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ENZYMATIC FORMATION AND CHEMICAL SYNTHESIS OF AN ACTIVE METABOLITE OF 3β-HYDROXY-5α-CHOLEST-8(14)-EN-15-ONE, A POTENT REGULATOR OF CHOLESTEROL METABOLISM*

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Summary: The enzymatic (rat liver mitochondria) conversion of 3β-hydroxy-5α-cholest-8(14)-en-15-one to 5α-cholest-8(14)-ene-3β,26-diol-15-one is described. The enzymatic product was judged, on the basis of ^{1}H and ^{13}C NMR studies, to be a 4:1 mixture of its 25R and 25S isomers. (25R)-5α-Cholest-8(14)-ene-3β,26-diol-15-one was prepared through a five-step synthesis from (25R)-26-hydroxycholesterol. The (25R) isomer of the new compound was found to be highly active in the suppression of the levels of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in cultured mammalian cells and to inhibit the esterification of cholesterol in jejunal microsomes.

3β-Hydroxy-5α-cholest-8(14)-en-15-one (I; Figure 1) is a potent inhibitor of sterol synthesis in mammalian cells in culture and lowers the levels of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase activity in these cells (1-3). Dietary administration of I to rats causes a marked inhibition of the absorption of exogenous cholesterol (4,5). I has been shown to serve as an alternative substrate for the enzyme acyl CoA:cholesterol acyl transferase of microsomes of rat liver and jejunum and to inhibit the oleoyl CoA-dependent esterification of cholesterol in hepatic and jejunal microsomes (6). I has significant hypocholesterolemic activity upon oral administration to rodents (7) and nonhuman primates (8,9). The lowering of total serum cholesterol in Rhesus monkeys has been found to be associated with a lowering of the levels of low density lipoprotein (LDL) cholesterol and LDL protein and with an elevation of the levels of high density lipoprotein (HDL) cholesterol

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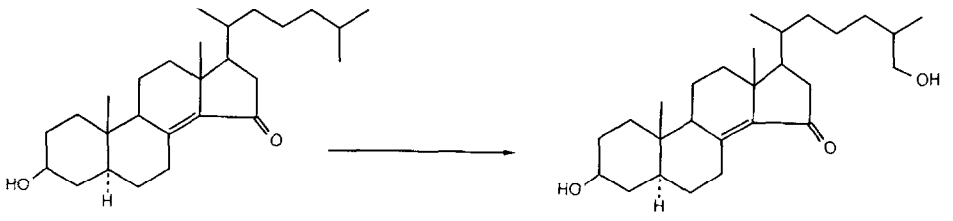

I II

Figure 1. Conversion of 5α-cholest-8(14)-en-3β-ol-15-one (I) to 5α-cholest-8(14)-ene-3β,26-diol-15-one (II).

and HDL protein (9). The elevation of HDL cholesterol levels has been found to be associated with a shift in the HDL lipoprotein profile to one in which the HDL_2 species predominates (9). All of these changes induced by I are believed to be beneficial for potential use in the treatment and/or prevention of coronary artery disease. An additional novel characteristic of I is its metabolism to cholesterol, a conversion which has been demonstrated *in vitro* in rat liver homogenate preparations (10,11) and *in vivo* upon oral and intravenous administration to rats and baboons (5,12-15). Although the major metabolites of I found in blood and tissues at 48 h after the intravenous administration of I to rats are cholesterol and cholesterol esters, a quantitatively more important metabolic fate of I is very rapid metabolism of I (and/or of cholesterol formed from I) to polar metabolites which are excreted in bile (14). As an initial step in investigations of this matter, we have studied the metabolism of I in rat liver mitochondrial preparations.

The purpose of this communication is to describe the enzymatic (rat liver mitochondria) conversion of I to 5α-cholest-8(14)-ene-3β,26-diol-15-one (II), the chemical synthesis of the 25R isomer of II and the effects of (25R)-II on the levels of HMG-CoA reductase activity in CHO-K1 cells and on the esterification of cholesterol in jejunal microsomes.

