 (10)	457 (12)	457 (14)
M-H_2O	382 (20)	382* (10)	382* (9)	384* (41)	454 (10)	454 (1)	454 (2)	454 (3)
M-H_2O-CH_3	367 (56)	367* (100)	367* (90)	369* (66)	439 (2)	439 (1)	439 (1)	439 (1)
M-ROH	[382]	[382]	[382]	[384]	382 (16)	382 (7)	382 (8)	382 (27)
M-ROH-CH_3	[367]	[367]	[367]	[369]	367 (100)	367 (100)	367 (100)	367 (100)
M-ROH-H_2O	364 (1)			366* (7)	364 (11)	364 (2)	364 (5)	364 (6)
M-ROH-H_2O-CH_3	349 (3)	349* (21)	349* (21)	351* (33)	349 (10)	349 (13)	349 (12)	349 (11)
M-C1..3-H[d]	339 (2)	339 (3)	339 (3)		339 (5)	339 (2)	339 (3)	339 (3)
M-pSC-ROH[d]	297 (4)	297 (4)	297 (4)	299* (22)	297 (11)	297 (2)	297 (3)	297 (3)
M-SC[d]	287 (26)	287* (16)	287* (13)	289* (1)	359 (10)	359 (3)	359 (6)	359 (8)
M-Ring A-H	[287]	[287]	[287]		287 (37)	287 (2)	287 (4)	287 (5)
M-H_2O-SC	269 (84)	269* (9)	269* (12)	271* (45)	341 (48)	341 (2)	341 (8)	341 (4)
M-ROH-SC	[269]	[269]	[269]	[271]	269 (63)	269 (4)	269 (7)	269 (15)
m/z 261[f]	261 (19)	261 (<1)	261 (1)		261 (24)	261 (1)	261 (1)	261 (3)
M-SC-28[d]	259 (8)	259 (1)	259 (2)		331 (12)	331 (0.4)	331 (1)	331 (2)
M-ROH-H_2O-SC	251 (21)	251 (5)	251* (6)	253* (13)	251 (76)	251 (3)	251 (6)	251 (15)
M-ROH-SC-28[d]	241 (6)	241 (1)	241 (1)	243 (3)	241 (8)	241 (1)	241 (1)	241 (6)
m/z 232	232 (3)	232 (10)	232 (10)	232 (30)				
m/z 107	107 (100)	107 (39)	107 (47)	107 (71)	107 (20)	107 (4)	107 (7)	107 (17)
m/z 105	105 (75)	105 (100)	105 (100)	105 (100)	105 (54)	105 (6)	105 (7)	105 (11)
Other ions	276[f] (8)	233 (28)	250 (8) 233 (24)	325[g] (2) 281[g] (4) 218 (33)	276[f] (14)	305 (10)	305 (7)	288 (25) 120 (73)
	R = H	R = H	R = H	R = H	R = TMS	R = TMS	R = TMS	R = TMS

[a]Major ions above *m/z* 100 mass spectra acquired at 70 eV by either direct-probe (free sterols; Shimadzu instrument) or GC-MS (TMS ethers; Extrel instrument). Relative intensities as % of base peak.

[b]Ions also observed in the high resolution mass spectrum are indicated by an asterisk; all such ions were compatible (± 3.5 millimass units) with the suggested assignments.

[c]Sterols **2** and **3** also showed ions at *m/z* 199, 209, 213, and 215 of 10–35% relative abundance; **1** and the TMS ethers showed the same ions in somewhat lower abundance.

[d]See footnote 4 for definition of SC, pSC, SC-28, and C1. .3.

[e]Unidentified sterol presumed to be a β,γ-unsaturated 15-ketosterol of 14α configuration (see experimental).

[f]See ref. 15 for suggested assignments.

[g]High resolution MS data indicted the following formulas: *m/z* 325, $C_{24}H_{37}$; *m/z* 281, $C_{21}H_{29}$.

TABLE 3. ^{1}H NMR chemical shifts of 15-oxygenated sterols[a,b,c,d]

	$\Delta^{8(14)}$ 3β-OH 15-keto	14β-Δ^7 3β-OH 15-keto	14β-Δ^7 3β-OBz 15-keto	14β-Δ^8 3β-OH 15-keto	14β-Δ^8 3β-OAc 15-keto	14β-Δ^8 3β-OH 15β-OH	14β-Δ^8 3β-QAc 15β-OAc	14β-Δ^8 3β-OH 15α-OH
	1	**3**	**7**	**2**	**6**	**4**[e]	**8**	**5**
H-1α	1.20	1.02	1.15	1.14		1.11		
H-1β	1.72	1.78	1.85	1.80		1.76		
H-2α	1.85	1.79	1.97	1.85		1.84		
H-2β	1.38	1.38	*1.56*	1.46		1.45		
H-3α	3.64	3.59	4.93	3.61	4.70	3.63	4.70	3.64
H-4α	1.68	1.74	*1.92*	1.66		1.67		
H-4β	1.28	1.25	*1.47*	1.33		1.32		
H-5α	1.41	1.53	1.66	1.35		1.36		
H-6	1.48	1.95	*1.93*	1.54		*1.48*		
	1.34	1.73		1.39		*1.20*		
H-7(α)	1.58	5.42	5.43	2.18		2.38		
H-7β	4.13			1.87		1.82		
H-9α	1.86	1.44	1.51					
H-11	1.65	1.54	1.53	2.05		*1.84*		
	1.53	*1.35*		2.05		*1.84*		
H-12α	1.25	1.25*	*1.29**	1.29		*1.15*		
H-12β	2.11	1.45*	1.46*	1.89		1.69		
H-14β		2.71	2.72	2.30	2.30	1.72		1.97
H-15						3.72	4.72	4.08
H-16	2.36	2.43	2.45	2.41	2.42	2.09		
	2.06	2.26	2.27	1.99		1.40		
H-17α	1.46	1.80	1.80	1.99		1.46		1.73
H-18	0.972	1.131	1.142	0.969	0.965	0.990	0.994	0.909
H-19	0.715	0.753	0.824	0.946	0.960	0.919	0.934	0.966
H-20	1.57	1.93	1.93	1.54		1.50		1.50
H-21	0.998	0.888	0.895	0.986	0.984	0.916	0.924	0.941
H-22	1.08	0.98	*0.94*	0.99		*1.00*		
	1.33	1.32	*1.37*	1.30		1.42		
H-23	1.18		1.18	1.14		1.14		
	1.33	*1.35*		1.39		1.40		
H-24	1.11	1.13	1.14	1.13		1.13		1.14
	1.15	1.13	1.14	1.13		1.13		1.14
H-25	1.51	1.50	1.50	1.52		1.52		1.52
H-26	0.861	0.862	0.865	0.862	0.861	0.861	0.861	0.864
H-27	0.863	0.865	0.868	0.864	0.863	0.863	0.865	0.868
Signals for acetate or benzoate groups			7.43 (m) 7.54 (p) 8.04 (o)		2.021		2.014 2.026	

