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Formation of Cyclopropanone during Cytochrome P450-Catalyzed N-Dealkylation of a Cyclopropylamine

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Abstract: The role of single electron transfer (SET) in P450-catalyzed N-dealkylation reactions has been studied using the probe substrates *N*-cyclopropyl-*N*-methylaniline (**2a**) and *N*-(1′-methylcyclopropyl)-*N*-methylaniline (**2b**). In earlier work, we showed that SET oxidation of **2a** by horseadish peroxidase leads exclusively to products arising via fragmentation of the cyclopropane ring [Shaffer, C. L.; Morton, M. D.; Hanzlik, R. P. *J. Am. Chem. Soc.* **2001**, *123*, 8502−8508]. In the present study, we found that liver microsomes from phenobarbital pretreated rats (which contain CYP2B1 as the predominant isozyme) oxidize [1′-^{13}C, 1′-^{14}C]-**2a** efficiently (80% consumption in 90 min). Disappearance of **2a** follows first-order kinetics throughout, indicating a lack of P450 inactivation by **2a**. HPLC examination of incubation mixtures revealed three UV-absorbing metabolites: *N*-methylaniline (**4**), *N*-cyclopropylaniline (**6a**), and a metabolite (**M1**) tentatively identified as *p*-hydroxy-**2a**, in a 2:5:2 mole ratio, respectively. 2,4-Dinitrophenylhydrazine trapping indicated formation of formaldehyde equimolar with **6a**; 3-hydroxypropionaldehyde and acrolein were not detected. Examination of incubations of **2a** by ^{13}C NMR revealed four ^{13}C-enriched signals, three of which were identified by comparison to authentic standards as *N*-cyclopropylaniline (**6a**, 33.6 ppm), cyclopropanone hydrate (**11**, 79.2 ppm), and propionic acid (**12**, 179.9 ppm); the fourth signal (42.2 ppm) was tentatively determined to be *p*-hydroxy-**2a**. Incubation of **2a** with purified reconstituted CYP2B1 also afforded **4**, **6a**, and **M1** in a 2:5:2 mole ratio (by HPLC), indicating that all metabolites are formed at a single active site. Incubation of **2b** with PB microsomes resulted in *p*-hydroxylation and N-demethylation only; *no* loss or ring-opening of the cyclopropyl group occurred. These results effectively rule out the participation of a SET mechanism in the P450-catalyzed N-dealkylation of cyclopropylamines **2a** and **2b**, and argue strongly for the N-dealkylation of **2a** via a carbinolamine intermediate formed by a conventional C-hydroxylation mechanism.

Introduction

Over the past 30 years, the mechanism of the cytochrome P450-catalyzed N-dealkylation of amines has been of great interest and debate. It is commonly observed that the alkyl group lost from an amine during N-dealkylation appears as an aldehyde or ketone arising from the dissociation of a carbinolamine intermediate.[1,2] One early and widely accepted mechanism for carbinolamine formation derives from the long-held consensus mechanism for aliphatic hydroxylation reactions that invokes an oxo-iron (Fe═O) intermediate and a hydrogen atom transfer (HAT) step followed by hydroxyl recombination. For example, the HAT mechanism was originally invoked to explain the striking observation that *N*-benzyl-*N*-cyclopropylamine (**1a**, see Chart 1 for structures) *inactivates* cytochrome P450 as it undergoes P450-catalyzed oxidation (Scheme 1).[3] However, the subsequent finding that analogue **1b**, which lacks an α-hydrogen, also inactivates P450 as efficiently as **1a**[4,5] requires a mechanism not involving HAT (or any alternative insertion-like process[6,7] requiring an α-hydrogen) leading to a carbinolamine intermediate.

The unique electronic structure of the cyclopropyl group as compared to that of ordinary aliphatic groups,[8] and the manifest unimportance of an α-hydrogen on the cyclopropane ring to their activity as suicide substrates, prompted the proposal, over 20 years ago, of a single electron transfer (SET) mechanism for the suicide substrate activity of **1a** and **1b** (Scheme 2).[4,5] It was suggested that such a mechanism could account not only for N-dealkylation of both cyclopropyl and ordinary aliphatic amines, but also many experimental observations regarding the P450-catalyzed N-dealkylation of amines which were not well accounted for by a HAT mechanism, such as low kinetic

