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INHIBITION OF STEROL BIOSYNTHESIS BY 14α-HYDROXYMETHYL STEROLS*

George J. Schroepfer, Jr.,**, Robert A. Pascal, Jr.***,
Roger Shaw****, and Andrew A. Kandutsch

Departments of Biochemistry and Chemistry
Rice University, Houston, Texas 77001
and The Jackson Laboratory, Bar Harbor, Maine 04609

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Summary: 14α-Hydroxymethyl-5α-cholest-7-en-3β-ol (I) and 14α-hydroxymethyl-5α-cholest-6-en-3β-ol (II) have been prepared by chemical synthesis from 3β-acetoxy-7α,32-epoxy-14α-methyl-5α-cholestane. Compound I, previously shown to be efficiently convertible to cholesterol upon incubation with rat liver homogenate preparations, has been found to be a potent inhibitor of sterol synthesis in animal cells in culture. Compound I caused a 50% reduction of the levels of HMG-CoA reductase activity in cultures of L cells and fetal liver cells at concentrations of 3×10^{-6} M and 8×10^{-6} M, respectively. Compound II, the Δ^6-analogue of I, caused a 50% suppression of the enzyme activity in the two cell types at even lower concentrations, 5×10^{-7} M and 2×10^{-6} M, respectively. Concentrations of I and II required to specifically inhibit sterol synthesis from acetate were similar to those required to suppress the levels of HMG-CoA reductase activity.

It has generally been considered that the first step in the enzymatic removal of each of the "extra" methyl groups of lanosterol is an oxidation to yield the corresponding hydroxymethyl derivative (1-3). In the case of the 14α-methyl group of such precursors of cholesterol, 14α-hydroxymethyl Δ^8- and Δ^7-sterols have been shown to be convertible to cholesterol upon incubation with rat liver homogenate preparations (4-7). We have previously noted that 14α-hydroxymethyl-5α-cholest-7-en-3β-ol is convertible to cholesterol upon incubation with the 10,000 x g supernatant fraction of rat liver homogenates (5). We now wish to report that this 14α-hydroxymethylsterol is a potent inhibitor of sterol synthesis in cultures of L cells and of fetal mouse liver cells.

Oxygenated derivatives of cholesterol have been considered as potential regulators of cholesterol biosynthesis (8-10). While we have previously shown

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** To whom inquiries should be directed at Rice University.

*** Recipient of a predoctoral fellowship from the National Science Foundation.

**** Present address, Department of Biochemistry, St. Louis University School of Medicine.

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that several oxygenated sterols which are enzymatically convertible to cholesterol are also potent inhibitors of sterol synthesis (11,12), the intermediary role of these sterols in the normal biosynthesis of cholesterol is far from established.

Since hydroxylation of the 14α-methyl group of lanosterol and other 14α-methyl substituted sterol precursors of cholesterol can be considered to be an obligatory step, our finding that a 14α-hydroxymethyl substituted sterol is a potent inhibitor of sterol biosynthesis raises the possibility that the normal regulation of sterol biosynthesis may occur not only by oxygenated metabolites of cholesterol but also by oxygenated sterol precursors of cholesterol.

Methods and Materials

Melting points were recorded in sealed evacuated capillary tubes using a Thomas Hoover melting point apparatus. Infrared (i.r.) spectra were recorded on a Beckman IR-9 spectrometer using KBr pellets. Nuclear magnetic resonance (n.m.r.) spectra were recorded in $CDCl_3$ solutions of the sterols on a Perkin-Elmer HR-12 spectrometer at 60 MHz or on a Varian Associates EM-390 spectrometer at 90 MHz using tetramethylsilane (TMS) as an internal standard. Peaks are reported as p.p.m. (δ) downfield from the TMS standard. Optical rotations were measured using a JASCO DIP-4 digital polarimeter with chloroform solutions of the sterols. Low resolution mass spectral (m.s.) analyses were made using an LKB-9000S spectrometer under operating conditions described previously (13). Thin layer chromatographic (t.l.c.) analyses were made on plates of silica gel G (E. Merck, Darmstadt). Components on the plates were visualized after spraying with molybdic acid (14). Gas-liquid chromatographic (g.l.c.) analyses were made using a Hewlett-Packard Model 402 unit equipped with dual flame ionization detectors. The columns (6 ft. x 0.25 in., o.d.) were packed with 3% OV-1 on Gas-Chrom Q (100/120 mesh) and maintained at 255°. 3β-Acetoxy-7α,32-epoxy-14α-methyl-5α-cholestane (m.p. 136-137° [literature: 136-137° (15)]; single component on t.l.c. and characterized by i.r., n.m.r., and m.s.) was prepared by a modification of the procedure described by Knight et al. (15).

