Silicon-Mediated Suicide Inhibition: An Efficient Mechanism-Based Inhibitor of Cytrochrome P-450$_{scc}$ Oxidation of Cholesterol

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Received September 6, 1983

The first and rate-determining step in the biosynthesis of steroid hormones is the side-chain cleavage of cholesterol, catalyzed by a three-enzyme system composed of a flavoprotein dehydrogenase, an iron–sulfur protein, and cytochrome P-450$_{scc}$.[1] The cytochrome, present in the inner mitrochondrial membrane of adrenal cortex, catalyzes the six-electron oxidation of cholesterol to pregnenolone and isocaproaldehyde.[2] Three discrete steps are proposed to occur within the same active site.[3] The first two involve hydroxylations at C-22 and C-20 to produce (20*R*,22*R*)-20,22-dihydroxycholesterol followed by an oxidative cleavage of the bond between C-20 and C-22 in the last step. Three NADPH and three O_2 are consumed in the process[4] (Scheme I).

The mechanism of oxygen activation by cytochrome P-450, the conversion of molecular oxygen to a reactive species capable of attacking a C–H bond, is not completely understood, but several experimental approaches suggest the participation of either a high-valent oxenoid such as the ferryl species $[FeO]^{2+}$ or, alternatively, a Fenton type radical.[5]

Previously, compounds containing acetylenic functional groups were shown to act as effective mechanism-based inhibitors of microsomal P-450's.[6] Recently we reported the inhibition of P-450$_{scc}$ by acetylenic side-chain steroids.[7] Elegant structural studies by Ortiz de Montellano et al.[8] suggest the participation of highly reactive oxirene intermediates, generated during oxygen atom insertion into the carbon–carbon triple bond. These intermediates appear to react with the prosthetic heme group. In the present study, we have devised a novel mechanism-based inhibitor for P-450$_{scc}$ using a well-known organosilicon chemistry of β-cation or β-radical.[9] We hoped that the hydroxylating species produced at the active-site heme might generate in situ the stabilized cation β to the trimethylsilyl (Me_3Si) group. If it remained sufficiently long in the active site, the Me_3Si β-cation could silylate nearby nucleophiles, thus covalently modifying the enzyme. We have synthesized 20-(2-(trimethylsilyl)ethyl)-5-pregnen-3β,20α-diol (**1**)[10] and examined its inhibitory activity toward purified P-450$_{scc}$.[11]

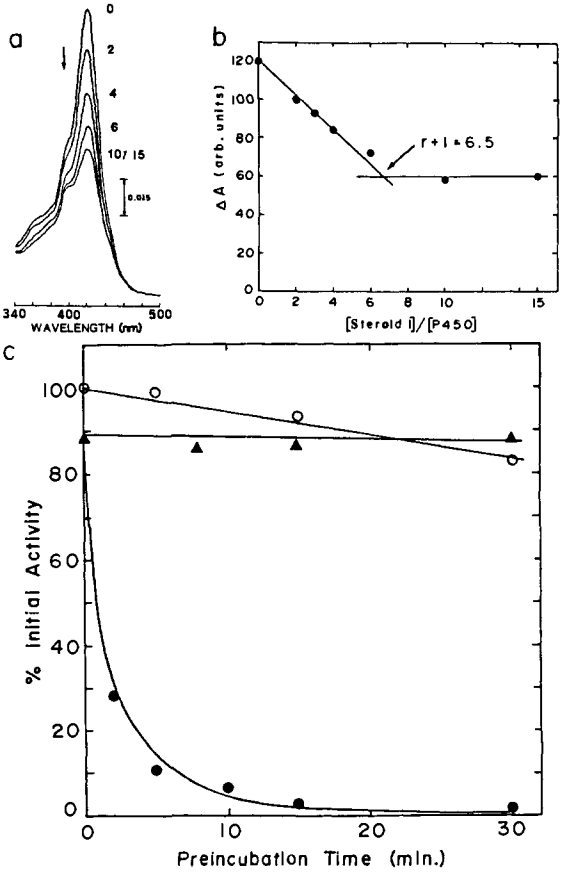
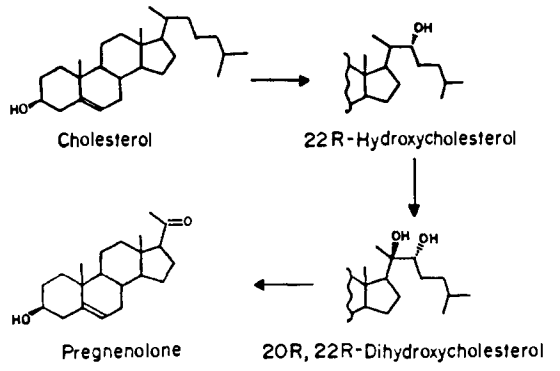


