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INHIBITION OF STEROL BIOSYNTHESIS BY 9α-FLUORO and 9α-HYDROXY DERIVATIVES OF 5α-CHOLEST-8(14)-EN-3β-OL-15-ONE*

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Summary: 5α-Cholest-8(14)-en-3β,9α-diol-15-one has been prepared by chemical synthesis in two steps from 5α-cholest-8(14)-en-3β-ol-15-one. 9α-Fluoro-5α-cholest-8(14)-en-3β-ol-15-one was prepared in 91% yield from the corresponding 9α-hydroxysterol. 9α-Fluoro-5α-cholest-8(14)-en-3,15-dione was prepared, in 84% yield, from the corresponding 3β-hydroxy compound. These compounds have been found to be potent inhibitors of sterol synthesis in animal cells in culture. The concentrations of the steroids required to cause a 50% inhibition of the synthesis of digitonin-precipitable sterols from labeled acetate were comparable to those required to cause a 50% reduction in the levels of HMG-CoA reductase activity in the same cells.

A number of 15-oxygenated sterols have been shown to be potent inhibitors of sterol biosynthesis in animal cells in culture (1-7). Of special importance was the demonstration that one of these 15-oxygenated sterols, 5α-cholest-8(14)-en-3β-ol-15-one (I), and a number of its derivatives have significant hypocholesterolemic activity in intact animals (8-11). The purposes of this communication are to describe the syntheses of the 9α-fluoro and 9α-hydroxy analogs of compound I and to present the results of studies indicating that these compounds are potent inhibitors of sterol biosynthesis in animal cells in culture. The chemical synthesis of the 3β-acetate derivative of 5α-cholest-8(14)-en-3β,9α-diol-15-one by an independent method has also been reported recently by Anastasia et al. (12).

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Materials and Methods

The recording of melting points (m.p.), proton magnetic resonance (p.m.r.), and mass spectra (m.s.) were carried out as described previously (13) as were thin layer chromatographic (t.l.c.) analyses. Ultraviolet (u.v.) spectra were recorded on ethanol solutions of the sterols. High resolution mass spectral analyses were made on a Varian CH-5 spectrometer. 5α-cholest-8(14)-en-3β-ol-15-one (I) was prepared as described previously (2,8). Perchloryl fluoride was purchased from Ozark-Mahoning Company (Tulsa, Oklahoma). HF-pyridine (70% HF in pyridine) was purchased from Aldrich Chemical Company (Milwaukee, Wisconsin).

15-Ethoxy-5α-cholest-8,14-dien-3β-ol (II)

Compound I (6.00 g; 15.0 mmol) in dry dioxane (60 ml) was treated with p-toluenesulfonic acid monohydrate (480 mg) and triethyl orthoformate (12 ml) and the resulting mixture was stirred at 25° for 24 h. A mixture of triethylamine (15 ml) and water (400 ml) was slowly added and the resulting mixture was poured into ether (1,000 ml). The ether solution was thoroughly washed with water, dried over anhydrous $MgSO_4$, and evaporated to dryness under reduced pressure to give II (5.26 g; 82% yield) as a light yellow glass which resisted all attempts at crystallization; p.m.r., 0.82 (s, 3H, C-19-CH_3), 0.97 (s, 3H, C-18-CH_3), 3.62 (m, 1H, C-3-H), and 3.80 (q, 2H, ethoxy); m.s., 428 (92%; M); high resolution m.s., 428.3649 (calc. for $C_{29}H_{48}O_2$: 428.3655); u.v., λ_{max} 254 nm (ε = 15,300). The product showed a single component on t.l.c. in 2 solvent systems.

