initial samples. This procedure gives a reasonable assurance that we have located the absolute minimum.

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Registry No. Proflavine complex with deoxy(cytidylyl-3,5'-guanosine), 73113-31-2.

Inactivation of Cytochrome P-450 by a Catalytically Generated Cyclobutadiene Species

Ralph A. Stearns and Paul R. Ortiz de Montellano*

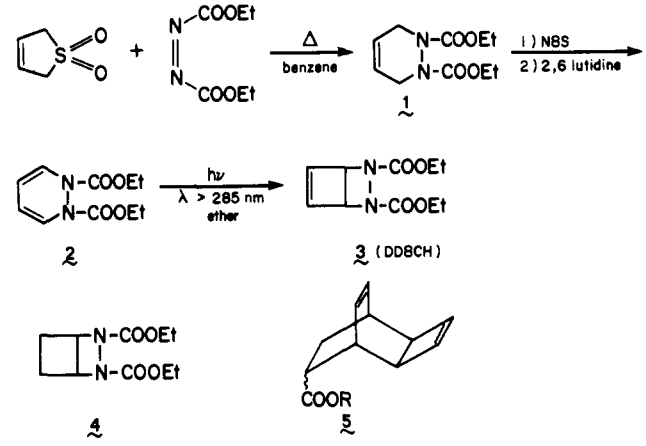
Contribution from the Department of Pharmaceutical Chemistry, School of Pharmacy and Liver Center, University of California, San Francisco, California 94143. *Received May 24, 1984*

Abstract: 2,3-Bis(carbethoxy)-2,3-diazabicyclo[2.2.0]hex-5-ene (DDBCH), synthesized by a route analogous to that reported for the preparation of the bis(carbomethoxy) analogue, inactivates the phenobarbital-inducible cytochrome P-450 isozymes of rat liver in a time-, NADPH-, and oxygen-dependent manner. The enzymes are protected by carbon monoxide and competitive alternative substrates but not by glutathione. The cyclobutenyl π bond and the diazabicyclo[2.2.0] skeleton are required for destructive activity, but hydrolytic removal of the carbamate groups is not. Enzyme inactivation reflects alkylation of the prosthetic heme group of the enzyme by a catalytically generated reactive species. The alkylated prosthetic group has been isolated and has been characterized, after demetallation and esterification, as *N*-(2-cyclobutenyl)protoporphyrin IX. The *N*-alkyl group is primarily located on the nitrogen of pyrrole ring D. DDBCH is thus oxidized by cytochrome P-450 to a cyclobutadienoid species that alkylates the prosthetic heme group.

The inactivation of cytochrome P-450 enzymes by catalytically activated substrates is of toxicological, pharmacological, and mechanistic importance. The inactivation of cytochrome P-450 enzymes can alter patterns of metabolism, perturb heme and hemoprotein synthesis, and, in some instances, inhibit critical cellular processes.[1] The sedative hypnotics ethchlorvynol and novonal, for example, alkylate the prosthetic heme groups of cytochrome P-450 enzymes in a process that initiates uncontrolled porphyrin synthesis and causes, or exacerbates, the clinical symptoms of porphyria in genetically predisposed patients.[2-4] In contrast, the *highly specific* inhibition of individual cytochrome P-450 isozymes is a promising route to the design of drugs and other pharmacologically active agents. Two such agents now in use are miconazole, an antimycotic drug that inhibits the 14-demethylation of ergosterol in fungi,[5] and piperonyl butoxide, an insecticide synergist.[6] Finally, in a mechanistic context, suicide substrates have been employed by this laboratory to explore the electronic nature of the catalytic mechanisms and the topologies of the active sites of cytochrome P-450 enzymes.[7,8]

The construction of isozyme-specific suicide substrates depends on the availability of latent destructive functionalities that can be incorporated into substrates recognized by the target enzymes. We have established in earlier work that introduction of a terminal carbon–carbon double or triple bond into a substate conveys cytochrome P-450 destructive activity.[1,7] We have recently, in a departure from the electrophilic and radical species on which most suicide substrates are based, established that 1-aminobenzotriazole is catalyticaly oxidized to benzyne or a closely related

Scheme I. Synthesis of DDBCH



species and that this species inactivates cytochrome P-450 enzymes in animals, plants, and insects with exceptional efficiency.[9-12] The high activity and surprisingly low toxicity of this benzyne precursor led us to search for alternative stereoelectronically activated moieties that could be unmasked by the catalytic action of redox enzymes. We report here that cytochrome P-450-catalyzed oxidation of 2,3-bis(carbethoxy)-2,3-diazabicyclo[2.2.0]hex-5-ene (DDBCH) unveils a highly antiaromatic and reactive cyclobutadienoid moiety that efficiently alkylates the prosthetic heme group of the enzyme.

Results

Synthesis. 2,3-Biscarbethoxy-2,3-diazabicyclo[2.2.0]hex-5-ene (DDBCH) was synthesized from butadiene and ethyl azodicarboxylate by the sequence outlined in Scheme I. The individual steps in the sequence were adopted in modified form from liter-

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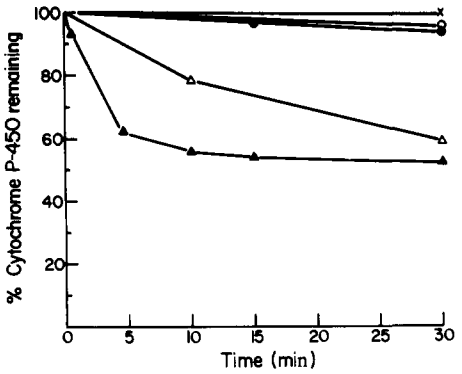

Figure 1. Destruction of microsomal cytochrome P-450 by DDBCH. The loss of cytochrome P-450, measured spectrophotometrically as the reduced-CO complex, is plotted as a function of the time of incubation of DDBCH (1 mM) with microsomes from phenobarbital-pretreated rats. The normal incubation mixture included the following substrates and modificiations: (X) no substrate, no NADPH; (○) no substrate; (▲) DDBCH (1 mM); and (△) DDBCH (1 mM) + benzphetamine (2 mM). The points for incubation of DDBCH (1 mM) with glutathione (10 mM) are superimposable on those obtained in the absence of glutathione. The results of incubating **4**, the saturated analogue of DDBCH (1 mM), with hepatic microsomes are also given (●).

