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Cytochrome P450-Catalyzed Oxidation of *N*-Benzyl-*N*-cyclopropylamine Generates Both Cyclopropanone Hydrate and 3-Hydroxypropionaldehyde via Hydrogen Abstraction, Not Single Electron Transfer

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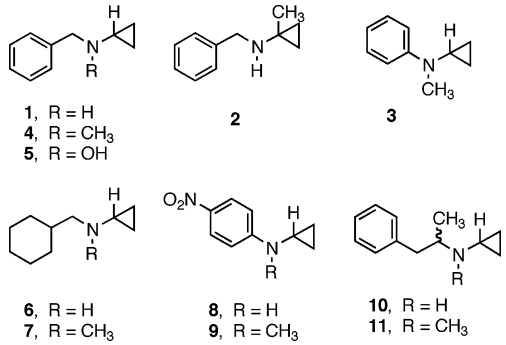
Abstract: The suicide substrate activity of *N*-benzyl-*N*-cyclopropylamine (**1**) and *N*-benzyl-*N*-(1′-methylcyclopropyl)amine (**2**) toward cytochrome P450 and other enzymes has been explained by a mechanism involving single electron transfer (SET) oxidation, followed by ring-opening of the aminium radical cation (protonated aminyl radical) and reaction with the P450 active site. Although the SET oxidation of *N*-cyclopropyl-*N*-methylaniline (**3**) by horseradish peroxidase leads exclusively to ring-opened (non-*cyclo*propyl) products, P450 oxidation of **3** leads to formation of cyclopropanone hydrate and no ring-opened products, and **3** does *not* inactivate P450. To help reconcile these discrepant behaviors we have determined the complete metabolic fate of **1** with P450 in vitro. 3-Hydroxypropionaldehyde (3HP), the presumptive "signature metabolite" for SET oxidation of a cyclopropylamine, was observed for the first time in 57% yield, along with cyclopropanone hydrate (34%), cyclopropylamine (9%), benzaldehyde (6%), benzyl alcohol (12%), and benzaldoxime (19%). Unexpectedly, *N*-benzyl-*N*-cyclopropyl-*N*-methylamine (**4**) was found *not* to inactivate P450 and *not* to give rise to 3HP as a metabolite without first undergoing oxidative N-demethylation to **1**. These and other observations argue against a role for SET mechanisms in the P450 oxidation of cyclopropylamines. We suggest that a conventional hydrogen abstraction/hydroxyl recombination mechanism (or its equivalent as a one-step "insertion" mechanism) at C−H bonds in **1**−**4** leads to nonrearranged carbinolamine intermediates and thereby to "ordinary" N-dealkylation products including cyclopropanone hydrate. Alternatively, hydrogen abstraction at the N−H bond of *secondary* cyclopropylamines **1** gives a neutral aminyl radical which could undergo rapid ring-opening leading either to enzyme inactivation or 3HP formation.

Introduction

The cyclopropylamine substructure is found in several biologically active natural products and is increasingly found within synthetic drug and drug candidate molecules. Since the initial reports that *N*-benzyl-*N*-cyclopropylamine (**1**, Chart 1) is a suicide substrate for cytochrome P450 enzymes,[1] as well as for monoamine oxidase,[2] cyclopropylamines have been widely applied as mechanistic probes of these and other oxidative enzymes.[3−12] The observation that compound **2**, the 1′-methyl derivative of **1**, inactivates both P450[13,14] and monoamine oxidase (MAO)[15] nearly as effectively as **1** ruled

Chart 1. Structures of Compounds **1**−**11**



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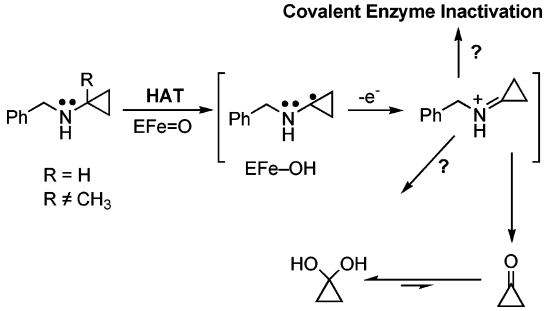
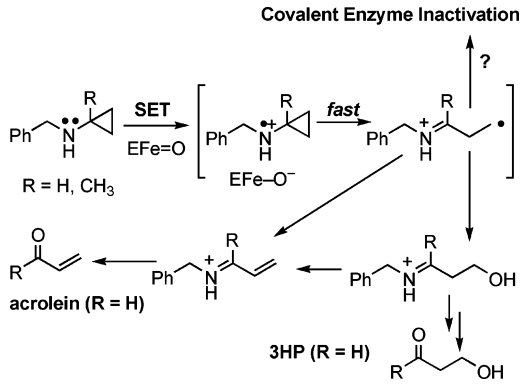
Scheme 1. Hydrogen Atom Transfer (HAT) Mechanism



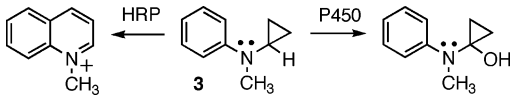
Scheme 2. Single Electron Transfer (SET) Mechanism



out the involvement of *N*-cyclopropylidene Schiff base intermediates in the inactivation process (Scheme 1) and focused attention on the single electron transfer (SET) mechanism shown in Scheme 2. Cyclopropyl aminium ions[16] are known to undergo rapid unimolecular ring-opening rearrangement to distonic iminium ion-radical species.[17,18] The latter have been suggested to account for the enzyme-inactivating ability of cyclopropylamines versus other types of amines in this regard (Scheme 2).

In addition to the suicide substrate activity of **1** and especially **2** toward cytochrome P450, several other observations have also been taken as support for an SET mechanism for the P450-catalyzed oxidation of amines.[19] For example, P450 oxidation of 4-alkyl-1,4-dihydropyridine derivatives leads to the extrusion of alkyl radicals that can be trapped by the P450 heme (leading to suicide inactivation) or by spin-traps in solution.[20] An SET mechanism was also invoked to explain the uniformly low kinetic deuterium isotope effects observed for P450-catalyzed N-dealkylation of amines and anilines.[21] In addition, the P450-inactivating potential of cyclopropylamines and other heteroatom-substituted cyclopropanes correlates to their one-electron oxidation potentials.[22] The P450-catalyzed N-demethylation of *p*-substituted *N*,*N*-dimethylanilines shows a Hammett ρ value of -0.6, indicating the development of partial positive charge in the transition state for carbinolamine formation.[23] Finally,

Scheme 3. SET versus P450 Oxidation of Probe **3**



Marcus analysis of similar data suggests that the active oxygen species of P450, presumed[24,25] to be the ferryl or iron−oxene complex FeO^{3+}, has a very much higher reduction potential than compound I of horseradish peroxidase, a heme enzyme well-known to perform single-electron oxidations of its substrates.