[a]Chemical shifts referenced to $Si(CH_3)_4$ signal at 0 ppm. Data obtained at 300.1 MHz in $CDCl_3$ solution at a concentration of 0.02–0.2 M. Data for **1** are taken from ref. 15.

[b]Chemical shifts of methylene protons between δ 0.9 and 2.0, obtained from HETCOR data, are generally accurate to ± 0.02 ppm. Values in italics are of lower accuracy (± 0.1 ppm).

[c]Asterisks indicate assignment of α and β stereochemistry may be interchanged. No stereochemical assignments are given for the protons of C-6, C-11, C-16, C-22, C-23, and C-24.

[d]Selected couplings: H-7β: **3**, **7**, br d, 5.5 Hz; H-14: **2**, **3**, **6**, **7**, s; **4**, d, 6 Hz; **5**, d, 4.7 Hz; H-15: **4**, **8**, ddd, ~8, ~7, ~6 Hz; **5**, t, 4 Hz; H-16 (downfield signal): **2**, dd, − 22.8, 12.7 Hz; **3**, **7**, dd, − 20.1, 9.9 Hz; **4**, ddd, 11.7, 6.6, 5.4 Hz; H-16 (upfield signal): **3**, **7**, ddd, − 20.0, 2.9, 1.5Hz; H-21, H-26, H-27: d, 6.6 ± 0.3 Hz.

[e]A spectrum of **4** in pyridine showed the following signals shifted downfield (ppm) relative to their position in $CDCl_3$: H-3 (0.28), H-7α (0.54), H-14 (0.45), H-15 (0.41), H-16 (downfield signal, 0.26), H-18 (0.17), H-19 (0.11), H-21 (0.10).

Table 2. MS and ^{1}H and ^{13}C NMR data are presented in Table 2, **Table 3**, and **Table 4**, respectively.

The material from fractions 243–272 of the MPLC column was subjected to preparative TLC (SS-3) to give an analytical sample of **3**: mp 92–95°C; $[\alpha]_D^{22}$ − 9.8° (*c*, 0.6); IR, 3300, 3000–2800, 1742, 1468, and 1044 cm^{-1}; TLC, single spots at R_f 0.33 (SS-5) and major spot at R_f 0.43 plus very minor impurities (SS-6); HPLC (methanol–water 92:8) showed purity of ~97% with retention time of 11.2 min; high resolution MS: ion at *m/z* 400 (M^+), 400.3336 (calc. for $C_{27}H_{44}O_2$: 400.3339), see also Table 2. MS and ^{1}H and ^{13}C NMR data are presented in Tables 2, 3, and 4, respectively. Chromatographic retention properties of **1**, **2**, and **3** are compared in **Table 5**.

In a similar isomerization of ketosterol **1** (277 mg) in methanol (10 ml) containing $HClO_4$, crystallization from

TABLE 4. ^{13}C NMR chemical shifts of 15-oxygenated sterols[a]

	$\Delta^{8(14)}$ 3β-OH 15-keto **1**[b]	14β-Δ^7 3β-OH 15-keto **3**	14β-Δ^7 3β-OBz 15-keto **7**	14β-Δ^8 3β-OH 15-keto **2**	14β-Δ^8 3β-OAc 15-keto **6**[c]	14β-Δ^8 3β-OH 15β-OH **4**	14β-Δ^8 3β-OAc 15β-OAc **8**	14β-Δ^8 3β-OH 15α-OH **5**
C1	36.49	36.41	36.19	34.08	33.85	34.17	34.86	34.91
C2	31.07	31.10	27.42	31.06	27.16	31.22	27.13	31.34*
C3	70.79	70.74	73.86	70.80	73.29	71.16	73.34	70.99
C4	37.69	37.55	33.61	37.79	33.68	37.91	33.79*	37.91**
C5	44.07	39.53	39.47	40.98	40.84	41.49	40.97	41.75
C6	29.10	29.88	29.87	25.06	24.96	25.36	24.53	25.29
C7	27.55	126.80	126.61	29.13	29.01	30.52	29.70	31.07*
C8	150.68	132.17	132.22	121.50	121.70	129.45	128.17	125.07
C9	50.82	47.28	47.18	139.00	138.73	137.17	137.52	144.73
C10	38.69	33.77	33.90	36.92	36.91	36.33	36.31	36.62
C11	19.52	20.50	20.48	21.03	21.05	20.73	20.61	21.06
C12	36.91	37.63	37.61	31.97	31.94	35.57	34.40	37.02**
C13	42.51	42.17	42.18	40.74	40.78	41.82	41.30	40.67
C14	140.23	64.46	64.43	65.34	65.44	61.92	58.21	60.39
C15	208.16	219.49	219.07	218.17	218.15	79.61	80.29	70.48
C16	42.41	36.86	36.91	40.51	40.59	37.36	33.67*	37.60**
C17	50.75	49.01	49.08	42.27	42.22	46.52	46.30	50.08
C18	18.73	19.60	19.69	22.27	22.34	25.39	25.07	24.69
C19	12.87	12.77	12.76	17.28	17.21	16.96	16.83	17.20
C20	34.43	33.25	33.32	34.44	34.52	32.79	33.13	33.00
C21	19.17	19.22	19.26	18.84	18.87	19.96	19.58	19.95
C22	35.76	31.63	31.70	35.36	35.44	35.57	35.39	35.77
C23	23.46	25.40	25.44	24.26	24.27	24.40	24.35	24.25
C24	39.31	39.15	39.19	39.15	39.18	39.38	39.28	39.44
C25	27.90	27.94	27.98	27.80	27.85	27.91	27.81	27.94
C26	22.48	22.46	22.49	22.42	22.46	22.52	22.43	22.50
C27	22.68	22.65	22.68	22.65	22.72	22.74	22.69	22.80
Signals for acetate or benzoate groups			165.99 (C=O) 132.65 (p) 130.81 (q) 129.47 (o) 128.21 (m)		21.38 170.65		21.33 21.36 170.38 170.68	