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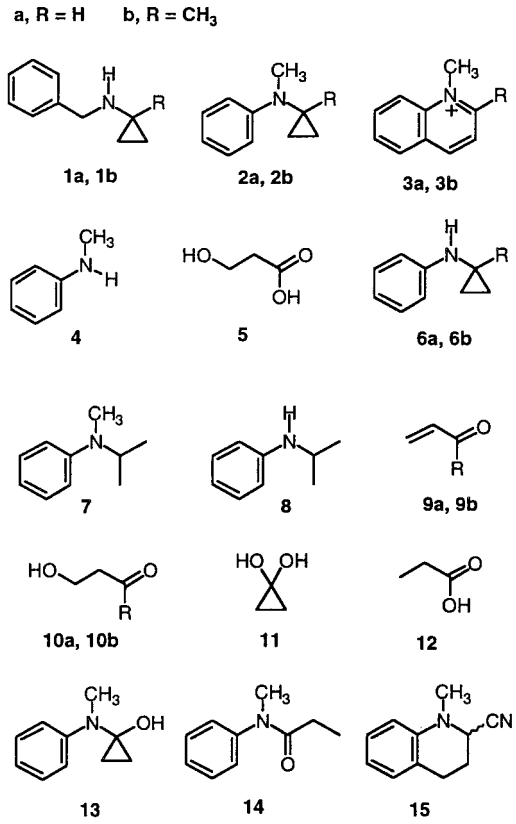
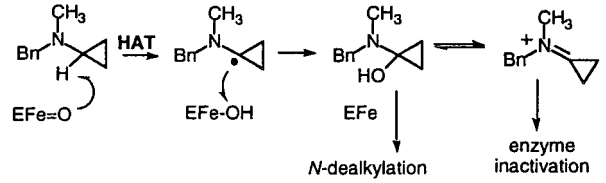
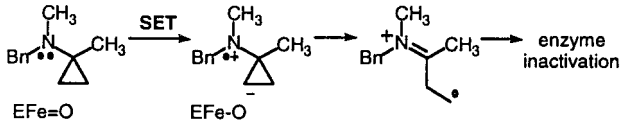
Chart 1



Scheme 1. HAT Mechanism

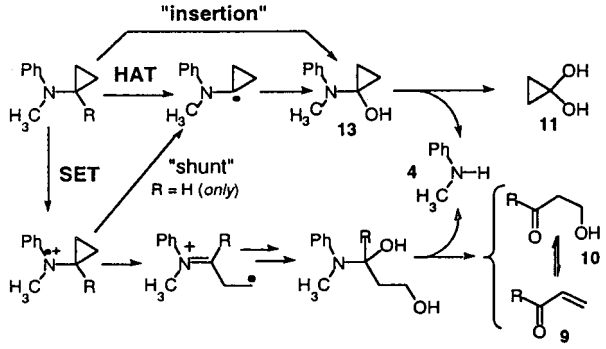


Scheme 2. SET Mechanism



deuterium isotope effects,[9,10] the extrusion of alkyl radicals during oxidative aromatization of 4-alkyl-1,4-dihydropyridines,[11] and Hammett and Marcus relationships in the N-dealkylation of a series of para-substituted *N*,*N*-dimethylanilines.[12,13] Numerous subsequent studies have utilized the SET mechanism to rationalize results for the metabolism of a variety of amine substrates by P450 and non-P450 enzymes.[14−19]

Scheme 3



Although P450-catalyzed N-dealkylations are now widely believed to occur by SET,[20−23] the experimental evidence supporting this mechanism (summarized by Zhao et al.[24]) remains largely indirect, and acceptance of this view is by no means universal.[25] In particular, structural identification of the cyclopropyl ring metabolites of **1a** and **1b** (or any other *N*-cyclopropylamine) following P450-catalyzed N-dealkylation has never been achieved,[24,26] although Kuttab et al. report cinnamaldehyde formation during microsomal oxidation of a *trans*-2-phenylcyclopropylamine.[27] This is a crucial point because, as compared to the HAT mechanism (or the modern "insertion" alternative to it[6,7]), the SET mechanism predicts very different metabolites from the cyclopropyl group (Scheme 3).