Table I. Destruction of Cytochrome P-450 by DDBCH and Its Analogues

substrate (incubation conditions)	cytochrome P-450 loss, %[a] 15 min	30 min
no substrate		5 ± 1
1 (1 mM)		6 ± 1
2 (1 mM)		13 ± 3
DDBCH		
(1 mM)	34 ± 2	43 ± 1
(under argon)	ND	3
DDBCH (10 mM)	44 ± 3	61 ± 3
DDBCH (10 mM) + BNPP (0.5 mM)	44 ± 2	65 ± 3
BNPP (0.5 mM)		6 ± 1
5 (5 mM)		20 ± 5

[a] Incubations were carried out as described in the Experimental Section. Modifications of the normal incubation system are given in parentheses. No entry indicates the experiment was not done; ND stands for not detectable.

ature procedures employed in the synthesis of the corresponding bis(carbomethoxy) analogue.[13-17] The saturated analogue of DDBCH was readily obtained by catalytic hydrogenation. The structures assigned to synthetic intermediates and final products are in full accord with complete spectroscopic and analytical data. Our initial intent was to remove the ethyl carbamate moieties by hydrolysis and decarboxylation because the release of cyclobutadiene with chemical oxidants only occurs with the unesterified bicyclic structure.[12-14] Hydrolytic removal of the diethylcarbamate groups, however, proved to be unexpectedly difficult and eventually unnecessary. In retrospect, the oxidizability of the underivatized cyclic hydrazine makes it unattractive as a suicide substrate for redox enzymes because autoxidation in the biological system would result in nonspecific enzyme inactivation.

Cytochrome P-450 Inactivation. Incubation of DDBCH with hepatic microsomes from phenobarbital-pretreated rats in the presence of NADPH causes a time- and concentration-dependent

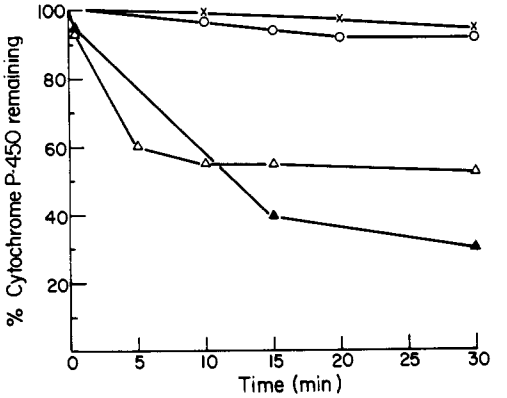

Figure 2. Destruction by DDBCH of liver microsomal cytochrome P-450 from rats induced with different agents. The spectrophotometrically measured loss of cytochrome P-450 is plotted as a function of time of incubation of DDBCH (1 mM) with hepatic microsomes from (X) untreated rats, (○) 3-methylcholanthrene-pretreated rats, (△) phenobarbital-pretreated rats, and (▲) a purified, reconstituted fraction of cytochrome P-450 isozymes from phenobarbital-pretreated rats.[22]

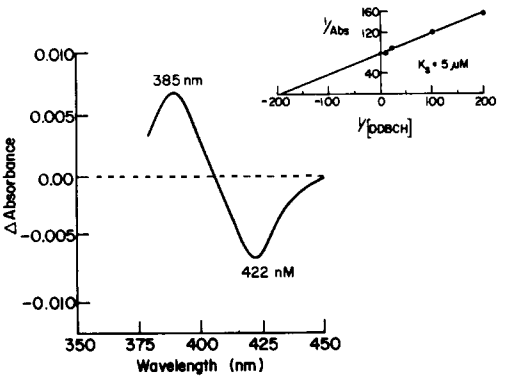

Figure 3. Difference spectra resulting from the interaction of DDBCH with unreduced microsomal cytochrome P-450 from phenobarbital-pretreated rats. A double reciprocal plot of the DDBCH concentration against the magnitude of the 420–390-nm peak-to-trough absorbance difference is presented in the inset.

decrease in the spectroscopically measured enzyme concentration (Figure 1). The enzyme is only inactivated by DDBCH if NADPH is present in the incubation. Approximately 43% of the enzyme is lost in 30 min with 1 mM DDBCH whereas 61% is lost when the DDBCH concentration is raised to 10 mM (Table I) (enzyme loss occurs more rapidly with the higher concentration). It thus appears that something in the order of 60% of the total cytochrome P-450 isozymes in phenobarbital-induced rat liver microsomes are vulnerable to destruction by this agent. In contrast, very little cytochrome P-450 loss is observed when DDBCH is incubated with microsomes from uninduced or 3-methylcholanthrene-induced rats (Figure 2).

Cytochrome P-450 loss is inhibited by carbon monoxide (a general cytochrome P-450 inhibitor) and benzphetamine (an alternative substrate for the phenobarbital-inducible isozymes)[18,19] but not by glutathione (a trap for reactive electrophiles) (Figure 1). The observation of a type I difference spectrum when DDBCH is added in the absence of NADPH to hepatic microsomes from phenobarbital-induced rats (Figure 3) demonstrates that the heme-iron ligation state is perturbed by DDBCH, presumably because DDBCH is bound in the active-site region of the enzyme.[20] A spectroscopic binding constant $k_s = 5\ \mu M$ is obtained by plotting the reciprocal of the DDBCH concentration against the reciprocal of the 422–385-nm peak-to-trough absorbance difference (Figure

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3, inset). The cofactor, inhibitor, and substrate binding studies imply that catalytic turnover of DDBCH precedes or accompanies the inactivation of vulnerable cytochrome P-450 isozymes. Catalytic activation of DDBCH to a reactive species that diffuses into the medium is unlikely, however, in view of the fact that glutathione does not attenuate the destructive process. Further evidence against the intervention of a diffusible reactive species is provided by the fact that the cytochrome b_5 concentration is not affected when microsomes are incubated with DDBCH (data not shown).