[a]Chemical shifts referenced to the $CDCl_3$ signal at 77.0 ppm. Data obtained at 75 MHz in $CDCl_3$ solution at a concentration of 0.05 - 0.2 M. Assignments marked with an asterisk may be interchanged within a column.
[b]Shieldings for **1** are essentially identical to those given in ref. 15.
[c]Chemical shifts for **6** are ±0.05 ppm.

water-methanol gave **1** (210 mg). The mother liquor was diluted with water and extracted with ether to afford a mixture of sterols, of which a portion was silylated with BSTFA-pyridine 1:1 overnight at room temperature.

TABLE 5. Chromatographic retention properties of unsaturated 15-ketosterols **1**, **2**, and **3**[a]

Technique	1	2	3
TLC[b]	0.26	0.35	0.22
HPLC (normal phase)[c]	7.4	5.0	8.6
HPLC (reversed phase)[d]	13.1	10.1	10.0
GC-MS[e]	12.37	10.68	11.12

[a]Retention times (HPLC and GC-MS) or R_f (TLC).
[b]SS-4.
[c]2-Propanol-hexane 3:97 at 2 ml/min, 5 μm Spherisorb silica (4.6 mm ID × 250 mm).
[d]Water-methanol 7:93 at 1 ml/min, 5 μm Spherisorb ODS-II (4.6 mm ID × 250 mm); **2** and **3** were not resolved.
[e]GC-MS condition B; samples analyzed as their TMS ethers.

GC-MS analysis (condition C) showed the following components (yields are based on the original 277 mg sample of **1**): 5.0% **2** (t_R 7.0 min), 0.2% **3** (t_R 7.5 min), 17.5% **1** (t_R 8.9 min), an unidentified isomer (0.2%, t_R 7.2 min) that was presumed to be a β,γ-unsaturated sterol of 14α configuration (MS, Table 2), and small amounts of unidentified oxidized sterols.

3β-Acetoxy-5α,14β-cholest-8-en-15-one (**6**) was prepared by treatment of **1** with pyridine-acetic anhydride 1:1 overnight at room temperature followed by dilution with methanol and evaporation to dryness. ^1H and ^{13}C NMR data are given in Tables 3 and 4, and LIS data are given in **Table 6**. Samples of [7,7,14,16,16-$^2H_5$]-**2** and [7,7,14,16,16-$^2H_5$]-**6** were prepared and isolated as described above for **2** and **6**. Their ^{13}C NMR spectra showed the absence of signals for C-7, C-14, and C-16. Other signals showed deuterium isotope shifts (δ(sterol-d_5)-δ(sterol-d_0, ppm): C-6 (−0.2), C-8 (−0.2), C-9 (0.2), C-13 (−0.1), C-15 (0.2), C-17 (−0.2); all other isotope shifts were <0.1 ppm.

TABLE 6. Lanthanide-induced shifts for 3β-acetoxy-5α,14β-cholest-8-en-15-one (**6**) and 3β-(*tert*-butyldimethylsilyloxy)-5α,14β-cholest-8-en-15β-ol (**9**)

	6 Obsd LIS	9 δ	9 Obsd LIS	15β-OH (B)[a] Calcd LIS	15α-OH (A)[a] Calcd LIS
C-1	164	34.35	34	34	−11
C-2	307	31.73	14	12	−20
C-3	603	72.14	1	−2	−26
C-4	287	38.41	7	−1	−4
C-5	168	41.64	38	33	33
C-6	118	25.39	60	47	72
C-7	251	30.55	216	236	199
C-8	275	129.16	251	275	300
C-9	169	137.50	137	135	153
C-10	156	36.38	63	62	49
C-11	116	20.72	106	106	101
C-12	118	35.49	132	141	170
C-13	213	41.89	229	228	276
C-14	419	62.07	438	455	448
C-15	1000	79.68	1000	1000	1000
C-16	424	37.38	468	463	434
C-17	181	46.45	221	215	262
C-18	146	25.41	163	143	126
C-19	125	17.01	48	48	39
C-20	67	32.79	107	108	69
C-21	30	19.96	59	67	15
C-22	18	35.59	65	62	7
C-23	−7	24.40	29	28	−9
C-24	−26	39.40	7	4	−22
C-25	−26	27.93	−1	1	−16
C-26	−27	22.55	−5	−2	−19
C-27	−29	22.77	−6	−2	−12
H-3		3.58	−14	−11	−53
H-5		1.33	22	19	3
H-14		1.72	437	426	265
H-15		3.71	612	596	597
H-17		1.46	157	157	207
H-18		0.993	147	125	106
H-19		0.916	42	44	36
H-20		1.50	92	105	72
H-21		0.919	48	59	5
H-24		1.13	−2	−4	−15
H-25		1.52	−1	4	−18
H-26		0.867	−6	−3	−20
H-27		0.870	−8	−2	−12

