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Mechanism-Based Inactivation of 4-Methylsterol Oxidase by 4α-(Cyanomethyl)-5α-cholestan-3β-ol

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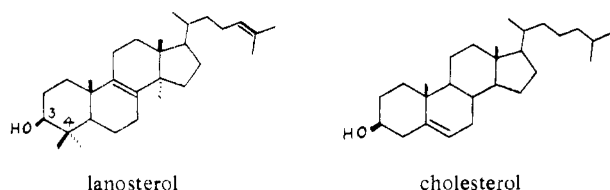
An important part of cholesterol biosynthesis in rat liver is the removal of the 4,4-dimethyl groups of lanosterol as CO_2, through oxidative demethylation. We report here the first mechanism-based inactivator of this key enzymatic process.

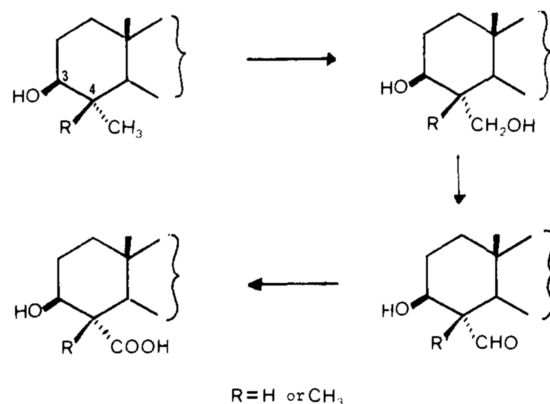


The enzyme-catalyzed oxidative loss of the 4,4-dimethyl grouping in lanosterol has been the subject of extensive studies,[1] including the use of model substrates such as 4,4-dimethyl- and 4α-methyl-5α-cholest-7-en-3β-ol and the corresponding 7,8-dihydro compounds.[2] These investigations led to the proposals summarized in Schemes I and II. Thus the rat liver microsomal system termed 4-methylsterol oxidase catalyzes the oxidation of the 4α-methyl group in either a 4,4-dimethyl or a 4α-methyl substrate to, successively, the 4α-hydroxymethyl, 4α-formyl, and 4α-carboxylic acid grouping (Scheme I). Each of these steps requires 1 mol equiv each of NAD(P)H and oxygen, and the rate of oxidation appears to increase with each successive step. The oxidation of the 4α-methyl group to the corresponding 4α-carboxylic acid occurs rapidly with no accumulation of intermediates. The demethylation process is then completed by an NAD^+-linked dehydrogenase/decarboxylase, via the β-keto acid (Scheme II).[3]

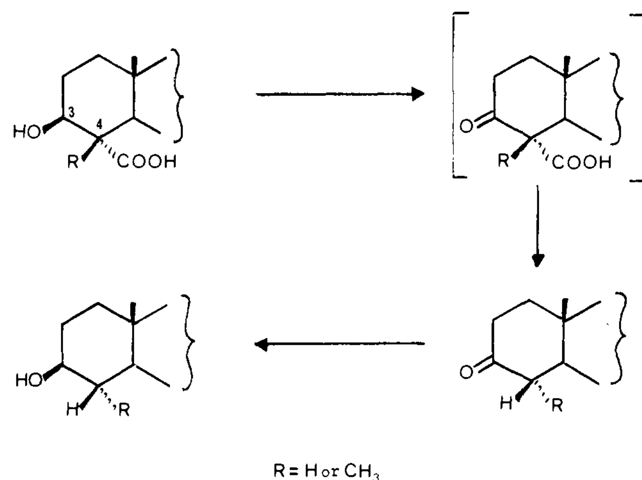
The microsomal 4-methylsterol oxidase system that converts the 4α-methyl group to a 4α-carboxy group is an NAD(P)H–O_2-requiring system but appears not to be a cytochrome P-450 dependent complex. We have devised a mechanism-based inactivator for 4-methylsterol oxidase, based on the reaction sequence of Scheme I and using the rationale shown in Scheme III. It was hoped that the enzymatically catalyzed hydroxylations normally occurring at the 4α-methyl group might generate the electrophilic acyl cyanide grouping from the 4α-cyanomethyl analogue **1**. Reaction of this enzyme-generated electrophile with a nucleophilic residue at the enzyme's active site could then lead to inactivation of the enzyme via covalent bond formation.