While these observations appear to make a persuasive argument in favor of a role for SET reactions in the P450-catalyzed oxidations of amines (and perhaps other substrates), more careful consideration[26] indicates that alternate explanations are possible, even probable, in some of these cases. Evidence against the role of SET reactions in the P450-catlalyzed N-dealkylations comes from studies of the regioselectivity of N-dealkylation of *N*-ethyl-*N*-fluoroethylanilines $Ph(Et)NCH_2$-CH_nF_{3-n}.[27] With HRP, loss of the fluoroethyl group increases with the degree of fluorine substitution and the acidity of the α hydrogens, indicating an important role for a C-deprotonation reaction following the initial SET oxidation at nitrogen. In contrast the opposite trend is observed with P450; loss of the fluoroethyl group decreases from 68% to only 8% across the series from $-CH_2CH_2F$ to CH_2CF_3.

Clearly, the role of SET mechanisms in P450-catalyzed N-dealkylations in general, and more specifically their corollary role in the suicide substrate activity of cyclopropylamines, is far from settled. Until recently, one very important type of evidence relevant to this latter question had been entirely missing, namely, the unambiguous identification of the fate of the three cyclopropyl carbons lost from the nitrogen center. Shaffer et al. first reported[28,29] that the oxidation of *N*-methyl-*N*-cyclopropylaniline (**3**) by horseradish peroxidase (HRP), a well-known SET oxidant, leads exclusively to fragmentation of the cyclopropane ring. The initial nitrogen-centered cation radical fragments to a distonic open-chain form which then recyclizes via radical addition to the phenyl ring; further oxidation generates the ultimate product, *N*-methylquinolinium, quantitatively (Scheme 3). In sharp contrast, the P450-catalyzed oxidation of **3** was shown to lead exclusively to ring-intact metabolites including cyclopropanone hydrate, *N*-cyclopropylaniline, and *p*-hydroxy-**3**, with no signs of either acrolein or 3-hydroxypropionaldehyde (3HP) and no signs of P450 inactivation by **3**, as would be predicted by Scheme 2.[30]

In view of the disparate behavior of cyclopropyl probe **3** with SET versus P450 enzymes, we undertook a detailed exploration of the interaction of **1** with P450 enzymes, both as a substrate and as an enzyme inactivator. For this work we used the original source of P450 enzymes in which the suicide substrate activity

(16) The neutral species $R_2N^{\bullet}$ (with three electrons on nitrogen) is an aminyl radical, while its protonated form (a nitrogen radical cation) is an aminium ion.
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of **1** was discovered, namely, liver microsomes from phenobarbital-treated rats (PB-microsomes), as well as several expressed, purified, and reconstituted P450 isoforms. Based on this work we recently reported that a significant portion of the inactivation of P450 enzymes during metabolism of **1** occurs via formation of a robust, spectroscopically distinct metabolic intermediate complex between α-nitrosotoluene (a metabolite of **1** but *not* of benzylamine) and the heme iron of P450.[31] In this Article we describe the complete metabolic fate of **1** in PB-microsomes. Significantly, we report for the first time that 3HP, long anticipated as the "signature" metabolite for SET oxidation of a cyclopropylamine by cytochrome P450 (Scheme 2), is indeed a major metabolite of **1**. Surprisingly, however, tertiary amine **4**, the *N*-methyl derivative of **1**, is *not* an inactivtor of P450 until it is N-demethylated to form **1**. These and other observations are discussed in terms of SET versus HAT mechanisms of stable metabolite formation as well as P450 inactivation by cyclopropylamines.

Results

Metabolite Identification. Since previous work in our laboratory[28−30] demonstrated the power and convenience of ^{13}C NMR for identification of metabolites of **3** in quenched incubation mixtures, we adopted a similar approach for the analysis of metabolites of **1**. Thus, comparison of the ^{13}C NMR spectrum of a complete microsomal incubation of [1′-^{13}C]-**1** (Figure 1S in the Supporting Information) to that of a blank incubation in which only substrate was omitted (Figure 2S in the Supporting Information) readily revealed a major peak from the C-1′ carbon of unmetabolized substrate (29.5 ppm) plus smaller peaks for the nonenriched carbons (i.e., the aromatic carbons at 128.6−130.6 ppm, the benzylic carbon at 51.5 ppm, and C-2′ and C-3′ of the cyclopropyl group which appear as a doublet (due to the ^{13}C-enrichment at C-1′; $J = 10$ Hz) at 3.00 ppm). Closer examination revealed five additional peaks at 22.8, 79.4, 88.1, 88.7, and 206.9 ppm that were not present in the blank and were not associated with the substrate. Two peaks at 22.7 and 79.4 ppm were identified as arising from cyclopropylamine and cyclopropanone hydrate, respectively, by their chemical shifts and by spiking the incubation sample with authentic standards and rerecording the spectrum (not shown).

Two peaks at 88.1 and 206.9 ppm, which were more intense than those at 22.7 or 79.4 ppm, were initially puzzling. However, the carbonyl carbon of 3HP and its corresponding gem-diol hydrate have been reported to have chemical shifts of 206.3 and 88.6 ppm, respectively.[32] To confirm the formation of 3HP as a metabolite of **1** the incubation mixture was treated with 2,4-dinitrophenylhydrazine (DNPH) reagent,[29] extracted, and the extract was analyzed by HPLC, ^{13}C NMR, and LC−MS comparison to an authentic standard. HPLC analysis of incubation extracts showed a peak at 11.5 min, the t_R of the standard, that increased with incubation time. LC−MS analysis of material collected from this HPLC peak from incubations of **1** and [1′-^{13}C]-**1** showed apparent molecular ions of *m*/*z* 255 and 256, respectively, and fragmentation patterns identical to that of the authentic standard (*m*/*z* 255). Finally, the ^{13}C NMR of the extract showed a single ^{13}C-enriched peak corresponding to the carbonyl (imine) carbon of the authentic DNP derivative (152.6 ppm in MeOH-d_4); this peak was absent in extracts from control incubations. Thus, it is clear that 3HP was formed as a microsomal metabolite of **1**; as described below, 3HP proved to be a major metabolite of **1**.

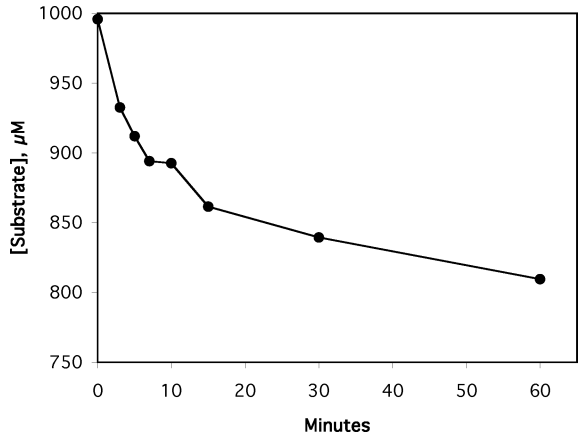

Figure 1. Kinetics of oxidation of **1** by PB-microsomes.