14α-Hydroxymethyl-5α-cholest-7-en-3β-ol and 14α-Hydroxymethyl-5α-cholest-6-en-3β-ol

3β-Acetoxy-7α,32-epoxy-14α-methyl-5α-cholestane (475 mg; 1.04 mmol) was heated under reflux with pyridine hydrochloride (900 mg) and acetic anhydride (20 ml) for 5 hours under nitrogen. After cooling to room temperature, the mixture was diluted with water and extracted 3 times with ether (100 ml portions). The combined extracts were washed successively with 2.5% HCl, water, a saturated solution of sodium carbonate, and water, dried over anhydrous magnesium sulfate, and evaporated to dryness under reduced pressure. To the resulting white solid in ether was added lithium aluminum hydride (600 mg). After standing 20 min at room temperature, ice and a 2.5% HCl solution were successively added and the resulting mixture was extracted 3 times with ether (100 ml portions). The combined extracts were successively washed with water, a saturated solution of sodium bicarbonate, and water, dried over anhydrous magnesium sulfate, and evaporated to dryness under reduced pressure. Thin layer chromatographic analysis (solvent: 50% U.S.P. ether in benzene) showed 3 components (R_f values of 0.32, 0.27 and 0.23). The mixture was subjected to medium pressure silica gel (Woelm; 0.032-0.063 mm) column (100 cm x 1.5 cm) chromatography using a mixture of ether and benzene (2:3) as the eluting solvent at a flow rate of 3.1 ml per min. Fractions 12.4 ml in volume were collected. At fraction 68 the eluting solvent was changed to a mixture of ether and benzene (1:1).

The contents of fractions 75 through 98 were pooled and, after evaporation of the solvent, recrystallized from acetone to give 14α-hydroxymethyl-5α-cholest-6-en-3β-ol (58 mg; 13% yield). Examination by t.l.c. indicated a trace of contamination by the material of R_f 0.27. The product was subjected to further purification on a silica gel (60-200 mesh) column (45 cm x 1.0 cm) using a mixture of benzene and ether (3:2) as the eluting solvent. The purified product, crystallized from acetone, melted at 204-205°; i.r., ν_{max} 3320, 2920, 1460, 1373, 1050, 1029, and 752 cm^{-1}; m.s., 416 (M, 1%), 398 (M-H_2O; 41%), 385 (M-CH_2OH; 100%), and 367 (M-H_2O-CH_2OH; 57%); n.m.r., 0.82 (s, 3H, C-19-CH_3), 0.92 (s, 3H,

C-18-CH_3), 3.46 (d, 1H, C-32-H; J=12 Hz), 3.65 (m, 1H, C-3-H), 4.19 (d, 1H, C-32-H; J=12 Hz), 5.35 (d, 1H, C-7-H; J=10 Hz), and 5.76 (d, 1H, C-6-H; J=10 Hz). The compound showed a single component on t.l.c. and g.l.c.

The contents of fractions 99 through 188 from the medium pressure chromatographic column were subjected to repeated (3) chromatography on the same column. After recrystallization from acetone, pure 14α-hydroxymethyl-5α-cholest-7-en-3β-ol (185 mg; 43% yield) was obtained which melted at 185-186°; $[\alpha]_D$ + 18.6° (c. 0.42, $CHCl_3$); i.r., 3310, 2910, 1463, 1373, and 1040 cm^{-1}; m.s., 416 (M; 2%), 401 (M-CH_3; 2%), 398 (M-H_2O; 1%), 385 (M-CH_2OH; 100%), and 367 (M-H_2O-CH_2OH; 44%); n.m.r., 0.73 (s, 3H, C-18-CH_3), 0.83 (s, 3H, C-19-CH_3), 3.22 (dd, 1H, C-32-H; J=10 Hz, 10 Hz; collapsed to d, 1H, J=10 Hz on exchange with D_2O), 3.56 (m, 1H, C-3-H), 3.66 (d, 1H, C-32-H; J=10 Hz), and 5.30 (m, 1H, C-7-H). The compound showed a single component on t.l.c. and g.l.c.

Cell Culture Studies

Mouse L cell (a subline of NCTC clone 929 mouse fibroblasts) cultures and primary cultures of fetal mouse liver cells were grown in serum-free medium as described previously (8). The preparation of steroid-containing media, procedures for assaying the conversion of [1-^{14}C]-acetate into digitonin-precipitable sterols, carbon dioxide, and fatty acids and methods for measurement of DNA and protein were as described previously (8,9,12). The cultures were preincubated with the test compound for 4 hours in the case of the L cells and for 12 hours in the case of the liver cell cultures; then [1-^{14}C]-acetate was added at a concentration of 4 μmoles (4 μCi) per ml and the incubations were continued for 2 more hours. To determine the effects of the sterols on HMG-CoA reductase of cultured L cells and liver cells, the sterols were incubated with the cultures for 5 hours and 12 hours respectively prior to harvesting for the determination of HMG-CoA reductase activity by a modification of the method of Brown, Dana, and Goldstein (16) using a higher concentration (80 μM) of RS-HMG-CoA and a 30 minute incubation period.

