ELSEVIER SCIENCE IRELAND

Chemistry and Physics of Lipids
70 (1994) 163–178

Chemistry and Physics of LIPIDS

Inhibitors of sterol synthesis: effects of a 7α-alkyl analog of 3β-hydroxy-5α-cholest-8(14)-en-15-one on 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in cultured mammalian cells and on serum cholesterol levels and other parameters in rats

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(Received 7 September 1993; revision received 22 December 1993; accepted 22 December 1993)

Abstract

The 7α-methyl analog (**II**) of 3β-hydroxy-5α-cholest-8(14)-en-15-one (**I**) was prepared by chemical synthesis and evaluated with respect to its effects on HMG-CoA reductase activity in CHO-K1 cells and on serum cholesterol levels in rats. The 7α-methyl substitution had no detectable effect on the potency of **I** in lowering HMG-CoA reductase activity in the cultured cells. In contrast, the 7α-methyl substitution had a marked effect on the action of **I** in the suppression of food consumption in rats. Whereas **II** was less potent than **I** in lowering serum cholesterol levels in rats, it did so at dosage levels at which only slight or moderate effects on food consumption were observed. Full ^{1}H and ^{13}C-NMR assignments for **II** and intermediates in its synthesis have been presented. Conformational analysis, based on ^{1}H-^{1}H coupling constants, NMR shieldings and force-field calculations, indicated that the 7α-methyl substitution had virtually no effect on the conformation of the 15-ketosterol apart from minor distortions of ring B.

Key words: 15-oxygenated sterols; ^{1}H and ^{13}C-NMR; Mass spectrometry; Conformational analysis

1. Introduction

3β-Hydroxy-5α-cholest-8(14)-en-15-one (**I**) (Fig. 1) is a potent inhibitor of cholesterol biosynthesis in cultured mammalian cells and lowers the levels of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase activity in these cells [1–5]. **I** has significant hypocholesterolemic action upon oral administration to rodents [6–8] and non-human primates [9,10]. **I** serves as an alternative substrate for acyl coenzyme A:cholesterol acyltransferase (ACAT) and inhibits the oleoyl-

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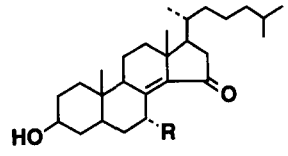
SSDI 0009-3084(94)002295-G



I R = H
II R = CH_3

Fig. 1. Structures of 3β-hydroxy-5α-cholest-8(14)-en-15-one (I) and 3β-hydroxy-7α-methyl-5α-cholest-8(14)-en-15-one (II).

CoA-dependent esterification of cholesterol in jejunal microsomes [11]. Oral administration of I to rats lowers the levels of ACAT activity in jejunal microsomes [12] and inhibits the intestinal absorption of cholesterol [13,14].

As part of a program designed to establish structure-activity relationships for 15-oxygenated sterols, we have evaluated the effects of 7α-methyl substitution on the actions of I. 3β-Hydroxy-7α-methyl-5α-cholest-8(14)-en-15-one (II), prepared by chemical synthesis (Fig. 2), was studied with respect to its effects on the levels of HMG-CoA reductase activity in CHO-K1 cells and on serum cholesterol levels in rats.

2. Experimental procedures and results

2.1. Materials and methods

3β-Hydroxy-5α-cholest-8(14)-en-15-one (I) and 3β-benzoyloxy-14α,15α-epoxy-5α-cholest-7-ene

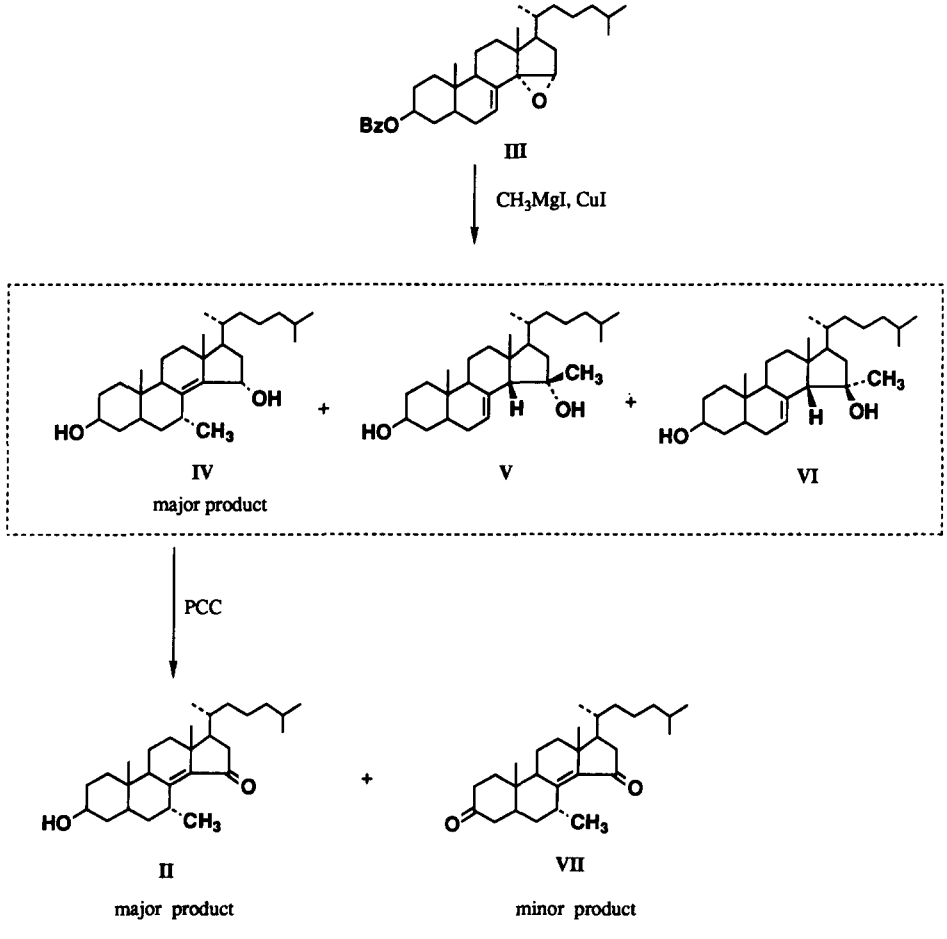


Fig. 2. Chemical synthesis of 3β-hydroxy-7α-methyl-5α-cholest-8(14)-en-15-one (II).

(III) were prepared as described previously [15]. Cholesterol was purified by way of its dibromide derivative [16]. [7(n)-^{3}H]Cholesterol (15 Ci per mmole) and [1,2,6,7(n)-^{3}H]cholesteryl oleate (65.8 Ci per mmole) were obtained from Amersham (Arlington Heights, IL). [2,4-^{3}H]3β-Hydroxy-5α-cholest-8(14)-en-15-one (13.5 mCi per mmole) was prepared by a minor modification of a procedure described previously [17].

Phosphate-buffered saline (PBS) (KCl, 2.7 mM; KH_2PO_4, 1.2 mM, NaCl, 137 mM; and Na_2HPO_4, 8.1 mM) and trypsin were purchased from Gibco Laboratories (Grand Island, NY). Ham's F12 medium [18] was obtained from Irvine Scientific (Irvine, CA). Fetal calf serum was purchased from Whittaker M.A. Bioproducts (Elkhart, IN). Lux tissue culture plasticware was from Miles Scientific (Elkhart, IN). (3RS)-[3-^{14}C]HMG-CoA (56 mCi per mmol) and (3RS)-[2-^{3}H]mevalonolactone (176 mCi per mmol) were purchased from Amersham. Chinese hamster ovary (CHO-K1) cells were obtained from the American Type Culture Collection (Rockville, MD).

