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Inactivation of Cytochrome P-450 during Catalytic Oxidation of a 3-[(Arylthio)ethyl]sydnone: *N*-Vinyl Heme Formation via Insertion into the Fe–N Bond

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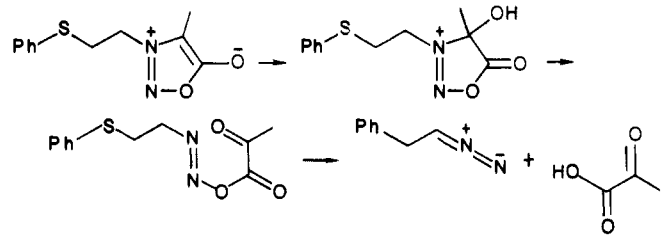
Abstract: A 3-[(2-arylthio)ethyl]-4-methylsydnone has been synthesized and shown to destroy hepatic microsomal cytochrome P-450 in a time- and NADPH-dependent manner. Enzyme destruction is accompanied by the formation of pyruvic acid and a green hepatic pigment. The pigment has been purified and identified by analytical and spectroscopic methods as a mixture of the four isomers of *N*-vinylprotoporphyrin IX. The results indicate that enzyme-catalyzed hydroxylation of the sydnone anionic carbon fragments the heterocyclic ring into pyruvic acid and a 2-(arylthio)ethyl diazonium species that reacts with the prosthetic heme group. Internal elimination of the arylthio moiety from a bridged Fe–CHR–N adduct rationalizes the formation of *N*-vinylprotoporphyrin IX. The results provide strong support for the biological formation of bridged Fe–C–N species and an explanation for some of the biological activities of the sydnones.

Sydnones, first synthesized at the University of Sidney,[1] have analgesic, antimalarial, antifungal, and antiinflammatory activities.[2,3] The development of clinically useful sydnone antiinflammatory drugs, however, has been stymied by the finding that the most active agents cause massive accumulation in rats and dogs of a fluorescent hepatic pigment tentatively identified as protoporphyrin IX.[4-6] The biochemical mechanisms responsible for the pharmacological activities of the sydnones are not known, but sydnones have specifically been shown to inhibit monoamine oxidase[7] and to prolong the sedative action of barbiturates. The latter activity presumably reflects inhibition of the metabolism of the barbiturates,[8] but little is actually known about the interaction of sydnones with metabolic enzymes beyond the fact that a number of the urinary metabolites of 3-[2-[(4-methylphenyl)-thio]ethyl]sydnone have recently been identified.[9] Model studies have demonstrated that sydnones are oxidized by a variety of oxidants, including oxygen, ozone, and peracids,[10-12] to products that can be rationalized by the reaction sequence in Scheme I.[10] We report here that the sydnones are oxidized by cytochrome P-450, essentially as proposed in Scheme I, to a reactive diazo intermediate that alkylates the prosthetic heme group and inactivates the enzyme. The structure of the resulting heme adduct provides compelling evidence for insertion of a carbene into one of the iron–nitrogen bonds to give a transient bridged Fe–CHR–N intermediate.

Results

Destruction of Rat Liver Cytochrome P-450. Incubation of liver microsomes from phenobarbital-pretreated rats with 3-[[2-(2,4,6-trimethylphenyl)thio]ethyl]-4-methylsydnone (TTMS), synthesized according to the general procedure of Wagner and Hill,[4] causes the loss of approximately 20% of the microsomal cytochrome P-450 within the first 2 min (Figure 1). Essentially the same NADPH- and TTMS-dependent enzyme loss is observed after a 30-min incubation as after a 2-min incubation, and only a small and gradual loss of cytochrome P-450 is observed if either TTMS or NADPH is omitted from the incubation. The NADPH requirement indicates that P-450 loss does not simply result from direct interaction of TTMS with the enzyme or its lipid environment. The time course for destruction of the enzyme is the same in the presence as in the absence of 10 mM glutathione (Figure 1). The finding that the prosthetic heme of only a minor fraction of the total microsomal P-450 is vulnerable to destruction, in conjunction with the failure of glutathione to protect the enzyme, implies a degree of isozyme specificity inconsistent with generalized destruction of microsomal proteins. TTMS thus appears to be a mechanism-based irreversible inhibitor that is catalytically oxidized to the species responsible for enzyme destruction.[13,14]

Scheme I. Mechanism Proposed for the Oxidative Fragmentation of Sydnones (Initial Oxidative Step Promoted by a Chemical Oxidant in the Published Model Studies[10] and by Cytochrome P-450 in the Microsomal System Described Here)



Heme Adduct Formation.[13,14] The administration of TTMS to phenobarbital-pretreated rats causes the accumulation of a green (red-fluorescent) pigment with the physical properties and absorption spectra of an *N*-alkylprotoporphyrin IX. Isolation of the green pigment by a procedure that demetalates *N*-alkylprotoporphyrin IX complexes and methylates porphyrin carboxyl groups yields an abnormal porphyrin fraction that is resolved by high-pressure liquid chromatography into four isomeric structures (Figure 2). The electronic absorption spectra of the individual isomers, numbered I–IV in the order of their elution from the column, are virtually identical. The spectra of the zinc complexes