The hitherto undescribed 4α-cyanomethyl compound (**1**) was synthesized[4] via 3β-benzyloxy-4α-(hydroxymethyl)-5α-cholestane

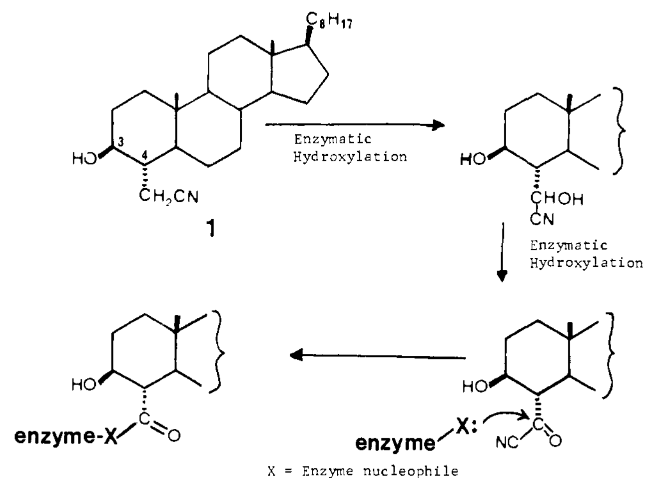
Scheme I



Scheme II



Scheme III



(**2**), mp 122–123.5 °C, derived in turn from the known[5] 4α-(carbomethoxy)-5α-cholestan-3β-ol (**3**) by benzylation, followed by $LiAlH_4$ reduction. Thus, compound **2** was converted to the *p*-toluenesulfonate (pyr–TsCl). Treatment with NaCN/DMF/acetone followed by removal of the 3β-benzyloxy group (Pd/C–H_2) then gave the desired 4α-(cyanomethyl)-5α-cholestan-3β-ol (**1**): mp 188–189 °C (acetone); IR ($CHCl_3$) 3630, 3460, 2240 cm^{-1}; NMR (300 MHz) δ 3.45 (m (7), 1, 3α-H), 2.90 (dd, J = 17, 4 Hz, 1, CH_2CN), 2.46 (dd, J = 17, 4 Hz, 1, CH_2CN); mass spectrum, m/e 427 (M^+), 412, 287, 272.

Our enzymatic studies, carried out with rat liver microsomal

(1) Porter, J. W.; Spurgeon, S. L. "Biosynthesis of Isoprenoid Compounds"; Wiley: New York, 1981; Vol. 1, pp 502–507.
(2) Sharpless, K. B.; Snyder, T. E.; Spencer, T. A.; Maheshwari, K. K.; Guhn, G.; Clayton, R. B. *J. Am. Chem. Soc.* **1968**, *90*, 6874–6875.
(3) Rahimtula, A. D.; Gaylor, J. L. *J. Biol. Chem.* **1972**, *247*, 9–15.
(4) Newly described compounds showed appropriate IR, NMR, and mass spectra. Compounds **1** and **4** showed correct elemental analyses.
(5) Czarny, M. R.; Maheswari, K. K.; Nelson, J. A.; Spencer, T. A. *J. Org. Chem.* **1975**, *40*, 2079–2085.

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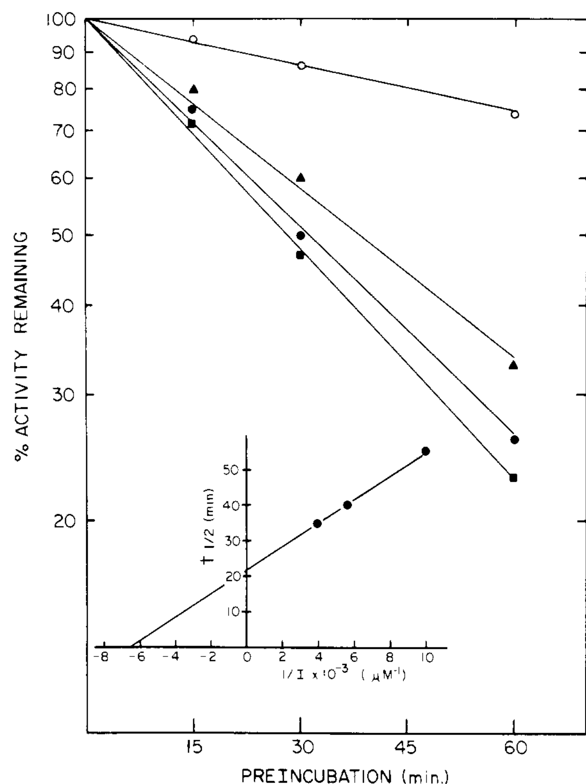
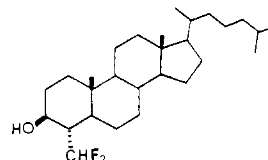


