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7α,15β-DICHLORO-5α-CHOLEST-8(14)-EN-3β-OL, A NOVEL INHIBITOR OF STEROL BIOSYNTHESIS IN ANIMAL CELLS*

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Summary: 7α,15β-Dichloro-5α-cholest-8(14)-en-3β-ol has been prepared by controlled lithium aluminum hydride reduction of 3β-benzoyloxy-7α,15β-dichloro-5α-cholest-8(14)-ene. The latter compound was prepared by treatment of 3β-benzoyloxy-14α,15α-epoxy-5α-cholest-7-ene with gaseous HCl in chloroform at -40°. The structure of the dichloro compound was unequivocally established by X-ray crystallographic analysis of the 3β-p-bromobenzoate derivative. 7α,15β-Dichloro-5α-cholest-8(14)-en-3β-ol was found to be a potent inhibitor of sterol synthesis in animal cells in culture. The dichlorosterol caused a 50% reduction of the level of HMG-CoA reductase in L cells in culture at a concentration of 6×10^{-7} M and a 50% inhibition of the synthesis of digitonin-precipitable sterols from labeled acetate in the same cells at a concentration of 2×10^{-6} M.

We have recently reported that a number of 15-oxygenated sterols are very potent inhibitors of sterol synthesis in animal cells in culture (1-5). In addition, several of these 15-oxygenated sterols have been found to have significant hypocholesterolemic activity in animals (6-9). A key intermediate in the chemical syntheses of a number of these 15-oxygenated sterols is 3β-benzoyloxy-14α,15α-epoxy-5α-cholest-7-ene (10) (Ia; Figure 1) whose structure has been unambiguously established by X-ray crystallographic analysis of the corresponding 3β-p-bromobenzoate ester (11) (Ib). We now wish to report that treatment of Ia with gaseous HCl in chloroform at -40° gives, in 87% yield, 3β-benzoyloxy-7α,15β-dichloro-5α-cholest-8(14)-ene (IIa). Evidence permitting unambiguous assignment of structure was provided by the results of X-ray crystallographic analysis of the corresponding

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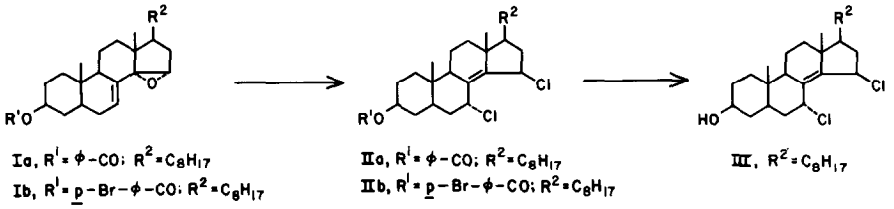


Figure 1. Synthesis of 7α,15β-dichloro-5α-cholest-8(14)-en-3β-ol (III).

3β-p-bromobenzoate ester (IIb) which was prepared in 93% yield from Ib in an analogous fashion. Treatment of IIa with lithium aluminum hydride under controlled conditions gave 7α,15β-dichloro-5α-cholest-8(14)-en-3β-ol (III) in 86% yield. The latter sterol was found to be a potent inhibitor of sterol synthesis in L cells grown in serum-free media and to reduce the levels of HMG-CoA reductase activity in the same cells.

Materials and Methods

The recording of melting points, infrared (i.r.) spectra, nuclear magnetic resonance (n.m.r.) spectra, optical rotations, and mass spectra (m.s.) were carried out as described previously (12) as were thin layer chromatographic (t.l.c.) analyses. 3β-Benzoyloxy-14α,15α-epoxy-5α-cholest-7-ene (Ia) was prepared as described previously (10). The preparation of 3β-p-bromobenzoyloxy-14α,15α-epoxy-5α-cholest-8(14)-ene (Ib) has been described previously (11).