In parallel with the above experiments, a microsomal incubation of [7-^{13}C]-**1** was carried out and analyzed by ^{13}C NMR. After incubation, acidification to pH 1 and extraction with ether to remove acidic and neutral metabolites, the ^{13}C NMR of the aqueous incubation mixture (Figure 3S in the Supporting Information) showed peaks corresponding to the nonenriched carbon atoms of unmetabolized substrate at 3.1, 29.6, and 128.7−130.4 ppm, as well as a large peak for the benzylic carbon of the substrate (51.7 ppm). The only metabolite peak detected in the aqueous concentrate was benzylamine, identified by its chemical shift (43.2 ppm). Analysis of the ether extract by ^{13}C NMR revealed three significant peaks at 65.5, 149.1, and 192.2 ppm that were not present in blank incubations (data not shown). The peaks at 65.5 and 192.2 ppm were consistent with benzyl alcohol and benzaldehyde, respectively, but the identity of the peak at 149.1 ppm was not immediately apparent. Hence, the ether extract was submitted to analysis by GC/MS, which yielded three peaks having retention times of 5.8, 6.9, and 9.4 min associated with *m*/*z* values of 107, 109, and 122, respectively. The injection of authentic standards of unlabeled benzaldehyde and benzyl alcohol resulted in peaks with retention times of 5.8 and 6.9 min and *m*/*z* values of 106 and 108, respectively (reflecting the absence of ^{13}C-enrichment in the standards). The apparent molecular weight of the 9.4 min peak, together with its large ^{13}C chemical shift, led us to suspect that it might be benzaldoxime, and comparison with an authentic standard confirmed this identification. The appearance of benzaldoxime among the metabolites of **1** was unexpected and led us to investigate separately[31] the mechanism of its formation and the role of its nitroso tautomer in the inactivation of P450 by **1**.

Quantitation of Metabolites. Incubation of **1** with PB-microsomes resulted in a rapid initial rate of consumption that decreased gradually but did not stop, even after 30 min when the rate had slowed to ≤10% of the initial rate (Figure 1). A replot of log[**1**] versus time (not shown) is strongly convex downward, reflecting substantial enzyme inactivation concurrent with substrate depletion. Despite the enzyme inactivation taking place during turnover of **1**, approximately 20% of the substrate (initially 1 mM) is consumed over 60 min of incubation.

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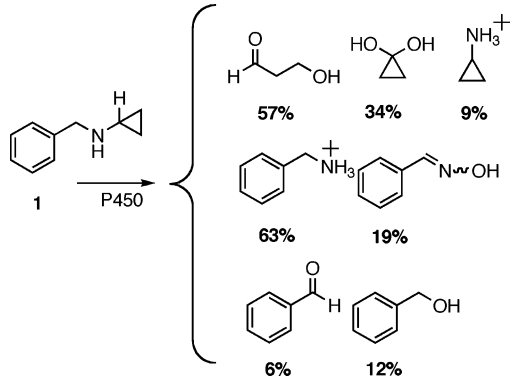
Scheme 4. Metabolites of **1** Formed by PB-Microsomes



To quantitate the cyclopropane-derived metabolites identified by ^{13}C NMR experiments the microsomal metabolism of [1′-^{14}C]-**1** (initial concentration 1.0 mM) was monitored by reversed-phase HPLC with radiochemical detection. All non-substrate-associated radioactivity was found to elute in the solvent front, indicating the highly hydrophilic nature of the metabolites, i.e., cyclopropylamine, cyclopropanone hydrate, and 3HP. Integration of the solvent front ^{14}C peak area showed these metabolites to be present at a total concentration of 199 μM after 60 min, which agrees with the 20% consumption of substrate mentioned above. Because HPLC failed to resolve the solvent front constituents, quantitation of the individual metabolites necessitated prior derivatization to enable separation (see Methods). Thus, cyclopropylamine, analyzed as its 4-nitrobenzamide derivative,[33] accounted for 9% of total metabolites, while 3HP, analyzed as its DNP derivative, accounted for 57% of total metabolites; cyclopropanone hydrate was estimated by subtraction to account for 34% of total metabolites. Similar studies using [7-^{14}C]-**1** showed that after 60 min of incubation, benzylamine was also a major (63%) metabolite while benzyl alcohol, benzaldehyde, and benzaldoxime accounted for 12%, 6%, and 19% of total metabolites, respectively). Thus the overall metabolism of **1** is as shown in Scheme 4.

Comparative Metabolism of 1 and Its *N*-Methyl Derivative (4). The observed formation of substantial amounts of the ring-opened metabolite 3HP was consistent with the possible involvement of an SET mechanism in the microsomal metabolism of **1** (viz., Scheme 2). To explore this possibility further, we submitted the tertiary cyclopropylamine **4** (1 mM) to metabolism by PB-microsomes. Because tertiary amines are much more susceptible than secondary or primary amines to oxidation by electron-abstracting agents,[34,35] we anticipated that **4** should be at least as susceptible to SET oxidation as **1** and that it would therefore also give a good yield of 3HP metabolite. At an initial concentration of 1 mM, compound **4** was an excellent substrate for metabolism by microsomal P450, being 50% oxidized after 60 min, even with microsomes from noninduced rats. Surprisingly, however, microsomal metabolism of **4** and [1′-^{13}C]-**4** generated only traces of 3HP as determined, respectively, by DNPH trapping and HPLC and by ^{13}C NMR. Moreover, in contrast to incubations with **1** which slow

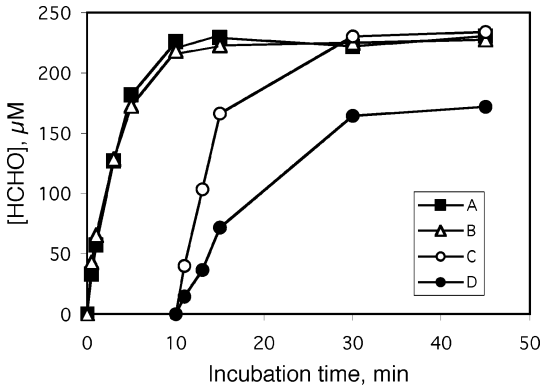


Figure 2. Formaldehyde production from microsomal incubation of **4** (500 μM) at 33 °C in the absence (curve A, closed squares) and presence (curve B, open triangles) of 250 μM **1**. The effect of preincubating microsomes plus NADPH in the absence and presence of **1** (250 μM) for 10 min prior to adding substrate **4** is shown by curves C (open circles) and D (closed circles), respectively. For each data point a 500 μL aliquot of the incubation mixture was removed and analyzed for formaldehyde formation as described in the Methods.

continuously due to enzyme inactivation (see above), the disappearance of **4** followed strictly linear (zero-order) kinetics throughout the 60 min incubation. ^{13}C NMR studies showed that compound **1**, cyclopropanone hydrate, and *N*-methylcyclopropylamine were the only ^{13}C-enriched metabolites of **4**. The gem-diol hydrate of formaldehyde was also clearly detectable at 85.0 ppm, even though this carbon was not ^{13}C-enriched. Thus, the principal route of microsomal metabolism of **4** is N-dealkylation, with N-demethylation to form **1** greatly predominating.

