drogen atom parameters, and anisotropic thermal parameters (8 pages). Ordering information is given on any current masthead page.

Scheme I







Scheme II





R= Hor CH

Scheme III



(2), mp 122-123.5 °C, derived in turn from the known⁵ 4 α -(carbomethoxy)-5 α -cholestan-3 β -ol (3) by benzylation, followed by LiAlH₄ reduction. Thus, compound 2 was converted to the *p*-toluenesulfonate (pyr-TsCl). Treatment with NaCN/DMF/acetone followed by removal of the 3 β -benzyloxy group (Pd/C-H₂) then gave the desired 4 α -(cyanomethyl)-5 α -cholestan-3 β -ol (1): mp 188-189 °C (acetone); IR (CHCl₃) 3630, 3460, 2240 cm⁻¹; NMR (300 MHz) δ 3.45 (m (7), 1, 3 α -H), 2.90 (dd, J = 17, 4 Hz, 1, CH₂CN), 2.46 (dd, J = 17, 4 Hz, 1, CH₂CN); mass spectrum, m/e 427 (M⁺), 412, 287, 272.

Our enzymatic studies, carried out with rat liver microsomal

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

Désirée L. Bartlett and Cecil H. Robinson*

Department of Pharmacology and Experimental Therapeutics The Johns Hopkins University, School of Medicine Baltimore, Maryland 21205

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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 4 α -methyl group in either a 4,4-dimethyl or a 4 α -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-O₂-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 synthesized⁴ via 3β -benzyloxy- 4α -(hydroxymethyl)- 5α -cholestane

⁽⁵⁾ Czarny, M. R.; Maheswari, K. K.; Nelson, J. A.; Spencer, T. A. J. Org. Chem. 1975, 40, 2079-2085.

Porter, J. W.; Spurgeon, S. L. "Biosynthesis of Isoprenoid Compounds";
 Wiley: New York, 1981; Vol. 1, pp 502-507.
 Sharpless, K. B.; Snyder, T. E.; Spencer, T. A.; Maheshwari, K. K.;

⁽²⁾ Snarpiess, K. D.; Snyder, I. E.; Spencer, I. 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.



Figure 1. Kinetics of NADPH-dependent inactivation of 4-methylsterol oxidase by various concentrations of 4α -(cyanomethyl)- 5α -cholestan- 3β -ol (1): \blacksquare , 250 μ M; \bullet , 175 μ M; \blacktriangle , 100 μ M; 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 $K_m = 125 \ \mu$ M in this system, and competition experiments⁷ revealed that 1 is a competitive inhibitor of 4-methylsterol oxidase with $K_i = 180 \ \mu$ M. Preincubation studies⁸ 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

(7) The final incubation mixture contained 0.1 M KH₂PO₄ (pH 7.4), 0.1 mM MgCl₂, 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 -[¹⁴C]methyl- 5α -cholestan- 3β -01 (20000 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 μ M substrate and 0, 25, 50, 75, 100 μ M inhibitor and 4 mg/mL Triton WR-1339 in a final volume of 1 mL. Following a 20-min incubation under O₂, the reaction was terminated via acidification, [¹⁴C]CO₂ 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 μ M inhibitor was also present. After 0, 15, 30, 60 min of preincubation (37 °C under O₂) 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 (4 α -¹⁴CH₃ \rightarrow 4 α -¹⁴COOH) was coupled to the NAD⁺ dependent 4 α -carboxylic acid decarboxylase (4 α -¹⁴COOH \rightarrow 4 α -1H + ¹⁴CO₂). 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 4 α -¹⁴COOH and 4 α -¹⁴CH₃ from the incubation mixture, the ratio of the radioactivity of product to substrate was measured to determine product formation.

(Figure 1) and were analyzed by the method of Kitz and Wilson⁹ to give $K_i = 142 \ \mu M$ and a $t_{1/2}$ of 22 min for saturating inhibitor concentration. The active site directed nature of the enzymeinactivation process was demonstrated by the following substrate-protection experiment. With 100 μM 4 α -cyanomethyl compound 1 the enzyme half-life was 54 min, which was increased to 81 min when 100 μM 4 α -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 μ M sodium cyanide concentrations, showing that cyanide ion is not responsible for the enzyme inactivation observed with compound 1. Formation and reaction of the 4 α -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



4 is a competitive inhibitor of 4-methylsterol oxidase, with $K_i = 280 \,\mu$ M, but although the normal enzymatic hydroxylation process could generate an acyl fluoride¹² from **4**, no enzyme inactivation occurs.

In summary, we have described a novel mechanism-based inactivator for the important cholesterol biosynthetic enzyme 4methylsterol 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.

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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.

(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/CH₂Cl₂ 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-H₂) in ETOH gave 4α -(difluoromethyl)- 5α -cholestan- 3β -ol (4): mp 154–155 °C (acetone/H₂O); IR (CHCl₃) 3630 cm⁻¹; NMR (300 MHz) δ 6.22 (t, J = 56 Hz, 1, CHF₂), 3.64 (m (7), 1, 3α -H]; mass spectrum, m/e 438 (M⁺), 423, 406, 284.

(12) The mechanism-based inactivation of the NADPH-O₂-linked human placental enzyme estrogen synthetase (aromatase) by 10β -(difluoro-methyl)-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.

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