5α-Cholest-8(14)-en-3β,9α-diol-15-one (III)***

Compound II (4.00 g; 9.3 mmol) in dry pyridine (75 ml) was cooled to -35° to -40° in a dry ice-acetone bath and a slow stream of perchloryl fluoride was bubbled through the stirred solution for 20 min. After bubbling nitrogen through the solution for 5 min, the mixture was poured into water and extracted with ether containing CH_2Cl_2 (10%). The extract was washed successively with water, cold 5% HCl, 5% $NaHCO_3$, and water and it was dried over anhydrous $MgSO_4$. Evaporation of the solvent under reduced pressure gave a yellow residue which, after medium pressure liquid chromatography on a silica gel column and recrystallization from acetone-water, gave III (1.34 g; 35% yield); m.p., 214.5-215.5°; p.m.r., 0.80 (s, 3H, C-19-CH_3), 0.97 (s, 3H, C-18-CH_3), 3.64 (m, 1H, C-3-H), and 3.98 (m, 1H, C-7β-H); m.s., 416 (14%; M); elem. anal., C, 77.70, H, 10.55 (calc. for $C_{27}H_{44}O_3$: C, 77.84, H, 10.64); u.v., λ_{max} 254 nm (ε = 13,500). The product showed a single component on t.l.c. in 2 solvent systems.

3β-Acetoxy-5α-cholest-8(14)-en-9α-ol-15-one (IV)

Compound III (500 mg; 1.20 mmol) in dry pyridine (15 ml) was treated with acetic anhydride (15 ml) for 24 h under nitrogen. After standard workup the crude product was recrystallized twice from acetone-water to give IV (476 mg; 86% yield), m.p. 194.0-195.5° [lit., 194-195° (12)]; p.m.r., 0.81 (s, 3H, C-19-CH_3; calc. 0.82), 0.96 (s, 3H, C-18-CH_3; calc. 0.97), 1.90 (s, 3H, methyl of acetoxy function), 3.96 (m, H, C-7β-H), and 4.86 (m, 1H, C-3-H);

*** CAUTION: While this reaction was repeated 9 times, on one occasion the reaction was accompanied by a violent explosion. Accordingly, we do not recommend the use of perchloryl fluoride for the preparation of III. The procedure recently described by Anastasia et al. for the preparation of the 3β-acetate derivative of III (12) or a new procedure which we have developed (E. J. Parish, M. Tsuda, and G. J. Schroepfer, Jr., manuscript in preparation) should be employed.

m.s., 458 (21%; M); high resolution m.s., 458.3396 (calc. for $C_{29}H_{46}O_4$: 458.3396); u.v., λ_{max} 254 nm (ε = 13,800) [lit., 254 nm (ε = 13,000) (12)]. The product showed a single component on t.l.c. in 2 solvent systems.

9α-Fluoro-5α-cholest-8(14)-en-3β-ol-15-one (V)

Compound III (2.00 g; 4.80 mmol) in CH_2Cl_2 (30 ml) was slowly added to a cooled (-35°) mixture of CH_2Cl_2 (20 ml) and HF-pyridine (20 ml). After stirring for 30 min, the mixture was poured into water and thoroughly extracted with ether. The combined extracts were dried over anhydrous $MgSO_4$ and the residue obtained upon evaporation of the solvent was subjected to silica gel column chromatography and recrystallization from acetone-water to give V (1.82 g; 90.5% yield) melting at 139.5-140.5°; p.m.r., 0.80 (s, 3H, C-19-CH_3), 0.96 (s, 3H, C-18-CH_3), 3.70 (m, 1H, C-3-H), 3.90 (m, 1H, C-7β-H); m.s., 418 (12%; M); high resolution m.s., 418.3247 (calc. for $C_{27}H_{43}O_2F$: 418.3246); u.v., λ_{max} 248 nm (ε = 13,600). The product showed a single component on t.l.c. in 3 solvent systems.

9α-Fluoro-5α-cholest-8(14)-en-3,15-dione (VI)

To compound V (500 mg; 1.19 mmol) in CH_2Cl_2 (40 ml) pyridinium chlorochromate (1.5 g) and sodium acetate (0.5 g) were added. After stirring for 30 min, the mixture was poured into a saturated solution of NaCl and thoroughly extracted with CH_2Cl_2. The combined extracts were dried over anhydrous $MgSO_4$ and the residue obtained upon evaporation of the solvent under reduced pressure was subjected to silica gel column chromatography and recrystallization from acetone-water to give VI (418 mg; 84% yield) melting at 155.0-156.5°; p.m.r., 0.98 (s, 6H, C-19-CH_3 and C-18-CH_3), 3.90 (m, 1H, C-7β-H); m.s., 416 (5%; M); high resolution m.s., 416.3090 (calc. for $C_{27}H_{41}O_2F$: 416.3090); λ_{max} 248 nm (ε = 13,700). The product showed a single component on t.l.c. in 3 solvent systems.