Since **1** is an inactivator of microsomal P450, yet **4** is oxidized to **1** with linear (i.e., zero-order) kinetics, we also examined the interaction of these compounds with P450 by direct photometric titration and by coincubation under several conditions. Interestingly, microsomal metabolism of **4** in the absence and presence of **1** showed that the presence of **1** had no effect of N-demethylation of **4** (Figure 2, curves A and B). However, preincubation of PB-microsomes with **1** alone, followed by addition of **4**, led to a considerable decrease in N-demethylation activity toward **4** compared to a control in which the preincubation was performed in the absence of **1** (Figure 2, curves D and C, respectively). These results suggest that **4** is indeed *not* an inactivator of P450 but first must be converted to **1** before P450 inactivation occurs and that the presence of substrate **4** actually *protects* P450 from inactivation by **1**. To determine if **4** had a higher affinity for P450 than **1** we examined their interaction with P450 directly by spectrophotometric titration. Compound **4** interacted very strongly with P450 in PB-microsomes and showed a classical type I (substrate-like) binding spectrum and an apparent dissociation constant (K_s) of 8.3 μM (data not shown). Such difference spectra arise from ligand-induced displacement of an iron-coordinated water molecule and are quite distinct from the type II spectral change which occurs with nitrogenous ligands that coordinate directly to the heme iron. In contrast, compound **1** had little if any observable effect on the P450 difference spectrum at concentrations up to 1.4 mM. Although this result cannot distinguish whether **1** binds only very weakly to ferric P450, or binds without displacing the iron-coordinated water in the axial position above the heme, the former interpretation would be

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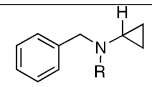
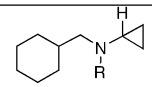
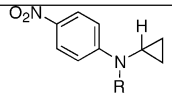
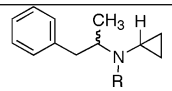
Table 1. Formation of 3HP from Incubation of Cyclopropylamines

Substrate incubated[a]		3HP formation (nmol/30 min)
	**1**, R = H	23
	4, R = CH_3	2
	**6**, R = H	5
	7, R = CH_3	13
	**8**, R = H	19
	9, R = CH_3	0
	**10**, R = H	48[b]
	11, R = CH_3	18[b]

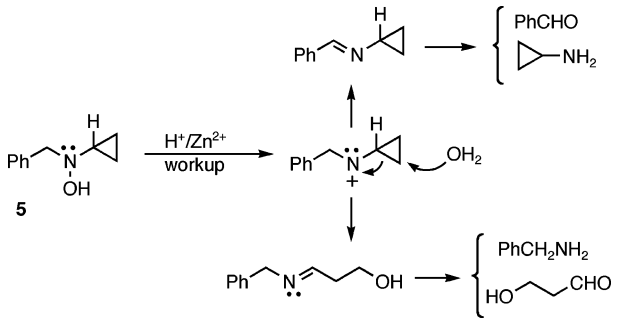
[a] Incubations were conducted at 37 °C with 0.5 mg of PB-microsomal protein, 0.5 μmol of substrate, and 0.5 μmol of NADPH in a total volume of 0.5 mL. [b] Values represent nmol of 3HP formed/20 min.

consistent with its inability to interfere with the metabolism of **4**, as shown by curves A and B in Figure 2.

Formation of 3HP from Secondary and Tertiary Cyclopropylamines and Secondary Hydroxylamine 5. In view of the significant difference in metabolism of **1** versus **4**, we examined several other pairs of secondary and tertiary cyclopropylamines to determine if other secondary cyclopropylamines were also more readily converted to 3HP than their tertiary N-methylated derivatives. As indicated in Table 1, for three of the four pairs tested, production of 3HP occurred to a much greater extent with the secondary cyclopropylamine than with its tertiary *N*-methyl analogue. This suggests that formation of 3HP from these compounds may actually *require* the N−H bond of a secondary amine. In this context it is interesting to note that of the several other cyclopropylamine-based P450 inactivators described in the literature, all are secondary amines.[7−9,36] Furthermore, whereas oxidation of 4-alkyl-1,4-dihydropyridine Hantzsch esters by P450 enzymes leads to alkyl radical extrusion and enzyme inactivation, their *N*-alkyl derivatives are also inactive in this regard until first undergoing N-deaklylation.[37] The significance of these observations is that, collectively, they cast doubt on the role of SET mechanisms in the formation of stable metabolites from **1** and possibly in the mechanism of suicide inactivation of P450 enzymes by **1**.

The generally more extensive production of 3HP from secondary cyclopropylamines versus their tertiary *N*-methyl derivatives, as well as our previous demonstration of the involvement of the microsomal flavin-containing monooxygenase (FMO) in the N-oxidation of **1**,[31] led us to consider that production of 3HP might also occur via a secondary cyclopropylhydroxylamine intermediate such as **5** as shown in Scheme 5. Using ^{13}C NMR, we demonstrated that, whereas [1′-^{13}C]-**5** is stable in incubation buffer for >4 h, addition of 15% zinc sulfate solution to simulate the workup of a microsomal

Scheme 5. Possible Role of N-Hydroxylation in Formation of 3HP



incubation results in complete loss of **5** and generation of [1′-^{13}C]-3HP as identified by DNPH trapping and ^{13}C NMR. Thus it is conceivable that a portion of the 3HP observed in actual microsomal incubations of **1** could have arisen via acid-catalyzed decomposition of **5** formed by the FMO enzyme but only if **5** were able to accumulate to a significant extent (up to 57% of substrate metabolized) during the microsomal incubations of **1**. However, such extensive accumulation seems unlikely for two reasons. First, the FMO enzyme in rat liver microsomes efficiently oxidizes secondary hydroxylamines to nitrones,[38] and second, hydroxylamine **5** reacts rapidly with both FMO and P450 in PB-microsomes.[31] Thus, it is likely that most if not all of the 3HP observed is formed as a true metabolite of **1** and not as an artifact from the chemical decomposition of **5** during workup of incubations.

Discussion

A central part of the concept behind mechanism-based or suicide substrate enzyme inactivators is that they resemble normal substrates, bind to the active site of an enzyme, and undergo early steps of the catalytic cycle as if they were a normal substrate. However, having unique chemical features not found in ordinary substrates, suicide substrates can, at least part of the time, exit the normal catalytic cycle prematurely in a way that leads to covalent inactivation of the enzyme. Thus, the study of suicide substrates can potentially illuminate at least part of the catalytic mechanism followed by normal substrates whose turnover does not lead to enzyme inactivation. A case in point is the P450-catalyzed N-dealkylation of tertiary amines, which has been conclusively shown to occur via the intermediacy of a carbinolamine which decomposes solvolytically to a carbonyl compound and a dealkylated amine.[39−41] Carbinolamine formation had been assumed to occur by a reaction analogous to aliphatic hydroxylation, but with the discovery that both **1** and **2** are suicide substrates for P450, the SET mechanism was proposed as a mechanism that could potentially account for both the N-dealkylation of normal amines and the suicide substrate activity unique to cyclopropylamines.[13,14]

