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4,4′-Dimethylcholesta-7,9,14-trienol is an Intermediate in the Demethylation of Dihydroagnosterol

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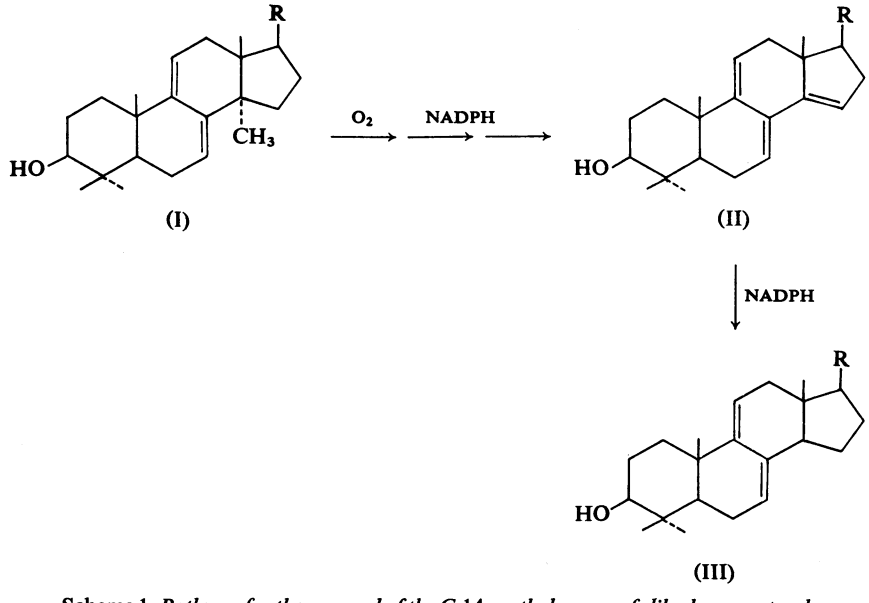
1. 4,4′-Dimethylcholesta-7,9,14-trienol is an intermediate in the metabolism of dihydroagnosterol to cholesterol by rat liver homogenate. 2. This triene is reduced by a rat liver microsomal preparation in the presence of NADPH to give 4,4′-dimethylcholesta-7,9-dienol under anaerobic conditions. 3. Reduction of the triene in the presence of [4-$^3H_2$]NADPH resulted in the incorporation of ^3H into the product. 4. Under aerobic conditions the triene is converted into cholesterol by a rat liver homogenate.

The conversion of lanosterol into cholesterol is accompanied by the removal of the C-14- and subsequently the C-4-methyl groups of lanosterol. The elimination of each C-4-methyl group is a sequential process in which each group is oxidized, first to an alcohol and then via the aldehyde to the carboxylic acid, which finally undergoes decarboxylation (Olson *et al.*, 1957; Pudles & Bloch, 1960). In contrast, the 14α-methyl group is removed as formic acid from the sterol nucleus at the oxidation state of an aldehyde (Alexander *et al.*, 1972). The C-14-demethylation is accompanied by the loss of the C-15α-hydrogen and results in the formation of a 14,15-double bond to give 4,4′-dimethylcholesta-8,14-dienol. The further metabolism of this 8,14-diene to cholesterol proceeds by the direct reduction of the 14,15-double bond to give 4,4′-dimethylcholest-8-enol.

The preceding paper has shown that in the conversion of dihydroagnosterol (I) into cholesterol, the 14α-methyl group is removed without apparent modification of the $\Delta^{7,9}$ system and leads to the accumulation of 4,4′-dimethylcholesta-7,9-dienol (III) (Tavares *et al.*, 1977). The mechanism of this demethylation is now studied and is shown to involve the introduction of a 14,15-double bond to give 4,4′-dimethylcholesta-7,9,14-trienol (II). The triene (II) is subsequently reduced by an NADPH-linked microsomal enzyme to give 4,4′-dimethylcholesta-7,9-dienol (III).



Scheme 1. *Pathway for the removal of the C-14-methyl group of dihydroagnosterol*

Experimental

Materials were obtained, supernatants from rat liver were prepared and radioactivity was measured as described by Bloxham *et al.* (1971). The incubations were performed and processed and cholesterol was isolated and purified by the methods described by Wilton *et al.* (1968). Chromatographic analyses of the steryl acetates by $AgNO_3$ t.l.c. were as described by Rahimtula *et al.* (1969).

Preparation of 4,4'-dimethylcholesta-7,9,14-trienol

This was prepared by modification of the method of Fieser & Ourisson (1953). 4,4'-Dimethylcholesta-7,9-dienyl acetate (25mg) in benzene and acetic acid (0.5ml+1ml) was treated with selenious acid (10.6mg) in acetic acid (0.8ml) at 0°C for 4h. Diethyl ether (25ml) was added and the mixture washed free of acetic acid, with water (3×25ml), conc. $NaHCO_3$ and NaCl. It was dried over anhydrous Na_2SO_4 and evaporated to dryness. The triene acetate was purified chromatographically on silica-gel GF_{254} plates developed in acetone/light petroleum (b.p. 60–80°C) (1:4, v/v) and subsequently by t.l.c. on silica gel GF_{254}/10% (w/w) $AgNO_3$ developed in benzene/light petroleum (b.p. 60–80°C) (1:1, v/v).