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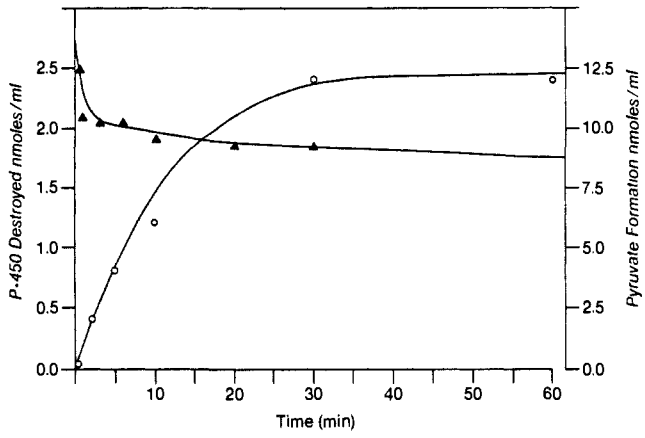


Figure 1. Time course for the destruction of cytochrome P-450 (▲) and the formation of pyruvic acid (○) in incubations of TTMS with hepatic microsomal cytochrome P-450. Each value for the loss of P-450 has been corrected for minor losses observed at the corresponding time point in the absence of TTMS (approximately 4%) or NADPH (approximately 2%). The points for the loss of cytochrome P-450 in the presence of 10 mM glutathione are essentially superimposable on those shown in the plot and are therefore not shown separately.

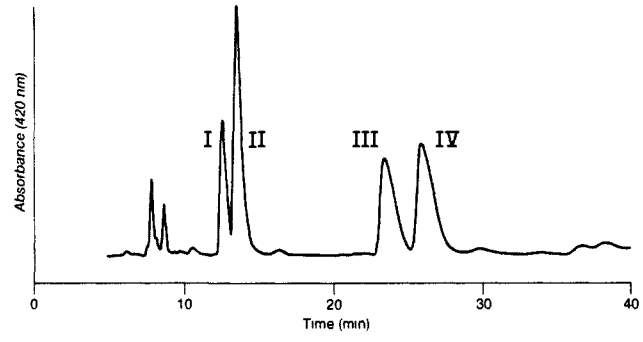


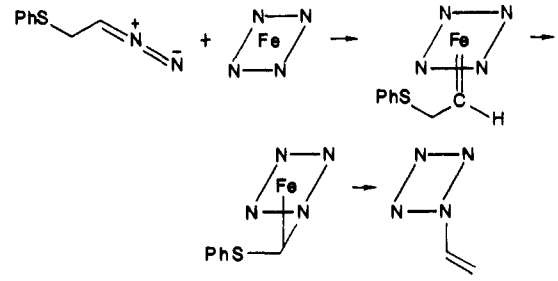
Figure 2. High-pressure liquid chromatographic analysis of the TTMS-derived pigment. The porphyrin isomers are labeled in order of their elution from the column. The analytical conditions are given in the Experimental Section.

of isomers I and II are the same as those of the zinc complexes of isomers III and IV (Figure 3) except for slight shoulders on the Soret bands of the first two isomers. The spectra of the free bases and zinc complexes are indistinguishable from those of the corresponding *N*-methylprotoporphyrin IX isomers.[15,16] It is clear from the spectroscopic data that isomeric *N*-alkylprotoporphyrin derivatives are formed in the destructive process. Earlier studies have shown that zinc-complexed *N*-alkylprotoporphyrin IX isomers with a shoulder on the Soret band bear the *N*-alkyl group on a vinyl-substituted pyrrole ring (ring A or B).[15,16] The inference that isomers I and II are alkylated on the nitrogens of pyrrole rings A and B is supported by the fact that the A- and B-ring isomers of *N*-methyl- and *N*-ethylprotoporphyrin IX also have lower retention times than the C- and D-ring isomers.

Structures of the Isomeric Heme Adducts. The field desorption mass spectrum of the isomeric porphyrins exhibited a strong protonated molecular ion peak ($M^+ + 1$) at m/z 617. This corresponds, if the alkyl group is attached to the dimethyl ester of protoporphyrin IX (molecular weight 590), to structures in which the *N*-alkyl group has a molecular weight of 27. The only alkyl moiety with such a molecular weight is a vinyl group. The inference that the porphyrin bears an *N*-vinyl group is confirmed by the following NMR studies.

Individual 500-MHz NMR spectra have been obtained for isomers II–IV. Isomer I was not obtained in sufficient quantity

Scheme II. Mechanism Proposed for *N*-Vinylprotoporphyrin IX Formation (Prosthetic Heme of Cytochrome P-450 Abbreviated as an Iron in a Square of Pyrrole Nitrogens)



for NMR analysis. Spin-decoupling studies were only carried out with isomer II, but sufficient data were obtained on isomers III and IV to confirm their structural relationship to isomer II. The NMR spectrum of isomer II exhibits the signals expected for the protoporphyrin IX skeleton of an *N*-alkylprotoporphyrin IX:[15-17] (a) singlets at 10.53, 10.38, 10.27, and 10.20 ppm due to the four meso protons (Figure 4); (b) six singlets in the 3.3–3.8 ppm region due to the four porphyrin and two ester methyl groups (not shown); (c) two doublets of doublets at 8.23 and 8.07 ppm (J = 11.4, 17.7 Hz) due to the internal protons of the porphyrin vinyl substituents; (d) doublets at 6.45 and 6.32 (J = 17.7 Hz) and at 6.29 and 6.23 ppm (J = 11.4 Hz) due, respectively, to the trans and cis terminal vinyl protons (Figure 4); (d) multiplets at 4.37, approximately 3.6, and 3.32 ppm due, respectively, to the internal and external methylene protons of the propionic acid side chains (not shown).

