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5α,6α-METHANOCHOLESTAN-3β-OL AS A PROBE OF THE MECHANISM OF ACTION OF CHOLESTEROL 7α-HYDROXYLASE

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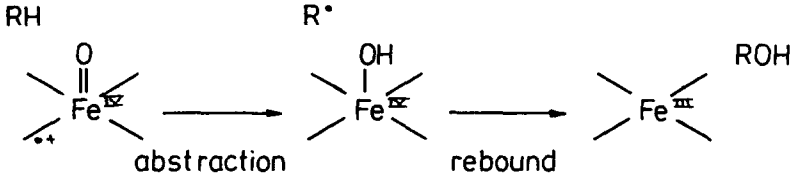
Abstract: Reduction of 3β-acetoxy-5α,6α-methano-7-oxocholestane with sodium in ether/liquid ammonia takes place with the expected opening of the three-membered ring but oxidation of 5α,6α-methanocholestan-3β-ol by cholesterol 7α-hydroxylase proceeds with the formation of the 7α-hydroxy derivative without opening of the cyclopropane.

Cholesterol 7α-hydroxylase catalyses the first and rate limiting step in the biosynthesis of bile salts from cholesterol[1] and is a mixed function oxidase of the cytochrome P-450 class.[2] The mechanism by which such enzymes hydroxylate their substrates has been a topic of continuous interest in recent years[3,4] but there have been few investigations of enzymes with such precise substrate specificity as cholesterol 7α-hydroxylase. It is widely thought that aliphatic hydroxylation occurs by a so-called 'oxygen rebound' mechanism in which the new carbon oxygen bond is formed by the coupling of a haem bound oxygen radical and a carbon radical (Scheme 1).



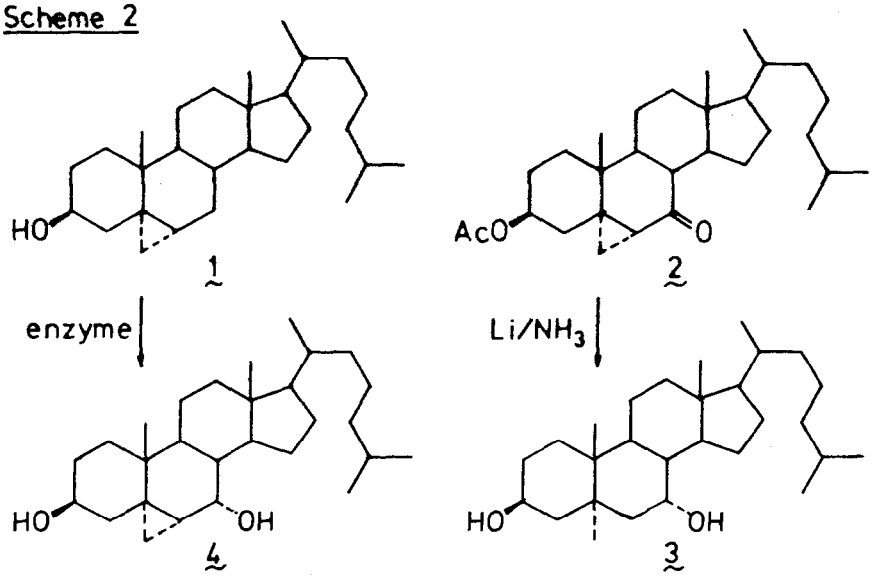
Scheme 1

Present address: Smith Kline and French Research Ltd., The Frythe, Welwyn, Hertfordshire AL6 9AR, England

One line of evidence leading to this conclusion has come from the study of model reactions using tetraphenyl porphyrin derivatives and Groves[5] has shown that hydroxylation of cyclopropane derivatives occurs with partial ring opening consistent with the intermediacy of a radical. We have studied the behaviour of a suitably substituted cyclopropane derivative of cholesterol, 5α,6α-methanocholestan-3β-ol 1 (Scheme 2) as a mechanistic probe for the enzyme. The synthesis of this compound together with those of a number of reference compounds for potential oxidation products have been described.[6]

