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INHIBITION OF STEROL SYNTHESIS IN ANIMAL CELLS BY 15-OXYGENATED STEROLS WITH THE UNNATURAL *CIS*-C-D RING JUNCTION— 5α,14β-CHOLEST-7-EN-15α-OL-3-ONE AND 5α,14β-CHOLEST-7-EN-15β-OL-3-ONE*

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Abstract—5α,14β-Cholest-7-en-15α-ol-3-one was prepared in 72 per cent yield by selective oxidation of the 3β-hydroxyl function of 5α,14β-cholest-7-en-3β,15α-diol by cholesterol oxidase. 5α,14β-Cholest-7-en-15β-ol-3-one was prepared in a similar manner from 5α,14β-cholest-7-en-3β,15β-diol in 66 per cent yield. The new compounds were found to inhibit sterol synthesis in mouse fibroblasts in culture. The 15α-hydroxy-3-ketosterol and the 15β-hydroxy-3-ketosterol caused a 50 per cent inhibition of the incorporation of [1-^{14}C]acetate into digitonin-precipitable sterols at concentrations of 2.0×10^{-6} M and 2.5×10^{-7} M, respectively, and suppressed the level of activity of 3-hydroxy-3-methylglutaryl Coenzyme A reductase activity by 50 per cent at concentrations of 3.3×10^{-7} M and 2.5×10^{-7} M respectively.

The results of recent studies from these laboratories have indicated that a number of 15-oxygenated sterols are potent inhibitors of sterol biosynthesis in animal cells in culture as judged by their effects on the incorporation of the label of [1-^{14}C]acetate into digitonin-precipitable sterols in L cells and in primary cultures of fetal mouse liver cells [1–3]. Most of the 15-oxygenated sterols which were found to serve as potent inhibitors had the normal *trans*-C-D ring juncture. However, in the course of our initial studies we observed that two 15-oxygenated sterols with the "unnatural" *cis*-C-D ring junction, 5α,14β-cholest-7-en-3β,15α-diol and 5α,14β-cholest-7-en-3β,15β-diol, were also potent inhibitors of sterol synthesis in the same cell culture systems [2]. Our previous research has defined the formation of 3β-benzoyloxy-5α,14β-cholest-7-en-15-one upon treatment of 3β-benzoyloxy-14α,15α-epoxy-5α-cholest-7-ene with boron trifluoride-etherate [4, 5]. Reduction of the former compound with lithium aluminum hydride gave 5α,14β-cholest-7-en-3β,15β-diol and 5α,14β-cholest-7-en-3β,15α-diol [4, 5]. The structures of these 15-oxygenated Δ^7-sterols with the "unnatural" *cis*-C-D ring junction were based upon spectral studies [4, 5] and unequivocally established by the results of X-ray crystallographic analysis of 3β,15β-bis-(*p*-bromobenzoyloxy)5α,14β-cholest-7-ene [4, 6].

Described herein are syntheses of 5α,14β-cholest-7-en-15α-ol-3-one and 5α,14β-cholest-7-en-15β-ol-3-one. These sterols were prepared from their corresponding 3β-hydroxysterols through selective oxidation with cholesterol oxidase. The two new 15-oxygenated Δ^7-sterols with the "unnatural" *cis*-C-D ring junction were found to be more potent inhibitors of sterol synthesis in mouse fibroblast cells in culture than the corresponding 3β-hydroxysterols from which they were prepared.

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EXPERIMENTAL

Materials and methods

Melting points were recorded in sealed evacuated capillary tubes using a Thomas Hoover melting point apparatus. Nuclear magnetic resonance (n.m.r.) spectra were recorded on a Varian Associates EM-390 spectrometer at 90 MHz using $CDCl_3$ as solvent. Resonances are reported as ppm (δ) downfield from the tetramethylsilane internal standard. Infrared (i.r.) spectra were recorded on a Beckman IR-9 spectrometer using KBr pellets. Low resolution mass spectral (m.s.) analyses were recorded using an LKB model 9000S single focusing instrument under the conditions described previously [7]. High resolution mass spectral analyses were recorded on a Varian CH-5 spectrometer through the courtesy of Professor C. C. Sweeley. Thin-layer chromatographic analyses were performed using silica gel G (E. Merck, Darmstadt). Visualization of components on analytical plates was made after spraying the plates with a molybdic acid spray [8] and brief heating at 80°. Gas–liquid chromatographic analyses were performed using a Hewlett-Packard model 402 instrument equipped with dual flame ionization detectors. The columns (6 ft × 0.25 in., o.d.) were packed with 3% OV-17 on Gas Chrom Q (100–120 mesh; Applied Science Laboratories, Inc., State College, PA) with a column temperature of 250°. Helium was used as the carrier gas at a flow rate of 66 ml/min. The trimethylsilyl ethers of the sterols were prepared by treatment of the sterol (~0.1 mg) with a mixture of hexamethyldi-