The destructive activity of DDBCH is lost when its carbon–carbon π bond is saturated (Figure 1). The double bond is thus clearly essential for the destructive process. The inactivity of diethyl 1,2,3,6-tetrahydropyridazine-1,2-dicarboxylate (**1**) and diethyl 1,2-dihydropyridazine-1,2-dicarboxylate (**2**), the monocyclic synthetic precursors of DDBCH, furthermore indicates that the bicyclic structure of DDBCH is also essential for destructive activity. The possibility that the cyclobutene structure alone is responsible for the destructive activity is essentialy excluded by the structure of the heme adduct (see below). Further evidence against localization of the destructive activity in the cyclobutene double bond is provided by the fact that the cyclobutene-containing structure **5**, despite detectable in vitro activity as a destructive agent (Table I), does not cause the accumulation of a characteristic heme adduct when administered in vivo to phenobarbital-pretreated rats (data not shown).

The possibility that hydrolysis of the carbamate groups of DDBCH by microsomal esterases precedes inactivation of cytochrome P-450, a possibility suggested by the requirement for removal of these groups prior to the *chemical* generation of cyclobutadiene from this system,[12-14] is ruled out by four complementary experiments. First, the experimental lag expected in the onset of cytochrome P-450 destruction if carbamate group hydrolysis is a prerequisite is not observed (Figure 1). Second, addition of an esterase (Sigma Type I carboxylic acid hydrolase) to the incubation mixture does not detectably alter the rate of cytochrome P-450 inactivation (not shown). Third, bis(*p*-nitrophenyl)phosphate (BNPP), an inhibitor of microsomal esterases,[21] does not detectably decrease the rate of cytochrome P-450 inactivation (Table I). Finally, and most definitively, DDBCH readily inactivates cytochrome P-450 in a purified, reconstituted preparation that is free of esterases (Figure 2).[22]

Isolation and Structure of the Prosthetic Heme Adduct. We have previously described the isolation of N-alkylated heme adducts from the livers of rats treated with cytochrome P-450 suicide substrates.[23-26] The livers, after in situ perfusion to remove hemoglobin, are homogenized, and the homogenate is allowed to stand in 5% H_2SO_4/methanol (v/v). The acidic methanol treatment esterifies the carboxylic acid groups of heme and removes the iron atom. The esterified porphyrins are then extracted with methylene chloride and are purified by thin-layer and high-pressure liquid chromatography A characteristic green (red-fluorescing) porphyrin fraction is obtained by this procedure from the livers from phenobarbital-induced rats 4 h after administration of DDBCH, although the green porphyrin fraction is unusually unstable. Even though as much as 1.5 mg of pigment (estimated from spectroscopic analysis of the crude extract) is obtained from 30 rats, only about 300 μg is obtained after the purification sequence. These quantitative estimates are based on the assumption that the molar absorbance of the Soret band of the DDBCH adduct is the same as that of *N*-methylprotoporphyrin IX (ϵ = 124 000).[25] The dismal stability of the adduct and the need to purify it from large amounts of liver have prevented its isolation in a form that is completely free of impurities. We have nevertheless succeeded in determining its structure by the combined use of electronic absorption, mass spectrometric, and high-resolution NMR techniques.

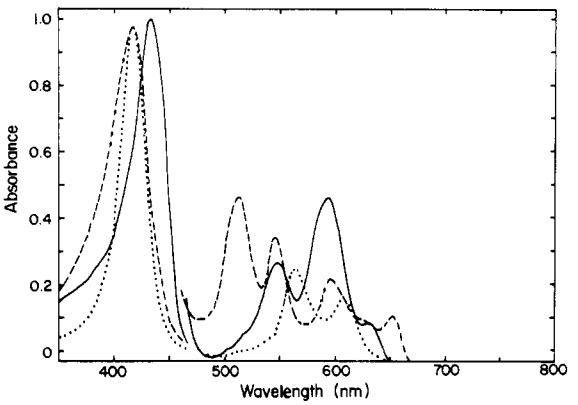

Figure 4. Electronic absorption spectra of the N-alkylated porphyrin isolated from DDBCH-treated rats: (---) free base, (—) chlorozinc complex, and (...) diprotonated. The spectra were taken in CH_2Cl_2.

The electronic absorption spectra of the free-base, the chlorozinc complex, and the mono- and diprotonated froms of the abnormal porphyrin isolated from rats treated with DDBCH (Figure 4) are very similar to the corresponding spectra of previously isolated *N*-alkylprotoporphyrin IX derivatives.[23,24] The free base has an etio porphyrin spectrum with a Soret band at 414 nm and bands of progressively decreasing intensity at 514, 546, 594, and 652 nm. The present porphyrin, however, is much more basic than other *N*-alkylprotoporphyrin IX derivatives. It is difficult to generate the free base, and, once generated it is difficult to preserve in the unprotonated state. The DDBCH adduct more closely resembles an *N*-aryl than an *N*-alkyl derivative in this respect. The spectrum of the chlorozinc complex has a Soret band at 432 nm and weaker bands at 548, 594, and 630 nm. The 630-nm band of the zinc complex, in particular, is a signature of an *N*-alkylprotoporphyrin IX structure.[23,24] It is to be noted (Figure 4) that the Soret band of the zinc complex does not have the shoulder at longer wavelengths that we have shown is characteristic of structures with the *N*-alkyl group on the nitrogen of one of the vinyl-substituted pyrrole rings.[25] The electronic absorption spectra thus point to an N-alkylated protoporphyrin IX structure with the *N*-alkyl group on pyrrole ring C or D. Formulation of the adduct as an *N*-alkylprotoporphyrin IX derivative is supported by the fact that reaction of the free base of the adduct with cupric acetate in methanol rapidly generates a porphyrin with the absorption spectrum (404, 538, 570, and 650 nm) of copper protoporphyrin IX. Rapid dealkylation of *N*-alkylporphyrins under these conditions has been demonstrated.[27-29]

The field desorption mass spectrum of the porphyrin exhibits few peaks other than a molecular ion at m/e 642 and a monoprotonated molecular ion at m/e 643 (not shown). A structure assembled from the dimethyl ester of protoporphyrin IX (M_w = 590) and cyclobutadiene (M_w = 52) is predicted to have a molecular weight of 642. The mass spectrometric data thus indicate that the *N*-alkyl moiety in the protoporphyrin IX adduct has the molecular weight of a cyclobutenyl residue.

