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AGENTS AFFECTING LIPID METABOLISM—I. ON THE INHIBITION OF CHOLESTEROL BIOSYNTHESIS BY ESTROGENS

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Abstract—The 16,16-difluoro analogues of equilin and equilenin methyl ethers have been synthesized. These and other natural and synthetic estrogens have been tested *in vitro* as inhibitors of cholesterol biosynthesis. All the compounds tested exhibited marked activity, with 16,16-difluoroequilin methyl ether being the most potent, inhibiting by 36 per cent at a concentration of 1×10^{-4} M. The possible role of inhibition of cholesterol biosynthesis in the mechanism of estrogen-induced hypocholesteremia is discussed.

ESTROGENS have long been known to affect lipid metabolism, as demonstrated by reductions in plasma cholesterol and β-lipoprotein levels.[1, 2] Feminizing effects have been a limiting factor in the use of these materials clinically[3, 4] and this has led to the development of synthetic estrogen analogues in an attempt to separate lipid-shifting from feminizing effects.[5–8] The most potent compounds of this type are derivatives of 16,16-difluoroestrone which have good cholesterol-lowering properties and very low estrogenic potencies.[8] This report and the availability of equilin and equilenin to us, prompted us to prepare the corresponding 16,16-difluoromethyl ethers.

It is known that the major portion of the serum cholesterol pool is derived from endogenous biosynthesis;[9] thus inhibition of this biosynthesis should result in marked lowering of serum cholesterol levels.[10]

The compound triparanol, [1-(*p*-diethylaminoethoxyphenyl)-1-(*p*-tolyl)-2-(*p*-chlorophenyl)-ethanol], related structurally to the nonsteroidal synthetic estrogens,[11] is reported to be a potent inhibitor of cholesterol biosynthesis[12] and is being used clinically as a cholesterol-lowering agent.[13] The clinical success obtained with this compound led us to examine estrone, equilin, and equilenin as well as their methyl ethers and 16,16-difluoromethyl ethers for their ability to inhibit the biosynthesis *in vitro* of cholesterol from mevalonate-2-^{14}C. These results form the basis of this report.

MATERIALS

I. Inhibitors

Estrone, equilin, and equilenin were the natural biological materials obtained from equine sources.* They were more than 99·5 per cent pure. The corresponding methyl ethers were prepared according to the usual procedures.[14] 16,16-Difluoroestrone methyl ether was prepared as described by Robinson *et al.*[8] The difluoroequilin and

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equilenin methyl ethers were prepared through the intermediate 16-hydroxymethylene derivatives as described below.

(+)-16-*Hydroxymethyleneequilin methyl ether.* A mixture of (+)-equilin methyl ether (21 g), ethyl formate (49 ml), and sodium methoxide (from 3·5 g of sodium) was refluxed in a 1:1 mixture of benzene:ether for 2·5 hr, then allowed to stir at room temperature for 12 hr. Filtration yielded the sodium salt of the title compound as a gel-like solid. It was suspended in a mixture of methylene chloride and water and acidified with 5% hydrochloric acid. The methylene chloride layer was separated, washed with water, dried, and evaporated to yield 22 g of an orange-coloured oil which, by treating with methanol, yielded 10 g of a white crystalline substance which, on further crystallizations from methylene chloride-methanol, had m.p. 178–180 °C $[\alpha]_D$ ($CHCl_3$): + 247·0 °.

$C_{20}H_{22}O_3$ requires: C, 77·40; H, 7·14 per cent.
Found: C, 76·85; H, 7·06 per cent.

(+)-16:16-*Difluoroequilin methyl ether.* (+)-16-Hydroxymethyleneequilin methyl ether (9·0 g) was dissolved in *tert*-butanol containing potassium *tert*-butoxide. Into this mixture perchloryl fluoride was bubbled for 16 min. During this period three additional portions of potassium *tert*-butoxide were added. At the end of the reaction the butanol was removed *in vacuo*, and the residue was distributed between water and chloroform. The chloroform layer was washed with water, dried, and evaporated to yield the crude title product as an orange-coloured solid. It was chromatographed on alumina. Elution with 1:1 benzene:chloroform yielded 3 g of the title compound. A sample was sublimed for analysis. It had m.p. 172–176 °C, $[\alpha]_D$ ($CHCl_3$): + 192·4 °.