To address the issue of identifying the C_3 metabolite(s) from a cyclopropylamine undergoing N-dealkylation specifically via a SET process, we turned to horseradish peroxidase (HRP) as a model oxidant. Because HRP is not as high potential of an oxidant as P450,[13] we used *N*-cyclopropyl-*N*-methylaniline (**2a**) as a more-easily oxidizable analogue of **1**. Under standard peroxidatic conditions,[28] HRP oxidation of **2a** affords 1-methylquinolinium (**3a**, 0.8 mol), *N*-methylaniline (**4**, 0.2 mol), and 3-hydroxypropionic acid (**5**, 0.2 mol). Under *anaerobic* peroxidatic conditions, HRP oxidation of **2a** generates **3a** *exclusively*, indicating that the 3-hydroxy group in **5** originates from molecular oxygen. These results demonstrate that $\mathbf{2a}^{+\bullet}$ reacts *exclusively* by unimolecular ring opening and *not* via C-1′ deprotonation (Scheme 3, "shunt"). The key to our success in the above studies was the use of dual [^{14}C/^{13}C] labeling to facilitate metabolite quantitation and identification, respectively.

We have now applied this approach to elucidate the action of cytochrome P450 on **2a**, the related compound *N*-(1′-

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methylcyclopropyl)-*N*-methylaniline (**2b**), and the noncyclopropyl analogue *N*-methyl-*N*-isopropylaniline (**7**). On the basis of the structural analogy of **2a** to **1a**, and the much higher reduction potential of ferryl P450 versus HRP compound I,[13] we anticipated that **2a** would undergo SET oxidation and inactivate P450 in parallel with its conversion to products lacking an intact cyclopropane substructure. *Surprisingly, both of these predictions proved to be totally incorrect.* In this manuscript, we describe the identification and quantitation of the products arising from the very efficient oxidation of **2a** and **2b** by cytochrome P450 enzymes present in liver microsomes from phenobarbital-induced rats (PB microsomes), and by purified reconstituted CYP2B1. While this manuscript was undergoing review and revision, Bhakta and Wimalasena[29] reported kinetic deuterium isotope effects for the P450-catalyzed N-dealkylation of the *p*-chloro analogues of **2a** and **2b**. Although they did not identify the key metabolites arising from the cyclopropyl group, the isotope effects were large enough to be consistent with a HAT mechanism for loss of the *N*-cyclopropyl substituent.

Results and Discussion

Prior to incubations with **2a**, we first investigated the microsomal oxidation of *N*-isopropyl-*N*-methylaniline (**7**), a model compound whose three-carbon *N*-substituent lacks the reactivity of a cyclopropyl moiety but otherwise has similar size and lipophilicity. Upon incubation with PB microsomes, **7** disappears in a first-order manner ($k_{obs} = 0.06$ min^{-1}) with ca. 90% of **7** being consumed within 45 min. At this time, the only metabolites observed are *N*-isopropylaniline (**8**) and formaldehyde, although at longer incubation times (60−90 min), trace amounts of aniline and acetone are also detected in the incubation mixtures. The preference for N-demethylation over N-dealkylation is a common theme in P450-catalyzed amine oxidations,[2,22] and a similar preference has been reported for HRP-catalyzed N-dealkylations of other *N*-alkyl-*N*-methylanilines.[30] We also found HRP to oxidize **7** solely by N-demethylation to **8**, but even after 60 min, 10% of **7** was consumed, presumably because of adverse steric interactions between the isopropyl moiety and the HRP active site.[31,32]

Turning our attention to compound **2a**, we found that HPLC analysis (A_{254}) of periodic incubation aliquots showed that P450 enzymes present in PB microsomes oxidize [1′-^{13}C]-**2a** in a first-order fashion ($k_{obs} = 0.03$ min^{-1}) with >80% consumption of **2a** after 90 min. Metabolite peaks corresponding to *N*-methylaniline (**4**), *N*-cyclopropylaniline (**6a**), and an unknown metabolite (**M1**) were detected in increasing amounts but with a constant 22:56:22 ratio over time, along with traces of aniline from further oxidation of **4** and/or **6a**. GC/EIMS analysis of an ether extract of incubation aliquots over time also detected the formation of the same four metabolites, and revealed that the peak corresponding to **M1** had *m*/*z* 164 (corresponding to [1′-^{13}C]-**2a** + ^{16}O). After 90 min of oxidation, HPLC analysis of the incubation solution at 320 nm, which is an optimal analytical wavelength for detecting **3a**, the major metabolite formed when **2a** is totally oxidized by HRP (ca. 80% yield under air; 100% under anaerobic conditions), revealed that **3a** was completely absent from the microsomal incubation of **2a**.