The structure of the *N*-alkyl fragment has been elucidated from the NMR data with the help of spin-decoupling experiments. The decoupling experiments have been particularly helpful because lipid impurities obscure some of the regions of interest in the NMR spectrum (Figure 5). The proton designations used in the following analysis of the NMR spectrum are given in the structure

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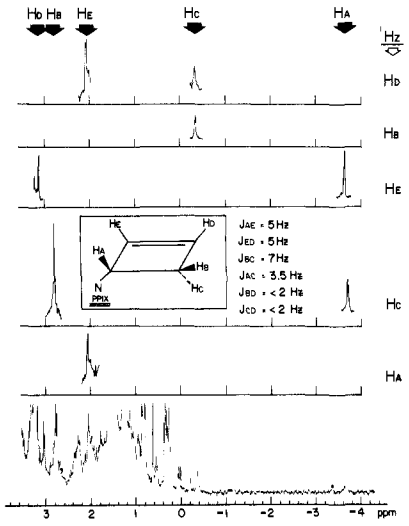

Figure 5. *N*-Alkyl proton region of the 240-MHz NMR spectrum of chlorozinc-complexed, dimethyl-esterified, *N*-(2-cyclobutenyl)protoporphyrin IX. The locations of the *N*-alkyl group protons (inset) in the NMR spectrum (bottom tracing) are indicated at the top of the figure. The change in the spectrum caused by irradiating the proton(s) identified in the right-hand margin is given in the associated tracing. A number of the peaks in the parent spectrum are due to lipid impurities.

in the inset of Figure 5. The doublet at −3.65 nm is assigned to H_A because its unique position requires the proton in question to sit in the center of the porphyrin ring current. A chemical shift of −3.65 is consistent with those for analogous protons in other *N*-alkylporphyrins (around −4.5 to 5.5 ppm) if allowance is made for the downfield shift associated with the fact that H_A is on a trisubstituted allylic rather than simply on a disubstituted carbon. Irradiation of the proton at approximately 2.05 ppm, which is attributed to H_E, causes the broad adduct doublet due to H_A (J_{AE} = 5 Hz) to narrow into a sharp doublet (Figure 5). The vinylic protons H_D and H_E are found at 3.07 and 2.05 ppm, respectively, rather than further downfield because they also experience the porphyrin ring current. Irradiation of H_E, thus, also reduces the doublet at 3.07 ppm to a singlet (J_{DE} = 5 Hz) (Figure 5). Coupling of H_D with H_B and H_C is characterized by a coupling constant smaller than 2 Hz and is only detected as a sharpening of the broad singlet due to H_C when H_D is irradiated. The dihedral angle between the protons in question is presumably such that little spin coupling occurs. H_B (multiplet at 2.8 ppm) and H_C (broad singlet at −0.37 ppm), however, are clearly coupled to each other (J_{BC} = 7 Hz) because irradiation of H_B sharpens the broad singlet assigned to H_C and irradiation of H_C results in collapse of H_B to a singlet (Figure 5). The difference in the chemical shifts of H_B and H_C reflects their different positions (cis or trans to the nitrogen) with respect to the porphyrin ring current. Finally, H_A is shown to be weakly coupled to H_C (J_{AC} = 3.5 Hz) because irradiation of H_C sharpens the broad doublet at high field attributed to H_A. The coupling between H_A and H_B, however, is apparently too small to be detectable. The decoupling experiments, thus, identify the five protons of the *N*-alkyl moiety predicted to be present when the molecular mass of dimethyl-esterified protoporphyrin IX is substracted from the mass spectrometrically obtained molecular ion. The chemical shifts of the five protons and their coupling patterns, which are consistent with, but as expected not identical with, those in cyclobutene,[30] can only be rationalized by the cyclobutadienoid structure in the inset of Figure 5.

The 240-MHz NMR spectrum firmly identifies the porphyrin skeleton of the adduct as a protoporphyrin IX moiety with methyl-esterified carboxylic acid groups. The protoporphyrin IX protons of the DDBCH adduct are readily located by comparing its NMR spectrum with the spectra of other *N*-alkylprotoporphyrin IX derivatives.[25,26,29,31,32] The methylene protons of the propionic acid side chains adjacent to the porphyrin ring give rise to a three-proton multiplet at 4.3 ppm and a one-proton multiplet at 4.05 ppm. The side-chain methylene protons vicinal to the carboxyl groups are found as a multiplet at 3.2 ppm (Figure 6) and as two doublets at 3.0 and 2.8 ppm. The chemical shifts of these two types of side-chain methylene protons and their appearance as multiple clearly resolved signals are characteristic of protoporphyrin IX derivatives with the *N*-alkyl group on pyrrole ring C or D but not A or B. In agreement with this is the observation that the internal protons of the vinyl substituents on pyrrole rings A and B give rise to one signal at 8.09–8.25 ppm rather than to two well-resolved signals (Figure 7).[31] The four terminal vinyl

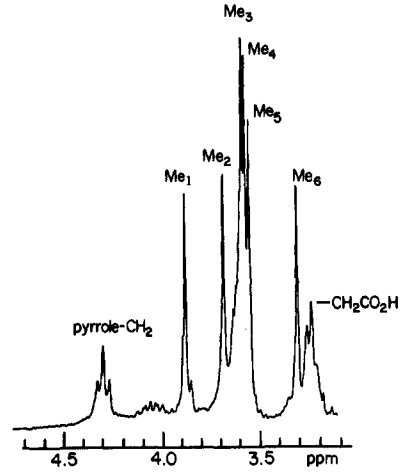

Figure 6. Region of the 240-MHz spectrum of the chlorozinc-complexed N-alkylated porphyrin in which the nonvinylic or aromatic protons of the protoporphyrin IX skeleton are found. The methyl group signals are numbered in order of decreasing chemical shift. The signals assigned to propionic acid side-chain methylene groups are indicated.

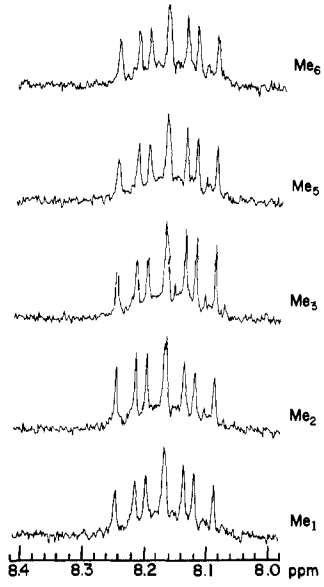

Figure 7. Sharpening of the NMR signals of the internal protons of the two protoporphyrin IX vinyl groups upon sequential irradiation of the methyl and methylene groups of the porphyrin skeleton. The signal pattern observed for the internal vinyl protons when the methyl group identified on the right side was irradiated is given in the associated tracing. The methyl groups are numbered as in Figure 6. Sharpening is only observed when methyls Me_2 (left-side sharpens) and Me_3 (right-side sharpens) are irradiated.