The triene acetate was hydrolysed by refluxing with $LiAlH_4$ in dry ether (10ml) for 30min, washed in water and by drying over anhydrous Na_2SO_4 gave the 4,4'-dimethylcholesta-7,9,14-trienol.

4,4'-Dimethyl[3α-^{3}H]cholesta-7,9,14-trienol was prepared as above except that 4,4'-dimethyl[3α-^{3}H]-cholesta-7,9-dienyl acetate (25mg; 10^9d.p.m.) was used as a starting material.

Similarly 4,4'-dimethyl[2-$^3H_2$]cholesta-7,9,14-trienol was prepared from 4,4'-dimethyl[2-$^3H_2$]-cholesta-7,9-dienyl acetate (20mg, 3×10^6d.p.m.).

The yield was approximately 20%. Mass-spectral analysis in an AEI MS30 combined g.l.c.–mass spectrophotometer of the 4,4'-dimethylcholesta-7,9,14-trienyl acetate gave ions at *m/e* 452 (M^+), 392 ($M^+-CH_3CO_2$), 377 ($M^+-CH_3CO_2-CH_3$), 339 (M^+−side chain; most intense ion), 279 (M^+−side chain−CH_3CO_2).

The 7,9,14-triene had a u.v.-absorption maxima at 263nm. It should be noted that, although a number of trienes are possible with mol.wt. 452, the following may be ruled out because they have different u.v.-absorption maxima; $\Delta^{5,7,9(11)}$, $\Delta^{6,8(14),9(11)}$, $\Delta^{5,7,14}$ (Fieser & Fieser, 1959, pp. 118, 167).

[4-$^3H_2$]NADPH was prepared by the method of Wilton *et al.* (1968).

Results

Trapping of radioactivity from an incubation of [3α-^{3}H]dihydroagnosterol in a fraction corresponding to 4,4'-dimethylcholesta-7,9,14-trienol

[3α-^{3}H]Dihydroagnosterol (I) (100μg; 2×10^6d.p.m.) was incubated under aerobic conditions with a 10000$g_{av.}$ supernatant of rat liver homogenate with non-radioactive cholesta-8,14-dienol (250μg) as 'trap'. The advantage of using cholesta-8,14-dienol to 'trap' the presumptive triene intermediate was that it could be easily removed from the radioactive 4,4'-dimethylcholesta-7,9,14-trienol (II) by separation into lanosteryl and cholesteryl bands on t.l.c. The radioactive lanosteryl band was acetylated and again subjected to t.l.c. After separation, 9.0% of the recovered radioactivity was associated with a band of material that was more polar than the 4,4'-dimethylcholesta-7,9-dienyl acetate and that was tentatively identified as 4,4'-dimethylcholesta-7,9,14-trienyl acetate. The 4,4'-dimethylcholesta-7,9-dienyl acetate accumulated in 7.3% yield, and 33% of the material applied to the chromatogram was recovered as dihydroagnosteryl acetate.

Analysis of the presumptive trapped 7,9,14-trienyl acetate by combined g.l.c.–mass spectrometry gave a molecular ion at *m/e* 452, which is what would be expected for this compound. Insufficient material prevented a full fragmentation spectrum from being obtained.

The trapped 4,4'-dimethylcholesta-7,9,14-trienyl acetate, after hydrolysis to the alcohol, was re-incubated with at 105000$g_{av.}$ rat liver microsomal fraction (2ml) anaerobically under N_2 with added NADPH (4μmol). Of the recovered radioactivity, 10.7% was associated with 4,4'-dimethylcholesta-7,9-dienol (III). Under similar conditions 4,4'-dimethyl[3-^{3}H]-cholesta-8,14-dienol was reduced to 4,4'-dimethyl-cholest-8-enol in 36% yield. The trapping experiments described above demonstrate the presence in rat liver of an enzyme system capable of biosynthesizing 4,4'-dimethylcholesta-7,9,14-trienol (II) from dihydroagnosterol (I). Also present in the microsomal fraction is an enzyme system capable of converting this triene into the reduced derivative 4,4'-dimethyl-cholesta-7,9-dienol.