The fact that the two internal protons of the porphyrin vinyl substituents appear as well- resolved doublets of doublets (Figure 4) confirms the earlier conclusion that the *N*-alkyl group in isomer II is on pyrrole ring A or B. We have previously shown that clean separation of the internal vinyl protons in the NMR, which indicates that the two vinyl groups are in distinctly different positions with respect to the porphyrin ring current, is diagnostic for alkylation of pyrrole ring A or B.[15-17] The presence of a single unresolved multiplet for the internal vinyl protons in the NMR of isomer IV (not shown), the fact that the propionic acid methylene protons of this isomer are clearly resolved in the NMR (not shown), and the absence of a shoulder in the Soret band of its zinc complex (Figure 3) indicate that isomer IV bears the *N*-alkyl group on pyrrole ring C or D. The NMR data on the porphyrin skeletons are thus exactly analogous to those for all other known *N*-alkylprotoporphyrin structures.[14] This unambiguously identifies the porphyrin skeleton as protoporphyrin IX and establishes that isomers I and II are alkylated on pyrrole rings A and B and isomers III and IV on pyrrole rings C and D.

Identification of the *N*-alkyl group is complicated by the fact that two of the three protons of the C_2H_3 moiety indicated by the molecular weight of 27 are obscured by signals in the 0–2 ppm region due to trace impurities carried through the purification sequence (each NMR spectrum was obtained on 40 μg of adduct). This problem was circumvented by decoupling experiments. Each of the three isomers for which an NMR spectrum is available exhibits a one-proton doublet of doublets, as required for the internal proton of an *N*-vinyl group, centered at −1.90 to −1.96 ppm. This doublet of doublets is at −1.96 ppm (J = 15.2, 8.5 Hz) in isomer II (Figure 5). Location of this signal downfield from that of the *N*-methylene protons of *N*-methyl- and *N*-ethylprotoporphyrin IX, which are found at −4 to −5 ppm,[15-17] is consistent with its identification as a vinyl proton because vinyl protons normally appear 2–3 ppm downfield from the analogous alkyl protons. Irradiation of a proton at −0.13 ppm causes the signal at −1.96 ppm to collapse to a doublet (J = 8.5 Hz) (Figure 5). Irradiation of a proton at 0.85 ppm similarly causes the signal at −1.96 ppm to collapse to a doublet (J = 15.2 Hz). The signal at −0.13 ppm is assigned to the terminal vinyl proton trans to the

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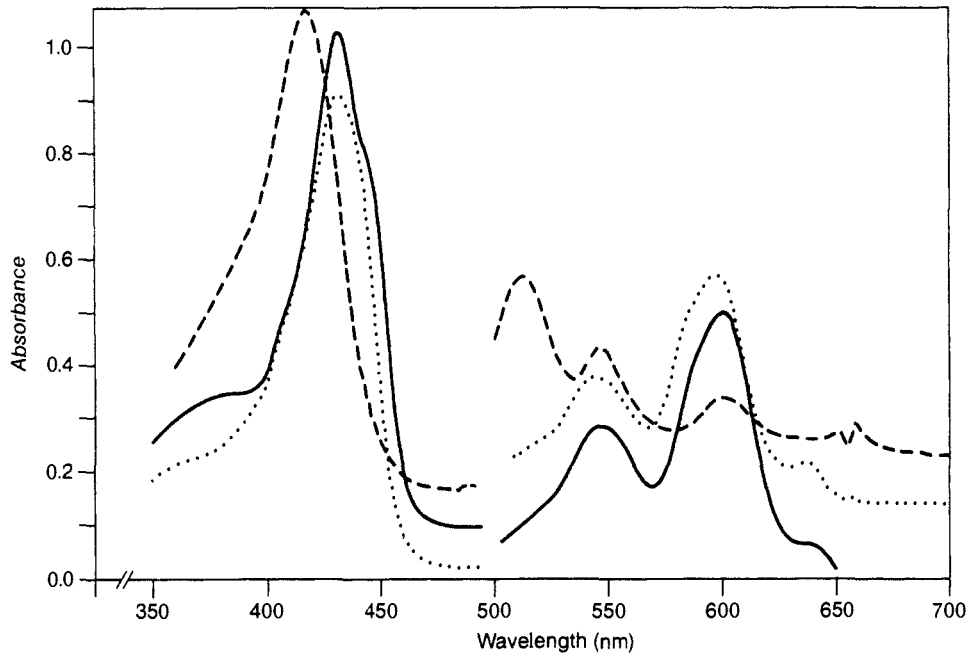

Figure 3. Electronic absorption spectra of the free base (---) and zinc-complexed forms (—) of dimethyl-esterified isomer II and of the zinc-complexed form (···) of dimethyl-esterified isomer IV.