3β-Benzoyloxy-7α,15β-dichloro-5α-cholest-8(14)-ene (IIa)

Dry HCl gas was passed through a solution of Ia (2.00 g) in chloroform at -40° for 3 h. The reaction mixture was repeatedly washed with cold water and dried over anhydrous magnesium sulfate. The residue obtained upon evaporation of the solvent at reduced pressure at 30° was recrystallized 3 times from acetone-water to give IIa (1.97 g; 87% yield); m.p., 116-117° (clearing at 138-139°); single component on t.l.c.; m.s. (relative intensity), 524 and 522 (M-HCl; 2% and 5%), base peak at m/e 349 (M-2HCl-CH_3-benzoic acid); high resol. m.s. on ion at m/e 522, 522.3275 (calc. for $C_{34}H_{47}O_2{}^{35}Cl$: 522.3264); elem. anal., calc. for $C_{34}H_{48}O_2Cl_2$: C, 72.97; H, 8.64; found: C, 72.95; H, 8.81; $[\alpha]_D$ -218.4° (c, 0.25; $CHCl_3$); n.m.r., 0.79 (s, 3H, C-19-CH_3), 1.12 (s, 3H, C-18-CH_3), 4.95 (m, 2H, C-3-H and C-7-H), 5.40 (m, 1H, C-15-H), and 7.85 (m, 5H, aromatic).

3β-p-Bromobenzoyloxy-7α,15β-dichloro-5α-cholest-8(14)-ene (IIb)

Treatment Ib with gaseous HCl as described above gave, in 93% yield, IIb; m.p. 148-149° (clearing at 164-165°); single component on t.l.c. in three solvent systems; m.s. (relative intensity), 604, 602 and 600 (M-HCl; 2%, 5%, and 4%), base peak at m/e 349 (M-CH_3-2HCl-bromobenzoic acid); high resol. m.s. on ion at m/e 600, 600.2406 (calc. for $C_{34}H_{46}O_2{}^{79}Br{}^{35}Cl$: 600.2370); elem. anal., calc. for $C_{34}H_{47}O_2Cl_2Br$: C, 63.95, H, 7.42; found: C, 63.80, H, 7.63; $[\alpha]_D$ -214.2° (c, 0.30, $CHCl_3$); n.m.r., 0.79 (s, 3H, C-19-CH_3), 1.10 (s, 3H, C-18-CH_3), 4.90 (m, 2H, C-3-H and C-7-H), 5.40 (m, 1H, C-15-H), and 7.85 (m, 4H, aromatic). Recrystallization from methylene chloride-ethanol (1:4) gave crystals suitable for X-ray analysis. A Syntex $P2_1$

diffractometer with niobium filtered MoKα radiation was used in the 20:θ scan mode (scan range 2 to 30 degrees minutes^{-1}) to collect diffraction data. A single crystal was used to collect 2182 unique reflections. A Patterson (13) map was used to locate the bromine and two chlorine atoms. The positions of the bromine and chlorine atoms were used to approximate the phases in a Fourier synthesis. From this and subsequent Fourier synthesis all nonhydrogen atoms were located. The structure was refined by a least squares method using diagonal and block matrices with a Hughes (14) weighting scheme. Bromine, chlorine, and oxygen atoms were given anisotropic temperature factors. The final weighted R factor was 1.4% and unweighted R factor was 13.8%. The structural analysis established the configurations of the chlorine atoms at the 7 and 15 positions as α and β, respectively. Crystal data: $C_{34}H_{47}O_2Cl_2Br$, M=638.6, monoclinic, space group $P2_1$, a=6.22 Å, b=11.36 Å, c=23.18 Å, β=91.4°, V=1637 Å^3, D_c=1.29 g cm^{-3}, F(0,0,0,)=0.409eÅ^{-3}. Full details of the structural analysis will be presented in a separate publication.