Initially, the SET mechanism also appeared attractive as a way to rationalize the enigmatically small kinetic deuterium isotope effects observed for the N-dealkylation of low-$E_{1/2}$ amines ($1.0 < k_H/k_D < 1.8$),[21] which contrast the relatively large isotope effects observed for N-dealkylation of high-$E_{1/2}$ amides,

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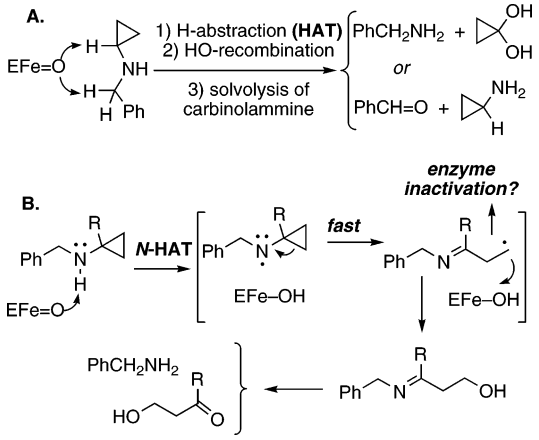
Scheme 6. Proposed Mechanisms for Interaction of **1** or **2** with Cytochrome P450



O-dealkylation of ethers, and C-hydroxylation of aliphatic systems ($4 < k_H/k_D < 10$).[42−45] However, isotope effect profiling studies of P450-catalyzed N-dealkylations and related HAT-type model reactions have been interpreted to favor the classical HAT (i.e., abstraction/recombination) mechanism,[46] and a moderately large (ca. 3.1) kinetic deuterium isotope effect more consistent with a HAT mechanism has recently been reported for P450-catalyzed N-decyclopropylation reactions.[6] The current consensus is that kinetic isotope effects do not provide support for SET involvement in N-dealkylation reactions.[6,26,27]

More recently the use of product studies to investigate the role of SET mechanisms in the oxidative metabolism of cyclopropylamines has been enabled by the development of facile synthetic methods for isotopic labeling of cyclopropylamines[28,47] and by the increased sensitivity of modern NMR spectrometers. In agreement with expectations for an SET reaction mechanism, oxidation of the low-$E_{1/2}$ substrate *N*-cyclopropyl-*N*-methylaniline (**3**) by horseradish peroxidase was found to generate exclusively cyclopropyl ring-opened products.[28,29] In contrast, oxidation of **3** by cytochrome P450 enzymes gave the ring-intact metabolite cyclopropanone hydrate as the *only* metabolite from the cyclopropyl moiety; moreover **3**, unlike **1**, does *not* inactivate P450.[30]

Because of the dichotomous behavior of **3** toward HRP versus P450, and because HRP does not oxidize **1**, we undertook to elucidate the complete P450 metabolic profile of **1**. As reported above, in addition to all of the anticipated normal N-dealkylation products (Scheme 6A), 3HP has for the first time been observed as a metabolite, indeed a *major* (57%) metabolite, derived from the cyclopropyl moiety of **1**. 3HP had long been anticipated as the "signature" metabolite for an SET mechanism for cyclopropylamine oxidation (Scheme 2). However, the fact that tertiary cyclopropylamines, which on the basis of oxidation potential should be more susceptible to SET oxidation than their secondary analogues,[43] apparently yield 3HP only *after* undergoing metabolism to a secondary cyclopropylamine is inconsistent with an SET mechanism. In fact, the formation of 3HP uniquely from *secondary* cyclopropylamines is entirely consistent with a variation of the HAT mechanism first proposed by Karki and Dinnocenzo,[26] as shown in Scheme 6B. In this scheme the enzymatic oxidant is depicted as the ferryl or iron−oxene derivative of P450 ($Fe{=}O^{3+}$),[24,25] but the N-HAT mechanism shown is not inconsistent with a radical-like Fe(III)−OO· precursor to the ferryl species being involved as the H-abstracting agent.

In conclusion, we believe the many results presented above argue persuasively in favor of the "traditional" HAT-type mechanism for most if not all P450-catalyzed N-dealkylation reactions, including those of cyclopropylamines. Other important questions remain, however. For example, at what point in the turnover cycle of P450 with **1** or **2** does the mechanism branch to suicide inactivation; is there *any* role for SET in amine metabolism by P450 enzymes; what isoforms of P450 in PB-microsomes are inactivated by **1**; in addition to metabolic intermediate complex formation,[31] how do **1** and **2** inactivate P450? Ongoing studies in our laboratory are directed toward answering these important questions.

Methods

Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and NADPH were purchased from Sigma (St. Louis, MO). Compounds **6**−**11** were available from earlier work in our laboratory. All other chemicals and reagents were of reagent grade or higher purity and were purchased from commercial suppliers. Liver microsomes from phenobarbital-treated rats were prepared, stored, and handled as described.[31,48,49] P450 ligand binding spectra were recorded as described by Chaurasia et al.[48] Other general laboratory instrumentation and procedures were as described.[31] Unless otherwise specified, the buffer used for all microsomal incubations was 0.1 M potassium phosphate, pH 7.5. Zinc sulfate solution (15% w/v) was used to precipitate proteins, but unlike many literature descriptions of this method we added barium hydroxide only when formaldehyde was the metabolite being measured. The syntheses of [1′-^{13}C]-**1**, [1′-^{14}C]-**1**, [7-^{13}C]-**1**, [7-^{14}C]-**1**, and **5** have been described previously.[31] 2,4-Dinitrophenylhydrazine derivatives of acrolein and 3-hydroxypropionaldehyde were prepared as described by Shaffer et al.[29]

***N*-Benzyl-*N*-methyl-[1′-^{13}C]-cyclopropylamine·HCl (4·HCl).** To a 30 mL culture tube (2.5 cm × 10 cm) was added *N*-benzyl-*N*-methylamine·HCl (0.79 g, 5.00 mmol), sodium [^{13}C]-formate (0.28 g, 4.00 mmol), and 10 mL of acetonitrile. The tube was capped with a Teflon-lined screw cap, and the contents were stirred at 82 °C for 48 h. The reaction was cooled to room temperature and diluted with ethyl acetate (15 mL), and the resulting solution rinsed with 1 M NaOH (15 mL) and 1 M HCl (15 mL). The ethyl acetate solution was then dried over magnesium sulfate, filtered, and the solvent was removed in vacuo yielding *N*-benzyl-*N*-methyl-[^{13}C]-formamide as a yellow liquid (0.55 g, 74% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.82 (d, 3H, J = 3.51 Hz), 4.46 (d, 2H, J = 3.83 Hz), 7.20−7.55 (m, 5H), 8.22 (dd, 1H, J = 62.81, 192.87 Hz). ^{13}C NMR (125.8 MHz, $CDCl_3$) δ 30.24, 34.87, 48.56, 54.30, 128.16, 128.41, 128.87, 128.99, 129.44, 129.65, 136.40, 136.66, 163.53 (^{13}C-enriched carbon), 163.69 (^{13}C-enriched carbon). GC/MS: t_R 14.5 min, m/z 150 [M^+].