Melting points were measured with a Thomas-Hoover apparatus in sealed, evacuated capillary tubes. Ultraviolet (UV) spectra were recorded on an IBM 9430 spectrophotometer using ethanol as the solvent. Infrared (IR) spectra were obtained on a Mattson Galaxy 6020 Fourier-transform IR spectrometer with KBr pellets. Thin-layer chromatography (TLC) was carried out on silica gel G plates (Analtech, Newark, DE) or on aluminum-backed silica gel 60 plates (EM Separations, Gibbstown, NJ) or, in the case of assays of HMG-CoA reductase activity, Whatman LK5D plates (American Scientific Products, Houston, TX). Components of the plates were visualized after spraying with 5% ammonium molybdate(VI) in 10% sulfuric acid followed by heating. Solvent systems were: SS-1, 40% ethyl acetate in hexane; SS-2, 50% ethyl acetate in hexane; SS-3, 50% ether in benzene; SS-4, 5% water in methanol. Unless otherwise specified, column chromatography was performed on silica gel (70–230 mesh; Aldrich Chemical Co.), medium-pressure liquid chromatography (MPLC) was done on a Lobar column (440 mm × 37 mm i.d., Lichroprep Si 60, 40–63 μm; EM Separations), and fraction volumes were 20 ml. Steroid samples were adsorbed onto silica gel by rotary evaporation from an ethyl acetate solution of the steroid containing silica gel (~3 g per g of steroid) and eluted from a small column onto the main MPLC column. High-performance liquid chromatography (HPLC) was performed isocratically on a Waters HPLC system with a 5-μm Spherisorb analytical column (250 mm × 4.6 mm i.d.; 1.0 ml/min; UV detection at 259 nm) or a Customsil C_{18} semipreparative column (250 mm × 9.4 mm i.d.; 3.0 ml/min; UV detection at 210 nm). The columns were obtained from Custom LC (Houston, TX). Solvents for analytical studies were HPLC grade. Colorimetric assay of cholesterol and cholesteryl esters in effluents of silicic acid-Super Cel columns was carried out using the color reagent described by Abell et al. [19].

Capillary gas chromatography (GC) was carried out using splitless injection on a Shimadzu GC-9A unit (1.3 kg per cm^2 nitrogen). The columns used were Rt_x 1701 (15 m × 0.25 mm i.d.; 14% cyanopropylphenyl, 86% methyl polysiloxane; 0.1 μm film thickness; Restek Corporation, Bellefonte, PA) and DB-5 (30 m × 0.25 mm i.d.; 5% phenyl, 95% methyl polysiloxane; 0.1 μm film thickness; J&W Scientific, Inc., Folsom, CA). The injector and flame-ionization detector were maintained at 290°C, and the column temperature was programmed as follows: 100°C for 3 min, 100–250°C at 20°C per min and 250°C for 15 min (Rt_x 1701 column) or 200°C for 3 min, 200–280°C at 20°C per min and 280°C for 15 min (DB-5 column). Trimethylsilyl (TMS) ether derivatives of the sterols were prepared using a 1:1 mixture (200 μl) of bis(trimethylsilyl)trifluoroacetamide (BSTFA) and pyridine for 1 h under nitrogen at room temperature, followed by evaporation to a residue that was dissolved in hexane (100 μl), from which aliquots (1 μl) were taken for GC analysis. Low-resolution mass spectra (MS) were recorded on a Shimadzu QP-1000 quadrupole spectrometer with an electron energy of 70 eV and direct-inlet sample introduction. GC-MS analyses were carried out as described previously [20,21] using a 15-m DB-5 capillary column with direct introduction of the effluent into the ion source of the mass spectrometer (Extrel ELQ-400). The definition of ions A, B, C and D have been presented previously [20,22].

Nuclear magnetic resonance (NMR) spectra

were measured on an IBM AF300 (75.5 MHz for ^{13}C, ~22°C) or a Bruker AMX500 (500.1 MHz for ^{1}H, 27°C) spectrometer in $CDCl_3$ solution and referenced to internal tetramethylsilane (^{1}H) or $CDCl_3$ at 77.0 ppm (^{13}C). Standard Bruker software was used to acquire DEPT (distortionless enhancement by polarization transfer), COSY-DEC (ω_1-decoupled ^{1}H-^{1}H correlation spectroscopy, τ_e 0.2 s) [23], HETCOR (^{1}H-^{13}C shift-correlated spectroscopy; ~50 increments, δ 0.6–2.6 in the ^{1}H dimension) and nuclear Overhauser effect (NOE) difference spectra (low-power irradiation for 1 s, 90° read pulse, 2.7-s acquisition time, 16 scans per cycle, non-degassed sample, no spinning). Saturation difference spectra [24,25] were acquired under NOE conditions. Coupling constants were derived from line spacings of 1D resolution-enhanced spectra and confirmed in some cases by spin simulation with NMR″ (Calleo Scientific Software, Ft. Collins, CO). Modeling of sterols by molecular mechanics was done with PCMODEL (Macintosh version 4.4, Serena Software, Bloomington, IN) and used to predict ^{1}H-NMR vicinal coupling constants. The purity of sterol samples was determined by HPLC, TLC and ^{1}H-NMR (500 MHz, after sufficient magnification of the vertical scale in the methyl and δ 2–6 spectral regions to detect a 1% impurity).

2.2. *Effects of added sterols on levels of HMG-CoA reductase activity in cultured mammalian cells*

CHO-K1 cells were maintained in a lipid-rich medium (Ham's F12 medium [18] supplemented with 5% fetal calf serum) 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), the cells were incubated for 18 h in lipid-deficient media (10 ml; Ham's F12 medium supplemented with 5% delipidated [26] fetal calf serum). The cells were then incubated for 4 h at 37°C with fresh lipid-deficient media (10 ml) containing various concentrations of the oxygenated sterols. Lipid-deficient media containing the oxygenated sterols were prepared by the addition of ethanol solutions of the sterols to the lipid-deficient medium, followed by equilibration at room temperature for at least 6 h prior to storage at 4°C. The ethanol concentration in all experimental media was constant. The media were removed, the plates were rinsed with ice-cold PBS and the cells were harvested by scraping into ice-cold PBS (5 ml) containing dithiothreitol (5 mM). Detergent-solubilized cell preparations were obtained for assay of HMG-CoA reductase activity using the method of Brown, Dana and Goldstein [27]. Replicate assays (n = 3) were carried out as described by Pinkerton et al. [3], except that the specific activity of the (3RS)-[3-^{14}C]HMG-CoA was 20 000 dpm per nmol.

2.3. *Effects of dietary administration of 3β-hydroxy-7α-methyl-5α-cholest-8(14)-en-15-one (II)*

Male rats of the Sprague-Dawley strain were purchased from Harlan Sprague-Dawley (Houston, TX) and housed in pairs for 6 days on a light (6:00–18:00 h)/dark cycle and fed a basal diet (Purina Formulab 5008) and water ad libitum. The rats were then divided into groups of eight animals each, so that the mean values of serum cholesterol and body weight were approximately the same for each group. The animals were then housed individually and, unless indicated otherwise, provided with diet and water ad libitum. The body weight and food consumption of the individual rats were determined daily. Blood for serum sterol determinations was obtained at ~8:00 h from tail vein on days 5 and 9, and 'neck blood' was obtained at the time of death on the morning of day 10. The experiments were terminated with ketamine anesthesia (0.2 ml, 100 mg/ml) followed by decapitation. Unless indicated otherwise, the following organs were removed, cleaned and weighed: liver, heart, small intestine, kidneys, adrenal glands, testes and spleen.

Two experiments (A and B) involved the dietary administration of the 7α-methyl-15-ketosterol **II**. In Experiment A one group of rats received **II** at a level of 0.10% by weight in diet (2.42 μmol per

g of diet). A second group, designated as ad libitum controls, had free access to the basal diet. The third group, designated as pair-fed controls, received basal diet but only in the amount consumed by its individual counterpart in the sterol-treated group on the previous day. Experiment B involved three groups of rats: an ad libitum control group and two groups receiving **II** at levels of either 0.15 or 0.20% by weight in diet (3.62 or 4.84 μmol per g of diet, respectively).