Materials and Methods

The recording of melting points (m.p.), ^1H and ^{13}C nuclear magnetic resonance (n.m.r.), and mass spectra (m.s.) were carried out as described previously (16). Infrared (i.r.) spectra were obtained using a Beckman 4230 spectrometer with KBr pellets. Ultraviolet (u.v.) spectra were recorded as described previously (16) on methanol solutions of the sterols. Thin layer chromatographic (t.l.c.) analyses were made on plates of silica gel 60 (EM Science, Cherry Hill, DE) and gas-liquid chromatography (g.l.c.) was carried out as described previously (16). Medium pressure liquid chromatography (m.p.l.c.) was carried out using alumina-$AgNO_3$ (17) or silica gel (32-63 microns). High pressure liquid chromatography (h.p.l.c.) was car-

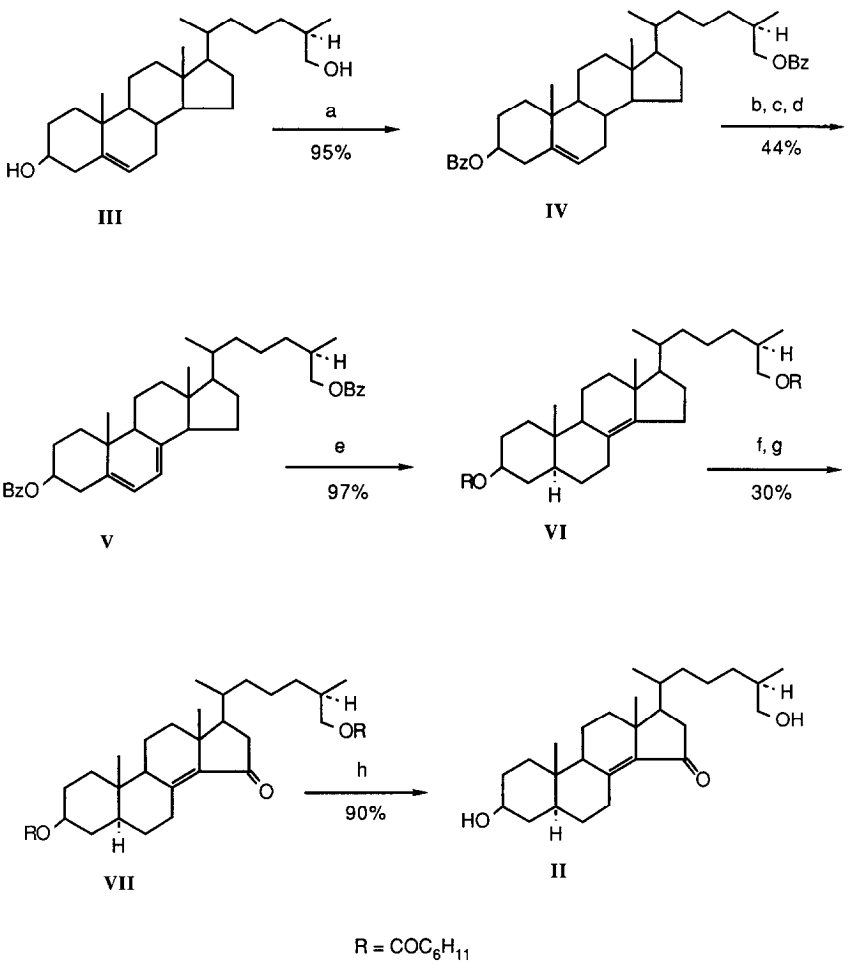

Figure 2. Chemical synthesis of (25R)-5α-cholest-8(14)-ene-3β,26-diol-15-one ((25R)-II). (a), BzCl-pyridine, room temp., 2 h; (b), 1,3-dibromo-5,5-dimethylhydantoin, reflux, 2 min; (c), triethyl phosphite, o-xylene, reflux, 3 h; (d), alumina-$AgNO_3$ m.p.l.c.; (e), H_2-PtO_2, HOAc-EtOAc, room temp., 16 h; (f), CrO_3-3,5-dimethylpyrazole, CH_2Cl_2, -20°, 1 h; (g), silica gel chromatography; (h) conc. H_2SO_4-methanol-H_2O.

ried out on a Spherisorb ODS-II column (4.6 mm x 250 mm; Custom LC, Houston, TX). Diosgenin was purchased from Steraloids, Inc. (Wilton, NH). (25R)-26-Hydroxycholesterol (III), melting at 177-178° (lit. 176° (18), 176-178° (19), 178-179° (20), 175-177° (21), 172-173° (22), 177-178° (23)) and showing a single component on t.l.c. and h.p.l.c., was prepared from diosgenin by modifications of the approach of Seo et al. (18) and was characterized by i.r., ^1H and ^{13}C n.m.r., and m.s. Compound I, prepared as described previously (1,24), had a purity in excess of 99% as judged by t.l.c. and g.l.c. [4-^{14}C]-I was a generous gift from the American Cyanamid Company.