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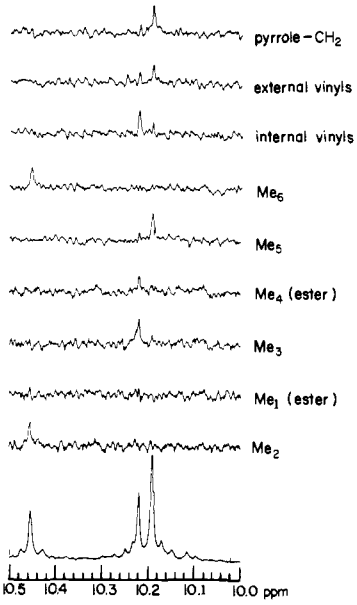

Figure 8. *meso*-Porphyrin proton region of the 360-MHz NMR spectrum of the chlorozinc-complexed N-alkylated porphyrin. The meso proton region of the spectrum is given in the bottom tracing. The nuclear Overhauser signal enhancements observed on irradiation of the methyl or methylene protons identified in the right margin are given in the associated tracing. The methyls are numbered as in Figure 6.

protons are found at 6.1–6.4 ppm, as in other *N*-alkylprotoporphyrin IX analogues (not shown). The methyl groups adjacent to the two vinyl groups have been identified by long-distance decoupling experiments (Figure 7). Irradiation of the methyl groups labeled in Figure 6 as Me_2 causes the left half of the internal vinyl proton multiplet to sharpen, whereas irradiation of Me_3 causes the right half to sharpen. No sharpening of the internal vinyl multiplet is observed when the other methyl groups are irradiated. Methyls Me_2 and Me_3, thus, are at positions 1 and 3 of the protoporphyrin IX skeleton, although additional information is required to differentiate between the two possible assignments.

The methyl group signals can be assigned by nuclear Overhauser experiments that establish their relationship to the meso protons. Three strong signals are observed for the four meso protons (Figure 8), so the signals for two of the meso protons must be superimposed. The weak signals in the meso proton region presumably are due to the meso protons of a minor regioisomer. The γ-meso proton signal is most readily identified because it exhibits a nuclear Overhauser enhancement on irradiation of the propionic acid side-chain methylene protons vicinal to the porphyrin ring (Figure 8). The δ-meso proton signal is identified by the fact that it exhibits a nuclear Overhauser enhancement on irradiation of two different methyl groups (Me_2 and Me_6). Methyl Me_2, therefore, is at position 1 of the porphyrin because it is one of the methyl groups already shown to to be weakly coupled to an internal vinyl proton (Figure 7). Methyl Me_6, therefore, must be at position 8 of the porphyrin ring. Methyls Me_1 and Me_4 do not give rise to nuclear Overhauser enhancements of meso proton signals and, thus, can be assigned to the two methyl ester groups. This only leaves methyl groups Me_3 and Me_5. Methyl Me_3 must be that at position 3 of the porphyrin ring because, as we have already shown, it is also weakly coupled to one of the internal vinyl protons (Figure 7). Methyl Me_5, therefore, by a process of elimination, is at position 5 of the porphyrin ring.

Complete assignment of all the porphyrin signals confirms protoporphyrin IX as the framework of the abnormal porphyrin and defines the pyrrole ring that is alkylated. We have previously demonstrated that the methyl substituent of the N-alkylated pyrrole ring is displaced upfield relative to its position when the ring is not alkylated.[31] In general, the methyl group on the alkylated ring appears at the highest field. In the present instance, the highest field methyl is Me_6, the methyl at position 8 of the porphyrin ring. The *N*-alkyl group consequently is on the nitrogen of pyrrole ring D. The complete structure of the predominant isolated porphyrin is given in Figure 9.

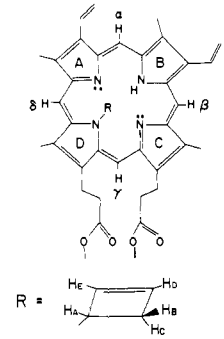

Figure 9. Ring D regioisomr of dimethyl-esterified *N*-(2-cyclobutenyl)-protoporphyrin IX isolated from rats treated with DDBCH. The pyrrole rings and the meso positions are labeled.

Discussion

The cytochrome P-450 isozymes induced by phenobarbital (but not those induced by 3-methylcholanthrene) oxidize DDBCH to a species that inactivates them. Enzyme inactivation, coupled to the accumulation of a porphyrin identified as *N*-(2-cyclobutenyl)protoporphyrin IX, presumably reflects alkylation of the prosthetic heme moiety by a catalytically activated form of the substrate. The porphyrin is alkylated with surprising regiospecificity on the nitrogen of pyrrole ring D, the position also specifically alkylated when cytochrome P-450 is inactivated by terminal olefins.[26,33] Heme alkylation by DDBCH is clearly distinguished from that mediated by terminal olefins, however, by the fact that the catalytically activated oxygen atom is incorporated into the alkylating moiety derived from the olefins but not into that generated from DDBCH. The carbon–carbon double bond of DDBCH is nevertheless essential for the activation process because destructive activity is lost when the double bond is saturated. The isolated cyclobutene moiety itself is insufficient, however (note the absence of a pigment with **5**). These results, in conjunction with the inactivity of the monocyclic analogues **1** and **2** and the demonstration that hydrolysis of the carbamate groups is not required, establish that the inhibitory potential resides in the integral structure of DDBCH rather than in an isolated structural feature (i.e., the π bond, the diazo function, the carbamate groups, or the bicyclic ring system).

The N-alkylated porphyrin obtained with DDBCH is unique because the *N*-alkyl moiety is attached to the porphyrin nitrogen through a secondary (allylic) rather than primary carbon. All the other heme adducts so far characterized, with the exception of *N*-aryl derivatives,[34] reflect reaction of the porphyrin with a terminal unsubstituted carbon in the substrate. The observation that internal acetylenes inactivate cytochrome P-450 but do not yield detectable N-alkylated porphyrins has led us to suggest that the reaction of secondary carbon centers with the heme nitrogens is sterically disfavored.[23,24] The unusual instability of the DDBCH adduct agrees with this postulate but its formation clearly demonstrates that whatever steric constraints are in force can be circumvented if the reactive center is sufficiently activated or compact.