Serum cholesterol was measured using a commercial assay kit ('Single Vial', Boehringer Mannheim Diagnostics, cat. no. 236691). The use of this method was justified by the demonstrated absence of significant levels of sterols other than cholesterol in serum which might affect the validity of the assay (see below). For example, the levels of **II** in serum on day 10 from animals treated with the 7α-methyl-15-ketosterol at 0.10, 0.15 and 0.20% were very low, i.e. 8.2, 6.7 and 8.7 μg per ml (see below). Accordingly, although **II** acts as a substrate for cholesterol oxidase, corrections for the presence of the low levels of **II** in serum in the determination of cholesterol levels were not made.

Statistical comparisons were made using a paired Student's *t* test. Unless indicated otherwise, variation is expressed as standard error of mean.

2.4. 7α-Methyl-5α-cholest-8(14)-ene-3β,15α-diol (IV)

Compound **IV** was prepared by the following minor modification of our previously published procedure [28]. Methyl magnesium iodide was prepared by the addition of methyl iodide (21.12 g, 149 mmol) to a suspension of magnesium turnings (3.62 g, 149 mmol) in ether (50 ml). Copper (I) iodide (2.83 g, 14.9 mmol) was added, the mixture was cooled to 0°C, and a cooled solution of epoxide **III** (5 g, 9.92 mmol) in tetrahydrofuran-benzene (250 ml, 1:1 ratio) was added dropwise over 2 h. The mixture was stirred for 3 h at 0°C and stored overnight at 4°C. The reaction mixture was poured into saturated aqueous NH_4Cl, followed by extraction with ethyl acetate. The combined organic layers were dried over anhydrous sodium sulfate and evaporated to a yellow solid (5 g). This residue was adsorbed onto silica gel (15 g) and subjected to MPLC (elution with 20% ethyl acetate in hexane). Evaporation of fractions 202–286 gave, in addition to other components (see below), **IV** as a white solid (2.1 g, 51% yield): m.p. 191–192.5°C, lit. m.p. 192.5–193.5°C [28]; TLC, single component in SS-1 (R_f 0.55) and SS-3 (R_f 0.43); HPLC in SS-4, t_R 5.0 min (98% purity); ^{1}H-NMR, ^{1}H-^{1}H-NMR coupling constants and ^{13}C-NMR, Tables 1, 2, and 3.

A similar reaction of epoxide **III** (10 g) with methyl iodide gave a crude product, of which 5 g was subjected to MPLC (elution with 1000 ml each of increasing concentrations of ethyl acetate in hexane: 5:95, 10:90 and 15:85, followed by 3400 ml of ethyl acetate-hexane 20:80). Fractions 166–188 gave the benzoate ester of **IV** (140 mg), fractions 204–286 gave **IV** (2.05 g), and fractions 290–318 gave 15β-methyl-5α,14β-cholest-7-ene-3β,15α-diol (**V**; 780 mg): ^{1}H-NMR, ^{1}H-^{1}H-NMR coupling constants and ^{13}C-NMR, Tables 1, 2, and 3.

Fractions 319–351 contained a mixture (780 mg) of **V** and 15α-methyl-5α,14β-cholest-7-ene-3β,15β-diol (**VI**). Further elution with ethyl acetate-hexane (30:70, 200 ml) gave **VI** (368 mg): ^{1}H-NMR, ^{1}H-^{1}H-NMR coupling constants and ^{13}C-NMR, Tables 1, 2, and 3.

In subsequent preparations of **IV** from **III**, the crude product was recrystallized from ethyl acetate to give **IV** of ~98% purity in 51–55% yield.

2.5. 3β-Hydroxy-7α-methyl-5α-cholest-8(14)-en-15-one (II)

Compound **II** was prepared by the following modification of our previously published procedure [28]. Pyridinium chlorochromate (8.75 g, 40.5 mmol) was added to a solution of **IV** (5.0 g, 12 mmol) in dichloromethane:pyridine (50:1,500 ml) cooled to 2–3°C, and the mixture was stirred at 2–3°C for 75 min under nitrogen. Ether (200 ml) was added, and the mixture was filtered through Florisil (50 g) and eluted with additional ether (200 ml). The ether solution was evaporated to a greenish oil (4.87 g) that was adsorbed onto silica gel (15 g) and subjected to MPLC (elution with 10% ethyl acetate in hexane (1000 ml), 15% ethyl acetate in hexane (1000 ml) and 20% ethyl acetate). Fractions 75–101 gave 7α-methyl-5α-cholest-

Table 1
^{1}H-NMR chemical shifts for 7α-methyl-15-ketosterol **II**, synthetic intermediates and 15-ketosterol **I**[a,b]

	IV	V	VI	VII	II	I
H-1α	1.15	1.06	1.06	1.57	1.19	1.20
H-1β	1.68	1.85	1.86	2.02	1.71	1.72
H-2α	1.83	1.80	1.81	2.33	1.85	1.84
H-2β	1.37	1.40	1.41	2.37	1.37	1.37
H-3α	3.65	3.60	3.61		3.66	3.64
H-4α	1.56	1.73	1.73	2.11	1.57†	1.68
H-4β	1.25	1.25	1.26	2.21	1.23	1.27
H-5α	1.61	1.47	1.42	2.05	1.64	1.42
H-6α	1.14	1.89	1.88	1.26	1.19	1.48
H-6β	1.52	1.77	1.77	1.62	1.56	1.35
H-7(α)		5.35	5.46			1.58
H-7β	2.88			4.38	4.38	4.14
H-9α	2.04	1.83†	1.66	2.22	2.11	1.85
H-11α	1.61	1.55	1.51	1.69	1.64	1.64
H-11β	1.53	1.26	1.21	1.61	1.53	1.54
H-12α	1.16	1.71	1.39	1.26	1.22	1.25
H-12β	1.95	1.64	1.78	2.13	2.09	2.10
H-14		1.76	2.02			
H-15β	4.71					
H-16α	1.81	1.84	1.81	2.38	2.35	2.35
H-16β	1.71	1.35	1.48	2.08	2.07	2.05
H-17α	1.46	1.72	1.35	1.44	1.42	1.46
H-18	0.816	0.944	1.000	1.011	0.974	0.973
H-19	0.729	0.734	0.731	0.927	0.715	0.717
H-20	1.46	1.51	1.55	1.58	1.57	1.58
H-21	0.951	0.920	0.903	1.005	0.994	0.998
H-22R	1.38	1.31	1.35	1.34†	1.33	1.33
H-22S	1.08	0.97	0.98	1.07	1.06	1.07
H-23R	1.37	1.37	1.34	1.34†	1.33	1.33
H-23S	1.16	1.17	1.18	1.19	1.19	1.19
H-24	1.12†	1.11	1.10	1.10	1.10	1.10
H-24	1.15†	1.15	1.15	1.15	1.15	1.15
H-25	1.52	1.52	1.52	1.52	1.52	1.51
H-26	0.865	0.866	0.864	0.869	0.865	0.862
H-27	0.869	0.870	0.866	0.872	0.868	0.865
CH_3[c]	1.180	1.187	1.133	1.011	1.008	

[a]Data obtained at 500.1 MHz in $CDCl_3$ solution at a concentration of 0.01–0.1 M. Chemical shifts referenced to $Si(CH_3)_4$ signal.
[b]Chemical shifts are generally accurate to 0.01 ppm, except for values marked by † (±0.02 ppm). Pro-R and pro-S assignments of C-22 and C-23 protons are based on comparisons with assignments in Refs. 33 and 35.
[c]Substituent at C-7 or C-15.