Thus, with respect to regioselectivity, the microsomal oxidation of **2a** (predominant N-demethylation) more closely parallels the microsomal oxidation of **7** (exclusive N-demethylation) than the HRP oxidation of **2a** (exclusive loss of the cyclopropyl group). The exclusive loss of the cyclopropyl group in the HRP reaction of **2a** can logically be attributed to the extreme reactivity[33] of the SET-generated cyclopropylaminium radical cation, which undergoes ring opening much more quickly than deprotonation at either of the α-carbons.[28,34−38] Another difference between the HRP versus microsomal oxidation of **2a** is that whereas microsomal incubation supernatants remain colorless throughout the course of the reaction, HRP incubations with **2a** are always colored (i.e., pale yellow at early timepoints progressing to dark yellow or even light blue at high levels of oxidation), possibly because of the presence of cation radicals and/or quinoid intermediates generated by SET. Because of differences in rate-limiting steps in the two enzyme systems, the absence of color in the P450 reactions may not be mechanistically significant. On the other hand, both HRP- and P450-catalyzed oxidations of 1,2,4,5-tetramethoxybenzene generate colored species in solutions, and radicals have been detected by spin trapping.[39]

To analyze carbonyl metabolites formed during microsomal oxidation of **2a**, EtOAc extracts of microsomal supernatants from various incubation timepoints were treated with 2,4-dinitrophenylhydrazine (DNPH) trapping reagent and analyzed by GC/EIMS.[38] In this way, we readily detected an increase in formaldehyde formation paralleling an increase in *N*-cyclopropylaniline (**6a**) formation as the oxidation of **2a** progressed. At no time during the incubation were the originally anticipated SET metabolites acrolein (**9a**) and/or 3-hydroxypropanal (**10a**) detected, although controls showed they were stable and detectable as DNP adducts if added to mock incubation mixtures. Interestingly, acrolein has been reported to cause a decrease in the content and activity of cytochrome P450 in microsomes in vitro.[40,41] Because we observe no significant decrease in microsomal P450 activity toward **2a**, even after extensive substrate oxidation, this result too suggests that acrolein is not being generated. Unfortunately, DNPH does not react with cyclopropanone hydrate (**11**).[38] Thus, we had to resort to using ^{13}C NMR to search for the presence of **11** in the incubation mixture.

To investigate the metabolites of **2a** further, a large-scale (10 mL) microsomal incubation with *dual-labeled* [1′-^{13}C, 1′-^{14}C]-**2a** was conducted to confirm quantitation of metabolites and determine the overall mass balance. After a 90 min incubation, a 1 mL aliquot analyzed by HPLC (see Supporting Information)

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contained the same four UV-absorbing metabolite peaks as described above, while ^{14}C coeluted within the solvent front (22%), **M1** (27%), **6a** (19%), and unmetabolized **2a** (32%), giving a 100% mass balance for ^{14}C; as expected, aniline and **4** were observed but were not radioactive. Throughout the course of the incubation, the amount of ^{14}C eluting within the solvent front was nearly stoichiometric with the amount of **4** detected by UV. The remaining 9 mL of this incubation mixture was quenched with 15% $ZnSO_4$, centrifuged, and the supernatant (ca. 9.9 mL) was removed and concentrated in vacuo to ca. 500 μL for ^1H-decoupled ^{13}C NMR analysis. When compared to the ^{13}C NMR spectrum of a 90 min control incubation of microsomes containing MeCN vehicle and cofactor but no substrate, the ^{13}C NMR spectrum of the microsomal incubation with [1′-^{13}C]-**2a** showed four additional ^{13}C signals: two major peaks at δ 42.2 and δ 79.2, and two minor peaks at δ 33.6 and δ 179.9 (see Supporting Information). On the basis of the comparison to the ^1H-decoupled ^{13}C spectra of authentic standards of probable metabolites of **2a** (spiked directly into a substrate-free microsomal incubation matrix), three of the metabolite ^{13}C signals correspond to **6a** (δ 33.6), **11** (δ 79.2), and propionic acid (**12**, δ 179.9). Structural assignments of **11** and **12** were also confirmed by spiking the actual incubation sample with authentic standards and remeasuring the NMR spectra. After each individual addition of a standard compound, a concomitant increase in its respective metabolite ^{13}C signal was observed. Again, none of the ^{13}C signals observed correspond to either **3a** (δ 149.1) or **5** (δ 176.2), confirming that these known major products of SET oxidation of **2a** are *not* formed in the P450 system.