The effects of I and (25R)-II on the levels of HMG-CoA reductase activity in CHO-K1 cells were determined as described previously (2,3). Effects of the sterols on ACAT activity (oleoyl CoA-dependent esterification of cholesterol) in rat jejunal microsomes were determined as described previously (6).

Synthesis of (25R)-5α-Cholest-8(14)-ene-3β,26-diol-15-one ((25R)-II, Figure 2)

Treatment of III with benzoyl chloride-pyridine gave, after m.p.l.c. on silica gel and crystallization from methanol-CH_2Cl_2, (25R)-cholest-5-ene-3β,26-diol 3β,26-

dibenzoate (IV), m.p. 136.5-137.5°[1]. Treatment of IV with 1,3-dibromo-5,5-dimethylhydantoin in a 1:4 mixture of benzene and hexane, followed by treatment with triethyl phosphite in o-xylene gave, after m.p.l.c. on alumina-$AgNO_3$ and crystallization from acetone-CH_2Cl_2, (25R)-cholesta-5,7-diene-3β,26-diol 3β,26-dibenzoate (V), m.p. 130.0-130.5°[1]. Hydrogenation (platinum oxide) of V in a 19:1 mixture of ethyl acetate and acetic acid gave, after crystallization from methanol-CH_2Cl_2, (25R)-5α-cholest-8(14)-ene-3β,26-diol 3β,26-dicyclohexylate (VI), m.p. 113.5-114.5°[1]. Oxidation of VI with CrO_3-3,5-dimethylpyrazole in CH_2Cl_2 at -20° for 30 min gave, after m.p.l.c. on silica gel and crystallization from (25R)-5α-cholest-8(14)-ene-3β,26-diol-15-one 3β,26-dicyclohexylate (VII), m.p. 124.5-125.5°[1]. Treatment of VII with sulfuric acid-methanol-water at 85° for 6 h gave, after m.p.l.c. on silica gel and crystallization from methanol, (25R)-5α-cholest-8(14)-ene-3β,26-diol-15-one (II), m.p. 197-198°; i.r., ν_{max} 3430, 3390, 2970, 2935, 2860, 1687 (conjugated C=O), 1613, 1583, 1465, 1377, 1325, 1228, 1215, 1120, 1085, and 1040 cm^{-1}; u.v., λ_{max} 259 nm (ε 14,300); ^1H n.m.r., 0.72 (s, 19-CH_3), 0.91 (d, J = 6.7 Hz, 27-CH_3), 0.97 (s, 18-CH_3), 1.00 (d, J = 6.5 Hz, 21-CH_3), 3.42 (dd, J_{AB} = 10.5 Hz, J_{BX} = 6.4 Hz, 26-H_B), 3.50 (dd, J_{AB} = 10.5 Hz, J_{AX} = 5.9 Hz, 26-H_A), 3.65 (m, 3α-H), 4.13 (br d, J = 13.9 Hz, 7β-H); m.s., 416 (58%; M), 401 (16%; M-CH_3), 398 (13%; M-H_2O), 383 (26%; M-CH_3-H_2O), 365 (10%; M-CH_3-H_2O), 287 (19%; M-side chain), 277 (10%; ring C-D fragment + 2H-CH_3) (16), 269 (94%; M-side chain-H_2O), 259 (11%; ring C-D fragment + 2H-H_2O-CH_3) (16), 251 (30%; M-side chain-H_2O-H_2O), and 55 (100%); high resolution m.s. on ion at m/z 416: 416.3274 (calc. for $C_{27}H_{44}O_3$: 416.3291).