7α,15β-Dichloro-5α-cholest-8(14)-en-3β-ol (III)

To IIa (1.00 g) in ether (100 ml) was added lithium aluminum hydride (0.24 g). After stirring for 20 min at 25°, the mixture was cooled to 0° and ice was cautiously added to decompose the unreacted hydride. The mixture was poured into a saturated solution of ammonium chloride and thoroughly extracted with ether containing methylene chloride (10%). The extracts were dried over anhydrous magnesium sulfate and the residue obtained upon evaporation of the solvent was subjected to chroma tography on a silica gel (60-200 mesh) column (40 cm x 3 cm) using 10% ether in benzene as the eluting solvent. Fractions 20 ml in volume were collected. The contents of fractions 7 through 17 were pooled and, after evaporation of the solvent under reduced pressure, recrystallized from acetone-water to give III (0.70 g; 86% yield; m.p., 98-100°; single component on t.l.c.; m.s. (relative intensity) 420 and 418 (M-HCl; 4% and 10%), base peak at m/e 349 (M-CH_3-H_2O-2HCl); high resol. m.s. on ion at m/e 418, 418.3002 (calc. for $C_{27}H_{43}O^{35}Cl$: 418.3000); elem. anal., calc. for $C_{27}H_{44}OCl_2$: C, 71.19; H, 9.74; found, C, 71.22, H, 9.76; $[\alpha]_D$ -246.1° (c, 0.38, $CHCl_3$); n.m.r., 0.72 (s, 3H, C-19-CH_3), 1.10 (s, 3H, C-18-CH_3), 3.70 (m, 1H, C-3-H), 4.96 (m, 1H, C-7-H), and 5.32 (m, 1H, C-15-H).

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 decribed previously (15). The preparation of steroid-containing media, procedures for assaying the conversion of [1-^{14}D]-acetate into digitonin-precipitable sterols and fatty acids and methods for measurement of DNA and protein were as described previously (2,15,16). The cultures were preincubated with the test compound for 4 hours; 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 the sterols were incubated with the cultures for 5 hours prior to harvesting for the determination of HMG-CoA reductase activity by a modification of the method of Brown, Dana, and Goldstein (17) using a higher concentration (80 μM) of RS-HMG-CoA and a 30 minute incubation period.

Results

The effects of 7α,15β-dichloro-5α-cholest-8(14)-en-3β-ol on the rates of incorporation of [1-^{14}C]-acetate into digitonin-precipitable sterols and fatty acids are shown in Figure 2. Studies of the rates of acetate metabolism to fatty

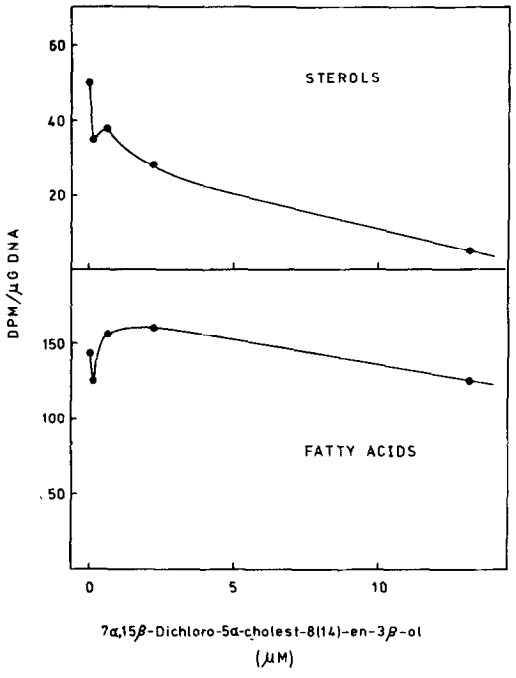
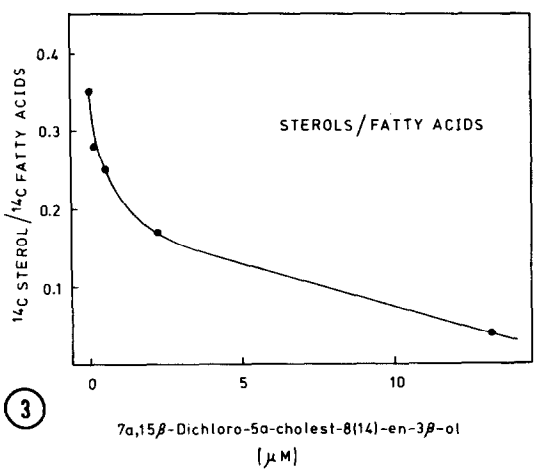