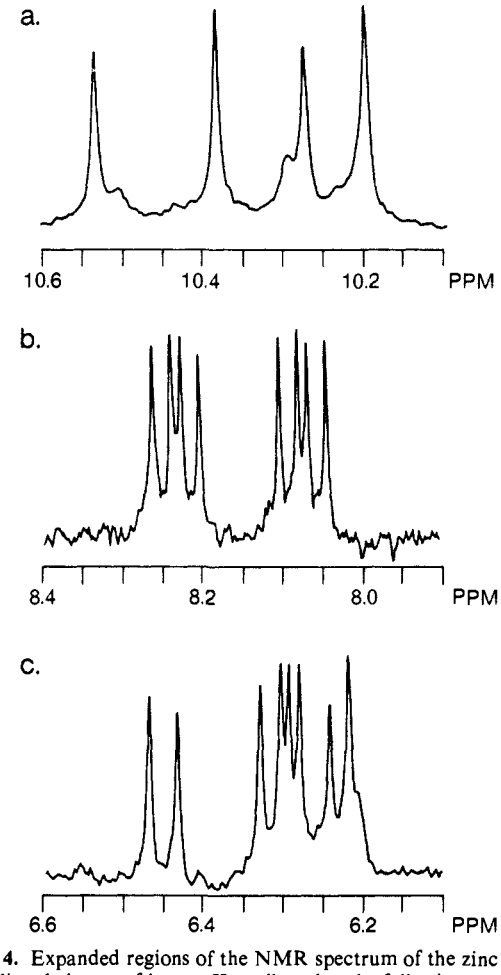

Figure 4. Expanded regions of the NMR spectrum of the zinc complex of the dimethyl ester of isomer II attributed to the following protons: (a) meso protons; (b) internal protons of the protoporphyrin IX vinyl substituents; (c) terminal protons of the protoporphyrin IX vinyl substituents.

internal vinyl proton at −1.96 ppm and that at 0.85 ppm to the cis proton because the coupling constant of the former is larger and because its placement closer to the porphyrin ring explains its larger upfield chemical shift. The NMR data, supported by the chromatographic, electronic absorption, and mass spectrometric results, establish that the adducts isolated from the livers of TTMS-treated rats are the four isomers of *N*-vinylprotoporphyrin IX (Figure 6).

Catalytic Release of Pyruvic Acid from TTMS. Identification of the heme-derived adduct as *N*-vinylprotoporphyrin IX suggests activation of TTMS by the sequence in Schemes I and II, which predict that pyruvic acid should be generated as a byproduct. Incubation of TTMS with microsomes from phenobarbital-pretreated rats results, indeed, in the time-dependent production of pyruvic acid (Figure 1). Pyruvic acid formation depends on the presence of both NADPH and TTMS in the incubation mixture. The accumulation of pyruvic acid lags behind cytochrome P-450 inactivation since P-450 destruction levels off after 5 min whereas pyruvic acid formation continues for 20–25 min (Figure 1). The final ratio of pyruvic acid formed to P-450 lost indicates that approximately 1 P-450 molecule is lost for every 16 molecules of pyruvic acid formed. This difference in the time course of inactivation and metabolite formation can be reconciled with a common oxidative event if the rates of heme destruction and pyruvic acid formation are governed by steps subsequent to the oxidative event. The lag in pyruvic acid accumulation is thus compatible with Scheme I if the bulk of the hydroxylated TTMS decomposes to the diazoalkane in a relatively slow secondary reaction or if two isozymes are involved, only one of which loses its chromophore.

Discussion

The formation of pyruvic acid in the cytochrome P-450 catalyzed oxidation of TTMS implicates the reaction mechanism in Scheme I. This mechanism is consistent with the model chemistry observed when sydnones are oxidized by ozone or peracids and with the urinary metabolites obtained in vivo from a different sydnone,[9-12] although a different reaction sequence was proposed to explain the urinary metabolites. The negative charge on the ring carbon of the sydnone ring makes it a particularly attractive site for enzymatic hydroxylation because the reactive oxygen species produced by cytochrome P-450 is electrophilic.[18] The continued formation of pyruvic acid for some time after P-450 chromophore loss is complete, and the fact that 16 molecules of pyruvic acid are formed per P-450 molecule destroyed indicates

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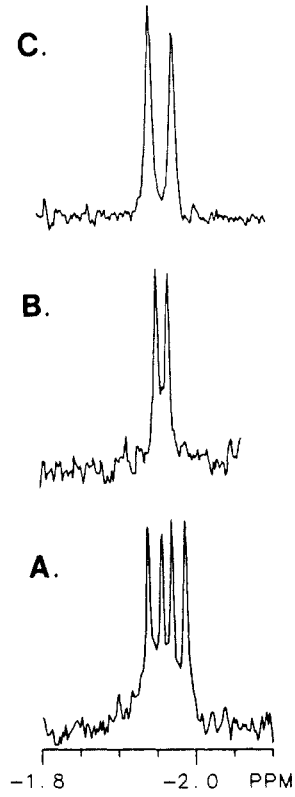

Figure 5. Internal proton of the pyrrole *N*-vinyl group. The change in the signal pattern of the *N*-vinyl proton (A) when the protons at −0.13 and 0.85 ppm, respectively, are irradiated are shown in (B) and (C). The proton at −0.13 ppm is assigned to the trans and that at 0.85 ppm to the cis terminal vinyl protons.