Figure 1. (a) Titration of Soret absorbance decreases after a 1-h turnover in the presence of steroid **1**. The P-450 concentration was 1 μM. The numbers in the graph indicate molar equivalents of steroid **1** present in the turnover solutions. (b) Final Soret absorbance (t = 1 h) as a function of the initial ratio os steroid **1** to P-450$_{scc}$. (c) P-450$_{scc}$ inhibition by Me_3Si steroid **1**. The P-450 concentration was 1.35 μM, and the steroid was 20 μM: ●, +NADPH; ▲, no NADPH; O, no inhibitor, +NADPH.

Scheme I



The addition of **1** to P-450$_{scc}$ produced a type II[12] (high spin → low spin) absorbance change. The binding constant, K_d, es-

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(10) Prepared in 76% yield by our previously described method: Wilson, S. R.; Shedrinsky, A. *J. Org. Chem.* **1982**, *47*, 1983–1984. IR 3300–3600 cm^{-1}; NMR ($CDCl_3$) 5.2 (1 H, brs) 3.3–3.7 (1 H, m), 1.26 (3 H, s), 1.04 (3 H, s), 0.88 (3 H, s), 0.0 (9 H, s); MS, *m/e* (% base) 418 (0.002, M^+), 400 (1.5, M – H_2O), 389 (7), 317 (25), 299 (21), 159 (22), 129 (23), 107 (15), 105 (19), 95 (16), 93 (15), 91 (18), 85 (12), 81 (25), 75 (46), 73 (100), 71 (30), 55 (32). Anal. Calcd for $C_{26}H_{44}SiO$ (M – 18): 400.316. Found: 400.306.
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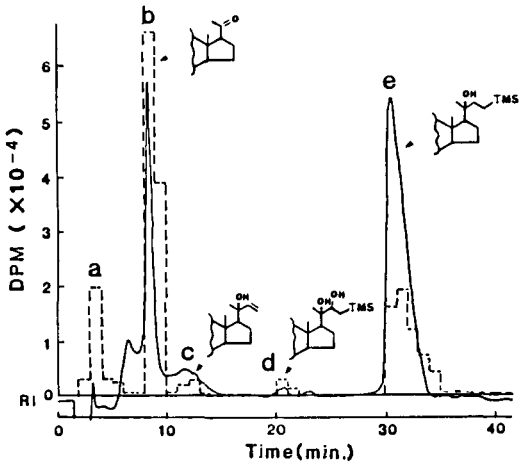
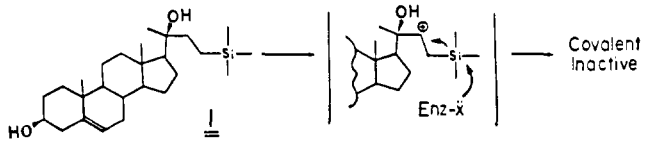


Figure 2. HPLC product analysis of P-450$_{scc}$ reaction with steroid **1** on a Whatman Partisil PXS: ODS-2 column. Methanol: water, 85:15, v/v; 1.5 mL/min. The solid line indicates refractive index response, and bars indicte radioactivity in dpm. The radioactive steroid **1** (tritium at C-7) was synthesized as in ref 10 except [7-^{3}H]pregnenolone (20 300 dpm/nmol) was used. The incubation contained 83 nmol P-450$_{scc}$, 50 nmol adrenodoxin, and 1.5 nmol adrenodoxin reductase. After a 4-h incubation, the reaction mixture was extracted exhaustively with ethyl acetate. The isolated steroids were dissolved in methanol before HPLC injection.

Scheme II



timated as earlier described,[13] was 0.78 μM. Incubation of **1** with P-450$_{scc}$ in the presence of an NADPH-generating system led to a time-dependent absorbance decrease in the Soret region ($t_{1/2}$ ~ 2 min, data not shown). A 10-fold molar excess of **1** was sufficient to titrate the system (Figure 1a). The partition ratio (r),[7] calculated from the final ΔA at each value of [steroid]/[P-450], is 5.5 ± 0.5 (Figure 1b).

Preincubation studies[14] with steroid **1** showed a time-dependent loss of enzyme activity ($t_{1/2}$ ~ 2 min) in the presence of NADPH and O_2 (Figure 1c). No inactivation was observed in the absence of NADPH and/or O_2, suggesting that inactivation is catalysed by P-450$_{scc}$. The irreversibility of the inhibition was suggested by the inability of inactivated enzyme to regain its activity after gel filtration. Furthermore, incubations were carried out in the presence of 2–10 mM β-mercaptoethanol, dithiothreitol, or NaF as a scavenger for reactive electrophiles. There was no protection from the inactivation, by these reagents, which suggested that the enzyme-generated inactivator does not become accessibile to other solutes prior to inactivation.