The structure of the heme adduct and the identification of destructive activity with the intact DDBCH structure suggests that cyclobutadiene, or a closely related species, is the alkylating agent. In this context it is important to note that cyclobutadiene has been liberated chemically not from DDBCH but from the diazo compound that results from carbamate group hydrolysis, decarboxylation, and oxidation of the hydrazine moiety.[13,14] The two-electron oxidation of a nitrogen in DDBCH presumably would

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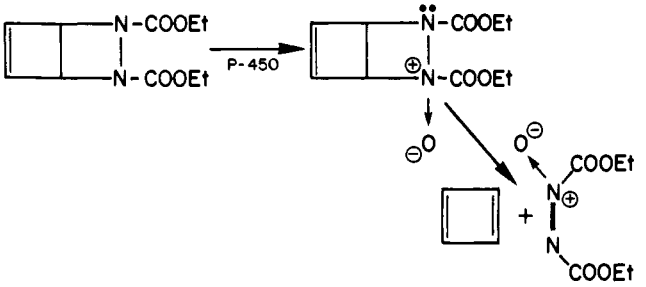
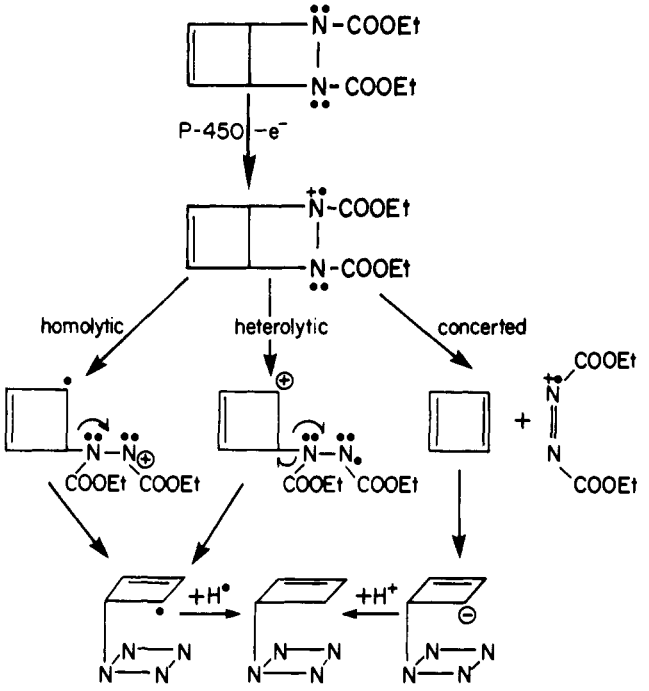
Scheme II. Possible Activation of DDBCH by Direct (Concerted) Transfer of an Oxygen Atom from Cytochrome P-450 to the Carbamate Nitrogen



yield the *N*-oxide of a carbamate (Scheme II). However, although the biological oxidation of trisubstituted amines and amides to the corresponding *N*-oxides and *N*-hydroxylamides is well documented,[35,36] the biological oxidation of N,N-disubstituted amides to *N*-oxide is unknown. The *N*-oxide of DDBCH nevertheless could, in principle, fragment to give cyclobutadiene plus the *N*-oxide of diethyl azodicarboxylate (Scheme II). The cyclobutadiene could alkylate the prosthetic heme nitrogen and pick up a proton to give the final adduct or could, alternatively, bridge the iron and a pyrrole nitrogen of the heme before the protonation step occurs. The hypothesis that the oxidation of DDBCH to its *N*-oxide initiates concerted (electrocyclic) unmasking of cyclobutadiene, however, is not supported by the fact that the diazo group is generally eliminated more readily than the corresponding azoxy moiety in concerted mechanisms.[37,38] The asymmetry introduced by the oxygen decreases rather than increases the pericyclic elimination reaction. If a similar relationship exists in the case of DDBCH and its *N*-oxide, the *N*-oxide would be expected to fragment more slowly by a concerted pathway. One alternative is for the carbamate carbonyl group to migrate to the *N*-oxide terminus in a reaction analogous to the biological rearrangement of aryl *N*-acyl-*N*-hydroxylamines to *N*-acyloxyamines.[36] It is not obvious how such a rearrangement would lead to the required release of a cyclobutadienoid species, however.

We have recently reported evidence that nitrogen is oxidized by cytochrome P-450, at least in some instances, by two one-electron steps rather than by a concerted two-electron transfer of the oxygen atom.[39] The oxidation of DDBCH to a radical cation by transfer of one electron to cytochrome P-450 is thus the mechanism favored for the activation step (Scheme III). The radical cation can undergo homolytic cleavage of the nitrogen–carbon bond to give a cyclobutenyl radical intermediate, heterolytic cleavage to yield a cyclobutenyl cation, or concerted elimination of the diethyl azodicarboxylate radical cation to give cyclobutadiene. Reaction of the prosthetic heme with one of these species would then provide the isolated heme adduct. If the heme is alkylated by the radical or cationic cyclobutenyl intermediates, elimination of the diethyl azodicarboxylate radical cation from the former and of the equivalent neutral species from the latter would unmask a radical that could abstract a hydrogen from the protein and yield the observed *N*-(2-cyclobutenyl) structure. The cyclobutenyl anion is expected, on the other hand, if cyclobutadiene is itself the alkylating agent (Scheme III). The *N*,*N*-bis(ethoxycarbonyl) derivatives of bicyclic hydrazines are oxidized electrochemically at potentials in the range of 1.4–1.5 V.[40] The resulting nitrogen radical cations are relatively stable, the oxidation process is reversible in the absence of allylic unsaturation, but the radical cations are unstable and the oxidation irreversible when

Scheme III. Reaction Alternatives Subsequent to One-Electron Oxidation of DDBCH by Cytochrome P-450



such unsaturation is present. A similar destabilization of nitrogen radical cations by allylic double bonds has been reported for the species generated electrochemically from *N*-acyl-*N*-alkoxyamines.[41] Enzymatic oxidation of DDBCH to a radical cation thus provides a reasonable route to the required cyclobutadienoid species and is consistent with the emerging view that cytochrome P-450 oxidizes nitrogen atoms by such a mechanism. Loss of destructive activity when the double bond in DDBCH is saturated is consistent with this mechanism.