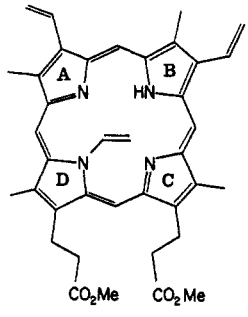

Figure 6. Dimethyl ester of one of the four isomers of *N*-vinylprotoporphyrin IX formed in the reaction of TTMS with cytochrome P-450. Isomers I and II bear the *N*-vinyl group on pyrrole rings A and B, whereas isomers III and IV bear it on pyrrole rings C and D (the pyrrole rings are labeled).

that the bulk of the hydroxylated sydnone decomposes to the diazoalkane and pyruvic acid in a process independent of the catalytic activity of the enzyme.

The destruction of cytochrome P-450 and its chromophore is consistent with the activation sequence in Scheme II, in which the prosthetic heme group reacts with the diazo intermediate unmasked by elimination of pyruvic acid from the hydroxylated TTMS product. The faster rate of cytochrome P-450 destruction than of pyruvic acid formation suggests that the ferric prosthetic group of the enzyme catalyzes conversion of the hydroxylated sydnone to the diazoalkane. It has been shown, in this context, that the decomposition of closely related aryldiazo alkyl ethers is subject to general-acid catalysis.[19]

The chemical reaction of ethyl diazoacetate or diazoacetaldehyde with cobalt tetraphenylporphyrin or cobalt octaethylporphyrin yields nitrogen gas and an adduct in which the activated carbon bridges the metal ion and one of the porphyrin nitrogens.[20-22] Vinylidene–carbene complexes generated reductively from the reaction of 2,2-bis(*p*-chlorophenyl)-1,1,1-trichloroethane (DDT) with iron(II) porphyrins[23] have furthermore been shown to rearrange on oxidation to structures in which the vinylidene carbon bridges the iron and a nitrogen of the porphyrin.[24-27] The latter complexes thus resemble those obtained in the reaction of diazocarbonyl compounds with cobalt porphyrins except that the bridging atom is a vinylidene carbon in the former and a carbonyl-substituted carbon in the latter. Finally, evidence is available that protonation of the bridging carbon converts the bridged porphyrins into the corresponding *N*-alkylporphyrins.[20-22,28] Analogous model studies have not been carried out with diazoalkyl compounds that are not stabilized by substitution, but the work with the carbonyl-stabilized analogues provides a clear *chemical* precedent for the iron–nitrogen-bridged intermediate postulated to precede *N*-vinylprotoporphyrin IX in Scheme II. The bridged intermediate may be preceded by a carbene complex, but the carbene complex, if formed, is highly transient. We have observed the formation of complexes that absorb at approximately 456 nm in the reactions of cytochrome P-450 with carbonyl-stabilized diazo compounds[29] but have been unable to detect similar absorption bands in incubations of TTMS with hepatic microsomes. The fact that the iron in the model vinylidene-bridged porphyrin complexes is in the ferric state[27,30] and that protonation of the carbon in the bridged complexes gives *N*-alkyl adducts[28] indicates that the carbon atom is negatively charged. A negative charge on the bridging carbon in the TTMS-derived adduct would be expected, as observed, to internally eliminate the β-thioaryl group. The internal elimination reaction thus provides the first clear evidence for the formation of carbon-bridged iron–nitrogen adducts in biological reactions. Experiments are under way to define the structural features of sydnones required for the destruction of cytochrome P-450 and to determine whether internal elimination can be used as a more general test for the intervention of bridged intermediates in the reactions of metalloporphyrins and hemoproteins.

The in vivo formation of an *N*-alkylprotoporphyrin IX with a relatively small *N*-alkyl moiety readily explains the observation that the administration of TTMS to dogs and rodents causes the hepatic accumulation of a fluorescent pigment.[6] We have recently demonstrated that TTMS specifically causes the accumulation of protoporphyrin IX in cultured chicken embryo liver cells[31] and have traced this accumulation to inhibition of ferrochelatase, the heme biosynthetic enzyme that inserts the iron into protoporphyrin IX. Previous work has demonstrated that *N*-alkylprotoporphyrin IX derivatives are powerful inhibitors of ferrochelatase when the *N*-alkyl group is a methyl or, if larger than a methyl, is located on pyrrole rings A or B.[32-34] The *N*-vinyl group in the TTMS

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adduct not only is relatively small but is present in isomers (I and II) in which it is bound to pyrrole rings A and B. The evidence thus strongly suggests that the *N*-vinylporphyrin formed in vivo in the reaction of TTMS with cytochrome P-450 inhibits ferrochelatase and causes the abnormal accumulation of protoporphyrin IX. The metabolic scheme proposed here furthermore suggests the possibility that the hepatotoxic or carcinogenic properties of sydnones[35] may result from release into the cellular compartment of the hydroxylated sydnone or the reactive alkyldiazo product derived from it.

Experimental Section

Materials and General Methods. NMR spectra of synthetic organic compounds were recorded in C^2HCl_3 on a Varian FT80 (80-MHz) instrument. NMR shifts are expressed in parts per million relative to tetramethylsilane. The electron-impact (70 eV) mass spectra of synthetic compounds were obtained on a Kratos MS-25 instrument. High-pressure liquid chromatography (HPLC) was carried out in a system with Beckman pumps and a Hewlett-Packard HP 1040A diode array detector. Infrared spectra were obtained on a Nicolet 5DX Fourier transform instrument. NADPH and glutathione were purchased from Sigma Chemical Co. Microanalyses were obtained by the Microanalytical Laboratory of the University of California, Berkeley.