The compound with δ 42.2 may be **M1**. Although the identity of **M1** has not been established with certainty, many observations suggest that it is *p*-hydroxy-**2a**. For example, **M1** elutes between aniline and *N*-methylaniline (**4**) on reverse-phase HPLC, which attests to its increased polarity relative to the much later eluting **2a**. GC/EIMS experiments demonstrate that **M1** survives GC conditions, has a similar GC t_R to acetaminophen, and has a MS fragmentation pattern similar to other related anilines. **M1** could conceivably be **13**, the putative carbinolamine metabolite precursor to both **11** and **4**, but this compound would not be likely to survive very long in solution, much less under GC conditions. By comparison to an authentic standard, **M1** was shown not to be *N*-methylpropionanilide (**14**), which could possibly have formed by acid-catalyzed rearrangement[42] of **13**. It is possible that **M1** is the *N*-oxide of **2a**, but its easy extractability and survival under GC conditions render this unlikely. Finally, given the propensity of CYP2B1 to *p*-hydroxylate monosubstituted benzenes, we believe **M1** is most likely *p*-hydroxy-**2a**.

The isomerization of cyclopropanone hydrate (**11**) to propionic acid (**12**) is well known.[43,44] Control experiments demonstrated that heating **11** at temperatures encountered during the concentration of incubation supernatants (ca. 70 °C) causes partial (ca. 20%) isomerization of **11** to **12**, whereas this isomerization occurs quantitatively upon acid reflux. Thus, as further confirmation of the presence of **11** in the incubations, 50 μL of concentrated HCl was added to the incubation NMR sample, and the solution was heated at 90 °C for 48 h. Subsequently, a ^1H-decoupled ^{13}C NMR spectrum showed the complete disappearance of the δ 79.2 signal of **11** *and a corresponding increase* in the δ 179.9 signal of **12**. Acid reflux also degraded the δ 42.2 signal, leaving that of **12** as the only ^{13}C-enriched signal detected in the acidified incubation mixture.

In our previous studies on the oxidation of **2a** by HRP, we observed that cyanide ion efficiently traps an iminium ion metabolite intermediate. The trapping product, identified as cyanotetrahydroquinoline **15**, is the major metabolite formed in the presence of 1 mM KCN.[38] Because cyanide has also been used to trap iminium intermediates in microsomal incubations,[45−47] we separately subjected **2a** and [1′-^{13}C]-**2a** to PB microsomes in the presence of 2 mM cyanide and looked for any cyanide adducts. Cyanide had no effect on either the rate or the extent of oxidation of **2a** relative to incubations conducted in the absence of cyanide, and no cyanide adducts (*m*/*z* 172, corresponding to **2a** − H + CN) were detected at any time during the incubation; only **M1**, **4**, and **6a** were observed in their usual proportions.

The above results, and most particularly the observation that cytochrome P450 generates *no* cyclopropane ring-opened metabolites from **2a** despite its otherwise very extensive oxidative N-dealkylation, strongly suggest that P450-catalyzed N-dealkylation of **2a** does *not* proceed via an aminium radical cation formed by an SET mechanism. Just as clearly, microsomal N-dealkylation of **2a** must occur via an intermediate carbinolamine that dissociates to cyclopropanone hydrate (**11**) and *N*-methylaniline (**4**). Despite the improbability of $\mathbf{2a}^{+\bullet}$ as an intermediate in the P450 oxidation of **2a**, because the former could *potentially* undergo proton loss leading to carbinolamine **13** (Scheme 3), we also examined the microsomal oxidation of compound **2b**, the C-1′ *methyl* homologue of **2a**, for which carbinolamine formation by *any* mechanism is not possible.

Under conditions identical to those used for **2a**, PB microsomes oxidize **2b** *completely* within 30 min. The disappearance of **2b** followed first-order kinetics throughout ($k_{obs} = 0.098$ min^{-1}), and only two metabolites were detected by both HPLC and GC/EIMS, *N*-(1′-methyl)cyclopropylaniline (**6b**) and an unknown metabolite (**M2**); neither aniline nor *N*-methylaniline (**4**) were detected. Use of DNPH reagent failed to detect either of the potential ring-opened metabolites methyl vinyl ketone (**9b**) or 4-hydroxy-2-butanone (**10b**), although, again, both were stable and easily detectable in spiked incubations at levels corresponding to 5% conversion. Metabolites **6b** and **M2** are formed in nearly equimolar amounts, and both are stable in the incubation mixture for at least 60 min after the full consumption of **2b**. GC/EIMS studies show that **M2** has *m*/*z* 177 (corresponding to **2b** + ^{16}O). On the basis of analogy to the conversion of **2a** to **M1**, and the similarity of **M1** and **M2** in terms of physical properties (i.e., HPLC and GC behavior), **M2** is believed to be *p*-hydroxy-**2b**. This also tends to reinforce the conclusion that **M1** is *p*-hydroxy-**2a**, because with **2b**, a carbinolamine analogous to **13** is not possible.