[a]Calculated geometries for the 12α,13β-half-chair conformers of 5α,14β-cholest-8-en-3β,15α-diol (A) and its 15β-OH isomer (B); *R*: 16.48% (A), 3.83% (B); Yb-O Distance (Å): 2.14 Å (A), 2.35 Å (B); Yb-O-C15 angle: 155° (A), 146° (B); Yb-O-C15-C14 torsion angle: 169° (A), 162° (B); magnetic axis deviation from Yb-O: 1.1° (A), 1.0° (B). Calculations for the twist (12β) conformers gave *R* values of 18.82% (A) and 4.06% (B). Calculations carried out for the 14α-H isomers showed *R* values of 14.33% (15β-OH) and 3.22% (15α-OH).

5α,14β-Cholest-8-ene-3β,15β-diol (4)

A solution of 3β-hydroxy-5α,14β-cholest-8-en-15-one (**2**, 1.0 g) in water (17.5 ml) and isopropyl alcohol (50 ml) containing $NaBH_4$ (0.5 g) was stirred under nitrogen at room temperature for 26 h. The mixture was concentrated to ~10 ml, diluted with water (25 ml), and extracted 3 times with CH_2Cl_2 (50 ml portions). The combined organic extracts were dried over anhydrous $MgSO_4$, and the white solid obtained upon evaporation of the solvent under reduced pressure was subjected to silica gel (30 g) column chromatography using SS-2 as the eluting solvent (fraction volume, 17 ml). The contents of fractions 18–54 were pooled to give, after evaporation of the solvent under reduced pressure, a white solid (693 mg). HPLC analysis (methanol–water 19:1; UV detection at 210 nm) showed two components with retention times of 6.4 min and 9.3 min in a 5:1 ratio. Purification of a portion (~300 mg) of this material by preparative HPLC (methanol–water 11:1) gave the minor component **5** (18 mg) and an analytical sample of **4** (189 mg): mp 117.0–117.5°C; $[\alpha]_D^{22}$ + 119° (*c*, 0.6); IR, 3360, 2980–2820, 1460, 1375, and 1040 cm^{-1}; TLC, single spots at R_f 0.32 (SS-5) and 0.38 (SS-6); single component on HPLC (methanol-water 92:8) with a retention time of 9.6 min; high resolution MS:ion at *m/z* 402 (M^+), 402.3491 (calc. for $C_{27}H_{46}O_2$: 402.3495), see also Table 2. MS data for **4** and ^1H and ^{13}C NMR data for **4** and **5** are presented in Tables 2, 3, and 4, respectively.

Derivatives of diol 4 (Fig. 2)

The trimethylsilyl ether derivative was prepared by treatment of **4** with BSTFA–pyridine 1:1 at room temperature. GC–MS showed a single peak at t_R 3.9 min (condition A); MS[4]: 546 (6, M^+), 531 (17, M–CH_3), 456 (48, M-TMSOH), 433 (3, M-SC), 431 (2, M-C-1...3-H), 371 (13, M-pSC-TMSOH), 366 (45, M-2TMSOH), 351 (58, M-2TMSOH-CH_3), 343 (40, M-SC-TMSOH), 304 (23), 290 (18), 289 (18), 287 (8), 281 (30, M-pSC-2TMSOH), 253 (100, M-SC-2TMSOH), 241 (77), 129 (86).

3β,15β-Diacetoxy-5α,14β-cholest-8-ene (**8**) was prepared by treatment of diol **4** (76 mg) with pyridine (1.2 g) and acetic anhydride (0.9 g) at room temperature for 24 h. Precipitation over ice and filtration gave a solid, which was purified by MPLC (elution with ethyl acetate–hexane 7:93; 20-ml fraction volumes; 2.5-ml/min flow rate). Fractions 69–80 were combined and evaporated to a solid (**8**) that gave a single peak (>98% pure) by HPLC (t_R 9.1 min, methanol) and by GC–MS (t_R 6.4 min, condition A); MS[4]: 486 (1, M^+), 426 (100, M–CH_3COOH), 411 (29, M–CH_3COOH–CH_3), 366 (38, M–2CH_3COOH), 351 (68, M–2CH_3COOH–CH_3), 341 (12), 313 (10, M–CH_3COOH–SC), 281 (7), 253 (36, M–SC–2CH_3COOH); TLC (SS-1): major spot (R_f 0.53) and very minor impurity (R_f 0.67); ^1H and ^{13}C NMR, Tables 2 and 3.

3β-(*tert*-Butyldimethylsilyloxy)-5α,14β-cholest-8-en-15β-ol (**9**) was prepared by heating diol **4** (78 mg) at 100°C for 5 h with imidazole (110 mg), dry DMF (1 ml), and sufficient TBDMS chloride (~90 mg) for formation of monosilylated derivatives as judged by TLC (SS-2). Chromatography

[4]Notation for mass spectral fragmentation: SC, C_8H_{17} side chain; pSC, C_6H_{13} part of side chain encompassing C-22 through C-27; -SC-28, loss of side chain together with C_2H_4 (C-16 and C-17); C1..3, fragment containing C-1, C-2, C-3 and any C-3 substituent.