2-[(2,4,6-Trimethylphenyl)thio]ethylamine.[36] To a solution of sodium metal (2.30 g, 0.1 mol) in 100 mL of absolute ethanol under nitrogen was added 2,4,6-trimethylbenzenethiol (7.61 g, 0.05 mol) followed by 2-bromoethylamine hydrobromide (10.24 g, 0.05 mol). The reaction mixture was refluxed 12 h before it was cooled, filtered, and concentrated on a rotary evaporator. The residue was taken up in 50 mL of diethyl ether and was washed with saturated aqueous NaCl. The organic layer was dried over Na_2SO_4 and the solvent removed under vacuum. Bulb-to-bulb distillation provided 8.61 g (88%) of the desired amine: IR 3366 (NH), 2921, 1604, 1058 cm^{-1}; ^1H NMR δ 6.85 (s, 2 H, aryl), 2.76 (t, 4 H, J = 2.8 Hz, SCH_2CH_2N), 2.51 (s, 6 H, 2,6 methyls), 2.26 (s, 3 H, 4 methyl), and 1.64 (s, 2 H, NH_2).

***N*-[2-[(2,4,6-Trimethylphenyl)thio]ethyl]alanine.**[4] To a solution of potassium *tert*-butoxide (4.80 g, 43 mmol) in 250 mL of freshly distilled *tert*-butyl alcohol was added 2-[(2,4,6-trimethylphenyl)thio]ethylamine (8.40 g, 43 mmol) followed by 2-bromopropionic acid (6.58 g, 43 mmol). The reaction mixture was refluxed under nitrogen for 14 h and was then cooled and the solvent removed under vacuum. The residue was taken up in 100 mL of 2% NaOH. The aqueous solution was washed with 100 mL of diethyl ether and was then acidified to pH 5 with concentrated HCl. Filtration followed by washing with ice-cold H_2O afforded 6.68 g (58%) of the desired amino acid. Stirring overnight in 5% (v/v) H_2SO_4 in methanol produced the methyl ester: IR 2926, 2852, 1720, 1261 cm^{-1}; ^1H NMR (of the ester) δ 6.90 (s, 2 H, aryl), 3.69 (s, 3 H, OCH_3), 3.32 (q, 1 H, J = 6.9 Hz, CHN), 2.72 (m, 4 H, SCH_2CH_2N), 2.49 (s, 6 H, 2,6 methyls), 2.24 (s, 3 H, 4 methyl), 2.12 (s, 1 H, NH), 1.49 and 1.27 (two d, 3 H total, each J = 6.9 Hz, CH_3CH). Anal. Calcd for $C_{14}H_{21}NO_2S$: C, 62.88; H, 7.92; N, 5.24. Found: C, 61.49; H, 7.65; N, 4.95.

***N*-Nitroso-*N*-[2-[(2,4,6-trimethylphenyl)thio]ethyl]alanine.**[4] A solution of *N*-[2-[(2,4,6-trimethylphenyl)thio]ethyl]alanine (4.68 g, 18 mmol) and $NaNO_2$ (1.32 g, 19 mmol) in 100 mL of 1:1 CH_2Cl_2–H_2 was stirred at 0 °C while 1.7 mL of concentrated HCl was added dropwise over 15 min. Stirring was continued for 2 h at room temperature, and the organic layer was then separated, washed with saturated aqueous NaCl, dried over Na_2SO_4, and concentrated under vacuum. Recrystallization from ethyl acetate–hexane afforded 4.60 g (89%) of the desired *N*-nitroso acid: mp 104–106; IR 2984, 2926, 1725, 1598, 1245 cm^{-1}; ^1H NMR δ 8.76 (s, 1 H, COOH), 6.93 (s, 2 H, aryl), 5.25 plus 4.52 (two quartets, 1 H total, 7.2 Hz, CHN), 4.22 (m, 2H, CH_2N), 3.04 (m, 2H, CH_2S), 2.51 plus 2.46 (two s, 6 H total, 2,6 methyls), 2.25 (s, 3 H, 4 methyl), 1.65 plus 1.36 (two d, 3 H total, J = 7.2 Hz each, CH_3 CH). Anal. Calcd for $C_{14}H_{20}N_2O_3S$: C, 56.73; H, 6.80; N, 9.45. Found: C, 57.55; H, 7.00; N, 8.93.