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 (14). The preparation of steroid-containing media, procedures for assaying the conversion of [1-^{14}C]-acetate into digitonin-precipitable sterols and fatty acids and methods for measurement of DNA and protein were as described previously (2,14,15). Studies of the rates of acetate metabolism to fatty acids were made so as to detect any possible effects of the inhibitors of sterol synthesis on general metabolism. As noted previously (2,14,15) minor variations in rates of fatty acid formation in experimental flasks as well as in control flasks were considered to be due to technical error and to variations in the metabolic characteristics of individual cultures. Accordingly, in an effort to correct for these sources of variation upon estimates of inhibitory potency, concentrations required to inhibit sterol synthesis by 50% were estimated from plots of the ratio of [^{14}C]-sterols to [^{14}C]-fatty acids as a function of the concentration of the inhibitor (2,14,15). In the case of the compounds studied in this paper, no significant effect on the rate of acetate metabolism to fatty acids was observed. HMG-CoA reductase activity in the cells was assayed by a minor modification (16) of procedures described previously (2,14,15).

Results

The effects of 5α-cholest-8(14)-en-3β,9α-diol-15-one on the synthesis of digitonin-precipitable sterols from labeled acetate in L cells and in primary cultures of fetal mouse liver cells are shown in Figure 1. These results indicate that

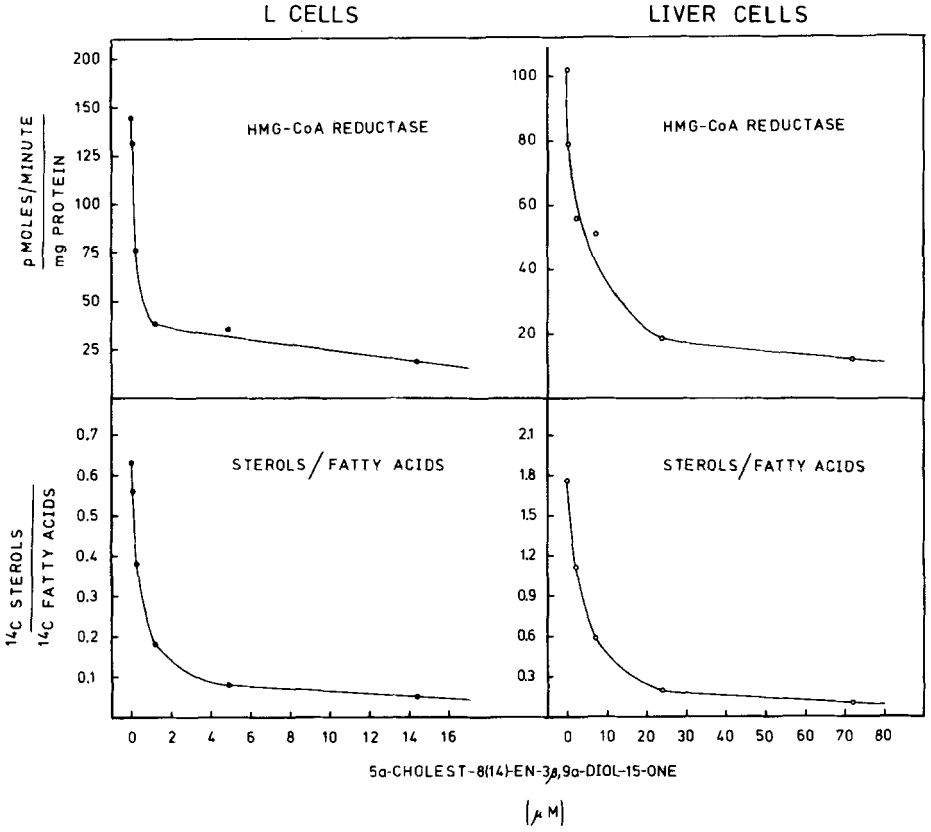

Figure 1. Effects of 5α-cholest-8(14)-en-3β,9α-diol-15-one on the synthesis of digitonin-precipitable sterols from labeled acetate and upon the levels of HMG-CoA reductase activity in L cells and in primary cultures of fetal mouse liver cells.