The results of the microsomal oxidation of **2b** parallel observations made with **2a** and demonstrate conclusively that

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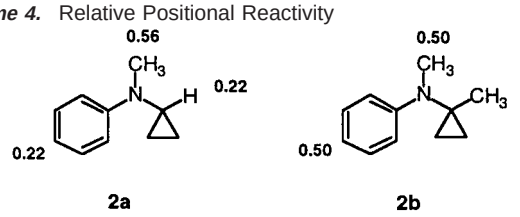
Scheme 4. Relative Positional Reactivity



2a **2b**

these *N*-cyclopropyl-*N*-methylanilines are metabolized via a non-SET mechanism. This contradicts the view that whenever there exists a mechanistic choice with a low $E_{1/2}$ amine, one-electron transfer is the preferred mechanism of oxidation by a high-valent hemoprotein or biomimetic system.[21,22,48] *N*-Cyclopropylanilines **2a** and **2b** are indeed low $E_{1/2}$ amines[49] and are readily oxidized by HRP to products arising exclusively via cyclopropyl ring fragmentation.[28,38,50] However, their metabolism by P450 is *totally* different. The absence of a hydrogen substituent on C-1′ of the cyclopropane ring in **2b** completely blocks P450 attack on the cyclopropane moiety, leaving N-demethylation and ring hydroxylation as the only alternatives; thus the ratio of *p*-hydroxylation to N-demethylation increases from 22:56 with **2a** to 1:1 with **2b**. This type of "metabolic switching" (viz., Scheme 4) is typical of P450 reactions in which a ferryl oxygen can select among different reactive positions in a substrate that can reorient itself within the active site cavity faster than it undergoes irreversible oxidation.[51−54]

Finally, because PB microsomes contain other P450 isozymes in addition to CYP2B1 (which can comprise 50−77% of total P450),[55,56] we carried out oxidations of **2a** using purified, reconstituted CYP2B1 isolated from PB-induced rat liver. GC/EIMS analysis of ether extracts of these incubations showed lower but quite significant overall conversion than with microsomes (ca. 20% after 60 min), but **4**, **6a**, and **M1** were again formed in the same ratio as observed with PB microsomes. Thus, at least in the purified reconstituted system, the formation of these three metabolites is characteristic of a single P450 active site.

Summary and Conclusions

The direct observation of cyclopropanone hydrate (**11**) as the only C_3 metabolite resulting from the P450-catalyzed N-dealkylation of **2a** is striking and completely unprecedented. Depending on one's view of the controversy over SET versus non-SET N-dealkylation mechanisms (viz., Scheme 3), the formation of **11** is either just as expected or completely unexpected. In our view, the complete lack of formation of *any* ring-opened metabolites from **2a** or **2b**, despite their relative ease of SET oxidation and the demonstrated overwhelming tendency of cyclopropyl aminium cation radicals to undergo cyclopropane ring-opening, suggests that the intermediacy of discrete aminium ion intermediates in the P450-catalyzed oxidation of these simple *N*-cyclopropylanilines would appear to be extremely unlikely. Several authors have noted that rates of diagnostic rearrangements of reactive intermediates ("radical clocks") in solution may be different in enzyme active sites.[57−59] An often-cited concern is the steric constraint that might be imposed by the amino acid side chains which define the size and shape of the active site, whereby a "tight" fit of the intermediate in the active site "pocket" might cause a radical clock to "run slow" and not give rearranged products that might otherwise be expected. In the present case, however, the occurrence of metabolic switching with **2a** and **2b** (Scheme 4), and with numerous deuterated medium-sized substrates,[51−54] suggests that substrates **2a** and **2b** have ample room and time to rotate and reorient within the P450 active site prior to undergoing any reaction committed to product formation. Thus, steric hindrance to rearrangement is unlikely to be the reason that ring-opened products from **2a** and **2b** are not observed. More likely, in our opinion, the absence of rearranged C_3 products signals the involvement of non-SET mechanism(s) in their oxidation by P450.