8(14)-ene-3,15-dione (**VII**, 1.13 g, 23% yield). Fractions 178–220 gave **II** (2.67 g, 54% yield), and fractions 221–276 gave **II** (0.67 g) of lower purity. Samples of **II** of ~99% purity from this and from multiple analogous preparations (total, 14 g) were combined and recrystallized from acetone-water to give **II** (13.1 g) used for biological testing: m.p. 121–122°C (lit. m.p. 120–121°C [28]); TLC, single

Table 2
^{1}H-^{1}H-NMR coupling constants for 7α-methyl-15-ketosterol **II**, synthetic intermediates and 15-ketosterol **I**[a]

	IV	V	VI	VII	II	I[b]
1α-1β	13.1	13.2	13.1	13.3	13.1	13.1
1α-2α	3.5*	3.5	3.2		3.7	3.5
1β-2β	13.8*	13.9	13.7*	12.4*	13.7	13.9
1β-2α	3.5*	3.3	3.6*	3.1	3.5*	3.3
1β-2β	3.5*	3.3	3.6*	5.6	3.5*	3.7
2α-2β	12.8	12.3*		15.3	12.8	12.7
2α-3α	4.7	4.5*	4.5		4.7*	5.0
2α-4α	2.1		2.3	1.6	2.1	1.9
2β-3α	11.1	11.2	11.1		11.2	11.2
3α-4α	5.0	4.5*	4.5		4.7*	4.9
3α-4β	11.1	11.1	11.0		11.1	11.0
4α-4β	12.3	12.2	12.3	14.9	12**	12.3
4α-5α	2.9*	3.7	3.6	4.0	3.1*	2.7*
4β-5α	12.4	12.6	12.4*	13.3	13.1**	12.2
5α-6α	3.0*	5.0	4.5*	3.5	3.1*	2.9*
5α-6β	12.7*	11.2		12.8	13.1**	12.0
6α-6β	13.0*	17.2	17.9	13.8	13.2**	12.4
6α-7(β)	1.8	5.3	5.3	1.5	1.5*	2.2
6β-7(β)	5.3	2.6*	2.3*	5.9	5.9	4.2
7β-CH_3	7.1			7.4	7.4	
9α-11α	8.3	4.7*		7.2	7.1	7.1
9α-11β	9.9	11.9*	13.1**	10.4	10.4	10.5
11α-11β	14.0*	13.1	13.1**	13.9	13.8	14.1
11α-12α		3.3		3.9	3.8*	3.8
11α-12β	3.1	4.6		3.4	3.3*	3.3
11β-12α	14.0*	12.6	13.1**	13.6	14.1	14.1
11β-12β	4.1	3.7	2.9	3.5	3.6	3.6
12α-12β	12.3	13.3		12.9	12.7	12.7
15β-16α	<1					
15β-16β	6.2					
16α-16β	13.6	12.9	11.7	18.4	18.3	18.5
16α-17α	5.6	6.9	6.3	7.7	7.7	7.8
16β-17α	12.4	12.4	13.2	12.5	12.6	12.4
17α-20		9.1	9.6	9.7	9.7	9.8
20-21	6.3	6.4	6.4	6.1*	6.7	6.6
20-22R		2.8*	2.9*			
20-22S		9.1*	9.1*			
24R-25	6.6*	6.7*	6.6*	6.6*	6.7*	6.6*
24S-25	6.6*	6.7*	6.6*	6.6*	6.7*	6.6*
25-26	6.6	6.7	6.6	6.6	6.6	6.6
25-27	6.6	6.6	6.6	6.6	6.6	6.6

[a]Coupling constants in Hz, generally accurate to ±0.2 Hz except for values marked by * (±0.5 Hz) or ** (±1 Hz).
[b]Additional couplings for **I**: 6α-7α, 5.6 Hz; 6β-7α, 4.2 Hz; 7α-7β, 14.3 Hz.

Table 3
^{13}C-NMR chemical shifts for 7α-methyl-15-ketosterol **II** and synthetic intermediates[a]

	IV	V	VI	VII	II
C-1	36.26	36.81	36.95	38.02	36.49
C-2	31.35	31.41	31.31	37.85	31.16
C-3	71.16	70.91	70.75	211.17	70.79
C-4	37.90	37.82	37.75	44.12	37.75
C-5	38.19	39.88	39.84	40.26	37.97
C-6	35.72	30.33	30.25	35.03	34.86
C-7	32.81	125.13	123.10	27.40	27.57
C-8	139.32	135.10	136.36	154.61	156.44
C-9	43.90	49.51	49.63	45.26	45.89
C-10	38.28	34.14	33.93	38.65	38.65
C-11	19.81	20.96	21.05	19.42	19.32
C-12	37.45	40.98	41.40	36.77	36.90
C-13	43.39	42.19	39.91	42.41	42.44
C-14	146.82	63.86	64.33	140.35	139.78
C-15	70.54	80.44	77.96	207.54	207.74
C-16	38.98	44.70	44.47	42.52	42.63
C-17	53.50	53.80	53.89	50.45	50.51
C-18	18.81	23.06	23.77	19.00	19.02
C-19	12.33	12.22	12.29	11.54	12.43
C-20	33.57	33.41	33.25	34.39	34.41
C-21	19.13	19.98	19.91	19.18	19.20
C-22	36.06	35.53	35.63	35.74	35.78
C-23	23.86	24.29	23.83	23.54	23.56
C-24	39.45	39.46	39.33	39.28	39.31
C-25	27.96	27.96	27.85	27.88	27.92
C-26	22.51	22.51	22.49	22.46	22.49
C-27	22.80	22.80	22.72	22.69	22.72
CH_3[b]	21.86	26.94	24.73	21.29	21.51

[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.
[b]Substituent at C-7 or C-15.

component in SS-2 (R_f 0.71) and SS-3 (R_f 0.68); HPLC in SS-4, t_R 10.5 min (≥99.5% purity by UV detection at 210 and 259 nm); UV λ_{max} 261 (ε 13 600) (lit. [28] λ_{max} 259 (ε 11 400)); MS, 414 (M^+; 51), 399 (M-CH_3; 15), 396 (M-H_2O; 61), 381 (M-H_2O-CH_3; 32), 363 (M-H_2O-H_2O-CH_3; 5), 311 (ion D-H_2O; 6), 301 (M-SC; 15), 283 (M-SC-H_2O; 100), 275 (ion B-CH_3; 5), 273 (ion C; 7), 269 (M-SC-H_2O-14; 6), 265 (M-SC-H_2O-H_2O; 17), 107(30) and 105(28). The MS of the TMS ether of **II** is presented in Table 4. ^{1}H-NMR, ^{1}H-^{1}H-NMR coupling constants and ^{13}C-NMR are presented in Tables 1, 2, and 3.

Dione **VII** [28] was identified by ^{1}H and ^{13}C-NMR spectroscopy (Tables 1, 2 and 3).

2.6. Effects of 3β-hydroxy-7α-methyl-5α-cholest-8(14)-en-15-one (II) and 3β-hydroxy-5α-cholest-8(14)-en-15-one (I) on levels of HMG-CoA reductase activity in CHO-K1 cells

The 7α-methyl analog (**II**) of the 15-ketosterol was highly active in lowering the elevated levels of HMG-CoA reductase activity induced by transfer of the CHO-K1 cells to lipid-deficient media (Table 5). The potency of **II** did not differ significantly from that of the 15-ketosterol **I**.

Table 4
Mass spectral data of TMS derivative of synthetic 3β-hydroxy-7α-methyl-5α-cholest-8(14)-en-15-one (**II**) along with those of **II** found in free and esterified forms in rat liver after its administration to rats at a level of 0.2% in diet for 10 days[a]

Ion (*m/z*)	Suggested assignment	Relative abundance in mass spectra of TMS derivative of **II**		
		Synthetic	Liver	
			Free	Ester
486	M^+	100	74	74
471	M-CH_3	29	23	20
468	M-H_2O	46	57	51
453	M-CH_3-H_2O	11	9	12
396	M-TMSOH	1	9	7
381	M-TMSOH-CH_3	32	30	28
378	M-TMSOH-H_2O	12	12	12
373	M-SC	9	10	10
363	M-TMSOH-CH_3-H_2O	22	20	19
355	M-SC-H_2O	48	60	57
341		4	6	7
301	Ion A	4	7	6
287	Ion A-14	2	6	8
283	M-SC-TMSOH	27	19	17
275	Ion B-CH_3	5	6	8
273	Ion A-28	6	10	11
265	M-SC-TMSOH-H_2O	71	100	100
107		24	44	44
105		28	42	40

[a]Ion abundances by GC-MS expressed as percentage of base peak (*m/z* > 100). Ions A and B are defined in Refs. 20 and 22.