Catalytic release of cyclobutadiene from DDBCH and benzyne from 1-aminobenzotriazole, or of a species equivalent in net reactivity to these structures, demonstrates the potential of stereoelectronically activated intemediates in the design of mechanism-based enzyme inhibitors. The reactivity inherent in such species has been extensively investigated in chemical systems but is only now being explored in the design of biologically active agents.

Experimental Section

Materials and General Methods. Gas–liquid chromatographic analyses were performed on a Varian 2100 flame ionization instrument fitted with a 6-ft. glass column packed with 3% OV-225 on 100/120 mesh Supelcoport. Routine mass spectra were obtained on a Kratos MS 25 instrument under electron impact conditions (EIMS) at 70 eV. Routine NMR spectra, recorded on a Varian FT 80 instrument in C^2HCl_3, are given in parts per million relative to trimethylsilane. NADPH, NADP, glucose 6-phosphate dehydrogenase, glucose 6-phosphate, and BNPP [bis(*p*-nitrophenyl) phosphate] were purchased from Sigma Chemical Co.

Diethyl 1,2,3,6-Tetrahydropyridazine-1,2-dicarboxylate. The procedure reported in the literature for synthesis of the dimethyl ester was used to prepare the diethyl analogue.[14-17] Butadiene, generated by heating butadiene sulfone in xylenes at 110 °C, was bubbled directly for 2 h into a separate flask containing a solution of ethyl azodicarboxylate (5 g, 28 mmol) in 60 mL of dry benzene at room temperature. The reaction was then allowed to stand in the dark for 2 days under a slight positive pressure of butadiene. The initial orange solution became faint yellow during this period. Removal of the benzene at a rotary evaporator and vacuum distillation gave 6.5 g (98%) of the title compound: bp 131–135 °C (0.1 torr); ^1H NMR 5.71 (d, J = 3 Hz, 2 H, —HC=CH—), 3.62, 3.81, 4.24, 4.43 (each a br 1 H s, C=CCH), 4.10 (q, J = 9 Hz, 2 H, O-CH_2-), and 1.17 ppm (t, J = 9 Hz, CH_3); EIMS, m/e 228 (M^+). The NMR data for the ring protons is essentially identical with that reported for the dimethyl derivative.[15]

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Diethyl 1,2-Dihydropyridazine-1,2-dicarboxylate. Diethyl 1,2,3,6-tetrahydropyridazine-1,2-dicarboxylate was brominated and subsequently dehalogenated by the procedure of Altman et al.[17] *N*-Bromosuccinimide (5.6 g, 32 mmol) was added to a solution of diethyl 1,2,3,6-tetrahydropyridazine-1,2-dicarboxylate (6.5 g, 28 mmol) in 800 mL of CCl_4, and the mixture was refluxed for 1.5 h, at which point all the solids float on the surface. Filtration and solvent removal gave approximately 5 g of brown oil that was taken up in dry toluene. The solution was then brought to rapid reflux before 3.8 mL (48 mmol) of 2,6-lutidine was added all at once. After refluxing a further 5 min, the solution was cooled, washed twice 3.7% HCl and once with saturated NaCl solutions, and dried ($MgSO_4$). Colored impurites were removed by column chromatography on silica gel 60 (1:3 ethyl acetate/hexane followed by ethyl acetate). Crystallization of the product from ether–hexanes provided 3.3 g (50%) of white crystals: mp 57–58 °C; ^{1}H NMR 6.7, 5.7 (AA′XX′ multiplet, 4 H, —HC=HC—CH=CH—), 4.13 (q, J = 9 Hz, 2 H, OCH_2), and 1.17 ppm (t, J = 9 Hz, 3 H, CH_3); EIMS, m/e 226 (M^+). The NMR of the ring protons is the same as that reported for the dimethyl analogue.[17] Anal. Calcd for $C_{10}H_{14}N_2O_4$: C, 53.1; H, 6.2; N, 12.4. Found: C, 53.01; H, 6.08; N, 12.23.

2,3-Bis(carbethoxy)-2,3-diazabicyclo[2.2.0]hex-5-ene. The photolysis was based on that of the bis(carbomethoxy) analogue by Altman et al.[16] A solution of diethyl 1,2-dihydropyridazine-1,2-dicarboxylate (3.3 g) in 600 mL of anhydrous diethyl ether was placed in a photolysis apparatus with an internal water-cooled Pyrex wall. The apparatus was immersed in a dry ice–acetone bath. The stirred solution was maintained under an argon atmosphere while it was irradiated for 4 h with a Hanovia 450-W high-pressure mercury lamp placed in the center well. The disappearance of starting material was monitored by gas chromatography (190 °C, retention time 5.13 min). The yellow-brown oil obtained on removal of the solvent at a rotary evaporator was separated by chromatography on a silica gel 60 column (CH_2Cl_2) into the desired *N*,*N*-bis(carbethoxy)-2,3-diazabicyclo[2.2.0]hex-5-ene and *N*-carbethoxy-2-(carbethoxyamino)pyrrole. These products have gas chromatographic retention times (190 °C) of 4.10 and 5.30 min, respectively, and are formed in a 6:4 ratio. The analogous two products were obtained in the work with the dimethyl ester.[16] Approximately 2 g of the bicyclic product was obtained after a final purification by low-pressure chromatography on a Lichroprep Si 60 40–64-μm column eluted with 1:1 ethyl acetate/hexanes: ^{1}H NMR 6.7, 5.1 (AA′XX′ multiplet, 4 H, —HC—CH=CH—CH—), 4.09 (q, J = 9 Hz, 2 H, OCH_2), and 1.15 ppm (t, J = 9 Hz, 3 H, CH_3); EIMS, m/e 226 (M^+).