the oxygenated sterol caused a 50% inhibition of sterol synthesis in the L cells and in the liver cells at 0.3 μM and 4.0 μM, respectively. A major site of the inhibition of sterol synthesis in both cell types appears to be at the level of HMG-CoA reductase since compound III caused a 50% reduction in the levels of HMG-CoA reductase activity in the L cells and in the liver cells at 0.4 μM and 5.0 μM, respectively (Figure 1). The effects of 9α-fluoro-5α-cholest-8(14)-en-3β-ol-15-one and of 9α-fluoro-5α-cholest-8(14)-en-3,15-dione on the synthesis of digitonin-precipitable sterols from labeled acetate in L cells and on the levels of HMG-CoA reductase activity in the same cells are shown in Figure 2. Both compounds caused a 50% inhibition of sterol synthesis at 0.5 μM. Both compounds

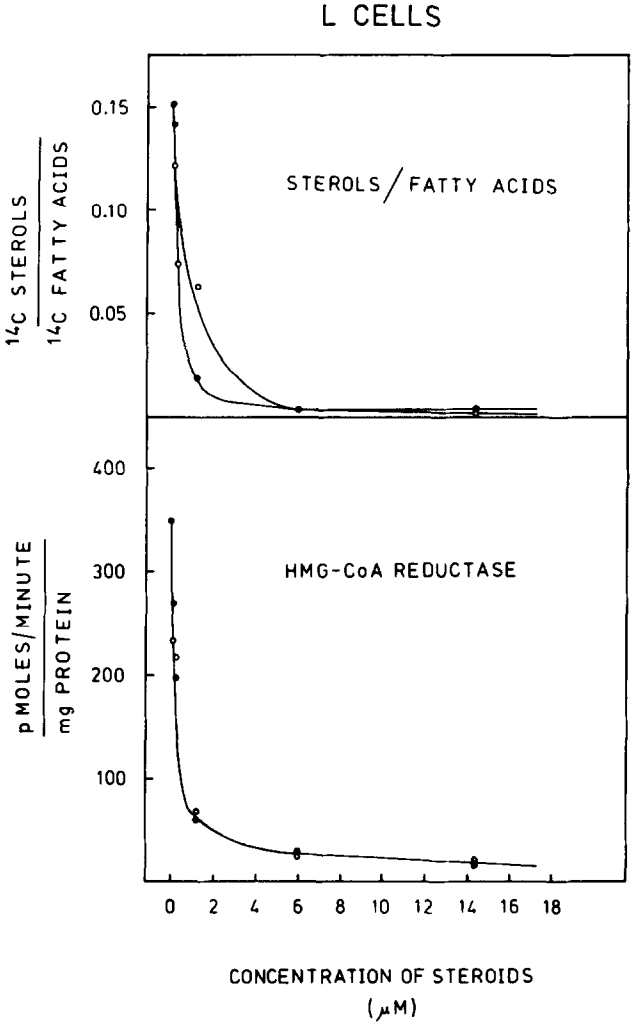

Figure 2. Effects of 9α-fluoro-5α-cholest-8(14)-en-3β-ol-15-one (o——o) and 9α-fluoro-5α-cholest-8(14)-en-3,15-dione (●——●) on the synthesis of digitonin-precipitable sterols from labeled acetate and upon the levels of HMG-CoA reductase activity in L cells.

caused a reduction in the levels of HMG-CoA reductase activity. The concentrations of the 3β-hydroxy compound and the 3-ketosterol required to cause a 50% reduction in the levels of HMG-CoA reductase activity were the same (0.2 μM).