The formation of **11** clearly requires a pathway involving C-hydroxylation (whether by HAT or an alternative insertion-like mechanism) and a carbinolamine intermediate, that is, **2a** → **13** → (**4** + **11**). In the case of **2b**, replacement of the critical α-hydrogen of **2a** by a methyl group results in classical "metabolic switching" to *p*-hydroxylation as a less-unfavorable alternative to attack on the cyclopropane moiety or its methyl substituent (Scheme 4). The observation that P450 apparently prefers to *hydroxylate* **2a**, despite its relatively low one-electron oxidation potential, poses a serious question for the long-standing *hypothesis*[4,5] that SET chemistry explains the mechanism-based inactivation of P450 by **1b** (and perhaps **1a**), because as *amines*, they are much less easily oxidized than *anilines* **2a** and **2b**. Perhaps suicide inactivation of P450 by **1b** (and perhaps **1a**) results from a minor SET side reaction off a major catalytic cycle involving C-hydroxylation via a non-SET mechanism. Additional studies to clarify these issues are under way in our laboratory.

Experimental Section

Materials. Chemicals and solvents of reagent or HPLC grade were supplied by Aldrich Fine Chemical Co. (Milwaukee, WI), TCI America (Portland, OR), and Fisher Scientific (Pittsburgh, PA). Ultima Gold XR liquid scintillation counter (LSC) cocktail was purchased from the Packard Instrument Co. (Meriden, CT). Water for incubation reagents and chromatography was distilled, followed by passage through a Millipore Milli-Q water system. Tetrahydrofuran (THF) was distilled under nitrogen from sodium and benzophenone. MeCN, Et_2O, and CH_2Cl_2 were distilled under nitrogen from CaH_2 prior to use. Compounds **3**, [1′-^{14}C]-**3**, [1′-^{13}C]-**3**, **7**, **11**, and **12** were prepared as described elsewhere and were fully characterized by TLC, NMR, HPLC, and capillary gas chromatography/electron-impact mass spectrometry (GC/EIMS).[38,50] Unless otherwise noted, all air- and moisture-sensitive reactions were performed under a nitrogen environment. Analytical thin-layer chromatography (TLC) was conducted on Analtech Uniplate 250

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μ silica gel plates with detection by UV light and I_2. For flash chromatography, Selecto Scientific 63-200 mesh silica gel was employed. ^{1}H NMR spectra were obtained at either 400 or 500 MHz with Bruker DRX 400 and DRX 500 spectrometers, while ^{1}H-decoupled ^{13}C NMR spectra were obtained at 100.6 MHz on a Bruker 400 spectrometer or 125.8 MHz on a Bruker 500 spectrometer. Proton chemical shifts are reported in ppm (δ) relative to tetramethylsilane as inferred from shifts of residual protons in the deuterated solvents (i.e., 7.26 ppm for $CDCl_3$, 4.80 ppm for D_2O, and 2.05 ppm for d_6-acetone); ^{13}C chemical shifts are in ppm relative to internal $CDCl_3$ (77.23 ppm).

Microsome Preparation. Male Sprague−Dawley rats (188−205 g), pretreated with sodium phenobarbital (80 mg/kg, ip for 3 days), were anesthetized with carbon dioxide and euthanized by decapitation. Their livers were immersed in ice-cold KH_2PO_4 buffer (50 mM, pH 7.4) containing 150 mM potassium chloride and 5 mM Na_2EDTA. The unperfused livers were individually sliced with scissors, and the mince was homogenized with a Teflon/glass homogenizer in ice-cold 50 mM phosphate buffer (pH 7.4) containing 150 mM potassium chloride and 5 mM Na_2EDTA using 3 mL of buffer/g liver. The homogenate was first centrifuged at 1000*g* for 10 min, and that supernatant was centrifuged again at 12 000*g* for 20 min. The supernatant was again decanted and centrifuged at 100 000*g* for 60 min, and the supernatant was discarded. The microsomes were removed from the glycogen pellet by swirling gently with cold phosphate buffer. After being rehomogenized in buffer, the microsomes were resedimented at 100 000*g* for 60 min. The resulting microsomal pellet was resuspended in ice-cold 100 mM KH_2PO_4 (pH 7.4) buffer containing 1 mM Na_2EDTA and glycerol (20%, v/v) and frozen at −70 °C. The protein concentration (80.1 mg/mL) was determined by the method of Bradford[60] using bovine serum albumin as a standard. The cytochrome P450 and b_5 contents (2.66 nmol/mg protein and 0.87 nmol/mg protein, respectively) were determined by difference spectra according to Omura and Sato.[61] Just prior to incubation, thawed microsomes were diluted 50% with 100 mM KH_2PO_4 (pH 7.4) buffer.