2,3-Bis(carbethoxy)-2,3-diazabicyclo[2.2.0]hexane. DDBCH (120 mg) in 50 mL of methanol was flushed briefly with argon before 10% Pd on carbon (200 mg) was added, and the mixture was shaken for 2 h on a Parr apparatus under hydrogen gas (40 lb/in^2 pressure). The solution was then filtered through diatomaceous earth to remove the catalyst. Solvent removal on a rotary evaporator gave a green oil that was purified by chromatography on a 1 × 20 cm column of silica gel 60 packed in 1:3 ethyl acetate/hexane. Elution with five column volumes of the same solvent yielded the reduced product in essentially quantitative yield: ^{1}H NMR 4.7 (br s, 2 H, H α to nitrogens), 4.15 (q, J = 9 Hz, OCH_2), 2.49 (br s, 4 H, —CH_2CH_2—), and 1.25 ppm (t, J = 9 Hz, CH_3); EIMS, m/e 228 (M^+). Decoupling experiments show that irradiation of the protons at 2.49 results in sharpening of the signal at 4.7 ppm. The data agree with that reported for the dimethyl ester obtained by a different procedure.[16]

Destruction of Cytochrome P-450 in Incubations with Rat Hepatic Microsomes. The livers of Sprague-Dawley 250–280-g male rats injected intraperitoneally once a day for 5 days with an 80 mg/kg dose of sodium phenobarbital in water were removed on the fifth day, and microsomes were prepared as described previously.[42,43] In certain experiments the rats were pretreated with 3-methylcholanthrene (20 mg/kg in corn oil) rather than phenobarbital as reported previously.[42] The loss of cytochrome P-450 was generally assayed in 10-mL incubations containing the desired substrate, microsomal protein (1 mg/mL), KCl (150 mM), DETAPAC (diethylenetriaminepentaacetic acid, 1.5 mM), and either NADPH (1 mM) or an NADPH regenerating system consisting of NADP (0.3 mM), glucose 6-phosphate (3.0 mM), glucose 6-phosphate dehydrogenase (0.5 units/mL), and $MgCl_2 \cdot H_2O$ (1 mM) all in 0.1 M NaK phosphate buffer (pH 7.4). Because of their insolubility, substrates were coated on the surface of the incubation flask by evaporation of an ether solution under argon while swirling. The incubations, prewarmed to 37 °C, were initiated by adding the NADPH or the NADPH-regenerating system. Control experiments were routinely carried out in the absence of NADPH or its regenerating system. Additions or deletions from the standard system are indicated in the text. Aliquots were taken at indicated times and were placed in test tubes immersed in ice until they could be assayed. Triplicate samples were taken for each time point.

Assays. Cytochrome P-450 content was assayed as the CO vs. reduced-CO difference spectrum by the procedure of Estabrook et al.[44] Cytochrome b_5 was quantitated by the method of Omura and Sato.[45] Difference binding spectra were determined by adding substrates to one of two spectrally balanced cuvettes containing unreduced microsomes.[44] An Aminco DW2a spectrophotometer was used for these biological assays. Protein concentrations were measured with Bio-Rad assay kits.

Destruction of Purified, Reconstituted Cytochrome P-450. This experiment was kindly performed by Drs. Regina Wang and Anthony Y. H. Lu (Merck Research). The incubation mixture contained 0.5 nmol/mL of purified rat liver phenobarbital-inducible cytochrome P-450,[18] 2000 units/mL of purified cytochrome P-450 reductase,[46] 20 μg/mL of dilauroylglyceryl-3-phosphorylcholine, and 1 mM NADPH, all in 0.1 M phosphate buffer (pH 7.4). The substrate was added as a solution in acetone (0.05 mL/mL final incubation). The assay protocol has been described.[42]

Isolation of the Prosthetic Heme Adduct. Sprague-Dawley male rats, pretreated with phenobarbital for 5 days as described above, were injected on the sixth day with DDBCH (22 mg in 0.1 mL of dimethyl sulfoxide per rat). Four hours later, the rats were decapitated and their livers, after perfusion with 1.15% KCl solution, were removed. The livers were homogenized in 5% H_2SO_4 (v/v) in methanol with a Waring blender. The homogenate was allowed to stand for 20 h at 4 °C. The mixture was then filtered, diluted with an equal volume of water, and extracted with methylene chloride. The combined extracts were washed with $NaHCO_3$ and were stirred with a saturated methanolic solution of 100 mg of zinc acetate before removal of the solvents at a rotary evaporator. The residue was taken up in CH_2Cl_2, and the solution was washed with water and saturated NaCl solution. Drying over anhydrous sodium sulfate and solvent removal gave a dark brown residue that was transferred to 2000-μm silica gel plates with approximately 5 mL of acetone. The plates were developed in 4:1 v/v chloroform/acetone. A green, red fluorescent, band with an R_f of approximately 0.6 was removed from the plate and was extracted with acetone. The dark green material thus obtained was further purified by high-pressure liquid chromatography on either a Whatman PXS 5/25 silica gel column eluted with a linear gradient of THF into a 1:2 THF hexane mixture or, in later work, on a 10-μm Porasil silica gel column first deactivated with 10% water in methanol and subseqently eluted with a linear gradient of acetone into CH_2Cl_2. The columns were monitored with a variable wavelength detector set at 590 nm. The metal ion can be removed from the zinc complex isolated above by stirring in 5% H_2SO_4/methanol for 15 min, extracting with CH_2Cl_2, washing several times with saturated $NaHCO_3$, drying over anhydrous Na_2SO_4, and removing the solvent. The spectrum of the free base, however, was only observed when triethylamine was added to the solution after acid treatment to remove the zinc and the spectrum was recorded immediately. The free base decomposes to an unidentified substance.

Spectroscopic Characterization of the Heme Adduct. Electronic absorption spectra were recorded in CH_2Cl_2 on a Hewlett-Packard Model 8450A spectrophotometer. Mass spectra were obtained on a Kratos AEI MS-9 instrument operating in the field desorption mode. NMR spectra were recorded on a Nicolet NT 360 instrument at the University of California at Davis or on a custom-built 240-MHz instrument in San Francisco. The pulse sequences and conditions have been reported.[31,32]

Acknowledgment. We are very much indebted to Drs. Reina Wang and Anthony Y, H. Lu (Merck Research) for data on the inactivation of purified, reconstituted cytochrome P-450 and to Dr. Kent L. Kunze of this laboratory for assistance with the preliminary NMR studies of the heme adduct. This investigation was supported by Grant GM 25515 from the National Institutes of Health. Support for core facilities employed in this work provided by Grants P30 AM-26743 (Liver Center) and RR-00719 (Berkeley Biomedical Mass Sepctrometry Resource).

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