Enzymatic Formation of 5α-Cholest-8(14)-ene-3β,26-diol-15-one

Three separate incubations of [4-^{14}C]-I (0.50, 0.50, and 0.59 μCi per μmol) with twice-washed rat liver mitochondria (1.8 mg protein per ml), prepared as described by Johnson and Lardy (25), were carried out. The reactions were conducted essentially as described by Taniguchi et al. (26) for 1 h at 37° in potassium phosphate buffer (0.04 M; pH 7.2) containing $MgCl_2$ (2 mM), RS-isocitrate (2 mM), KCN (1 mM), and NADPH (0.5 mM) in a total volume of 200 or 250 ml. The final concentration of [4-^{14}C]-I was 100 μM and the substrate was added in acetone (1% final concentration). The reactions were terminated by the addition of 1/10 volume of 1 N HCl and the incubation mixtures were extracted with $CHCl_3$-methanol (2:1). Recovery of radioactivity was 98%, 95%, and 92%, respectively, and the lipid extracts were subjected to preparative t.l.c. on silica gel G (two developments with hexane-ethyl acetate (1:1)) followed by reverse phase h.p.l.c. (Dynamax 60A or Spherisorb ODS-II columns; solvent, 80% methanol). Yields of 15.3, 13.8, and 8.6% of material corresponding to II (0.54, 0.49, and 0.60 μCi per μmol, respectively) were observed. This material, which showed a single component on t.l.c., reverse phase h.p.l.c., and g.l.c., was identified as II on the basis of spectral characteristics which were virtually identical with those of synthetic (25R)-II; m.s., 416 (74%; M), calc. for $C_{27}H_{44}O_3$ 416.3291, found 416.3306; 401 (19%; M-CH_3), 398 (49%; M-H_2O), calc. for $C_{27}H_{42}O_2$ 398.3185, found 398.3202, 383 (47%; M-H_2O-CH_3), 380 (49%; M-H_2O-H_2O), 365 (36%; M-H_2O-H_2O-CH_3), 287 (16%; M-side chain), 277 (14%; ring C-D fragment + 2H-CH_3) (16), 269 (100%; M-side chain-H_2O), calc. for $C_{19}H_{25}O$ 269.1905, found 269.1906, 259 (16%; ring C-D fragment + 2H-H_2O-CH_3) (16), 251 (73%; M-side chain-H_2O-H_2O). The metabolic samples of II were also characterized in the form of its diacetate derivative, m.s., 500 (100%; M), calc. for $C_{31}H_{48}O_5$ 500.3502, found 300.3502, 485 (11%; M-CH_3), 440 (68%; M-CH_3COOH), 425 (49%; M-CH_3COOH-CH_3), 407 (12%; M-CH_3COOH-CH_3-H_2O), 365 (22%; M-CH_3COOH-CH_3COOH-CH_3), 329 (10%; M-side chain), 311 (42%; M-side chain-H_2O), 269 (32%; M-CH_3COOH-side chain), 251 (97%; M-side chain-CH_3COOH-H_2O).

The n.m.r. chemical shifts for synthetic and enzymatic II differed by less than 0.1 ppm for ^{13}C n.m.r. and less than 0.01 ppm for ^1H n.m.r. However, careful analysis of the ^1H and ^{13}C n.m.r. spectra of metabolic samples of II revealed minor peaks adjacent to resonances corresponding to C22, C24, C26, C27, and H27. The ^1H n.m.r. spectrum of the diacetate derivative of metabolic samples of II showed especially good resolution in acetone-d_6 at 300 mHz; 0.74 (s, 19-CH_3), 0.905 (d, J = 6.7 Hz, 27-CH_3), 0.910 (d, J = 6.7 Hz, 27-CH_3 of (25S)-II), 1.00 (s, 18-CH_3), 1.02 (d, J = 6.6 Hz, 21-CH_3), 3.807 (dd, J_{AB} = 10.8 Hz, J_{BX} = 6.8 Hz, 26-H_B of (25S)-II, 3.812

[1] Sterols IV-VII gave ^1H and ^{13}C n.m.r., i.r., and mass spectra consistent with their assigned structures.