Figure 2. Effect of 7α,15β-dichloro-5α-cholest-8(14)-en-3β-ol on the rates of incorporation of [1-^{14}C]-acetate into digitonin-precipitable sterols and fatty acids in L cells grown in serum-free media (2,15,16).

acids were made so as to detect any possible effects of the inhibitor of sterol synthesis on general metabolism. In an effort to correct for variations of individual cultures upon the estimation of the inhibitor potency, the concentration required to inhibit sterol synthesis by 50% was estimated from a plot (Figure 3) of the ratio of [^{14}C]-sterols to [^{14}C]-fatty acids as a function of the concentration of the inhibitor (2,15,16). The results of this analysis indicated that 7α,15β-dichloro-5α-cholest-8(14)-en-3β-ol caused a 50% inhibition of sterol synthesis at a concentration of 2×10^{-6} M. A major site of this inhibition of sterol synthesis appears to be at the level of HMG-CoA reductase since the dichloro sterol caused a 50% reduction in the level of HMG-CoA reductase in the L cells at a concentration of 6×10^{-7} M (Figure 4).

Discussion

The results presented above indicate that treatment of 3β-benzoyloxy-14α,15α-epoxy-5α-cholest-7-ene (Ia) with gaseous HCl in $CHCl_3$ at -40° gives, in very high



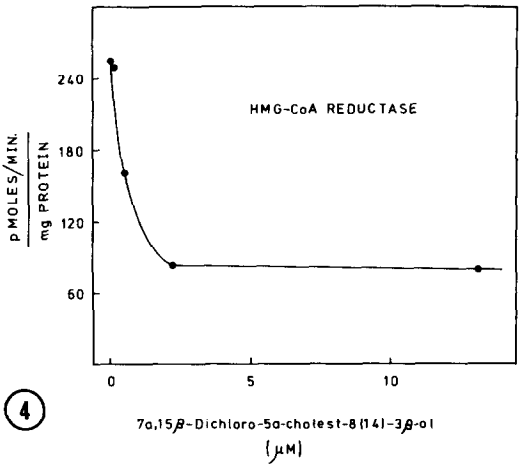


Figure 3. Effect of 7α,15β-dichloro-5α-cholest-8(14)-en-3β-ol on the ratio of the incorporation of [1-^{14}C]-acetate into digitonin-precipitable sterols to the incorporation of [1-^{14}C]-acetate into fatty acids in L cells grown in serum-free media (2,15,16).

Figure 4. Effect of 7α,15β-dichloro-5α-cholest-8(14)-en-3β-ol on the level of HMG-CoA reductase activity in L cells grown in serum-free media (2,15,16).

yield, 3β-benzoyloxy-7α,15β-dichloro-5α-cholest-8(14)-ene (IIa). The latter compound was fully characterized and evidence permitting unambiguous assignment of structure was derived from the results of X-ray crystallographic analysis of the corresponding 3β-p-bromobenzoate ester (IIb) which was prepared from Ib in an analogous fashion. The precise mechanism of the formation of IIa from Ia is not clear. One possible reaction course would involve initial opening of the epoxide to give a Δ^7-14α-hydroxy-15β-chloro derivative which, upon attack by chloride ion at the C-7α-position, could initiate an S_N2' rearrangement leading to the formation of the 7α,15α-dichloro-$\Delta^{8(14)}$-system. Alternatively, the overall reaction could be envisioned as proceeding via an initial introduction of chlorine at C-7 in the α configuration followed by ring opening of the 14α,15α-epoxide function. If the reaction follows an S_N2' reaction course analogous to that observed previously for this α,β-unsaturated epoxide upon reaction with hydroxide ion to give 5α-cholestan-3β,7α,15α-triol (18), the formation of the 7α-chloro-$\Delta^{8(14)}$-15α-hydroxy derivative