3-[2-[(2,4,6-Trimethylphenyl)thio]ethyl]-4-methylsydnone.[4] A solution of *N*-nitroso-*N*-[2-[(2,4,6-trimethylphenyl)thio]ethyl]alanine (4.60 g, 15.5 mmol) and 60 mL of acetic anhydride was allowed to stand at room temperature for 4 days. The reaction mixture was poured into 100 mL of water and was stirred for 12 h. It was then extracted with CH_2Cl_2 (2 × 100 mL), and the combined organic layers were washed with H_2O, saturated $NaHCO_3$, and saturated NaCl before they were dried over Na_2SO_4. Solvent removal under vacuum and recrystallization from ethyl acetate–hexane afforded 1.94 g (45%) of the desired sydnone: mp 65–66 °C; IR 4438, 2958, 1737 (C=O) 1450, 1375, 1250, 901 cm^{-1}; ^1H NMR δ 6.96 (s, 2 H, aryl), 4.23 (t, 2 H, J = 7.7 Hz, CH_2N), 3.10 (t, 2 H, J = 7.7 Hz, SCH_2), 2.48 (s, 6 H, 2,6 methyls), 2.27 (s, 3 H, 4 methyl), and 2.00 (s, 3 H, CCH_3); EIMS m/e 278 (M^+). Anal. Calcd for $C_{14}H_{18}N_2O_2S$: C, 60.40; H, 6.52; N, 10.07. Found: C, 60.39; H, 6.35; N, 9.98.

Destruction of Cytochrome P-450 in Incubations with Rat Liver Microsomes. The destruction of cytochrome P-450 was assayed as described previously.[37,38] Hepatic microsomes were prepared from rats injected intraperitoneally with phenobarbital (80 mg/kg) once a day for 5 days. The standard incubation mixture consisted of microsomal protein (2.5 nmol/mL), KCl (150 mM), EDTA (1.5 mM), NADPH (1 mM), and TTMS (5 mM) in 0.1 M sodium phosphate–potassium phosphate buffer (pH 7.4). Additions or omissions from this standard mixture are indicated in the text. The incubations were started by adding NADPH to the other components of the mixture after preequilibration at 37 °C. After incubation at that temperature for the indicated time, the incubations were ended by rapidly cooling the mixture to 0 °C.

Assays. The cytochrome P-450 concentration was determined from the CO vs. reduced CO difference spectrum on an Aminco DW2a spectrometer by the procedure of Estabrook et al.[39] Protein concentrations were measured with Bio-Rad kits. The formation of pyruvic acid was quantitated by the method of Friedemann.[40] A 10% solution of trichloroacetic acid (5 mL) was added to each 10-mL microsomal incubation mixture, and the mixture was centrifuged on a desktop centrifuge to sediment the protein. The protein was discarded, and 1 mL of a 0.1% solution of (2,4-dinitrophenyl)hydrazine in 2 N HCl was added to the supernatant. The mixture was allowed to stand 5 min before 3.0 mL of ethylbenzene was added, the mixture shaken, and the aqueous layer removed. The organic layer was shaken with 6.0 mL of 10% Na_2CO_3 solution, and 5 mL of the aqueous layer was added to 5.0 mL of 1.5 N NaOH. The pyruvate hydrazone in the solution, present as the disodium salt, was quantitated on an Aminco DW-2a spectrometer by measuring its absorbance at 435 nm. The reference cuvette in these measurements derived from an incubation from which both NADPH and TTMS were omitted. Standard curves were constructed by similarly analyzing liver microsomes to which known amounts of pyruvic acid had been added.

Isolation of the Prosthetic Heme Adduct. Sprague–Dawley (250–260-g) male rats, injected intraperitoneally once a day for 5 days with an 80 mg/kg dose of sodium phenobarbital in water, were injected on the sixth day with TTMS (165 mg/rat in 0.3 mL of dimethyl sulfoxide). Three hours later, the rats were decapitated and their livers removed after perfusion with 1.15% KCl solution. The livers were homogenized in 5% H_2SO_4 (v/v) in methanol with a Waring blender. The homogenate was allowed to stand in the dark for 18 h at 4 °C. The mixture was then filtered, combined with an equal volume of water, and extracted three times with methylene chloride. The organic extract, washed three times with water and dried over anhydrous sodium sulfate, was concentrated to a volume of 100 mL on a rotary evaporator before 0.5 mL of methanol saturated with zinc acetate was added. The remaining solvent was then removed, and the residue was flash chromatographed on a Merck silica gel 60 (230–240 mesh) column eluted first with 1:1 hexane–tetrahydrofuran and then, in order, with tetrahydrofuran, 1:1 $CHCl_3$–tetrahydrofuran, $CHCl_3$, and finally 3:1 $CHCl_3$–acetone. The green, red-fluorescent fraction thus obtained was further purified by high-pressure liquid chromatography on a Partisil-5 PAC column (4.6 × 250 mm) eluted with a 60-min linear gradient (0–100%) of methanol into 1:1 (v/v) hexane–tetrahydrofuran. Zinc was removed from the porphyrins thus isolated by treatment with 5% H_2SO_4/methanol as described before.[16,37,38] The metal-free porphyrin mixture was subjected to high-pressure liquid chromatography on the same Partisil-5 PAC column eluted isocratically with 50:50:1.5 hexane–tetrahydrofuran–methanol. The four porphyrin isomers separated in this last step were collected individually for spectroscopic characterization.

Spectroscopic Characterization of the Heme Adduct. Electronic absorption spectra were recorded in CH_2Cl_2 on a Hewlett-Packard Model 8450A spectrometer. Mass spectra were obtained on a Kratos MS 50 instrument operating in the field desorption mode. NMR spectra were recorded on a General Electric GN 500-MHz instrument with the fol-

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lowing instrumental parameters: spectral width, 4000 Hz; pulse width, 15 μs; delay between acquisitions, 500 μs; number of acquisitions, 800; size, 16K; acquisition time, 1.02 s; line broadening, 1.0 Hz; temperature, 25 °C.