(dd, J_{AB} = 10.7 Hz, J_{BX} = 6.8 Hz, 26-H_B), 3.904 (dd, J_{AB} = 10.7 Hz, J_{AX} = 5.9 Hz, 26-H_A), 3.918 (dd, J_{AB} = 10.8 Hz, J_{AX} = 5.8 Hz, 26-H_A of (25S)-II), 4.15 (ddd, J = 14.0, 4.2, 2.1 Hz, 7β-H), 4.66 (m, 3α-H). In addition to a doublet 0.005 ppm downfield from the H27 resonance, a second set of minor peaks (the AB portion of an ABX pattern) was observed adjacent to the analogous resonance for H26. In all of the above instances, the major peaks were ~4X the intensity of the minor peaks. The chemical shift differences between the major and minor peaks in the ^{13}C n.m.r. spectra were essentially identical to those observed for 25R and 25S epimers of other 26-hydroxysterols. The ^{13}C n.m.r. chemical shift differences (25R-25S) which we observed for our enzymatic sample of II were -0.07, -0.13, 0.11, and -0.19 ppm for C22, C23, C26, and C27. These values are in very good agreement with the corresponding values for C23, C26, and C27 reported by Seo et al. (18) for III (-0.12, 0.10, and -0.21) and for C22, C23, C26, and C27 reported by Batta et al. (27) for 5β-cholestane-3α,7α,26-triol (-0.10, -0.13, -0.16, and -0.20). Based on these results, the enzymatic product II was judged to be a 4:1 mixture of its 25R and 25S isomers.

Biological Activities of (25R)-5α-Cholest-8(14)-ene-3β,26-diol-15-one

At a concentration of ~0.1 μM, both I and (25R)-II caused a 50% reduction of the elevated levels of HMG-CoA reductase, induced by transfer of the cells to delipidized serum. (25R)-II also inhibited the oleoyl CoA-dependent esterification of cholesterol in jejunal microsomes. At a concentration of 10 μM, 55% inhibition was observed. Under the same conditions, I showed 53% inhibition at 2 μM.

Discussion

Described herein is the first chemical synthesis of (25R)-5α-cholest-8(14)-ene-3β,26-diol-15-one ((25R)-II; Figure 2). For this work, the known (25R)-26-hydroxycholesterol (III) was employed as the starting material. Benzoylation of III gave the Δ^5-3β,26-dibenzoate (IV) in 95% yield. Allylic bromination of IV with 1,3-dibromo-5,5-dimethylhydantoin followed by dehydrobromination (28) with triethyl phosphite gave, after purification by m.p.l.c. on alumina-$AgNO_3$, the $\Delta^{5,7}$-3β,26-dibenzoate (V) in 44% yield. Hydrogenation-isomerization (29) (H_2/PtO_2/ethyl acetate-acetic acid) of V gave the $\Delta^{8(14)}$-3β,26-dicyclohexanoate (VI) in a 97% yield. Oxidation of VI with an excess of CrO_3-3,5-dimethylpyrazole complex (30) in CH_2Cl_2 at -20°C gave, after silica gel column chromatography, the $\Delta^{8(14)}$-15-keto-3,26-dicyclohexanoate (VII) in 30% yield. Hydrolysis of VII with H_2SO_4-MeOH-H_2O gave the desired (25R)-II in 90% yield.

Incubations of I with washed rat liver mitochondria in the presence of NADPH gave II as the major product, which, on the basis of the results of n.m.r. studies, was shown to be a 4:1 mixture of the 25R and 25S isomers of II. The observed 4:1 ratio of the 25R and 25S isomers of II is the same as the ratio of the rates of formation of the 25R and 25S isomers of 5β-cholestane-3α,7α,26-triol observed by Shefer et al. (31) upon incubation of 5β-cholestane-3α,7α-diol with rat liver mitochondria. Atsuta and Okuda (32) reported that incubation of 5β-cholestane-

3α,7α,12α-triol with a reconstituted system containing a partially purified rat liver mitochondrial cytochrome P-450 gave exclusively the 25R isomer of 5β-cholestane-3α,7α,12α-tetrol. The TLC evidence presented for the absolute stereospecificity of the reaction does not appear to exclude the presence of significant amounts of the 25S isomer.

(25R)-II was found to be highly active in suppressing the levels of HMG-CoA reductase activity in CHO-K1 cells, with a potency which was indistinguishable from that of I. (25R)-II also inhibited the oleoyl CoA-dependent esterification of cholesterol in jejunal microsomes, with a 55% inhibition at 10 μM. Under the same conditions, I was considerably more active, with 53% inhibition at 2 μM. Thus, (25R)-II, a major mitochondrial metabolite of I, is not only highly active in the suppression of the levels of activity of a key regulatory enzyme in the biosynthesis of cholesterol but also inhibits an enzyme of considerable importance in the absorption of cholesterol and the general intracellular metabolism of cholesterol. Accordingly, to fully define the biological actions of I, the activities of (25R)-II and other potential metabolites of I must also be taken into consideration.

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