silazane, trimethylchlorosilane, and pyridine (0.1 ml; 13:8:10 by volume) at 50° for 20 min. Cholesterol oxidase (components 1 and 3 of the Cholesterol Auto Test) was purchased from Biodynamics (BMC Division) of Boehringer Mannheim (Indianapolis, IN). 5α,14β-Cholest-7-en-3β,15β-diol and 5α,14β-cholest-7-en-3β,15α-diol were samples whose syntheses were recently described elsewhere [5].

5α,14β-Cholest-7-en-15β-ol-3-one

To component 1 (200 ml) of the Bio-Dynamics (BMC Division) Cholesterol Auto Test was added "cholesterol oxidase" (6.0 ml; component 3 of the Cholesterol Auto Test) and the resulting mixture was diluted with distilled water (600 ml). 5α,14β-Cholest-7-en-3β,15β-diol (126 mg; 0.315 mmole) in isopropanol (35 ml) was added and the resulting mixture was incubated with shaking at 37° for 7 hr. The mixture was extracted four times with 100-ml portions of chloroform. The extracts were combined, dried over anhydrous magnesium sulfate, and the volume was reduced to ~6 ml under reduced pressure. The resulting material was subjected to preparative thin-layer chromatography on six silica G plates (750 μm in thickness) using ether as the developing solvent. The desired ketone was eluted from the plates with chloroform and, after evaporation of the solvent under reduced pressure, crystallized from acetone–water to give 5α,14β-cholest-7-en-15β-ol-3-one (83.2 mg; 66 per cent yield) as needles melting at 90.5–91.5°; i.r., ν_{max} 3475, 2955, 2930, 1716 (C=O stretch of six-membered ring ketone), 1468, 1363, 1184, 1108, 1090, 1071, 833 and 533 cm^{-1}; n.m.r., 0.98 (s, 6H, C-18-CH_3 and C-19-CH_3), 2.22 (m, 5H, C-2-H_2, C-4-H_2 and C-14-H), 3.81 (m, 1H, C-15-H), and 5.45 (m, 1H, C-7-H). Apart from the infrared data presented above, the location of the ketone function at carbon atom 3 was indicated by the appearance of the absorption at 2.22 ppm, by the disappearance of the C-3-H absorption found in the spectrum of the 3β,15β-diol [0.75 (s, 3H, C-19-CH_3), 0.94 (s, 3H, C-18-CH_3), 2.10 (m, 1H, C-14-H), 3.57 (m, 1H, C-3-H), 3.80 (m, H, C-15-H), and 5.42 (m, 1H, C-7-H)], and by the downfield shifts of the C-18-CH_3 and C-19-CH_3 absorptions from their positions in the spectrum of the 3β,15β-diol by 0.04 and 0.23 ppm respectively. Mass spectrum, 400 (56%; M), 385 (14%; M-CH_3), 382 (76%; M-H_2O), 367 (56%; M-CH_3-H_2O), 297 (42%), 269 (91%; M-side chain-H_2O), 255 (12%), base peak at *m/e* 230; high resolution m.s., 400.3334 (calculated for $C_{27}H_{44}O_2$: 400.3341). The compound showed a single component on thin-layer chromatographic analysis on a silica gel G plate (solvent: ether). Gas–liquid chromatographic analysis of the trimethylsilyl ether derivative on a 3% OV-17 column indicated the purity to be at least 98.9 per cent. Gas–liquid chromatography–mass spectral analysis of the trimethylsilyl ether derivative showed the following prominent ions in the high mass region of the spectrum: 472 (1.5%; M), 457 (14%; M-CH_3), 382 (64%; M-trimethylsilanol), 367 (40%; M-CH_3-trimethylsilanol), 304 (18%), 297 (37%), 269 (61%; M-trimethylsilanol-side chain), 243 (17%), and 241 (100%).