N-benzyl-*N*-methyl-[^{13}C]-formamide (0.55 g, 3.68 mmol) was dissolved in 8 mL of dry tetrahydrofuran in a three-neck round-bottom flask and stirred vigorously under nitrogen. Titanium isopropoxide (1.35 mL, 4.60 mmol) was added, followed by 3.68 mL of a 3.00 M solution

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of ethylmagnesium bromide in ether (11.05 mmol). Upon addition of the Grignard reagent, gas evolved and a yellow color formed. The mixture was heated for 15 min at 45 °C, whereupon the reaction mixture became black. The reaction mixture was then cooled to room temperature, stirred for 12 h, and quenched by addition of 15 mL of saturated aqueous ammonium chloride solution. After adding 20 mL of 1 M NaOH the resulting mixture was extracted with ether (3 × 25 mL). The extracts were pooled, dried over magnesium sulfate, filtered, and concentrated in vacuo giving a yellow liquid (0.41 g). This material was then applied to a silica gel column and eluted with 2.5% ether in pentane to obtain 0.21 g of *N*-benzyl-*N*-methyl-[1′-^{13}C]-cyclopropylamine as a clear colorless liquid. This liquid was then dissolved in 10 mL ether and bubbled with HCl gas resulting in the formation of a white precipitate. The white precipitate that formed was collected by suction filtration and dissolved in a minimum volume of hot absolute ethanol, cooled to room temperature, and the product was precipitated by addition of ether giving 0.15 g of a white crystalline solid (20%). ^{1}H NMR (400 MHz, D_2O) δ 0.80 (m, 2H), 0.91 (m, 2H), 2.81 (dm, 1H, J = 186.18 Hz), 2.94 (m, 2H) 7.50 (m, 5H). ^{13}C NMR (100.6 MHz, D_2O) δ 4.65, 39.28 (^{13}C-enriched carbon), 42.60, 61.31, 129.27, 129.44, 130.38, 131.67. GC/MS (free base): t_R 11.2 min, m/z 161 [M^+].

[1′-^{13}C]-*N*-Benzyl-*N*-cyclopropylhydroxylamine ([1′-^{13}C]-5). In a three-neck round-bottom flask was combined [1′-^{13}C]-**1** (56 mg, 0.38 mmol), sodium tungstate dihydrate (5 mg, 0.015 mmol), and 3 mL of water. Hydrogen peroxide solution (30%, 0.10 mL) was added over 30 min with constant cooling on ice. The reaction was allowed to warm to room temperature and stirred an additional 3 h. The reaction was halted by addition of 6 mg of sodium hydrogen sulfite and 48 mg of sodium chloride. The resulting solution was extracted with methylene chloride (4 × 10 mL), and the extracts were pooled, dried over magnesium sulfate, and concentrated in vacuo giving 0.048 g of the crude nitrone product as determined by TLC. Without purification the crude nitrone was dissolved in 2 mL of dry tetrahydrofuran and added dropwise over 30 min to a stirred ice-cold slurry of lithium aluminum hydride (0.045 g, 1.19 mmol) in 2 mL of tetrahydrofuran under an atmosphere of nitrogen with constant cooling. After addition of the nitrone solution was complete, the reaction was left to stir at 0 °C for 3 h. At this point TLC showed that no starting material remained. The reaction was quenched by the slow addition of 3 mL of water with continual cooling. The resulting mixture was extracted with ether (4 × 10 mL), and the extracts were pooled, dried over magnesium sulfate, and concentrated giving 0.025 g of a yellow liquid. After silica gel chromatography using 3:1 pentane/ether as solvent, 0.015 g of the desired product was obtained as an amorphous white solid (24% yield from [1′-^{13}C]-**1**). ^{1}H NMR (400 MHz, MeOH-d_4) δ 0.57 (m, 4H), 2.30 (dm, 1H, J = 171.77 Hz), 3.96 (s, 2H), 7.21−7.35 (m, 5H). ^{13}C NMR (100.6 MHz, MeOH-d_4) δ 6.86, 7.02, 41.65 (^{13}C-enriched carbon), 63.32, 128.18, 129.13, 130.81.

***N*-Cyclopropyl-4-nitrobenzamide.** Cyclopropylamine (5.00 mmol), triethylamine (10.00 mmol), and 5 mL of dry tetrahydrofuran were combined in a 50 mL culture tube. A 1 M solution of 4-nitrobenzoyl chloride (10 mL, 10.00 mmol) in dry tetrahydrofuran was added dropwise with stirring. The tube was capped with a Teflon-lined screwcap and heated at 65 °C for 4 h. The reaction was quenched by addition of water (10 mL), and the organic portion was separated and concentrated in vacuo. The resulting material was then dissolved in 40 mL of chloroform and rinsed with saturated aqueous sodium carbonate (2 × 30 mL) and H_2O (30 mL). The resulting solution was dried over magnesium sulfate, filtered, and the solvent was removed in vacuo to give *N*-cyclopropyl-4-nitrobenzamide. The crude product was dissolved in hot benzene, and hexane was added until a cloudy solution resulted. Upon standing in a refrigerator, the desired product formed as white needles (51% yield). Mp 175 °C (Lit. 178−179 °C).[50] ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.62−0.69 (m, 2H), 0.90−0.95 (m, 2H), 2.90−2.97 (m, 1H), 6.35 (s, 1H), 7.90−7.92 (m, 2H), 8.26−8.29 (m, 2H). GC/MS: t_R 16.3 min, m/z 206 [M^+].

***N,N*-Diethyl-4-nitrobenzamide.** *N*,*N*-Diethyl-4-nitrobenzamide, used as an internal standard for HPLC, was prepared and purified as described for *N*-cyclopropyl-4-nitrobenzamide giving the desired product as white needles (43% yield). Mp 64−66 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.11−1.14 (m, 3H), 1.26−1.31 (m, 3H), 3.21 (m, 2H), 3.57 (m, 2H), 7.55 (d, 2H, J = 8.77 Hz), 8.28 (d, 2H, J = 8.74 Hz). GC/MS: t_R 18.4 min, m/z 222 [M^+].

***N*-Cyclopropyl-*N*-methyl-*N*-cyclohexylmethylamine·HCl (7·HCl).** In a 16 mm × 100 mm culture tube was combined the hydrochloride salt of *N*-cyclopropyl-*N*-(cyclohexylmethyl)amine (0.47 g, 2.50 mmol), 5 mL of methanol, formaldehyde (12.7 M solution, 0.22 mL), and acetic acid (0.57 mL, 10 mmol) along with 3 Å molecular sieves. After stirring for 30 min, sodium cyanoborohydride (0.24 g, 3.75 mmol) was added and the tube was capped with a Teflon-lined screwcap. The reaction was allowed to stir overnight. The reaction was then quenched by addition of water (10 mL), and the resulting solution was extracted with ether (15 mL). The aqueous portion was then made alkaline by addition of 10 mL of 1 M NaOH and extracted with ether (3 × 15 mL). The extracts were pooled, dried over magnesium sulfate, and concentrated under reduced pressure. The colorless liquid obtained was dissolved in 5 mL of ether and bubbled with HCl gas resulting in the formation of a white precipitate (0.054 g, 11%). ^{1}H NMR (400 MHz, D_2O) δ 0.95−1.29 (m, 9H), 1.69 (m, 5H), 1.90 (m, 1H), 2.79 (m, 1H), 2.92 (s, 5H), 3.15 (s, 5H). ^{13}C NMR (100.6 MHz, D_2O) δ 25.20, 25.70, 30.56, 32.95, 40.62, 42.23, 64.64.