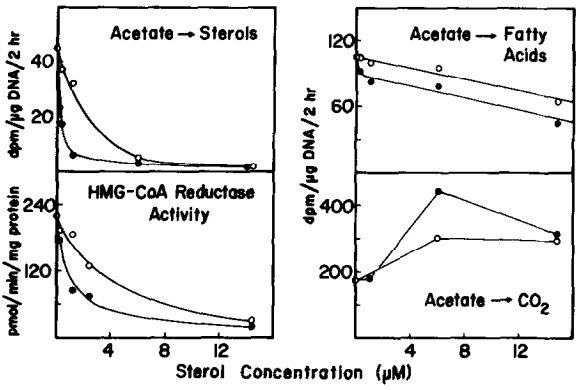


Figure 1. Effects of 14α-hydroxymethyl sterols on the metabolism of [1-^{14}C]-acetate to sterols, fatty acids CO_2 and on the levels of HMG-CoA reductase activity in L cells.

O———O, 14α-hydroxymethyl-5α-cholest-7-en-3β-ol

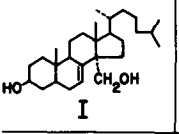
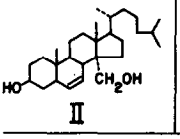
●———●, 14α-hydroxymethyl-5α-cholest-6-en-3β-ol

Results

The effects of 14α-hydroxymethyl-5α-cholest-7-en-3β-ol and 14α-hydroxymethyl-5α-cholest-6-en-3β-ol on the incorporation of acetate into digitonin-precipitable sterols, fatty acids, and CO_2 and on the levels of HMG-CoA reductase activity in the L cells are presented in Figure 1. The results indicate that the two sterols inhibited sterol synthesis from acetate at concentrations which had no significant effect upon the rates of fatty acid or CO_2 production from the same substrate. The effects of the two sterols on HMG-CoA reductase are essentially as expected if inhibition of sterol synthesis was due to suppression of the activity of this key enzyme in sterol biosynthesis.

A condensed summary of the inhibitory potencies of the two sterols in the two cell types is presented in Table I. The Δ^6-sterol was, in all tests, several fold more active than the Δ^7-sterol, a finding which may be the result of less ready metabolism of the Δ^6-sterol in these cells. These results indicate that the Δ^6-sterol is among the most potent of the inhibitory sterols identified to date (8-12).

Table I. Inhibition of sterol synthesis and reduction of HMG-CoA reductase activity by 14α-hydroxymethyl sterols.

Inhibitor	Concentrations (μM) required for 50% inhibition			
	L Cells		Primary Cultures of Liver Cells	
	Sterol Synthesis*	HMG-CoA Reductase	Sterol Synthesis*	HMG-CoA Reductase
 I	2	3	10	8
 II	0.2	0.5	3	2

* Calculated as the ratio of ^{14}C-sterols to ^{14}C-fatty acids (8).

Discussion

The preparation of 14α-hydroxymethyl-5α-cholest-7-en-3β-ol by another route has been reported by Anastasia et al. (17). While the value of the specific optical rotation and the n.m.r. data reported by the latter workers are in close agreement with those reported here, their reported melting point (124-125°) is considerably lower than that (185-186°) observed in the present study. The reason for this discrepancy is not readily apparent. In the present cases, the stereochemical orientation of the 14α-hydroxymethyl group in compounds I and II appears soundly established. The stereochemical orientation of carbon atom 32 of 3β-acetoxy-7α-, 32-epoxy-14α-methyl-5α-cholestane, and hence of the same carbon atom in compounds I and II, rests on a body of evidence, the most compelling of which is the unequivocal determination of the stereochemical orientation of the 14α-methyl group (carbon atom 32) of a derivative (18,19) of 3β-benzoyloxy-14α-methyl-5α-cholest-7-en-15-one by x-ray analysis. The latter compound can be considered as the starting material for the synthesis of the 7α,32-epoxide.

The high potencies of the 14α-hydroxymethyl sterols in the inhibition of sterol synthesis and in the reduction of the levels of HMG-CoA reductase activity in the two cell types is of interest in considering the normal regulation of cholesterol biosynthesis. Until very recently, hydroxylation and elimination of the 14α-methyl group of 4,4,14α-trimethyl sterol precursors of cholesterol has generally been considered to precede removal of the methyl groups at carbon atom 4. However, the reported isolation of a number of 14α-methyl sterols and 4α,14α-dimethyl sterols from a variety of sources (20-27) suggests that removal of the three "extra methyl" groups of lanosterol and related 4,4,14α-trimethyl sterols can be initiated by removal of either the 4α-methyl function (28-30 and references cited therein) or the 14α-methyl group. The findings reported here indicate that the 14α-hydroxymethyl sterols studied are potent inhibitors of sterol synthesis and suggest the possibility that others, such as the 14α-hydroxymethyl analogue of lanosterol, might also inhibit sterol biosynthesis. They further suggest the possibility that the normal regulation of sterol biosynthesis in animal cells involves not only oxygenated metabolites of cholesterol but also oxygenated sterol precursors of cholesterol.

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