Discussion

The ethyl enol ether (II; Figure 3) was prepared in 82% yield by treatment of compound I with triethyl orthoformate and an acid catalyst, an adaptation of

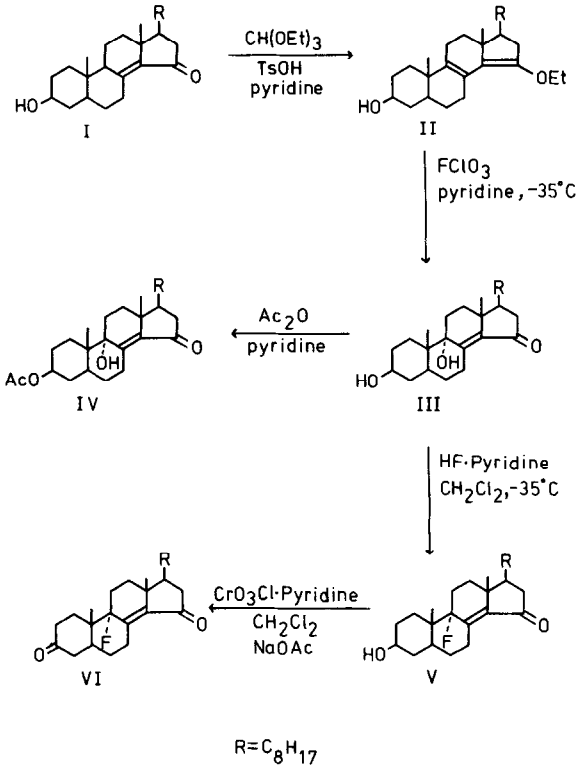

Figure 3. Chemical synthesis of 5α-cholest-8(14)-en-3β,9α-diol-15-one (III), 9α-fluoro-5α-cholest-8(14)-en-3β-ol-15-one (V), and 9α-fluoro-5α-cholest-8(14)-en-3,15-dione (VI).

procedures described previously for the preparation of steroidal enol ethers (17,18). The results of i.r., p.m.r., u.v., and high and low resolution mass spectral analyses were fully compatible with the assigned structure for II. Treatment of II with perchloryl fluoride gave 5α-cholest-8(14)-en-3β,9α-diol-15-one (III) in 35% yield. The results of i.r., p.m.r., u.v., and high and low resolution mass spectral analyses were compatible with the assigned structure for III. In addition, acetylation of III gave 3β-acetoxy-5α-cholest-8(14)-en-9α-ol-15-one (IV) in 86% yield. The i.r., p.m.r., u.v., and high and low resolution mass spectral properties indicated the assigned structure. Moreover, the melting point and p.m.r. and u.v. data compared favorably with those reported recently for IV prepared by an independent method (12). In addition, the results

of detailed ^{13}C n.m.r. studies of compounds III and IV were fully compatible with the assigned structures[§]. Compound V was prepared in 90% yield from compound III by an adaptation of the method of Ambles and Jacquesy (19). Oxidation of V with pyridinium chlorochromate gave the corresponding 3-ketone (VI) in 84% yield. The results i.r., u.v., p.m.r., and high and low resolution mass spectral analyses were fully compatible with the assigned structures for V and VI.

The results presented herein demonstrate that 5α-cholest-8(14)-en-3β,9α-diol-15-one is an effective inhibitor of sterol synthesis in L cells and in primary cultures of fetal mouse liver cells. The inhibitory potencies of the 9α-hydroxy compound in the two cell culture systems were similar to those of the parent compound, 5α-cholest-8(14)-en-3β-ol-15-one (2). The inhibitory potencies of the 9α-hydroxy compound on sterol synthesis in the two cell types correlated closely with its action in lowering the levels of HMG-CoA reductase activity in the same cells. It is noteworthy that 9α-hydroxylation of a number of C_{19} and C_{21}-steroids has been demonstrated in microorganisms (20,21, and references cited therein). However, to our knowledge, 9α-hydroxylation of C_{27} sterols has not been reported.

9α-Fluoro-5α-cholest-8(14)-en-3β-ol-15-one and 9α-fluoro-5α-cholest-8(14)-en-3,15-dione were also found to be potent inhibitors of sterol synthesis in L cells. The inhibitory potencies of the two 9α-fluoro sterols in inhibiting the synthesis of digitonin-precipitable sterols in L cells were similar, but not identical, to the potencies of the two sterols in causing a reduction of the levels of HMG-CoA reductase in the same cells. It is noteworthy that the concentrations (0.2 μM) of the two 9α-fluoro sterols required to cause a 50% reduction in the levels of HMG-CoA reductase activity were comparable to those of their 9α-protio parent compounds, i.e., 5α-cholest-8(14)-en-3β-ol-15-one (2; 0.3 μM) and 5α-cholest-8(14)-en-3,15-dione (7; 0.4 μM).