Table 5
Effect of 3β-hydroxy-7α-methyl-5α-cholest-8(14)-en-15-one (II) and 3β-hydroxy-5α-cholest-8(14)-en-15-one (I) on levels of HMG-CoA reductase activity in CHO-K1 cells

Sterol concentration (μM)	HMG-CoA reductase activity (% of control)	
	II[a] (7α-methyl-15-ketosterol)	I[b] (15-ketosterol)
0.0	100.0	100.0
0.1	68.2 ± 2.5	62.5 ± 2.8
0.25	40.0 ± 0.2	45.8 ± 2.0
0.50	32.9 ± 0.6	36.6 ± 1.6
1.0	29.4 ± 1.5	28.8 ± 1.4
2.5	22.1 ± 0.6	23.6 ± 1.6

[a]Mean ± S.D. for replicate ($n = 3$) assays of HMG-CoA reductase activity.
[b]Mean ± S.E.M. of 40 independent experiments in which triplicate determinations of enzyme activity were made at each concentration.

2.7. *Effects of dietary administration of 3β-hydroxy-7α-methyl-5α-cholest-8(14)-en-15-one to rats: studies of the chemical nature of sterols in blood and liver*

The liver (9.17 g) of one rat that received the 7α-methyl-15-ketosterol **II** (0.20% in diet) for 10 days was homogenized with a 2:1 mixture (300 ml) of $CHCl_3$ and methanol. The resulting mixture was filtered, and, after washing of the filter twice with 2:1 $CHCl_3$-methanol (50 ml portions), the filtrate was mixed with water (100 ml). The separated organic phase was washed twice with water and evaporated to dryness under reduced pressure. The residue was dissolved in toluene (10 ml) and applied to a 1:1 silicic acid-Super Cel column (100 cm × 2.2 cm) along with internal standards of [7(n)]^{3}H]cholesterol (10^6 dpm), [1,2,6,7(n)-^{3}H]-cholesteryl oleate (10^6 dpm) and [2,4-^{3}H]3β-hydroxy-5α-cholest-8(14)-en-15-one (10^6 dpm). The column was eluted with toluene, which, at fraction 60, was changed to toluene-ether (92:8). Fractions 11 ml in volume were collected, and aliquots were taken for assay of ^{3}H and for colorimetric assay of cholesterol and cholesteryl esters (Fig. 3).

Capillary GC (Rt_x 1701 column) analyses of the TMS derivative of aliquots of fractions 42–50 (corresponding to the mobility of cholesterol) showed one major component with the retention time of the TMS ether of cholesterol and trace amounts (<1%) of material with the retention times of the TMS ethers of β-sitosterol and campesterol. The remainder of the material in fractions 42–50 was pooled, and the residue obtained after evaporation of the solvent under reduced pressure was dissolved in methanol-ethyl acetate (9:1) and subjected to reversed-phase HPLC on a semi-preparative C_{18} column using methanol (3 ml per min) as the solvent and UV detection at 210 nm. The resulting chromatogram showed a single peak with the same retention time (22.5 min) as cholesterol.

The contents of fractions 14–20, corresponding to the location of cholesteryl esters as determined colorimetrically, were saponified with 10% ethanolic KOH (3 ml) at 70°C for 2 h, followed by extraction with hexane (3 × 4 ml). The resulting free sterols were subjected to reversed-phase HPLC on a semi-preparative C_{18} column using

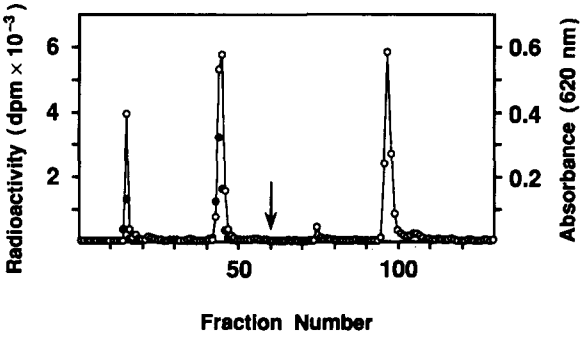


Fig. 3. Silicic acid-Super Cel column chromatographic analysis of total lipid extract of liver of rat treated with 3β-hydroxy-7α-methyl-5α-cholest-8(14)-en-15-one (0.2% in diet for 10 days) along with internal standards of (in order of elution) [1,2,6,7(n)-^{3}H]cholesteryl oleate, [7(n)-^{3}H]cholesterol and [2,4-^{3}H]3β-hydroxy-5α-cholest-8(14)-en-15-one; O——O, radioactivity; ●——●, cholesterol or cholesteryl esters determined colorimetrically. The arrow at fraction 60 marks the change in mobile phase from toluene to toluene-ether (92:8).

15-ketosterol (and of the 7α-methyl-15-ketosterol), were combined and subjected to mild alkaline hydrolysis by treatment with potassium carbonate (200 mg) in methanol (3 ml) for 3 h at 50°C, followed by extraction with methyl-t-butyl ether (3 × 5 ml). The resulting free sterols were subjected to reversed-phase HPLC on a semi-preparative C_{18} column using methanol-water (95:5) as solvent (3 ml per min). The material with the mobility of authentic 7α-methyl-15-ketosterol **II** (13.7 min) was analyzed by GC-MS in the form of its TMS derivative. The retention time and MS (Table 4) were essentially the same as those of the TMS derivative of an authentic sample of **II**. Independent GC analysis on an Rt_x 1701 column (using an internal standard of the TMS derivative of cholesterol) indicated a level of **II** (in the ester form) of 0.9 μg per g of liver.

The contents of fractions 86–102, corresponding to the mobility of the 15-ketosterol **I** (and a component with absorbance at 259 nm in fractions 88–93), from the silicic acid-Super Cel column were combined and subjected to reversed-phase HPLC on a semi-preparative C_{18} column (solvent methanol-water (95:5); 3 ml per min). The material with the mobility of the authentic 7α-methyl-15-ketosterol **II** (13.6 min) was subjected to GC-MS analysis in the form of its TMS derivative. The retention time and MS (Table 4) were essentially the same as that of the TMS derivative of an authentic sample of **II**. Independent GC analysis on a DB-5 column (using an internal standard of the TMS ether of cholesterol) indicated a level of **II** of 7.5 μg per g of liver.

The livers of four additional rats that received **II** at a level of 0.20% in diet for 10 days were analyzed in a similar fashion. The mean levels of free, esterified and total **II** in the livers of the five rats were 11.1 ± 2.0, 5.2 ± 1.2 and 16.3 ± 1.3 nmol per g. The liver of another rat that received **II** at a level of 0.10% in diet for 10 days was also studied by the same method. The levels of **II** in the free and esterified states were 5.1 and 1.2 nmol per g of liver, respectively.

The nature of sterols in serum was studied as follows. From the serum samples obtained at time

Table 6
Effect of dietary administration of 3β-hydroxy-7α-methyl-5α-cholest-8(14)-en-15-one (**II**) (at levels of 0.10, 0.15 and 0.20% in diet for 10 days) on mean values of daily food consumption (g ± S.E.M.; $n = 8$) in male Sprague-Dawley rats

Day	Food consumption (g)					
	Experiment A			Experiment B		
	Control (Ad libitum)	Compound **II** (0.1%)	Control (Pair-fed)	Control (Ad libitum)	Compound **II** (0.15%)	Compound **II** (0.20%)
1	15.1 ± 0.8	14.3 ± 0.6[a]	16.1 ± 0.2	15.1 ± 1.6	14.3 ± 0.8	14.5 ± 0.6
2	16.2 ± 0.3	15.6 ± 0.4	11.4 ± 1.8	17.2 ± 0.3	15.0 ± 0.6[b]	13.2 ± 0.5[c]
3	17.1 ± 0.3	16.1 ± 0.4	15.6 ± 0.4	17.2 ± 0.3	13.7 ± 0.5[b]	11.2 ± 0.6[c]
4	16.1 ± 0.2	16.2 ± 0.3	16.0 ± 0.4	16.7 ± 1.0	14.7 ± 0.5	13.6 ± 0.6[c]
5	17.0 ± 0.4	17.2 ± 0.3	16.2 ± 0.3	18.0 ± 0.6	15.8 ± 0.5[b]	12.9 ± 0.7[c]
6	17.0 ± 0.4	17.6 ± 0.3	17.2 ± 0.3	18.4 ± 0.5	16.4 ± 0.7	14.4 ± 0.4[c]
7	16.9 ± 0.5	17.5 ± 0.4	17.6 ± 0.3	18.1 ± 0.3	16.7 ± 0.6[b]	14.0 ± 0.7[c]
8	16.9 ± 0.4	17.4 ± 0.4	17.5 ± 0.4	19.3 ± 0.5	17.5 ± 0.7	16.8 ± 0.7[c]
9	16.1 ± 0.5	17.1 ± 0.4	17.4 ± 0.4	19.4 ± 0.4	16.4 ± 1.0[b]	15.7 ± 0.5[c]
10	15.6 ± 0.4	16.8 ± 0.4	17.1 ± 0.4	18.4 ± 0.4	17.9 ± 0.8	16.8 ± 0.9