Figure 1. Kinetics of NADPH-dependent inactivation of 4-methylsterol oxidase by various concentrations of 4α-(cyanomethyl)-5α-cholestan-3β-ol (**1**): ■, 250 μM; ●, 175 μM; ▲, 100 μM; O, no inhibitor (background loss of activity or control). Inset, analysis of inactivation rates (corrected for background).

preparations,[6] show that compound **1** is a mechanism-based inactivator of 4-methylsterol oxidase. The substrate 4α-methyl-5α-cholestan-3β-ol showed K_m = 125 μM in this system, and competition experiments[7] revealed that **1** is a competitive inhibitor of 4-methylsterol oxidase with K_i = 180 μM. Preincubation studies[8] with cyanomethyl compound **1** revealed time-dependent loss of enzyme activity in the presence of the cofactor NADPH. *No inactivation was seen in the absence of NADPH, which argues for an enzyme-catalyzed inactivation process.* The enzyme-inactivation rates followed pseudo-first-order kinetics (Figure 1) and were analyzed by the method of Kitz and Wilson[9] to give K_i = 142 μM and a $t_{1/2}$ of 22 min for saturating inhibitor concentration. The active site directed nature of the enzyme-inactivation process was demonstrated by the following substrate-protection experiment. With 100 μM 4α-cyanomethyl compound **1** the enzyme half-life was 54 min, which was increased to 81 min when 100 μM 4α-methyl substrate was also present. The irreversibility of the process was shown by the inability of inactivated microsomes to regain enzyme activity after centrifuging and washing. Finally, inactivation experiments were carried out in the presence of reduced glutathione (2 mM) as a scavenger for reactive electrophiles in solution. No change in enzyme half-life was observed, providing strong evidence that the inactivation process does not involve release into solution of electrophilic intermediates that later return to inactivate the enzyme.

Methylsterol oxidase is inhibited weakly (15%) by 1.5 mM cyanide ion.[10] The postulated cyanohydrin intermediate could release cyanide ions, which might inhibit the enzyme. However, the resulting concentrations of cyanide ion would be in the *picomolar* range, since the oxidase system is transforming substrate at the rate of 0.1 nmol/(min mg of microsomal protein). We found no loss of enzyme activity above control over a 60-min period at 1 μM sodium cyanide concentrations, showing that cyanide ion is not responsible for the enzyme inactivation observed with compound **1**. Formation and reaction of the 4α-acyl cyanide is therefore still a plausible inactivation pathway.

The inactivation process appears to be structure specific. Thus, we have synthesized and evaluated other 4α-substituted analogues such as 4α-(difluoromethyl)-5α-cholestan-3β-ol (**4**).[11] Compound



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4 is a competitive inhibitor of 4-methylsterol oxidase, with K_i = 280 μM, but although the normal enzymatic hydroxylation process could generate an acyl fluoride[12] from **4**, no enzyme inactivation occurs.

In summary, we have described a novel mechanism-based inactivator for the important cholesterol biosynthetic enzyme 4-methylsterol oxidase. The 4α-cyanomethyl compound **1** causes irreversible inactivation of the enzyme, possibly through generation of the electrophilic 4α-acyl cyanide at the active site.

This irreversible inhibitor should be useful for further study of the oxidative demethylation process in cholesterol biosynthesis, as well as for possible effects on cholesterol biosynthesis in vivo. Further studies on the mechanism of the inactivation process are in progress.

Acknowledgment. It is a pleasure to thank Dr. J. L. Gaylor for much helpful advice concerning 4-methylsterol oxidase. We also thank Dr. P. A. Marcotte for helpful discussions. This work was supported in part by NIH Grant AM 15918.

Registry No. 1, 82510-99-4; **2**, 82511-00-0; **3**, 82511-01-1; **4**, 82521-44-6; NADPH, 53-57-6; 4-methylsterol oxidase, 42616-26-2.