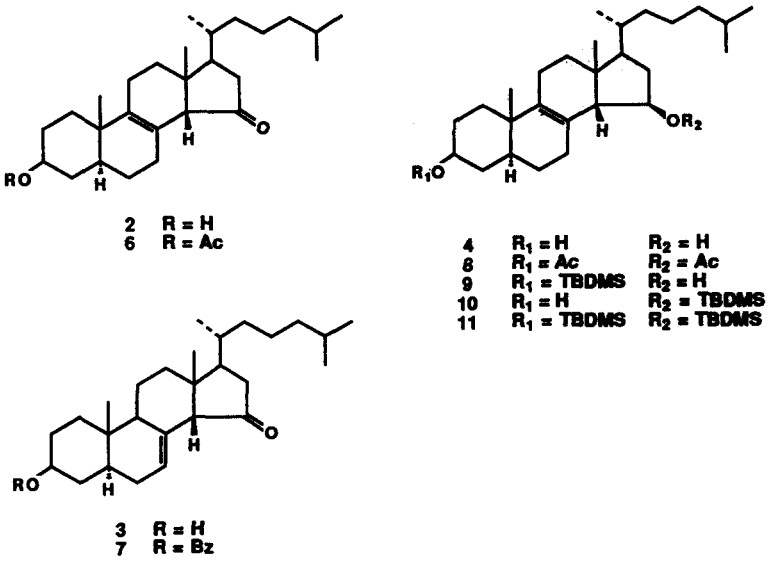

Fig. 2. Ester and TBDMS ether derivatives of 15-oxygenated sterols **2**, **3**, and **4**.

(silica gel, elution with ethyl acetate–hexane 3:97) afforded samples of 3β,15β-bis(TBDMS) ether **11**, and 3β-TBDMS ether **9**, and 15β-TBDMS ether **10**. Ethers **9–11** were distinguished by GC–MS and by their ^{13}C NMR silylation shifts (**Fig. 3**). Table 6 shows ^{13}C NMR data and relative LIS for ether **9**.

^{1}H and ^{13}C NMR assignments for 2, 3, and 4

Assignments in rings A and B and at the end of the side chain followed immediately from comparisons with previous ^{13}C assignments for Δ^{7} and Δ^{8} sterols (13) in conjunction with correlated ^{1}H signals from HETCOR experiments. After trivial assignments of the methyl and quaternary carbons, several methine and methylene carbons in and near ring D remained to be assigned. Of the methine carbons in **2** and **3**, C-14 was correlated to a ^{1}H singlet, and C-17 and C-20 were distinguished by their large chemical shift differences that reflect their different number of α and β carbon neighbors. In Δ^{7} sterol **3**, C-9 and C-17 were correlated to H-19 and H-18, respectively, in the long-range HETCOR spectrum, and this assignment was consistent with correlations of H-17 to H-16 and H-20 in the COSY spectrum. Of the methylene carbons, C-11 was recognized by its characteristic high-field shift ($\delta \sim 21$) and C-16 by its correlation to protons forming a characteristic doublet of an AB quartet at δ 1.9–2.5 with coupling constants similar to those of other 15-ketosterols (14, 15). The remaining methylene carbons, C-12 and C-22, were distinguished by the correlation of C-22 to signals at $\delta \sim 1.3$ and $\delta \sim 1.0$ that are characteristic of the C-22 protons in a C_8H_{17} side chain (15) and, in the case of **3**, by the long-range HETCOR pairing of C-12 to H-18. The LIS for Δ^{8}-15-ketosterol acetate **6** (Table 6) and the ^{13}C NMR spectra of **2**-d_5 and **6**-d_5 support the assignments for **2**. The assignments for **4**, made by similar arguments, were confirmed by the LIS data shown in Table 6 for its TBDMS ether **9**.

The ^{1}H NMR signals were assigned mainly from HETCOR spectra. Stereochemistry of CH_2 protons was assigned whenever possible by comparison with reported ^{1}H assignments of similar sterols (16). H-7α and H-7β were distinguished in Δ^{8}-14β-sterols **2** and **4** based on the width of $\sim$39 Hz for the 7α resonance, compatible only with that for an axial proton.

Effects of 3β-hydroxy-5α-cholest-8(14)-en-15-one (1), 3β-hydroxy-5α,14β-cholest-8-en-15-one (2), 3β-hydroxy-5α,14β-cholest-7-en-15-one (3), and 5α,14β-cholest-8-ene-3β,15β-diol (4) on the levels of HMG-CoA reductase activity in CHO-K1 cells

The effects of the two β,γ-unsaturated analogs (**2** and **3**) of the $\Delta^{8(14)}$-15-ketosterol **1** on the levels of HMG-CoA

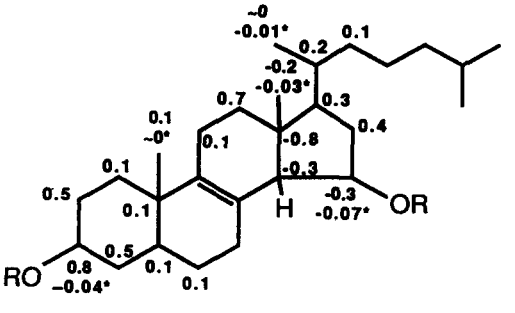

Fig. 3. ^{1}H and ^{13}C NMR substituent increments (TBDMS ether–free sterol) in ppm for 3β-TBDMS ether (rings A and B) and for 15β-TBDMS ether (rings C and D) of 3β,15β-dihydroxy-5α,14β-sterols. ^{1}H substituent increments are marked by an asterisk. Substituent increments were calculated from NMR shieldings for ethers **9**, **10**, and **11**.