***N*-Cyclopropyl-*N*-methyl-1-methyl-2-phenylethylamine·HCl (11·HCl).** In a 16 mm × 100 mm culture tube the hydrochloride salt of *N*-cyclopropyl-1-methyl-2-phenylethylamine (**10**) (0.53 g, 2.50 mmol), methanol (4 mL), formaldehyde (12.7 M solution, 0.22 mL, 2.79 mmol), and acetic acid (0.57 mL, 10 mmol) were combined over 3 Å molecular sieves. After stirring for 30 min, sodium cyanoborohydride (0.24 g, 3.75 mmol) was added, and the tube was capped with a Teflon-lined screwcap. The reaction was allowed to stir overnight, water was then added, and the resulting solution was extracted with ether (15 mL). The aqueous portion was then made alkaline by addition of 10 mL of 1 M NaOH and extracted with ether (3 × 15 mL). The extracts were pooled, dried over magnesium sulfate, and concentrated under reduced pressure. The colorless liquid obtained was dissolved in 5 mL of ether and bubbled with HCl gas resulting in formation of a white precipitate (0.149 g, 26%). ^{1}H NMR (400 MHz, D_2O) δ 0.87−1.07 (m, 4H), 1.26 (t, 3H, J = 5.71 Hz), 2.75−2.97 (m, 5H), 3.25 (dd, 1H, J = 4.07, 13.17 Hz), 3.75−3.84 (m, 1H), 7.30−7.59 (m, 5H). ^{13}C NMR (100.6 MHz, D_2O) δ 3.59, 3.80, 5.50, 5.77, 12.34, 13.76, 36.51, 36.57, 37.04, 37.55, 37.65, 38.13, 64.16, 64.45, 127.59, 127.67, 129.27, 129.30, 129.72, 136.48, 136.72.

Incubation and Metabolite Analysis by ^{13}C NMR. Concentrated PB-microsomes (729 μL, 30 mg of protein, 101 nmol of P450) were diluted with 8.9 mL of buffer and warmed to 37 °C. To this mixture was added 40 μL of either buffer or a solution of [1′-^{13}C]-**1** or [7-^{13}C]-**1** or [1′-^{13}C]-**4** in buffer (0.5 M, 20 μmol) and 53 μL of an NADPH regeneration system (0.13 units/μL glucose-6-phosphate dehydrogenase and 1 M glucose-6-phosphate in buffer). This mixture was vortexed thoroughly and preincubated for 3 min at 37 °C. The incubation was started by addition of 200 μL of a 50 mM solution of NADPH in buffer (10 μmol) bringing the total volume to 10 mL. The incubation was conducted for 90 min at 37 °C in an oscillating water shaker bath. The incubation was quenched by addition of 2 mL of 15% zinc sulfate solution (which dropped the pH to around 4), the precipitate was sedimented by centrifugation, and the resulting supernatant was removed. For the [7-^{13}C]-**1** incubations the supernatant was further acidified to pH 1 with 1 mL of 1 M HCl and extracted with ether (3 × 5 mL). The extracts were set aside for later analysis (see below). After extraction the supernatant was concentrated with gentle heating under vacuum to a volume of approximately 500 μL. This liquid was

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then transferred to a 5 mm NMR tube along with 50 μL of D_2O. These samples were then analyzed by ^{13}C NMR. After acquisition of the initial ^{13}C NMR spectra for the PB-microsomal incubation of [1′-^{13}C]-**1**, cyclopropylamine (5.9 μL of 7.61 M solution in H_2O, 45 μmol) and cyclopropanone hydrate (32.2 μL, 1.4 M in H_2O, 45 μmol)[30,51] were added, and the sample was reanalyzed by ^{13}C NMR.

Analysis of Ether Extracts for Non-Amine Metabolites of [7-^{13}C]-1 by ^{13}C NMR and GC/MS. A blank incubation and an incubation containing [7-^{13}C]-**1** were conducted in parallel as described above. The ether extracts of the quenched incubation supernatants were dried over magnesium sulfate and filtered. To avoid loss of volatile metabolites, the ether extracts were concentrated to <250 μL using a Vigreux distillation column. To this liquid was then added 1 mL of deuterated chloroform, and the sample transferred to a 5 mm NMR tube and analyzed by ^{13}C NMR. After acquisition of the NMR spectrum, an aliquot of the NMR solution was removed and without dilution analyzed by GC/EIMS (DB5 column) using the following oven program: 50 °C initial temperature increasing to 150 °C at 10 °C/min, then 30 °C/min to 250 °C, then hold at 250 °C for 10 min. To limit the loss of volatile compounds the injection port temperature was maintained at 150 °C. To confirm the retention times and mass spectral fragmentation patterns, authentic standards were injected using the same oven program.

Incubation of 1, [1′-^{14}C]-1, and [7-^{14}C]-1 for Metabolite Quantitation. These incubations were carried out essentially as described above, except that the incubations were scaled down to 1.5−2.0 mL and the microsomal protein concentration was decreased to 2 mg/mL. Aliquots (200 μL) were removed at various times for analysis as described below.

Incubations for amine analysis were quenched by addition to a mixture of 45 μL of 15% zinc sulfate solution and 20 μL of 2.75 mM 4-methoxybenzylamine (internal standard). The precipitate was removed by centrifugation for 10 min. The supernatant was removed, extracted with pentane/ether (9:1), and residual pentane/ether was removed by briefly blowing a stream of nitrogen over the solution. The quenched incubation samples were then analyzed by HPLC (100 μL injection) using an isocratic solvent system consisting of 15% acetonitrile in 100 mM $NaClO_4$ (acidified to pH 2.25 using perchloric acid) at a 1 mL/min flow rate on a Kromasil C4 column (5 μm, 4.6 mm × 150 mm).[52] The column effluent was passed through a UV detector (210 nm) in series with a Ramona radioactivity flow detector with a solid scintillant cell. The amounts of benzylamine formed and **1** remaining were quantified based on radiochemical peak integration or using a calibration curve prepared from standard solutions of nonradiolabeled benzylamine and **1**.

For analysis of non-amine metabolites incubations were quenched by addition of 15% zinc sulfate solution (45 μL) and acetophenone (20 μL, 1.25 mM in acetonitrile) as an internal standard. The quenched incubation samples were then analyzed by HPLC (100 μL injection) using an isocratic solvent system consisting of 27.5:72.5 acetonitrile/ammonium formate solution (50 mM, adjusted to pH 3.6) at a flow rate of 1 mL/min on a Vydac C18 column (5μ, 4.6 mm × 250 mm). Column effluent was passed through a UV detector (254 nm) in series with a Ramona radioactivity flow detector with a solid scintillant cell. Non-amine metabolite formation was quantified based on radiochemical peak area integration.