Further studies of the biological effects of the new 15-oxygenated sterols described herein are in progress. The two 9α-fluoro-15-ketosterols hold promise

§ (These studies will be reported in detail elsewhere along with a discussion of the mechanism of formation of III from II and the description of an alternative new method for the chemical synthesis of III; E. J. Parish, M. Tsuda, and G. J. Schroepfer, Jr., in preparation.)

of representing new hypocholesterolemic agents of high activity in view of the fact that their 9α-protio analogs have been found to possess significant hypocholesterolemic activity in animals (8,9,10).

REFERENCES

1. Schroepfer, G. J., Jr., Parish, E. J., Chen, H. W., and Kandutsch, A. A., (1976) Fed. Proc., 35, 1697.
2. Schroepfer, G. J., Jr., Parish, E. J., Chen, H. W., and Kandutsch, A. A., (1977) J. Biol. Chem., 252, 8975-8980.
3. Schroepfer, G. J., Jr., Parish, E. J., and Kandutsch, A. A., (1977) J. Amer. Chem. Soc., 99, 5494-5496.
4. Schroepfer, G. J., Jr., Raulston, D. L., and Kandutsch, A. A., (1977) Biochem. Biophys. Res. Commun., 79, 406-410.
5. Schroepfer, G. J., Jr., Pascal, R. A., Jr., and Kandutsch, A. A., (1978) Biochem. Pharmacol., 28, 249-252.
6. Schroepfer, G. J., Jr., Parish, E. J., Tsuda, M., Raulston, D. L., and Kandutsch, A. A., (1979) J. Lipid Res., in press.
7. Schroepfer, G. J., Jr., Parish, E. J., and Kandustch, A. A., (1979) Chem. Phys. Lipids, in press.
8. Raulston, D. L., Mishaw, C. O., Parish, E. J., and Schroepfer, G. J., Jr., (1976) Biochem. Biophys. Res. Commun., 71, 984-989.
9. Schroepfer, G. J., Jr., Monger, D., Taylor, A. S., Chamberlain, J. S., Parish, E. J., Kisic, A., and Kandutsch, A. A., (1977) Biochem. Biophys. Res. Commun., 78, 1227-1232.
10. Kisic, A., Monger, D., Parish, E. J., Satterfield, S., Raulston, D. L., and Schroepfer, G. J., Jr., (1977) Artery, 3, 421-428.
11. Kisic, A., Taylor, A. S., Chamberlain, J. S., Parish, E. J., and Schroepfer, G. J., Jr., (1978) Fed. Proc., 37, 1663.
12. Anastasia, M., Fiecchi, A., and Scala, A., (1979) J. Chem. Soc. Perkin I, 1821-1824.
13. Schroepfer, G. J., Jr., Pascal, R. A., Jr., Shaw, R., and Kandutsch, A. A., (1978) Biochem. Biophys. Res. Commun., 83, 1024-1031.
14. Kandutsch, A. A., and Chen, H. W., (1973) J. Biol. Chem., 248, 8408-8417.
15. Kandutsch, A. A., and Chen, H. W., (1974) J. Biol. Chem. 249, 6057-6061.
16. Saucier, S. E., and Kandutsch, A. A., (1979) Biochim. Biophys. Acta, 572, 541-556.
17. Marshall, C. W., Ralls, J. W., Saunders, F. J., and Riegel, B., (1957) J. Biol. Chem., 228, 339-348.
18. Bowers, A., Holton, P. G., Necoechea, E., and Kincl, F. A., (1961) J. Chem. Soc., 4057-4060.
19. Ambles, A., and Jacquesy, R. (1976) Tetrahedron Lett., 1083-1084.
20. Chang, F. N., and Sih, C. J., (1964) Biochemistry, 3, 1551-1557.
21. Wovcha, M. G., Antosz, F. J., Knight, J. C., Kominek, L. A., and Pyke, T. R., (1978) Biochim. Biophys. Acta, 531, 308-321.