5α,14β-Cholest-7-en-15α-ol-3-one

5α,14β-Cholest-7-en-15α-ol-3-one was prepared, in 72 per cent yield, from 5α,14β-cholest-7-en-3β,15α-diol as described for the case of the 15β-hydroxy compound. After preparative thin-layer chromatography and crystallization from acetone–water, the compound was obtained as fine needles melting at 121–122°; i.r., ν_{max} 3475, 2960, 2930, 2870, 1712 (C=O stretch of six-membered ring ketone), 1467, 1435, 1382, 1191, 1058, 1000, 899 and 570 cm^{-1}; n.m.r., 0.94 (s, 6H, C-18-CH_3 and C-19-CH_3), 2.20 (m, 5H, C-2-H_2, C-4-H_2 and C-14-H), 4.02 (m, 1H, C-15-H), and 5.45 (m, 1H, C-7-H). Apart from the infrared data presented above, the location of the ketone function at carbon atom 3 was indicated by the appearance of the absorption at 2.20 ppm, by the disappearance of the C-3-H absorption found in the spectrum of the 3β,15α-diol [0.72 (s, 3H, C-19-CH_3), 0.91 (s, 3H, C-18-CH_3), 2.14 (d, 1H, C-14-H, J = 5 Hz), 3.58 (m, 1H, C-3-H), 4.01 (m, 1H, C-15-H), and 5.44 (m, 1H, C-7-H)], and by the downfield shifts of the C-18-CH_3 and C-19-CH_3 absorptions from their positions in the spectrum of the 3β,15α-diol by 0.03 and 0.22 ppm respectively. Mass spectrum, 400 (23%; M), 382 (100%; M-H_2O), 367 (60%; M-CH_3-H_2O), 297 (50%), 269 (100%; M-H_2O-side chain), and 255 (13%); high resolution m.s., 400.3338 (calculated for $C_{27}H_{44}O_2$: 400.3341). The compound showed a single component on thin-layer chromatographic analysis on a silica gel G plate (solvent: ether). Gas–liquid chromatographic analysis of the trimethylsilyl ether derivative on a 3% OV-17 column (column temperature, 250°) indicated the purity to be at least 98.6 per cent. Gas–liquid chromatography–mass spectral analysis of the trimethylsilyl ether derivative showed the following prominent ions in the high mass region of the spectrum: 472 (1.7%; M), 457 (8%; M-CH_3), 382 (72%; M-trimethylsilanol), 367 (44%; M-CH_3-trimethylsilanol), 304 (17%), 297 (36%), 269 (63%; M-trimethylsilanol-side chain), and 241 (100%).

Cell culture studies

Mouse L cells (a subline of NCTC clone 929 mouse fibroblasts) were grown in serum-free media as described previously [2, 9, 10]. The preparation of steroid-containing media, procedures for assay of the rate of conversion of [1-^{14}C]acetate into digitonin-precipitable sterols, and fatty acids, and methods for measurement of DNA, protein and 3-hydroxy-3-methylglutaryl Coenzyme A (HMG-CoA) reductase were as described previously [2, 9, 10]. The L cell cultures were preincubated with the test compounds for 4 hr; then [1-^{14}C]acetate was added at a concentration of 4 μmoles (4 μCi) per ml. To determine the effects of the sterols on HMG-CoA reductase, the L cell cultures were incubated for 5 hr with the test compounds prior to harvesting for determination of microsomal HMG-CoA reductase activity.

RESULTS

The effects of 5α,14β-cholest-7-en-15α-ol-3-one and 5α,14β-cholest-7-en-15β-ol-3-one on the rates of incorporation of [1-^{14}C]acetate into digitonin-precipitable sterols and fatty acids are shown in Fig. 1. Studies of the rates of acetate metabolism to fatty acids were made so as to detect any possible effects