TABLE 7. Effects of 3β-hydroxy-5α-cholest-8(14)-en-15-one (1), 3β-hydroxy-5α,14β-cholest-8-en-15-one (2), and 3β-hydroxy-5α,14β-cholest-7-en-15-one (3) on levels of HMG-CoA reductase activity in CHO-K1 cells

Sterol Concentration	HMG-CoA Reductase Activity (% of Control Activity)[a]					
	Suppression of Rise Induced by Transfer of Cells to Lipid-Deficient Media			Suppression of Elevated Levels Induced by Transfer of Cells to Lipid-Deficient Media		
μM	1	2	3	1	2	3
0.0	100.0 ± 3.6[b]	100.0 ± 4.3[c]	100.0 ± 6.1[d]	100.0 ± 6.6[e]	100.0 ± 3.4[f]	100.0 ± 0.6[g]
0.1	52.7 ± 1.9	76.6 ± 4.8	71.1 ± 6.9	55.4 ± 2.6	64.5 ± 0.9	106.9 ± 7.0
0.25	50.8 ± 5.3	63.8 ± 7.0	59.2 ± 1.3	40.9 ± 1.2	51.4 ± 1.1	93.9 ± 2.7
0.5	31.1 ± 0.6	61.2 ± 5.5	35.9 ± 2.4	34.1 ± 0.9	34.8 ± 2.4	69.8 ± 1.5
1.0	29.1 ± 0.5	43.0 ± 2.1	34.7 ± 2.9	31.0 ± 1.8	28.7 ± 1.2	38.0 ± 0.7
2.5	27.5 ± 4.3	37.1 ± 1.6	27.4 ± 3.2	23.6 ± 0.7	17.0 ± 0.6	21.4 ± 2.2

[a]Variation is expressed as ± SD of replicate (n = 3) assays for the experimental values.
[b-g]Mean values for controls were 1190, 752, 836, 3370, 3870, and 2600 pmol/min per mg protein, respectively.

reductase activity in CHO-K1 cells are presented in **Table 7**. Both **2** and **3** suppressed the rise of HMG-CoA reductase activity induced by transfer of the cells to lipid-deficient media. However, **2** and **3** were less active than **1** in this regard; **1** caused a 47% inhibition of the rise in reductase activity at a concentration of 0.1 μM, whereas **2** and **3**, at the same concentration, caused a 23% and 29% suppression in the rise of reductase activity, respectively. The β,γ-unsaturated 15-ketosterols **2** and **3** also caused a decrease in the elevated levels of HMG-CoA reductase activity induced by transfer of the cells to lipid-deficient media. However, **2** and **3** were less active than **1** in this regard. At a concentration of 0.1 μM, **1** caused a 45% reduction in the levels of HMG-CoA reductase activity. At the same concentration, **2** caused a 35% decrease in reductase activity while **3** had no effect. At 0.5 μM, **3** caused a 30% decrease in reductase activity.

The effects of 5α,14β-cholest-8-ene-3β,15β-diol (**4**) on the levels of HMG-CoA reductase activity in CHO-K1 cells are presented in **Table 8**. The Δ^8-3β,15-diol **4** appeared to be slightly more active than the $\Delta^{8(14)}$-15-ketosterol **1** in blocking the rise in HMG-CoA reductase activity induced by transfer of the cells to lipid-deficient media. However, **4** and **1** appeared to be of equal potency in lowering the elevated levels of reductase activity induced by transfer of the cells to lipid-deficient media.

DISCUSSION

Treatment of the $\Delta^{8(14)}$-15-ketosterol **1** with a small amount of perchloric acid in refluxing methanol led to the formation of its β,γ-unsaturated analogs, 3β-hydroxy-5α,14β-cholest-8-en-15-one (**2**) and 3β-hydroxy-5α,14β-cholest-7-en-15-one (**3**). These 15-ketosterols, previously observed as byproducts in the acid-catalyzed deuterium labeling of **1** (15), have now been isolated by chromatography and characterized by melting point, optical rotation, IR, MS, and ^{1}H and ^{13}C NMR. The locations of the olefinic bond in **2** and **3** were based upon the absence

TABLE 8. Effects of 3β-hydroxy-5α-cholest-8(14)-en-15-one (1) and 5α,14β-cholest-8-ene-3β,15β-diol (4) on levels of HMG-CoA reductase activity in CHO-K1 cells

Sterol Concentration	HMG-CoA Reductase Activity (% of Control Activity)[a]			
	Suppression of Rise Induced by Transfer of Cells to Lipid-Deficient Media		Suppression of Elevated Levels Induced by Transfer of Cells to Lipid-Deficient Media	
μM	1	4	1	4
0.0	100.0 ± 2.0[b]	100.0 ± 3.3[c]	100.0 ± 4.1[d]	100.0 ± 3.7[e]
0.1	72.3 ± 3.4	50.2 ± 3.9	85.7 ± 2.1	73.3 ± 1.2
0.5	36.0 ± 2.7	29.2 ± 1.2	52.1 ± 0.9	51.5 ± 1.8
1.0	19.4 ± 1.5	31.6 ± 1.9	44.5 ± 1.7	48.7 ± 3.1
2.5	15.6 ± 0.3	18.5 ± 2.8	21.7 ± 3.7	34.7 ± 2.4
5.0	9.7 ± 1.1	13.0 ± 1.0	20.9 ± 3.0	21.1 ± 0.9

[a]Variation is expressed as ± SD of replicate (n = 3) assays for the experimental values.
[b-e]Mean values for controls were 4270, 4280, 760, and 674 pmol/min per mg protein, respectively.

and presence, respectively, of an olefinic proton in their ^{1}H NMR spectra.