(6) Miller, W. L.; Kalafer, M. E.; Gaylor, J. L.; Delwiche, C. V. *Biochemistry* **1967**, *6*, 2673–2678.

(7) The final incubation mixture contained 0.1 M KH_2PO_4 (pH 7.4), 0.1 mM $MgCl_2$, 30 mM nicotinamide, a NADPH-generating system of 0.25 mM NADPH, 3 mM isocitrate, and 0.5 unit/mL isocitrate dehydrogenase, a NAD^+-generating system of 0.7 mM NAD^+, 3 mM pyruvate, and 50 unit/mL lactic dehydrogenase, and 1 mg of microsomal protein/mL. For a typical experiment solutions of radiolabeled substrate 4α-[^{14}C]methyl-5α-cholestan-3β-ol (20 000 dpm nmol^{-1}) and inhibitor dissolved with the aid of Triton WR-1339 were added to the incubation mixture at 37 °C to give final steroid concentrations of 25 μM substrate and 0, 25, 50, 75, 100 μM inhibitor and 4 mg/mL Triton WR-1339 in a final volume of 1 mL. Following a 20-min incubation under O_2, the reaction was terminated via acidification, [^{14}C]CO_2 was collected as described by Gaylor (Gaylor, J. L. *J. Biol. Chem.* **1963**, *238*, 1649–1655), and the radioactivity was measured to determine product formation.

(8) The final incubation mixture was as in footnote 7 except 100–250 μM inhibitor was also present. After 0, 15, 30, 60 min of preincubation (37 °C under O_2) with inhibitor, radiolabeled substrate was added, to assay for enzyme activity. Incubations were for 20 min and the reaction was terminated as before. For convenience in routine assays, the NAD(P)H-dependent methylsterol oxidase (4α-$^{14}CH_3$ → 4α-^{14}COOH) was coupled to the NAD^+-dependent 4α-carboxylic acid decarboxylase (4α-^{14}COOH → 4α-H + $^{14}CO_2$). The oxidase is rate determining (Gaylor, J. L.; Delwiche, C. V. *J. Biol. Chem.* **1976**, *251*, 6638–6645). However, in our inactivation experiments it was clearly necessary to assay the oxidase independently of the decarboxylase. The NAD(P)H-dependent oxidase was assayed directly by an adaption of a procedure by Gaylor (Miller, W. L.; Gaylor, J. L. *J. Biol. Chem.* **1970**, 245, 5369–5374). Thus after extraction (acetone/ether) and isolation (thin-layer chromatography) of 4α-^{14}COOH and 4α-$^{14}CH_3$ from the incubation mixture, the ratio of the radioactivity of product to substrate was measured to determine product formation.

(9) Kitz, R.; Wilson, I. B. *J. Biol. Chem.* **1962**, *237*, 3245–3249.

(10) Miller, W. L.; Gaylor, J. L. *J. Biol. Chem.* **1970**, *245*, 5375–5381.

(11) Compound **4** was synthesized from intermediate **3**. Jones oxidation of **3** gave 3β-benzyloxy-4α-formyl-5α-cholestane, which was treated with DAST/CH_2Cl_2 to give 3β-benzyloxy-4α-(difluoromethyl)-5α-cholestane. DAST ((diethylamino)sulfur trifluoride) was prepared according to Middleton (Middleton, W. J. *J. Org. Chem.* **1975**, *40*, 574–578). Hydrogenolysis (Pd/C–H_2) in ETOH gave 4α-(difluoromethyl)-5α-cholestan-3β-ol (**4**): mp 154–155 °C (acetone/H_2O); IR ($CHCl_3$) 3630 cm^{-1}; NMR (300 MHz) δ 6.22 (t, J = 56 Hz, 1, CHF_2), 3.64 (m (7), 1, 3α-H]; mass spectrum, m/e 438 (M^+), 423, 406, 284.

(12) The mechanism-based inactivation of the NADPH–O_2-linked human placental enzyme estrogen synthetase (aromatase) by 10β-(difluoromethyl)-4-androstene-3,17-dione is postulated to involve acyl fluoride formation via enzymatic oxidation of the difluoromethyl group: Marcotte, P. A.; Robinson, C. H. *Biochemistry* **1982**, *21*, 2773–2778.