$C_{19}H_{20}O_2F_2$ requires: F, 11·94 per cent.
Found: F, 11·91, 12·01 per cent.

(±)-16-*Hydroxymethyleneequilenin methyl ether.* A mixture of (±)-equilenin methyl ether (9·4 g), ethyl formate (21·9 ml), and sodium methoxide (from 1·57 g of sodium) was refluxed in a 1:1 mixture of benzene:ether for 3·5 hr. The mixture was then allowed to stir overnight at room temperature; it was then extracted with water several times. The aqueous solution was acidified to pH 6 with dilute hydrochloric acid and extracted with methylene chloride to yield a yellow solid. Crystallization from methylene chloride-methanol yielded 7·5 g of material. A sample was sublimed for analysis. It had m.p. 186–187 °C.

$C_{20}H_{20}O_3$ requires: C, 77·9; H, 6·54 per cent.
Found: C, 78·25; H, 6·28 per cent.

(±)-16:16-*Difluoroequilenin methyl ether.* (±)-16-Hydroxymethyleneequilenin methyl ether (5·3 g) was dissolved in *tert*-butanol containing potassium *tert*-butoxide. Into this mixture perchloryl fluoride was bubbled for 16 min. During this period three additional portions of potassium *tert*-butoxide were added. At the end of the reaction the butanol was removed *in vacuo*, and the residue was distributed between water and chloroform. The chloroform layer was washed with water, dried, and evaporated.

Chromatography on alumina followed by sublimation yielded the title compound. It was crystallized from ether and had m.p. 220–224 °C.

$C_{19}H_{18}O_2F_2$ requires: F, 12·01 per cent.
Found: F, 12·00, 12·44 per cent.

II. Cofactors

DPNH (98–100%), ATP (disodium salt, 99–100%), and glucose-6-phosphate (dipotassium salt, 98–100%) were of the highest purity commercially available and were purchased from Sigma Chemical Co. TPN (sodium salt) was obtained from Pabst Laboratories.

III. Substrate

Labelled mevalonic acid was obtained from Nuclear-Chicago Corp. as (±)-mevalolactone-2-^{14}C. It was diluted with unlabelled (±)-mevalolactone to a specific activity of 0·25 μc/μmole, then saponified overnight at room temperature with 1·1 equivalents of alcoholic potassium hydroxide. The excess alkali was neutralized with dilute hydrochloric acid and the (±)-melvalonate-2-^{14}C was used in incubations as the potassium salt.

METHODS

The enzyme system employed, which actively incorporates mevalonate into cholesterol, was prepared from homogenates of rat liver according to the method of Bucher and McGarrahan.[15] It consisted of the combined microsomal and supernatant fraction of the homogenate. The incubation medium was essentially that of Popjak *et al.*[16] and contained enzyme fraction (4·0 ml), DPNH (2 μM), TPN (2 μM), ATP (5 μM), glucose-6-phosphate (4 μM), and (±)-mevalonate-2-^{14}C (2 μM containing 0·5 μc of ^{14}C) in a final volume of 5·0 ml. Test compounds were added at a final concentration of 1×10^{-3} or 1×10^{-4} M in a 2·0% gelatin suspension (0·1 ml).

Incubations were carried out at 37 °C for 1 hr in a Dubnoff metabolic shaker, with air as the gas phase. Enzyme action was arrested by addition of 1·0 ml of 20 N potassium hydroxide. Cholesterol carrier (200 mg) and ethanol (4·0 ml) were added to each flask and the samples saponified at 75–80 °C. The nonsaponifiable lipids were extracted with 3 × 25 ml of petroleum ether (b.p. 30–40 °C).