The 14β-stereochemistry of the Δ^7-15-ketosterol **3** was demonstrated by comparing its NMR spectra with those of an authentic sample (7, 8) of its benzoate ester **7**, which was prepared by an independent route and for which assignment of structure was based on X-ray crystal analysis of an appropriate derivative (7, 17). The NMR chemical shifts of **3** and **7** (Tables 3 and 4) agreed to $\leq$0.1 ppm for ^{13}C and $\leq$0.02 ppm for ^{1}H (except for the usual benzoylation shifts in ring A, polarizable sp^2 carbons, and H-9). The 14β-H configuration of the Δ^8-15-ketosterol **2** was apparent from its downfield ^{13}C NMR signal for C-18 (δ 22.27). The stereochemistry of **2** was established conclusively by reducing **2** to diol **4** and conducting an LIS experiment on the 3β-TBDMS ether derivative of **4** (see below). These results parallel closely those of Van Horn and Djerassi (18), who obtained Δ^7- and Δ^8-15-ketosteroids by acid-catalyzed isomerization of 5α-androst-8(14)-en-15-one and assigned the 14β-configuration to the β,γ-unsaturated ketones based on optical rotatory dispersion.

Treatment of Δ^8-15-ketosterol **2** with $NaBH_4$ resulted in a 5:1 mixture of two 15-hydroxy epimers **4** and **5** (Fig. 1).[5] Determination of the configuration of the 15-hydroxyl groups was complicated by the involvement of multiple conformers in the flexible C–D ring system (see below), a circumstance that would introduce ambiguity into a structure determination based on ^{1}H–^{1}H coupling constants, chemical shift comparisons, or solvent-induced shifts. Therefore, the 15-hydroxyl configuration of **4** was established by an LIS experiment carried out on its 3β-TBDMS ether derivative **9**. The LIS method is analogous to that used previously to determine the structure of 5β-cholest-8-ene-3β,15α-diol (5). Comparison of the observed LIS values for **9** with those for structures derived from molecular mechanics indicated reasonable agreement[6] for the 14β-H 15β-hydroxy isomer (R 3.83%) and very poor agreement (R 16.48%) for its 15α epimer (Table 6). Thus, **4** is the 15β-hydroxy isomer.

The NMR spectra of the 14β-Δ^8-sterols displayed many anomalies. Remarkably large ^{13}C chemical shift differences between the Δ^7 and Δ^8 15-ketosterol isomers **2** and **3** were observed for several remote carbons, including C-17 (6.7 ppm), C-20 (1.2 ppm), C-22 (3.7 ppm), and C-23 (1.1 ppm). Unexpectedly large ^{1}H chemical shift differences were also found for H-12β (0.44 ppm), H-20 (0.39 ppm), and H-21 (0.10 ppm). The chemical shifts of the Δ^7 isomer **3** showed the greatest deviation from the usual ranges for C_{27} 14α-sterols, but certain NMR chemical shifts (H-17α, C-22, and C-23) of the Δ^8 sterol **2** also differed somewhat from those of other 15-oxygenated sterols (5, 6, 14, 15). Comparison of the NMR spectra of **2** and **4** also revealed unexpectedly large chemical shift differences for such remote atoms as C-12, C-20, C-21, H-12β, and H-21. Smaller but significant chemical shift differences were observed between **4** and **5** for C-12, C-20, and H-21. These combined results suggested the involvement of variable populations of discrete conformers that differ in the flexible C–D ring system and in the side chain.

Several conformers are possible for these 14β-sterols. Ring C can exist in either a twist or chair conformation (Δ^7-14β-sterols) or in a distorted twist or 12α,13β-half-chair conformation (Δ^8-14β-sterols). Twist conformations of ring C have been observed in X-ray crystal structures of 3β,15β-bis(*p*-bromobenzoyloxy)-5α,14β-cholest-7-ene, a derivative of **3** (17), and of 3-methoxy-14-methyl-14β-estra-1,3,5(10),15-tetraen-17-one (20), and such conformations have been implicated in several 14β-steroids with various substituents (20, 21). Discrete conformers are not normally observed in ring D because of the low energy barriers in the pseudorotation circuit. However, side chain conformers were explored based on the results of previous work (21) and on the NMR chemical shift differences noted above. Three conformers are possible by rotation about the C17–C20 bond.

[5]The preparation of the 3β-pivaloyl ester of **4** has recently been reported by Araki, Eguchi, and Morisaki (19). Treatment of 3β-pivaloyloxy-14α,15α-epoxy-5α-cholestane with three equivalents of boron trifluoride–etherate in benzene for 1 h at room temperature gave 3β-pivaloyloxy-5α-14β-cholest-8-en-15α-ol in 75% yield. Oxidation of the latter compound with pyridinium chlorochromate in CH_2Cl_2 gave the corresponding 14β-Δ^8-15-ketone in 73% yield. Apart from the strongly negative Cotton effect observed for the 14β-Δ^8-15-ketone, used to establish the configuration at C-14, no other physical or spectral properties were presented in this communication, a situation which precludes comparisons with the compounds described in this manuscript. Araki et al. (19) also reported that treatment of 3β-pivaloyloxy-5α,14β-cholest-8-en-15-one with 5% KOH in refluxing methanol gave the $\Delta^{8(14)}$-15-ketosterol **1**.

[6]The agreement factor for **9** (R 3.83%) was somewhat higher than those usually observed in LIS experiments. This result may be attributable to deviations between the actual conformers of **9** and ones calculated from molecular mechanics as well as to the presence of multiple conformers. Such conformational complexity may also have contributed to the inability of the LIS method to rule out the 14α-H,15α-OH isomer (R, 3.22%), a known (5) compound with spectral properties distinctly different from those of **4**. Difficulty in differentiating a 14α-H,15α-OH sterol from its 14β-H,15β-OH isomer has been noted previously for the LIS method used with molecular mechanics structures (5).