DNPH Trapping and Quantitation of 3HP. Aliquots (200 μL) of an incubation mixture of **1** with PB-microsomes (2 mg of protein/mL) were removed at 0, 15, 30, and 60 min and placed into a mixture of 15% zinc sulfate solution (45 μL) and internal standard (20 μL × 2 mM acetaldehyde or glycolaldehyde in acetonitrile). Precipitated protein was removed by centrifugation for 10 min. The supernatant (200 μL) was removed and combined with 10 μL of a hexanes-extracted DNPH solution (0.15 M)[28] and allowed to stand at room temperature for 30 min. The resulting mixture was extracted with ethyl acetate (2 × 500 μL), and the combined extracts were dried in vacuo. The resulting orange residue was dissolved in 200 μL of acetonitrile, and the solution of DNPH-trapped material (20 μL) was analyzed by HPLC using a Vydac C18 column (5μ, 4.6 mm × 250 mm) with UV monitoring at 350 nm eluting at 1 mL/min with a gradient of solvent B (acetonitrile) in solvent A (MeOH/H_2O, 1:1 v/v) as follows: 0−13 min, 9% B; 13−18 min, 9−30% B; 18−21 min, 30% B; 21−23 min, 30−100% B; 23−26 min, 100% B; 26−27 min, 100−9% B; 27−35 min, hold at 9% B. Formation of 3HP was quantified using a calibration curve prepared from a standard solution of 3,3-diethoxypropanol (which generates 3HP, but not acrolein, upon hydrolysis under the above conditions).

Quantitation of Cyclopropylamine Formation. Incubation aliquots (200 μL) were removed at 0, 15, 30, and 60 min and combined with 45 μL of 15% zinc sulfate solution and 20 μL of 1 mM *N*,*N*-diethyl-4-nitrobenzamide (internal standard). After brief centrifugation the supernatant was removed and combined with 180 μL of 5 M potassium hydroxide and 400 μL of freshly prepared 100 mM 4-nitrobenzoyl chloride in tetrahydrofuran and allowed to stand at room temperature for 30 min. The resulting mixture was extracted with ethyl acetate (2 × 250 μL), the extracts were concentrated in vacuo, and the resulting material was dissolved in 100 μL of acetonitrile. HPLC analysis was performed by injection of 20 μL of this solution onto a Vydac C18 column (5μ, 4.6 mm × 250 mm) monitored by UV at 270 nm and eluted at a flow rate of 1 mL/min using a gradient of solvent B (acetonitrile) in solvent A (70:30 H_2O/acetonitrile) as follows: 0−8 min, 0% B; 8−15 min, 0−55% B; 15−20 min, hold at 55% B; 20−23 min, 55−0% B. Formation of cyclopropylamine was quantified using a calibration curve prepared from a standard solution of cyclopropylamine.

Effect of 1 on the N-Demethylation of 4. To examine the direct effect of coincubating **1** with **4**, a suspension of PB-microsomes (2 mg of protein/mL, 4436 μL) was added to each of two 25 mL Erlenmeyer flasks. To each of these flasks was also added 95 μL of a 25 mM solution of **4** (2.38 μmol) in buffer and 29 μL of an NADPH regenerating system containing glucose-6-phosphate dehydrogenase (0.13 units/mL) and glucose-6-phosphate (1.0 M) in buffer. One flask then received 95 μL of a solution of **1** (12.5 mM, 1.19 μmol) in buffer while the other received 95 μL of buffer alone. Prior to initiating the reaction by addition of NADPH (see below), a 490 μL "zero-time" aliquot was removed from each flask and added to a 1.7 mL Eppendorf tube containing 250 μL each of 15% zinc sulfate solution and saturated aqueous barium hydroxide solution. Each tube was then vortexed, 10 μL buffer was added, and the mixture was held for later analysis. The two incubation tubes were then warmed in an oscillating water bath at 33 °C for 3 min prior to initiating reaction by the addition of 85 μL of an NADPH solution (50 mM in 0.1 M KH_2PO_4 buffer). At 0.5, 1, 3, 5, 10, 15, 30, and 45 min after the addition, a 500 μL aliquot was removed from each flask and added to separate 1.7 mL Eppendorf tubes containing 250 μL of 15% zinc sulfate solution. The tubes were vortexed, a saturated solution of barium hydroxide was added (250 μL), and the tubes were vortexed again. After brief centrifugation, 800 μL of the supernatant was removed, combined with 400 μL of Nash reagent,[53] and heated at 60 °C for 10 min. The absorbance of each incubation sample was then measured at 415 nm versus a similarly treated buffer (blank) solution. Formaldehyde formation was quantified using a calibration curve prepared from standard solutions.

To examine the effect of preincubation of **1** on the N-demethylation of **4**, PB-microsomes were diluted with buffer to a protein concentration of 2 mg/mL and kept on ice until use. Diluted microsomes (3502 μL) were added to each of two 25 mL Erlenmeyer flasks along with 23 μL

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of an NADPH regenerating system as described above. To one of the two flasks was added a solution **1** in buffer (75 μL, 12.5 mM, 0.94 μmol), and to the other was added 75 μL of buffer alone. To each flask was then added 75 μL of an NADPH solution (50 mM in buffer), and the resulting mixtures were incubated in the absence of **4** in an oscillating water bath at 33 °C for 10 min. Prior to addition of **4**, a 490 μL aliquot was removed from each flask and added to a 1.7 mL Eppendorf tube containing 250 μL each of 15% zinc sulfate and a saturated aqueous barium hydroxide solution and 10 μL of buffer to generate a zero-time sample. After 10 min of preincubation time, a solution of **4** (65 μL, 25 mM in buffer, 1.63 μmol) was added to each flask. This was followed by further incubation in the oscillating water bath at 33 °C. At 1, 3, 5, 10, 20, and 25 min after the addition of **4**, a 500 μL aliquot was removed from each flask in parallel and added to a 1.7 mL Eppendorf tube containing 250 μL of 15% zinc sulfate solution; after vortexing to mix thoroughly, 250 μL of saturated aqueous barium hydroxide solution was added and the tubes were vortexed again. Samples were processed and analyzed for formaldehyde as described above.

Mock Incubation of [1′-^{13}C]-5. To a 16 mm × 100 mm culture tube was added 5394 μL of buffer, 56 μL of a 100 mM solution of [1′-^{13}C]-**5** (5.6 μmol) in methanol, and 100 μL of a 2.5 M solution of ethylene glycol (250 μmol, internal standard) in water. The mixture was thoroughly vortexed, and a 550 μL aliquot was removed, added to a 5 mm NMR tube along with 50 μL of D_2O, and analyzed by ^{13}C NMR after 30 min and again after 4 h. To the remaining solution was added 1 mL of a 15% solution of zinc sulfate which resulted in the precipitation of a white solid. Precipitate was sedimented by centrifugation, and the supernatant was removed and concentrated with gentle heating under vacuum to a volume of approximately 500 μL. This liquid was then transferred to a 5 mm NMR tube along with 50 μL of D_2O and analyzed by ^{13}C NMR.

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Supporting Information Available: ^{13}C NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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