Conversion of chemically synthesized dimethyl[3α-^{3}H]-cholesta-7,9,14-trienol (II) into dimethyl[3α-^{3}H]-cholesta-7,9-dienol (III)

A more definitive proof of the intermediacy of 4,4'-dimethylcholesta-7,9,14-trienol (II) required it to be chemically synthesized. This was accomplished by a modification of the method of Fieser & Ourisson (1953) (see the Experimental section). The chemically synthesized 4,4'-dimethyl[3α-^{3}H]cholesta-7,9,14-trienol (II), on incubation with a 105000$g_{av.}$ rat liver microsomal fraction under anaerobic conditions in the presence of NADPH, resulted in up to 39% accumulation of 4,4'-dimethyl[3α-^{3}H]cholesta-7,9-dienol (III). In the absence of NADPH or by using a boiled microsomal fraction there was no conversion

of triene into diene. The microsomal fraction, besides converting triene into diene, also decomposed the triene to a more polar compound in the presence or absence of NADPH. This unidentified polar compound was located at the baseline after t.l.c. of the acetylated sterols on silica gel/10% (w/w) $AgNO_3$.

To the accumulated 4,4'-dimethyl[3α-^{3}H]cholesta-7,9-dienyl acetate was added unlabelled diene (5mg) and five recrystallizations from cold diethyl ether/methanol were performed. The specific radioactivity of the 4,4'-dimethylcholesta-7,9-dienol was maintained over the recrystallizations and confirmed that the radioactivity was associated with this compound.

Enzymic reduction of the 14,15*-double bond of* 4,4'*-dimethylcholesta-*7,9,14*-trienol*

The incubation of unlabelled 4,4'-dimethylcholesta-7,9,14-trienol (II) (500μg approx.) and [4-$^3H_2$]-NADPH (6mg; 3.0×10^6 d.p.m.) with a 105000$g_{av.}$ microsomal preparation of rat liver homogenate under anaerobic conditions led to the accumulation of 4,4'-dimethyl[^{3}H]cholesta-7,9-dienol (III) (23500d.p.m.). If the substrate was omitted, or if the microsomal preparation had been boiled, there was no incorporation of radioactivity into the band corresponding to the 7,9-diene.

The use of unlabelled 4,4'-dimethylcholesta-8,14-dienol as substrate in a similar incubation led to the incorporation of radioactivity (43860d.p.m.) into 4,4'-dimethylcholest-8-enol. After addition of unlabelled 4,4'-dimethylcholesta-7,9-dienyl acetate to the presumptive ^{3}H-labelled 7,9-diene the specific radioactivity was maintained over five recrystallizations from methanol/diethyl ether. This provided strong evidence that the trapped radioactivity was associated with 4,4'-dimethylcholesta-7,9-dienol. Therefore there is a direct transfer of ^{3}H from NADPH during the reduction of this 14,15-double bond.

Conversion of 4,4'*-dimethylcholesta-*7,9,14*-trienol into cholesterol*

4,4'-Dimethyl[2-$^3H_2$]cholesta-7,9,14-trienol (II) was prepared chemically as described in the Experimental section and its metabolism to cholesterol compared with that of [2-$^3H_2$]dihydrolanosterol (I) and 4,4'-dimethyl[2-$^3H_2$]cholesta-7,9-dienol (III). In view of the fact that the triene is to a large extent decomposed by the liver homogenate, the 4.1% conversion of 4,4'-dimethylcholesta-7,9,14-trienol into cholesterol that was obtained compares well with the 12% conversion of 4,4'-dimethylcholesta-7,9-dienol. Under similar conditions dihydrolanosterol was converted into cholesterol in 32% yield.

Discussion

The trapping of 4,4'-dimethylcholesta-7,9,14-trienol (II) from incubations of dihydroagnosterol (I) with rat liver preparations and its conversion into 4,4'-dimethylcholesta-7,9-dienol (III) and into cholesterol provide very good evidence for the intermediary role of the 7,9,14-triene.

The further conversion of triene into cholesterol occurs through the reduction of the 14,15-double bond. The enzyme participating in the saturation of this system is present in the microsomal fraction of rat liver and catalyses the direct transfer of a 'hydride ion' from [4-^{3}H]NADPH to unlabelled triene to give 4,4'-dimethyl[^{3}H]cholesta-7,9-dienol (III). The reduction of the 14,15-double bond in both the 4,4'-dimethylcholesta-8,14-dienol and 4,4'-dimethylcholesta-7,14-dienol involves a proton-initiated Markownikoff addition mechanism in which the 15β-hydrogen arises from the medium whereas the 14α-hydrogen is derived from the 4*S*-position of NADPH (Wilton *et al.*, 1970). Because the same stereoelectronic factors apply to the reduction of the 14,15-double bond in the triene we would predict that the orientation of addition is maintained in this reduction in order that the Markownikoff rules are obeyed. Moreover, it is probable that the same enzyme is involved in this reduction, because it has been shown to have a broad specificity (Wilton *et al.*, 1970). We would also predict that the same enzymes that are responsible for the demethylation of the 14α-methyl group in lanosterol conversion into cholesterol are probably also involved in this demethylation.

On the basis of these assumptions the pathway suggested for the demethylation of dihydroagnosterol is summarized in Scheme 1. A more detailed discussion of the involvement of $\Delta^{7,9}$-diene in cholesterol biosynthesis is to be found in the preceding paper (Tavares *et al.*, 1977).

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