[7]Molecular mechanics calculations showed isomers **2** and **3** to be 1–2 kcal/mol higher in enthalpy than the conjugated 15-ketosterol **1**, a result generally compatible with the presence of small amounts of **2** and **3** in an equilibrated sample of **1**. Detection of small amounts of an additional isomer of **1** is consistent with calculations indicating that the 14α isomers of **2** and **3** are also only 1–2 kcal/mol higher in enthalpy than **1**. Precise estimation of relative free energies is complicated by the entropy of mixing for the conformers of **2** and **3**.

Combinations of the three side-chain rotamers with the twist and chair (half-chair) conformers of ring C gave six plausible conformations for each of sterols **2**–**5**. Preliminary molecular mechanics calculations showed at least two conformers of very similar energies (ΔH 0.1–1 kcal/mol) for each sterol.[7] The three side-chain rotamers and the twist and 12α,13β-half-chair (or chair) conformations of ring C were present in various low-energy conformers. Although definitive analysis of the conformations will require further investigation, this result is consistent with the observed anomalies in NMR chemical shifts. The absence of signal broadening in the NMR spectra of **2**, **3**, and **4** and their derivatives indicated that the interconversion barriers between their conformers are lower than those for the similar ring A–B conformers of 5β-Δ[8]-sterols (5, 22).

The reliability of the ^{13}C NMR signal assignments in the vicinity of rings C and D and C-20 was critical because they furnished the experimental evidence for conformational differences between **2**, **3**, and **4**. This conformational complexity severely limited the application of ^{13}C chemical shift comparisons with other 14β-sterols (23, 24) for distinguishing signals in these regions. Consequently, key assignments were made by correlations from HETCOR and COSY spectra. In the case of **2** and **4**, the assignments were confirmed by the LIS of derivatives, i.e., the Δ[8]-15-ketosterol acetate **6** and the TBDMS ether **9** (Table 6).

The mass spectra of the three isomers were very similar (Table 2). However, relative to **1**, both **2** and **3** showed higher abundances of ions due to losses of methyl and lower ion intensities corresponding to losses of side chain. The low resolution mass spectra of **2** and **3** were very similar with fragment ions of high abundance in the high mass region corresponding to M–CH_3 M–H_2O, M–H_2O–CH_3, M–H_2O–H_2O–CH_3, and M–SC. Exact mass measurements for each of these ions were in accord with these assignments. The mass spectra of the TMS ethers of **2** and **3** showed a dominant *m/z* 367 ion that easily distinguished their mass spectra from those of the TMS ether of **1**. Otherwise, the TMS ethers of **1**, **2**, and **3** showed similarities and differences comparable to those described above for the free sterols. The β,γ-unsaturated ketosterols **2** and **3** could not be reliably differentiated based on the mass spectral fragmentation patterns of the free sterols or their TMS ethers.

Table 5 summarizes the chromatographic properties of 15-ketosterols **1**, **2**, and **3**. Although the reversed phase HPLC conditions did not resolve **1** and **3**, normal phase HPLC, like TLC, provided a clean separation of the three 15-ketosterols. An important objective of the isolation of **2** and **3** was to establish methods for detecting possible contamination of samples of **1** by isomers **2** and **3**. Formation of these isomers is possible during the final acidic hydrolysis in the laboratory-scale synthesis of **1** (6), and the Δ[7]-15-ketosterol benzoate **7** has been indicated as an intermediate in an industrial-scale preparation of **1** (25). However, we have not detected either **2** or **3** in purified samples of **1** from either source. Tissue and blood samples containing **1** and its metabolites may also be subjected to acidic conditions during workup or derivatization. The chromatographic and spectral data presented herein provide the basis for differentiating actual metabolites from these potential artefacts of workup and separation.

3β-Hydroxy-5α-cholest-8(14)-en-15-one (**1**) and a large number of other 15-oxygenated sterols have been shown to be potent inhibitors of sterol synthesis and/or to lower the levels of HMG-CoA reductase activity in cultured mammalian cells (1–5, 14, 26–36). Almost all of the 15-oxygenated sterols studied to date have had either a Δ[8(14)]-double bond or a *trans* orientation of the C–D ring junction. Five synthetic 15-hydroxysterols with the unnatural *cis* C–D ring junction, i.e., 5α,14β-cholest-7-ene-3β,15α-diol (7, 8),5α,14β-cholest-7-ene-3β,15β-diol (7, 8), 15α-hydroxy-5α,14β-cholest-7-en-3-one (31),15β-hydroxy-5α,14β-cholest-7-en-3-one (31), and 15β-methyl-5α,14β-cholest-7-ene-3β,15α-diol (34), for which unequivocal establishment of the configurations at carbon atoms 14 and 15 was made by X-ray crystallographic analyses (7, 17, 34, 37), were shown to inhibit the synthesis of digitonin-precipitable sterols from labeled acetate and to lower the levels of HMG-CoA reductase activity in mouse L cells (1, 31, 34). 5α,14β-Cholest-7-ene-3β,15α-diol (30) was more potent than 5α,14β-cholest-7-ene-3β,15α-diol (1) in lowering the levels of HMG-CoA reductase activity in mouse L cells. In the present study we have shown that another 15-hydroxysterol with the unnatural *cis* C–D ring junction, 5α,14β-cholest-8-ene-3β,15β-diol (**4**), was highly active in lowering the levels of HMG-CoA reductase activity in CHO-K1 cells.

The Δ[8]- and Δ[7]-15-ketosterols with the *cis* C–D ring junction (**2** and **3**) also lowered the levels of HMG-CoA reductase activity in CHO-K1 cells. However, **2** and **3** were not as potent as the Δ[8(14)]-15-ketosterol **1**.

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