[a]The food consumption on day 1 was less than that of the pair-fed control group ($P = 0.035$).
[b]The values of the food consumption on day 2 ($P = 0.0013$), day 3 ($P = 0.001$), day 5 ($P = 0.030$), day 7 ($P = 0.040$) and day 9 ($P = 0.021$) were less than those for the ad libitum controls.
[c]The values of the food consumption on day 2 ($P = 0.0001$), day 3 ($P = 0.0002$), day 4 ($P = 0.036$), day 5 ($P = 0.0006$), day 6 ($P = 0.0006$), day 7 ($P = 0.0024$), day 8 ($P = 0.016$) and day 9 ($P = 0.0013$) were less than those for the ad libitum controls.

of death (day 10) from each of the animals in each group, an aliquot (200 μl) was taken and combined to give a pooled sample for each group. The pooled samples were subjected to mild alkaline hydrolysis as described above. A small aliquot of the free sterols, obtained by extraction with hexane (3 × 10 ml), was analyzed by GC in the form of its TMS derivative. In each group (including the ad libitum controls) one major sterol was observed with the retention time of the TMS derivative of cholesterol (14.4 min), as well as very low levels of two components with the retention times of the TMS derivative of β-sitosterol (16.9 min) and campesterol (15.6 min). In addition, the samples from the groups treated with **II** at levels of 0.1, 0.15 and 0.20% in diet showed very low levels of a component with the retention time of the TMS derivative of the 7α-methyl-15-ketosterol **II** (19.7 min). This component was absent in the sample obtained from control animals that did not receive **II**. The levels of **II** in serum were determined as follows. The free sterols from each of the groups treated with **II** were subjected to reversed-phase HPLC on a C_{18} column. The solvent system was methanol-water (95:5) for 17 min, followed by methanol for 25 min. Under these conditions the 7α-methyl-15-ketosterol and cholesterol eluted at 13.5 min and 36–38 min, respectively. The material with the mobility of **II** was analyzed by GC on an Rt_x 1701 column along with an internal standard of the TMS derivative of cholesterol (10 μg). The levels of **II** in the serum from the animals treated with **II** at levels of 0.10, 0.15 and 0.20% were low relative to cholesterol, i.e. 8.2, 6.7 and 8.7 μg per ml, respectively. In each case the identity of the 7α-methyl-15-ketosterol was further confirmed by GC-MS analyses with the same retention time and MS as that of the TMS derivative of authentic **II**.

2.8. Effects of dietary administration of 3β-hydroxy-7α-methyl-5α-cholest-8(14)-en-15-one (II) on food consumption, body weight, serum cholesterol levels and weights of selected organs in male Sprague-Dawley rats

Administration of the 7α-methyl-15-ketosterol **II** at a level of 0.10% in diet had no effect on food consumption (Table 6). Similarly, administration

Table 7
Effect of dietary administration of 3β-hydroxy-7α-methyl-5α-cholest-8(14)-en-15-one (**II**) (at levels of 0.10, 0.15 and 0.20% in diet for 10 days) on mean values of body weight (g ± S.E.M.; $n = 8$) in male Sprague-Dawley rats

Day	Body weight (g)					
	Experiment A			Experiment B		
	Control (Ad libitum)	Compound II (0.1%)	Control (Pair-fed)	Control (Ad libitum)	Compound II (0.15%)	Compound II (0.20%)
0	169.4 ± 4.4	172.4 ± 3.1	174.4 ± 2.0	156.4 ± 2.7	154.1 ± 3.8	153.6 ± 4.2
1	172.6 ± 4.4	175.0 ± 2.9	178.6 ± 1.9	162.6 ± 2.9	159.8 ± 3.7	160.0 ± 4.1
2	180.1 ± 4.1	181.0 ± 2.4	178.5 ± 2.7	170.8 ± 2.7	166.4 ± 4.1	162.5 ± 4.3
3	185.8 ± 4.4	187.6 ± 2.7	185.0 ± 2.4	177.6 ± 2.9	170.3 ± 4.2	163.9 ± 4.6[a]
4	192.9 ± 4.5	194.6 ± 2.9	190.9 ± 2.0	181.9 ± 3.7	175.0 ± 3.9	168.6 ± 5.0
5	198.9 ± 4.5	202.5 ± 2.6	198.3 ± 2.0	192.3 ± 3.3	185.0 ± 4.2	173.8 ± 5.3[a]
6	200.0 ± 4.7	204.5 ± 2.6	199.1 ± 2.1	194.4 ± 3.6	185.6 ± 4.5	175.9 ± 5.4[a]
7	203.6 ± 4.6	208.9 ± 2.6	202.6 ± 2.0	200.9 ± 4.0	192.8 ± 4.6	180.9 ± 5.6[a]
8	208.9 ± 5.0	214.1 ± 2.8	208.6 ± 2.1	207.3 ± 4.1	205.9 ± 9.6	186.1 ± 5.8[a]
9	215.9 ± 5.2	221.3 ± 3.0	216.6 ± 2.1	217.4 ± 4.4	206.3 ± 5.6	193.6 ± 6.4[a]
10	217.9 ± 7.1	223.8 ± 2.9	219.4 ± 1.7	220.8 ± 4.3	210.5 ± 5.5	197.0 ± 7.0[a]

[a]The mean values for animals receiving **II** at a level of 0.20% by weight in diet were less than those of ad libitum control animals on day 3 ($P = 0.031$), day 5 ($P = 0.019$), day 6 ($P = 0.023$), day 7 ($P = 0.019$), day 8 ($P = 0.018$), day 9 ($P = 0.016$) and day 10 ($P = 0.016$).

Table 8
Effect of dietary administration of 3β-hydroxy-7α-methyl-5α-cholest-8(14)-en-15-one (II) on levels of serum cholesterol in male Sprague-Dawley rats

Experiment	Group	Serum cholesterol (mg per dl)			Cholesterol lowering (% change from ad libitum controls)	
		Day 0	Day 5	Day 9	Day 5	Day 9
A	Control (ad libitum)	108.5 ± 2.5	103.3 ± 1.7	95.6 ± 2.6		
	Compound II (0.1%)	108.5 ± 2.1	99.1 ± 2.0	97.3 ± 1.4	−4.1[ns]	+1.8[ns]
	Control (pair-fed)	108.2 ± 2.1	98.4 ± 1.5	92.8 ± 1.9		
B	Control (ad libitum)	125.7 ± 3.2	102.5 ± 2.2	96.9 ± 2.6		
	Compound II (0.15%)	125.7 ± 3.0	86.6 ± 2.9	81.7 ± 2.5	−15.5	−15.7
	Compound II (0.2%)	126.5 ± 2.6	69.2 ± 1.4	68.3 ± 2.8	−32.5	−35.7

[ns]Not significant ($P > 0.05$).

of II at a level of 0.15% had only a slight effect on food consumption (with mean values significantly less than controls on days 2, 3, 5, 7 and 9). The 7α-methyl-15-ketosterol at a level of 0.20% resulted in a modest suppression of food consumption. The slight effects of II on food consumption are in marked contrast to the major suppression of food consumption consistently observed in rats upon administration of the parent 15-ketosterol I at 0.1% by weight in diet [6–8].