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Ynenol Lactones: Synthesis and Investigation of Reactions Relevant to Their Inactivation of Serine Proteases†

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Abstract: The syntheses of ynenol lactones [5(*E* or *Z*)-(3-R′-2-propynylidene)-3-R-tetrahydro-2-furanones and 6(*E*)-(3-R′-2-propynylidene)-3-R-tetrahydro-2-pyrones; R = H, alkyl, benzyl; R′ = H, alkyl, phenyl] which are designed as serine protease suicide substrates have been accomplished. *E* ynenol lactones are prepared via iodolactonization of ω-hexynoic and ω-pentynoic acids, followed by the $CuI/Et_3N/PdCl_2(PPh_3)_2$-mediated coupling of the resulting *E* iodo enol lactones with appropriate alkynes. Isomerization of *E* iodo enol lactones gives the *Z* isomers, which can be separated and coupled to give the *Z* ynenol lactones. We have shown that the alkaline hydrolysis of ynenol lactones parallels the reaction sequence that has been proposed to account for ynenol lactone inactivation of serine proteases, namely, lactone ring cleavage, formation of the allenone, and conjugate addition of a nucleophile to the β-carbon of the allenone. When the acetylene terminus of the ynenol lactone is unsubstituted, alkaline hydrolysis leads to the allenone without a detectable intermediate. When the terminus is alkyl or phenyl substituted, an intermediate (which is probably the propargyl ketone resulting from α protonation) is apparent in the reaction kinetics. Base-catalyzed isomerization of 1-hexyn-4-one and 2-hexyn-5-one to allenones indicates a profound substituent effect of γ substitution ($k_{\gamma\text{-H}}/k_{\gamma\text{-Me}} = 300$). Nucleophilic attack on the allenones by hydroxide and *n*-butylamine gives, respectively, 1,3-dione monoanions and *Z*-β-amino enones. When the allenone is γ-phenyl substituted and is treated with hydroxide, an intermediate consistent with an ynenolate anion is apparent in the kinetics and ultraviolet spectra; the intermediate is formed with a pK_a of 13.4. Similar pK_a values are observed in the reaction kinetics of hydroxide with γ-methyl-substituted allenones.

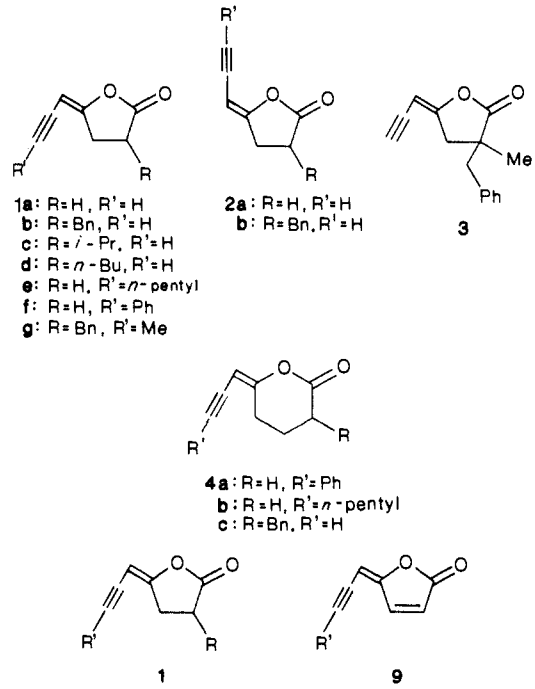
A contemporary and intellectually appealing strategy in drug design involves the use of latent reactive substrates as enzyme inactivators.[1] This strategy features a relatively unreactive "suicide substrate" S (Scheme I) that is designed initially to bind to a specific target enzyme E and then to be transformed during the normal course of enzyme catalysis to a highly reactive species S*. Ideally, S*, before it can escape from the active site, is trapped by an amino acid side chain or coenzyme to give an irreversibly inactivated enzyme E-X.

Electrophilic allenes have played an important role in the development of this strategy as the demonstrable or putative intermediates that cause enzyme inactivation.[2] The success of inhibitors that are designed to generate these Michael acceptors has led us to explore the use of allenones as the latent functional group in a series of novel serine protease inhibitors.[3] In this paper we discuss the synthesis and nonenzymatic reactions of ynenol lactones **1–4**, which are designed to produce electrophilic allenones at protease active sites.

Scheme II outlines the reactions that must occur to achieve enzyme inactivation by this strategy. These are enzyme acylation (**1** → **5**), formation of the allenone (**5** → **7** or **5** → **6** → **7**), and capture of an enzyme nucleophile (**7** → **8**). Nonenzymatic models of each of these reactions will be presented herein to demonstrate the chemical and kinetic feasibility of this scheme. The characterization of these compounds as protease inhibitors will be presented elsewhere.[3]

Synthesis

To our knowledge the ynenol lactones **1** have not been previously characterized,[4] although their unsaturated analogues **9** have been frequently documented as natural products in the biosynthesis of polyacetylenes.[5]



The ynenol lactones in this study are prepared by coupling *E* or *Z* iodo enol lactones **10** and **11** with appropriate alkynes

† Contribution No. 241 from the Institute of Bioorganic Chemistry. This paper is dedicated to the memory of Dr. Howard J. Ringold.

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