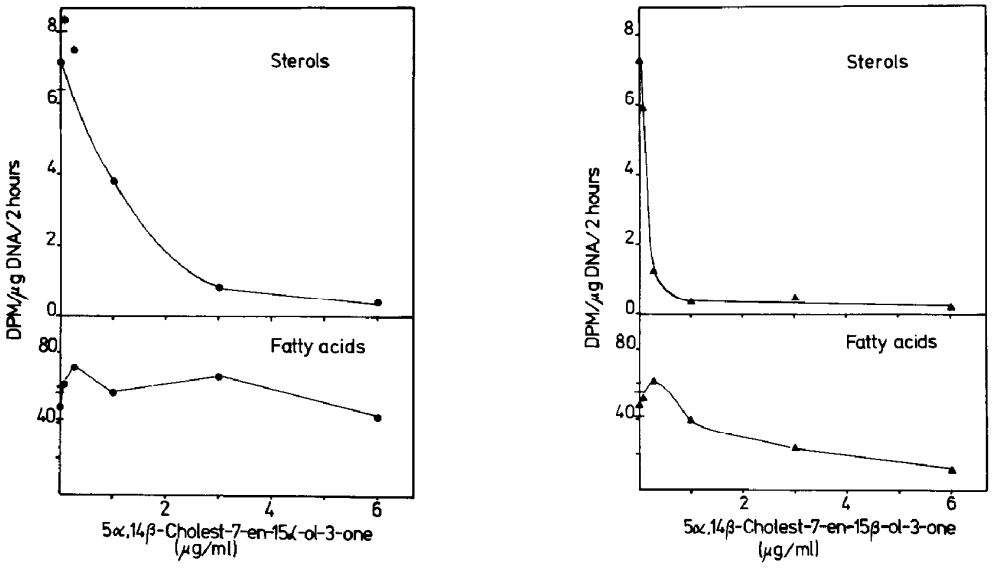

Fig. 1. Effects of 5α,14β-cholest-7-en-15α-ol-3-one (left) and 5α,14β-cholest-7-en-15β-ol-3-one (right) on rates of incorporation of [1-^{14}C]acetate into digitonin-precipitable sterols (above) and fatty acids (below).

of the inhibitors of sterol biosynthesis on general metabolism. In an attempt to correct for variations of the individual cultures upon the estimation of inhibitory potencies, the concentrations of the inhibitors required to inhibit sterol synthesis by 50 per cent were estimated from plots (Fig. 2) of the ratio of ^{14}C sterols to ^{14}C fatty acids as a function of the concentration of the inhibitors [2, 9]. The results of these analyses indicate that 5α,14β-cholest-7-en-15α-ol-3-one and 5α,15β-cholest-7-en-15β-ol-3-one caused a 50 per cent inhibition of sterol synthesis at concentrations of 2.0×10^{-6} M and 2.5×10^{-7} M respectively. A major site of the inhibitory action of these sterols appears to be at the level of HMG-CoA reductase since the 15α-hydroxy-3-ketosterol and the 15β-hydroxy-3-ketosterol caused a 50 per cent reduction of the level of microsomal HMG-CoA reductase activity in the L cells at concentrations of 3.3×10^{-7} M and 2.5×10^{-7} M respectively (Fig. 2).

DISCUSSION

Recent results from these laboratories indicated that two 15-oxygenated sterols with the unnatural *cis*-C-D ring junction, 5α,14β-cholest-7-en-3β,15β-diol and 5α,14β-cholest-7-en-3β,15α-diol, cause sig-

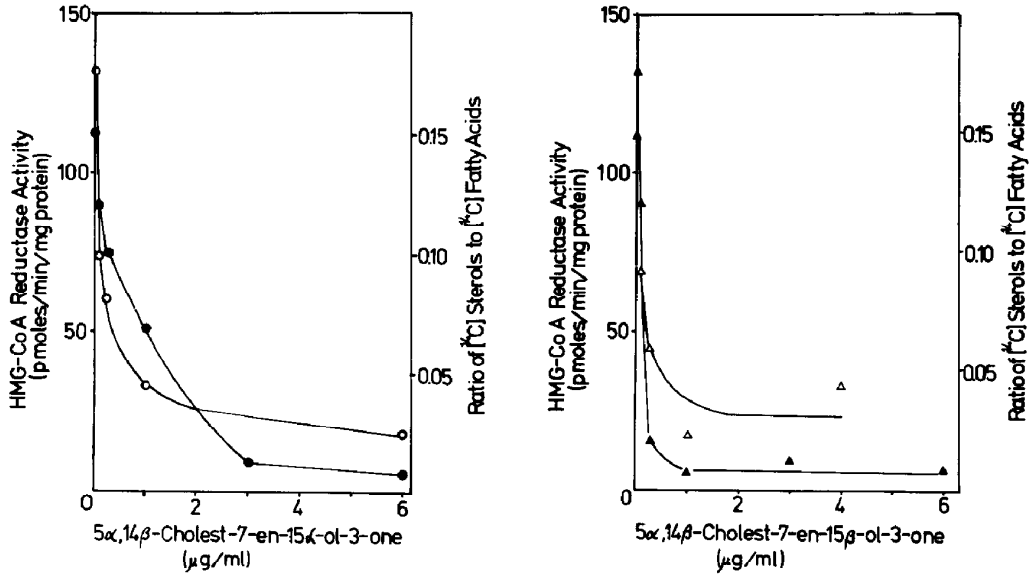

Fig. 2. Effects of 5α,14β-cholest-7-en-15α-ol-3-one (left) and of 5α,14β-cholest-7-en-15β-ol-3-one (right) on sterol synthesis (expressed as ratio of incorporation of [1-^{14}C]acetate into digitonin-precipitable sterols to incorporation of [1-^{14}C]acetate into fatty acids; ●——● and ▲——▲) and on levels of microsomal HMG-CoA reductase activity (○——○ and △——△) in L cells.