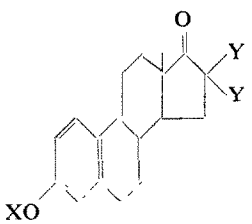
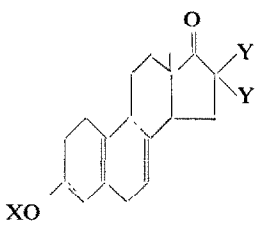
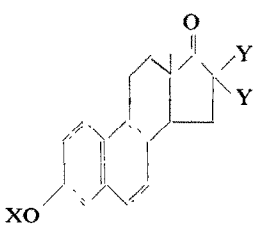
Cholesterol was purified to constant radioactivity via the dibromide procedure as described by Schwenk and Werthessen,[17] using hot methanol for recrystallization. Digitonin purification procedures were not employed, since the cholesterol digitonide may, in many instances, be contaminated by coprecipitation of "higher counting companions."[18] Suitable amounts of dibromocholestrol were plated on 1 in. × $\frac{5}{16}$ in. stainless steel planchets and counted in a gas-flow counter (Nuclear-Chicago Corp., model C 115, low background, micromil window). The counts were corrected for background and autoabsorption.[19]

RESULTS AND DISCUSSION

The results are presented in Table 1 as per cent of inhibitions, all compounds having been tested at a final concentration of 1×10^{-3} M. We find that the natural estrogens, their methyl ethers, and the methyl ethers of their 16,16-difluoro analogues all cause a

marked inhibition of cholesterol biosynthesis at the concentrations tested. Compared with the natural estrogens, their methyl ethers show a slight reduction in activity and, although there is a further diminution in potency of 16,16-difluoroequilenin methyl ether,* the corresponding derivatives of estrone and equilin show increased activity, with 16,16-difluoroequilin methyl ether being the most potent (inhibition of 36 per cent at a concentration of 1×10^{-4} M).

TABLE 1. INHIBITION OF CHOLESTEROL BIOSYNTHESIS FROM (±)-MEVALONATE-2-C^{14}

Inhibitors		% Inhibition*		
Series	Skeleton	X = H; Y = H	X = CH_3; Y = H	X = CH_3; Y = F
Estrone		78 (10^{-3} M)	44 (10^{-3} M)	87 (10^{-3} M)
Equilin		83 (10^{-3} M)	77 (10^{-3} M)	97 (10^{-3} M) 36 (10^{-4} M)
Equilenin		64 (10^{-3} M)	58 (10^{-3} M)	33 (10^{-3} M)

* Control is set at 100% activity. Under the conditions of the experiment above, the control cholesterol dibromide contained about 30,000 cpm.

This, to our knowledge, is the first demonstration of inhibition of cholesterol biosynthesis by estrogenic substances. Tomkins *et al.*[20] were the first to demonstrate the inhibition *in vivo* of hepatic cholesterol biosynthesis, by cholesterol itself and by other steroids of the cholestane and androstane series.[21] These results were subsequently confirmed, for Δ^4-cholestenone, by Steinberg and Fredrickson.[9] The only demonstration *in vitro* of cholesterol biosynthesis inhibition by steroids is that of Singer *et al.*[22] who

* This may be due to the use of synthetic (±)-equilenin as the starting material for the preparation of this compound.

showed that Δ^1 testolactone, at a concentration of $1{\cdot}2 \times 10^{-3}$ M, inhibited incorporation of mevalonate into cholesterol by 27 per cent and that Δ^4-androstene-17α-ol-3-one-17β-oic acid, at a concentration of $1{\cdot}1 \times 10^{-3}$ M, inhibited mevalonate by 73 per cent.

Noble and Boucek[23] have reported on the effect *in vivo* of estrogens on hepatic cholesterol biosynthesis. After oral administration of small doses of estrogens to rats for 21 days, they found that liver slices of the treated animals synthesized cholesterol from ^{14}C-acetate at normal (females) or slightly increased (males) rates. Similar results have been obtained with female rats by Rubin and White.[24] The slightly increased biosynthesis observed by Noble and Boucek with male rats cannot be explained on the basis of a direct action of the estrogens on the enzymes involved in cholesterol synthesis.

The mechanism by which estrogens affect lipid metabolism is not known. It has been reported that estrogens, synthetic and natural, cause stimulation of the phagocytic ability of hepatic reticuloendothelial cells,[25, 26] and it has been suggested that the effect of estrogens on lipid metabolism may be mediated through this property.[27] However, Friedman *et al.*[28] have postulated that the reticuloendothelial system acts only on cholesterol of exogenous origin. The demonstration of the inhibition of cholesterol biosynthesis by estrogens suggests that this property may contribute to their effect on lipid metabolism. However, since low doses of estrogens are used therapeutically, the degree to which cholesterol biosynthesis is inhibited *in vivo* under these conditions remains indefinite.

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