As we have emphasised,[7] it is important in the use of cyclopropanes as mechanistic probes to show that the selected probe does indeed undergo ring opening in response to the formation of a radical at the adjacent carbon. To investigate this point, we took 3β-acetoxy-7-oxocholestane 2[6] and reduced it with sodium in ethereal liquid ammonia following Dauben.[8] The product formed in greater than 90% yield after chromatography on a silica gel plate was characterised as 3β,7α-dihydroxy-5α-methylcholestan 3 on the basis of the following spectroscopic properties. The mass spectrum showed a molecular ion at m/z 418.3802 ($C_{28}H_{50}O_2$ requires m/z 418.3811) and prominent fragment ions at m/z 400, 385, 382, and 367 corresponding to initial loss of water followed by further loss of water and a methyl group; this behaviour is in contrast to that of cyclopropane-containing analogues which characteristically fragment by an initial competing loss of m/z 15 and 18.[6] The ^{1}H nmr spectrum (250 MHz) showed a new methyl group at δ 1.20 (s), and resonances for two protons on carbon atoms bearing oxygen atoms (δ 3.5 (m) 3-H, 4.15 (bd s) 7-H). No resonances characteristic of the cyclopropane ring[6] were observed and both spectra were in these respects readily distinguishable from those of the corresponding diol 4 in which the ring remains intact. This result is as expected on the basis of Dauben's experiments using cyclopropanes fused to ring A of the steroid nucleus.[8] The selected probe is therefore suitable for investigation of the enzyme.

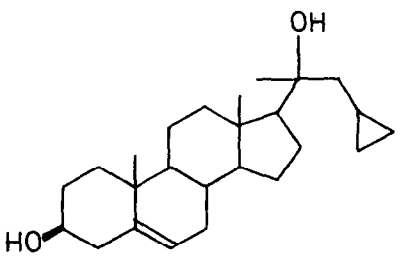
Scheme 2



A lipid-depleted microsomal powder was obtained from the liver of cholestyramine treated rats.[9] The enzyme activity assayed using [^{14}C]cholesterol as substrate and the

only significant product was cholestan-3β,7α-diol as shown by tlc comparison with authentic standard compounds. Cyclopropyl cholesterol 1 was found to inhibit the 7α-hydroxylation of cholesterol; kinetic analysis of the data showed competitive inhibition (K_i = 11 μM) with respect to cholesterol. The affinity of 1 for the enzyme is comparable with that observed by Schwartz and Margolis[10] for 7-keto- and 7α-hydroxy-cholesterol. No indications of time-dependent inhibition were obtained as had been previously found for the oxidation of aryl cyclopropanes by cytochrome P-450.[11] To investigate the products of oxidation of 1 by the enzyme, a tritiated sample was prepared by borohydride reduction of the corresponding 3-ketone. Only one tritium-containing product was isolated by tlc and its Rf was close to that expected for a cholestane diol. The product copurified by repeated crystallisation with an authentic sample of 5α,6α-methanocholestan-3β,7α-diol.[6] No other oxidation products were detected either radiochemically or by capillary gc-ms on the trimethylsilyl ethers of reaction products. We conclude that hydroxylation of 1 by cholesterol 7α-hydroxylase takes place without opening of the three-membered ring.

The most extensively studied series of cyclopropane-containing substrates to have been used to probe the mechanism of cytochromes P-450 are cyclopropylamines[3,12] and in all of these cases, ring opening has been found to lead to time-dependent inhibition of the enzyme. In contrast, steroids lacking an easily-oxidised nitrogen atom at the reaction site do not suffer ring opening and are not time-dependent inhibitors. For example Hoyte and Hochberg found that a cyclopropyl probe 5 for the mechanism of action of the cholesterol side chain cleavage enzyme, another specific cytochrome P-450 enzyme, underwent oxidation affording cyclopropane carboxylic acid.[13] As far as cholesterol 7α-hydroxylase is concerned, it has been established that cholestan-3β-ol is a good substrate[14] and hence the double bond in cholesterol does not appear to be critical for the hydroxylation mechanism. The lack of ring opening could therefore be accounted for either by insertion of an oxene or by a very short-lived radical intermediate. Since this enzyme is characterised by a particularly high substrate specificity[15] it seems probable that the precise binding of the substrate maintains the expected carbon radical intermediate so close to the oxygen radical at the haem ion atom that the rebound stage is too fast for ring opening to occur. We were recently informed that similar observations have been made by Ortiz de Montellano using methyl cyclopropane as a substrate for cytochrome P-450.[16]



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