Table 1. Inhibition of sterol synthesis and HMG-CoA reductase activity in L cells by 15-oxygenated sterols with unnatural *cis*-C-D ring junction

Inhibitor	Conc (μM) required for 50% inhibition	
	Sterol synthesis	HMG-CoA reductase
5α,14β-Cholest-7-en-3β,15β-diol (Ref. 2)	1.0	4.5
5α,14β-Cholest-7-en-3β,15α-diol (Ref. 2)	3.2	6.7
5α,14β-Cholest-7-en-15β-ol-3-one	0.25	0.33
5α,14β-Cholest-7-en-15α-ol-3-one	2.0	0.25

nificant inhibition of sterol synthesis and a reduction of the level of HMG-CoA reductase activity in mouse fibroblasts and in primary cultures of fetal mouse liver cells [2]. To further investigate the potential inhibitory action of 15-oxygenated sterols with the unnatural *cis*-C-D ring junction, we pursued the preparation of the corresponding 15β-hydroxy- and 15α-hydroxy-3-ketosterols. The use of the enzyme cholesterol oxidase for the selective oxidation of the 3β-hydroxyl functions of 5α,14β-cholest-7-en-3β,15β-diol and 5α,14β-cholest-7-en-3β,15α-diol proved extremely valuable.

The 15α-hydroxy- and 15β-hydroxy-3-ketosterols were both more potent in the inhibition of sterol synthesis in the L cells and in reduction of the levels of HMG-CoA reductase activity in the same cells than the corresponding 3β,15-hydroxysterols (Table 1). The 15β-hydroxy-3-ketosterol was more potent in the inhibition of the incorporation of [1-^{14}C]acetate into digitonin-precipitable sterols than was the 15α-hydroxy-3-ketosterol, a finding similar to that made previously [2] in the cases of the corresponding 3β,15-hydroxy-Δ^7-sterols (Table 1) and in the cases of the corresponding 3β,15-hydroxy-$\Delta^{8(14)}$-sterols [2]. These findings are in contrast to those observed previously in the cases of the 15-hydroxy epimers of 3β-methoxy-14α-methyl-5α-cholest-7-ene [2], 14α-methyl-5α-cholest-7-en-3β-ol [2], and 14α-ethyl-5α-cholest-7-en-3β-ol [3]. In each of these series of 14α-alkylated Δ^7-sterols with the *trans*-C-D ring junction, the 15α-hydroxy epimer was more potent in the inhibition of sterol synthesis in the L cells.

It is noteworthy that no significant difference was observed between 5α,14β-cholest-7-en-15α-ol-3-one and 5α,14β-cholest-7-en-15β-ol-3-one with respect to their potencies in reducing the levels of HMG-CoA reductase activity in the L cells (Table 1).

Further studies of the effects of the new compounds described herein are in progress.

REFERENCES

1. G. J. Schroepfer, Jr., E. J. Parish, H. W. Chen and A. A. Kandutsch, *Fedn Proc.* **35**, 1697 (1976).
2. G. J. Schroepfer, Jr., E. J. Parish, H. W. Chen and A. A. Kandutsch, *J. biol. Chem.* **252**, 8975 (1977).
3. G. J. Schroepfer, Jr., E. J. Parish and A. A. Kandutsch, *J. Am. chem. Soc.* **99**, 5494 (1977).
4. E. J. Parish, M. E. Newcomer, G. L. Gilliland, F. A. Quiocho and G. J. Schroepfer, Jr., *Tetrahedron Lett.* 4401 (1976).
5. E. J. Parish and G. J. Schroepfer, Jr., *Chem. Phys. Lipids* **19**, 107 (1977).
6. G. L. Gilliland, M. E. Newcomer, E. J. Parish, G. J. Schroepfer, Jr. and F. A. Quiocho, *Acta crystallogr. B* **33** 3117 (1977).
7. F. F. Knapp, Jr. and G. J. Schroepfer, Jr., *Chem. Phys. Lipids* **17**, 466 (1976).
8. F. F. Knapp, Jr. and G. J. Schroepfer, Jr., *Steroids* **26**, 339 (1975).
9. A. A. Kandutsch and H. W. Chen. *J. biol. Chem.* **248**, 8408 (1973).
10. A. A. Kandutsch and H. W. Chen, *J. biol. Chem.* **249**, 6057 (1974).