Incubation of C-7 tritium-labeled steroid **1** with P-450$_{scc}$ in the presence of NADPH and O_2 produced at least four steroid products. Figure 2 shows an HPLC analysis of the extracted steroids after a 4-h incubation. No products were found in the absence of NADPH. The assignments for the structure of materials in peaks b, c, and e were made on the basis of comigration during HPLC with authentic steroids under several solvent conditions and on GC-MS analyses. The peak d was assigned to be the diol because periodate treatment of the material in d produced pregnenolone. The partition ratio (r) estimated from the total products, assuming complete inactivation, was 5.8, agreeing with the titration study. While the products are consistent with the cationic mechanism shown in Scheme II, an alternative mechanism would involve oxidative cleavage to a Me_3Si radical plus ethylene as observed by Trahanovsky.[15] Ethylene is produced (0.6 mol/mol enzyme) in the turnover of steroid **1** with kinetics similar to those for inactivation.[16] In each alternative, trimethylsilylation of some group in the holoenzyme is predicted, a point under current investigation.

In summary, we have described a novel mechanism-based inhibition of P-450$_{scc}$ by steroid **1**. The rate of the Soret absorbasnce decrease, the enzyme activity loss, and ethylene formation are indistinguishable with a $t_{1/2}$ of 2 min. The partition ratio is estimated to be approximately 5.5 from the product analysis and the Soret absorbance titration. Detailed mechanistic studies of the inactivation process and in vivo inhibition studies are being conducted.[17] We are also investigating whether Me_3Si compounds will be generally useful as a new class of monoxygenase mechanism-based inhibitors.

Acknowledgment. This work was supported by NIH Grants GM28358 (W.H.O.-J.) and GM31482 (S.R.W.).

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(14) The final turnover mixture contained 2.5 μM adrenodoxin, 150 nM adrenodoxin reductase, 50 mM K$^+$MOPS (pH 7.2), 10 mM $MgCl_2$, 0.2% Tween 20, 1 unit/mL catalase, a NADPH-generating system of 16 μM NADPH, 3 mM G6P, and 1 unit/mL G6P dehydrogenase. Activity was assayed essentially according to the method of Takikawa et al. Takikawa, O.; Gomi, T.; Suhara, K.; Itagaki, E.; Takemori, S.; Katagiri, M. *Arch. Biochem. Biophys.* **1978**, *190*, 300–306.

(15) Trahanovsky, W. S.; Himstedt, A. L. *J. Am. Chem. Soc.* **1974**, *96*, 7974–7976.

(16) The incubation was carried out as in ref 14 except the reaction was done in a small vial fitted with a rubber septum. Ethylene production was assayed by using a gas chromatograph (GC) equipped with a Porapak N (Waters) column and a flame ionization detector. At various times, an aliquot of the atmosphere above the incubation mixture was removed and injected into the GC with gas-tight syringe.

(17) We (R. J. Krueger, A. Nagahisa, and W. H. Orme-Johnson) find that **1** inhibits corticoids formation by ACTH-stimulated adrenal cortical cells, work in progress.

On the Detailed Pathway of Methyl Loss from Ionized Methyl Isobutyrate in the Gas Phase

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Received October 31, 1983

There is now ample evidence,[1] both experimental and theoretical, that the unimolecular loss of alkyl radicals R· from gaseous cation radicals having the general structure **1** cannot be described in terms of a direct, radical-induced carbon–carbon bond cleavage (Scheme I, **1** → **2**). Instead, energetically more favored is the multistep reaction **1** → **3** → **5** → **6**, despite the fact that this sequence contains such unusual steps as consecutive [1,2] migrations of a protonated carboxyl group (**1** → **3**)[2] and hydrogen.[3] For R^3 = H the eventually generated product ion **6** differs from **2** in that in the former the protonated carboxyl group is attached

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(3) Although there are no authentic [1,2]-hydrogen migrations in monoradicals in solution (see, for review: Beckwith, A. L. J.; Ingold, K. U. In "Rearrangements in Ground and Excited States"; de Mayo, P., Ed.; Academic Press: New York, **1980**; Vol. 42–1, p 161) evidence in favor of such a process in the gas phase has been presented: Gordon, A. S.; Tardy, D. C.; Ireton, R. *J. Phys. Chem.* **1976**, *80*, 1400.

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