Administration of II at a level of 0.10 or 0.15% had no effect on the growth of the rats, expressed as mean values of total body weight (Table 7). Animals fed II at a level of 0.20% in diet had lower mean values of body weight (relative to ad libitum controls) on days 3 and 5–10 (Table 7). The values on days 9 and 10 were 6% less than that of control animals.

Administration of the 7α-methyl-15-ketosterol (II) at a dosage of 0.10% had no effect on the level

Table 9
Effect of dietary administration of 3β-hydroxy-7α-methyl-5α-cholest-8(14)-en-15-one (II) (at levels of 0.10, 0.15 and 0.20% in diet for 10 days) on weights of selected organs of male Sprague-Dawley rats

Organ	Organ weight (g ± S.E.M.)					
	Experiment A			Experiment B		
	Control (Ad libitum)	Compound II (0.1%)	Control (Pair-fed)	Control (Ad libitum)	Compound II (0.15%)	Compound II (0.20%)
Liver	9.45 ± 0.26	9.59 ± 0.26	9.29 ± 0.16	9.80 ± 0.21	9.44 ± 0.42	8.31 ± 0.55[f]
Small intestine	5.52 ± 0.13	6.85 ± 0.18[a,b]	6.12 ± 0.20[c]	6.11 ± 0.17	7.59 ± 0.41[d]	9.82 ± 0.38[e]
Spleen	0.60 ± 0.03	0.64 ± 0.02	0.60 ± 0.02	0.67 ± 0.02	0.59 ± 0.03	0.62 ± 0.03
Kidneys	1.70 ± 0.05	1.69 ± 0.04	1.71 ± 0.02	1.85 ± 0.05	1.76 ± 0.10	1.60 ± 0.07
Adrenals	0.038 ± 0.001	0.037 ± 0.001	0.039 ± 0.001	0.040 ± 0.001	0.040 ± 0.002	0.041 ± 0.002
Heart	0.78 ± 0.03	0.76 ± 0.02	0.72 ± 0.01	0.82 ± 0.03	0.80 ± 0.03	0.72 ± 0.03
Testes	2.57 ± 0.09	2.54 ± 0.10	2.65 ± 0.08	2.75 ± 0.08	2.74 ± 0.10	2.64 ± 0.06

[a]Compound II (0.1%) vs. ad libitum control, $P = 0.0006$.
[b]Compound II (0.1%) vs. pair-fed control, $P = 0.012$.
[c]Ad libitum control vs. pair-fed control, $P = 0.0014$.
[d]Compound II (0.15%) vs. control, $P = 0.015$.
[e]Compound II (0.20%) vs. control, $P = 0.0001$.
[f]Compound II (0.20%) vs. control, $P = 0.029$.

of serum cholesterol (Table 8). Higher dosages (0.15 and 0.20%) of **II** resulted in significant lowering of serum cholesterol levels. The magnitude of the lowering was greater at 0.20% dosage than at 0.15%. The extents of lowering of serum cholesterol by **II** on days 5 and 9 were very similar.

The effects of dietary administration of **II** on selected organ weights are presented in Table 9. The mean weight of livers of animals receiving **II** at a level of 0.20% in diet was lower than that of the ad libitum controls. However, no significant difference was observed when the liver weights were expressed as a percentage of total body weight. The only clear effect of **II** on organ weights was that on the weight of small intestine, for which a dose-dependent increase was observed.

3. Discussion

The 7α-methyl-15-ketosterol **II** has been prepared by modifications of reactions previously presented by this laboratory [28]. In the current study, reaction of the epoxide **III** with CH_3MgI in the presence of CuI gave the desired 7α-methyl-$\Delta^{8(14)}$-3β,15α-dihydroxysterol **IV** in consistent yields of 51–55% along with products of 1,2-addition, i.e. 15β-methyl-5α,14β-cholest-7-ene-3β,15α-diol (**V**) and 15α-methyl-5α,14β-cholest-7-ene-3β,15β-diol (**VI**). The 3β,15α-diol **V** had previously been obtained by Grignard reaction of the Δ^7-14α,15β-epoxide **III** with CH_3MgI in the absence of CuI [29] and identified by X-ray crystallography [29,30]. ^1H and ^{13}C-NMR data indicated that the other product **VI** was an isomer of **V**. A C-D *cis* ring junction (14β-H) was demonstrated by the downfield position of C-18 (δ 23.77) in the ^{13}C-NMR spectrum of **VI** and by a strong NOE for H-14 upon irradiation at H-18. These and other NMR results (Tables 1 and 3) led to the identification of **VI** as the 15β-hydroxy epimer of **V**. Byproducts **V** and **VI** were slightly more polar than **IV** on silica gel and could be removed from **IV** by MPLC. However, recrystallization from ethyl acetate provided a more efficient purification for large-scale syntheses and furnished **IV** in purity comparable to that obtained by MPLC. The desired 7α-methyl-15-ketosterol **II** was obtained from **IV** by oxidation with pyridinium chlorochromate, using a modification of a procedure previously described from this laboratory [28]. Surprisingly, the starting diol **IV** was not readily separable from the 15-ketosterol product **II** on silica gel in ethyl acetate-hexane solvent systems. Consequently, the oxidation was carried out until the starting diol was completely consumed, as judged by HPLC. The major by-product under these conditions, 7α-methyl-3,15-dione **VII**, was easily separated from **II** by MPLC.

Complete ^1H and ^{13}C-NMR signal assignments are presented for the 7α-methyl-15-ketosterol **II** and for intermediates and by-products in its synthesis (Tables 1 and 3). These assignments were established by standard methods [31] using a combination of DEPT, HETCOR, COSYDEC and 1D spectra together with chemical shift comparisons [15,32–35]. Accurate ^1H-NMR chemical shifts were obtained from COSYDEC spectra [23] with a narrow window (typically δ 0.5–2.5 or smaller in f_1 and f_2) and 256 increments, an experiment that required only ~30 min on samples of $\geq$2 mg. Most shieldings could be determined to a precision of $\pm$0.002 ppm from the COSYDEC spectra, although strong coupling effects and signal overlap occasionally reduced the accuracy to $\pm$0.02 ppm. Most chemical shifts could be conveniently read from the f_1 projection of the δ 1.0–2.5 region of the COSYDEC spectrum (excluding the t_1 noise of the methyl signals). Despite the presence of artefacts and intensity distortions, this projection served as a useful 'proton-decoupled' proton spectrum. Closely spaced resonances were not resolved in the partial f_1 projection, but signals differing by as little as 0.002 ppm could be distinguished in the COSYDEC spectrum if they showed correlations at different chemical shifts in f_2. Signals outside the spectral acquisition window were folded into predictable locations with full intensity in the f_1 dimension. These folded signals provided additional confirmation for assignments of protons adjacent to functionalized carbons.

^1H-^1H-NMR coupling constants (Table 2) were obtained by first-order analysis of multiplet patterns in resolution-enhanced 500-MHz ^1H spectra or from isolated multiplets observed in NOE and

saturation difference spectra [24,25]. The major sources of error in measuring coupling constants were second-order distortions of multiplet patterns and unresolved lines in multiplets having two or more couplings differing by <1 Hz. The coupling constants were used in conjunction with molecular modeling and Karplus relationships [36] to analyze the conformations of 14β-sterols **V** and **VI** and to determine any effect of the 7α-methyl substitution on the conformation of 15-ketosterols. The values for $J_{H17-H20}$ of the 14β-sterols **V** and **VI** (9.1 and 9.6 Hz) indicate that C-22 is anti to C-13 in the predominant conformation, unlike the case of some other 14β-sterols [34]. These results are in accord with force-field calculations with PCMODEL. The $J_{H20-H22R}$ and $J_{H20-H22S}$ values indicate that C-17 is anti to C-23 in the principal conformer but that another conformer (*+gauche*) is significantly populated. Other observed coupling constants were compatible with force-field calculations showing ring C of **V** and **VI** as a chair flattened at C-14 (asymmetry parameter $C_s(14) \sim 2$). A distorted chair[1] is observed in the X-ray structure of the 3β-*p*-bromobenzoate ester of **V** [30], but ring C was in a twist conformation in the crystal structure of the bis-*p*-bromobenzoate ester of 5α,14β-cholest-7-ene-3β,15β-diol [37]. The observed $J_{H16-H17}$ coupling constants were compatible with force-field calculations showing **VI** as a 15α,16β half-chair and **V** as intermediate between a 15α envelope and 15α,16β half-chair.[2] The coupling constants (observed in $CDCl_3$ solution) were in fair agreement with the predicted couplings based on the crystal structure of the 3β-*p*-bromobenzoate ester of **V**,[2] which showed ring D as a 15α envelope. These results suggest considerable conformational variability in rings C and D of the 14β-sterols.