would be anticipated. A stereospecific displacement of the 15α-hydroxyl function by chloride could thereupon yield the 7α,15β-dichloro-$\Delta^{8(14)}$-derivative. In the course of our investigations to date we have been unable to isolate any intermediates in the overall conversion of Ia to IIa. Treatment of IIa with lithium aluminum hydride under controlled conditions gave 7α,15β-dichloro-5α-cholest-8(14)-en-3β-ol (III) in 86% yield. The latter compound was fully characterized.

The results presented herein indicate that the dichloro-sterol (III) is a very potent inhibitor of sterol synthesis in L cells grown in serum-free media. This inhibitory activity appears to be specific for sterol synthesis and not due to an effect on the general metabolism of acetate inasmuch as no significant inhibition of the metabolism of acetate to fatty acids was observed. The site of action of this new inhibitor of sterol synthesis appears to be at the level of mevalonate formation since the dichloro-sterol was found to be very active in the reduction of the levels of HMG-CoA reductase activities in the same cells. It is noteworthy that, of a very large number of sterols and sterol derivatives found to have significant potency in inhibition of sterol synthesis in the L cells, all have been either dioxygenated or trioxygenated sterols (1-5,15-16,19). 3,7- and 3,15-dioxygenated (1-5,15-16,19) and 3,7,15-trioxygenated (2) sterols have previously been shown to be potent inhibitors of sterol synthesis in these cells. The present case constitutes the first example of an inhibitor of sterol synthesis at the level of mevalonate formation of this type in these cells (without affecting fatty acid synthesis) in which no oxygen substitution is present other than that at carbon atom 3.

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., in press.
6. Raulston, D.L., Mishaw, C.O., Parish, E.J., and Schroepfer, G.J., Jr., (1976) Biochem. Biophys. Res. Commun., 71, 984-989.
7. Kisic, A., Monger, D., Parish, E.J., Satterfield, S., Raulston, D.L., and Schroepfer, G.J., Jr., (1977) Artery, 3, 421-428.
8. 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.
9. Kisic, A., Taylor, A.S., Chamberlain, J.S., Parish, E.J., and Schroepfer, G.J., Jr., (1978) Fed. Proc., 37, 1663.
10. Parish, E.J., Spike, T.E., and Schroepfer, G.J., Jr., (1977) Chem. Phys. Lipids, 18, 233-239.
11. Conner, B.N., Parish, E.J., Schroepfer, G.J., Jr., and Quiocho, F.A., (1977) Chem. Phys. Lipids, 18, 240-257.
12. Schroepfer, G.J., Jr., Pascal, R.A., Jr., Shaw, R., and Kandutsch, A.A., (1978) Biochem. Biophys. Res. Commun., in press.
13. Patterson, A.L., (1935) Z. Krist., A90, 517-542.
14. Hughes, E.W., (1941) J. Amer. Chem. Soc., 63, 1737-1752.
15. Kandutsch, A.A., and Chen, H.W., (1973) J. Biol. Chem., 248, 8408-8417.
16. Kandutsch, A.A., and Chen, H.W., (1974) J. Biol. Chem., 249, 6057-6061.
17. Brown, M.S., Dana, S.E., and Goldstein, J.L., (1973) Proc. Nat. Acad. Sci., U.S.A. 70, 2162-2166.
18. Tsuda, M., Parish, E.J., and Schroepfer, G.J., Jr., submitted for publication.
19. Kandutsch, A.A., and Chen, H.W., J. Cell. Physiol., 85, 415-424 (1975).