Comparison of the ^{13}C-NMR spectrum of 7α-methyl-15-ketosterol **II** (Table 1) with that of the parent 15-ketosterol **I** [15] showed the expected downfield shifts for β-carbons C-6 and C-8 (5.8 ppm) and upfield shifts for γ-carbons C-5 and C-9 (6.1 and 4.9 ppm). Similar comparisons of the ^{1}H chemical shifts showed that introduction of the 7α-methyl group caused a downfield shift (~ 0.25 ppm) for H-5α, H-6β, H-7β and H-9α and upfield shifts for H-6α (0.3 ppm) and H-4α (0.1 ppm). The effect on other protons was <0.1 ppm. The substituent effects of the 7α-methyl group were comparable to those observed previously for a methyl group in rigid systems (Refs. 32, 38 and 39 and references therein). Except for relatively minor differences in ring B, the vicinal ^{1}H-^{1}H coupling constants of **II** were very similar to those of **I** (Table 2). These results together with ^{1}H and ^{13}C chemical shift comparisons indicate that, apart from minor distortions in ring B, introduction of the 7α-methyl group has essentially no effect on the conformation of the 15-ketosterol.

The 7α-methyl-15-ketosterol **II** was found to be highly active, equivalent in potency to the 15-ketosterol **I**, in lowering the levels of HMG-CoA reductase activity in CHO-K1 cells. Administration of **II** to rats resulted in a significant reduction of serum cholesterol levels at dosages of 0.15 and 0.20% by weight in diet. However, no effect was observed at a dosage of 0.10%. Thus, whereas the potency of **II** in lowering HMG-CoA reductase activity in CHO-K1 cells was comparable to the parent 15-ketosterol **I**, introduction of the 7α-methyl group reduced the potency of the 15-ketosterol with respect to effects on serum cholesterol levels. For example, **II** at a dosage of 0.10% by weight in diet (2.42 μmol per g of diet) had no effect on serum cholesterol, whereas **I** at 0.10% (2.50 μmol per g of diet) has consistently shown marked hypocholesterolemic action in rats [6–8].

Another consistent finding with the parent 15-ketosterol **I** upon its dietary administration to rats has been a substantial suppression of food consumption [5–8]. The decreased food consumption induced by **I** is associated with a striking decrease in the growth of rats [5–8]. It is important to note that these effects have not been observed upon oral administration of **I** to non-human primates

1. Re-examination of asymmetry parameters for the X-ray structure in Ref. 30 indicates that ring C is a distorted chair rather than a half-chair.

2. The following ^{1}H-^{1}H coupling constants were calculated by applying a Karplus equation to molecular models derived from force-field calculations: **V**, $J_{16\alpha-17\alpha}$ 6.6 Hz, $J_{16\beta-17\alpha}$ 11.3 Hz; **VI**, $J_{16\alpha-17\alpha}$ 6.3 Hz, $J_{16\beta-17\alpha}$ 11.4 Hz. Similar calculations using the X-ray co-ordinates for the 3β-*p*-bromobenzoate ester of **V** gave: $J_{16\alpha-17\alpha}$ 7.6 Hz, $J_{16\beta-17\alpha}$ 10.4 Hz.

[9,10]. In the present study no effect of **II**, at a dosage of 0.10%, was observed and only slight to moderate effects on food consumption were observed at higher doses (0.15 and 0.20%). Also in marked contrast to **I**, dietary administration of **II** had no effect on mean values of body weight except at the higher dose (0.20%), in which case only a slightly lower value (~11% relative to controls) was observed. It is especially noteworthy that the hypocholesterolemic action of **II** was observed under conditions in which only slight or moderate effects on food consumption were observed, and at the dose of 0.15%, little or no effect on body weight was observed.

The mechanism(s) involved in the action of **I** on food consumption has not been established. We have recently observed that administration of the 25,26,26,26,27,27,27-heptafluoro analog of **I**, even at high dosages, has little or no effect on food consumption in rats.[3] This finding suggests that the action of **I** on food consumption may be due to blockage of the formation of one or more products of the side chain oxidation of **I**, a very prominent process in the metabolism of **I** in rats [40,41], which appears to be initiated by oxidation at C-26.[4] The observation that **II** has little effect on food consumption may be due to a lower net conversion of **II** to products derived from side chain oxidation. A lower formation of such products from **II** could arise from a number of factors, including decreased absorption of **II**, lower efficiency of **II** as a substrate for side chain oxidation or lower activity of **II** or its metabolic products on the processes involved in the suppression of food consumption. Further studies will be required to elucidate the precise reason(s) for the effect of the 7α-methyl substitution in modifying the action of **I** on food consumption.

Administration of **II** had no clear effect on the weights of individual organs, except in the case of the small intestine, in which a dose-dependent increase in weight was observed. The effect of **II** on the weight of the small intestine appears to be less than that caused by **I**, for which detailed analyses of morphology have been presented [8].

The major fate of **I** after its administration to animals is the formation of polar metabolites, which are excreted in bile [40,41]. In addition, a significant fraction of **I** undergoes conversion to cholesterol [14,40–46]. A metabolic scheme has been presented for the overall conversion of **I** to cholesterol [46]. A suitably labeled form of **II** was not available for the detailed studies of its metabolism. However, studies of serum and liver obtained from animals treated with **II** showed no compounds corresponding to intermediates in the potential conversion of **II** to 7α-methyl cholesterol. The levels of the 7α-methyl-15-ketosterol **II** in serum after dietary administration of **II** to rats for 10 days were very low relative to cholesterol. At dosages of 0.10, 0.15 and 0.20% **II** in diet, the levels of **II** (free plus esterified) in pooled serum samples were 8.2, 6.7 and 8.7 μg per ml, respectively (corresponding to concentrations of 19.8, 16.2 and 21.0 μM, respectively). The identification of **II** in serum was based upon chromatographic data (HPLC and GC) and the results of GC-MS experiments. The presence of other metabolites of **II** in serum was not detected. Detailed analyses of the sterols of the liver of a rat fed the 7α-methyl-15-ketosterol **II** at a level of 0.10% in diet for 10 days indicated the presence of low but significant levels of free **II** (11.1 ± 2.2 nmol per g) and esterified **II** (5.2 ± 1.2 nmol per g) in liver. The identification of **II** in liver was based upon chromatographic data and the results of GC-MS analyses. No other metabolites of **II** were detected in liver. These combined results indicate little or no metabolism of the 7α-methyl-15-ketosterol to 7-methyl-cholesterol or other potential metabolites in this possible pathway.

In summary, introduction of the 7α-methyl substitution into the parent 15-ketosterol **I** had no detectable effect on its potency in lowering HMG-CoA reductase activity in CHO-K1 cells. In contrast, the 7α-methyl substitution had a marked effect on the action of **I** in the suppression of food consumption. While the 7α-methyl-15-ketosterol

3. N. Gerst, F.D. Pinkerton, A. Kisic, W.K. Wilson, S. Swaminathan and G.J. Schroepfer, Jr., J. Lipid Res., in press.
4. J.St. Pyrek, S. Numazawa and G.J. Schroepfer, Jr., unpublished data.

was less potent in lowering serum cholesterol levels in rats, it did so under conditions in which only slight or moderate effects on food consumption were observed.

4. Acknowledgments

This work was supported in part by the Ralph and Dorothy Looney Endowment Fund and the Robert A. Welch Foundation (Grant C-583). The Rice 500-MHz NMR Facility was established with the support of NIH grant RR05759 and the W.M. Keck Foundation.

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