Microsomal Incubations. Incubations (10 mL) were performed in a capped 25 mL Erlenmeyer flask under air at 37 °C in a shaking water bath. Each incubation contained 500 μL of microsomes (2 mg of protein/mL 0.1 M KH_2PO_4 buffer (pH 7.4)), an NADPH regeneration system composed of 100 μmol of glucose 6-phosphate and 13.3 units of glucose 6-phosphate dehydrogenase, 50 μL of 100 mM aniline substrate dissolved in acetonitrile (yielding an initial substrate concentration of 0.5 mM), and 5 μmol of NADPH. Sample aliquots (1 mL) were removed by micropipet at 0, 2, 5, 10, 15, 20, 30, 45, 60, and 90 min after the addition of NADPH and quenched with 15% $ZnSO_4$ (200 μL) to precipitate microsomal protein. The protein was sedimented by centrifugation, and the supernatant (1 mL) immediately underwent three analytical procedures: 20 μL was analyzed by HPLC (A_{254} and A_{320}); 400 μL was diluted with H_2O (100 μL), extracted with Et_2O (300 μL), and a 1 μL aliquot of the organic layer was analyzed by GC/EIMS; and 500 μL was treated with 15 μL of 0.15 M DNPH trapping solution for 30 min at room temperature, the solution was extracted with EtOAc (2 × 250 μL), and a 1 μL aliquot of the organic layer was analyzed by GC/EIMS. For the determination of ^{14}C within microsomal protein, the precipitated pellet was treated with 2 N KOH (200 μL) at room temperature for 48 h following supernatant removal. An aliquot (100 μL) of the basic solution was mixed with LSC-cocktail (6 mL) and analyzed by LSC.

Reconstituted CYP2B1 Incubation Procedure. CYP2B1[62] and NADPH-cytochrome P450 reductase[63] were isolated as described. For reconstitution of monooxygenase activity, P450 (0.75 nmol in 10 μL of buffer), reductase (1.5 nmol in 13 μL of buffer), and dilauroylphosphatidyl choline (32 nmol in 20 μL of buffer) were combined and vortexed occasionally over 40 min at room temperature. After this preincubation, 434 μL of phosphate buffer (0.1 M, pH 7.4) and 500 nmol of substrate (in 2.5 μL of MeCN) were added, and the mixture was incubated at 37 °C in a shaking water bath for 3 min. NADPH (1.0 μmol in 20 μL of buffer) was added to initiate the reaction and again after 30 min; after 60 min at 37 °C, the reaction was quenched with aqueous $ZnSO_4$ solution (15% w/v, 100 μL) and extracted with Et_2O (300 μL) for GC/EIMS analysis.

Metabolite Analysis by HPLC and GC/EIMS. Procedures for the direct analysis of metabolites and their derivatives by HPLC and GC/EIMS have been described in detail previously.[28] The preparation and analysis of standard solutions of potential carbonyl metabolites, and the characterization of authentic DNP-carbonyl standards for formaldehyde, acetone, acrolein (**9a**), and 3-hydroxypropanal (**10a**), are also described elsewhere;[28] this same procedure was employed to synthesize the DNP-adducts of the potential acyclic carbonyl metabolites of **2**. Thus, methyl vinyl ketone (**9b**, 420 μL, 5.1 mmol) was added dropwise with stirring to 0.15 M DNPH trapping solution (35 mL, 5.3 mmol); a bright orange precipitate formed instantaneously. After 30 min, the orange crystals were isolated by suction filtration, washed with H_2O (100 mL), and purified by flash chromatography (10% EtOAc in hexanes) to afford a cyclic DNP derivative (2-(2,4-dinitrophenyl)-(2-methyl)2,3-dihydropyrazole, 204 mg, 16%) as bright orange crystals. mp 148−149 °C. TLC (hexanes:EtOAc, 10:1) R_f = 0.32. ^{1}H NMR (400 MHz, d_6-acetone): δ 2.25 (s, 3H), 5.63 (d, J = 11.0 Hz, 1H), 5.90 (d, J = 17.8 Hz, 1H), 6.65 (m, J = 9.6 Hz, 1H), 8.08 (d, J = 9.6 Hz, 1H), 8.44 (dd, J = 8.3, 1.7 Hz, 1H), 9.02 (d, J = 2.6 Hz, 1H), 11.25 (s, 1H). GC t_R = 25.0 min. EIMS: $[M]^+$ = 250. Anal. Calcd for $C_{10}H_{10}N_4O_4$: C, 48.00; H, 4.03; N, 22.39. Found: C, 48.30; H, 4.20; N, 22.14. Similarly, 4-hydroxy-2-butanone (**10b**, 500 μL, 5.8 mmol) was used as described above and afforded the DNP derivative of methyl vinyl ketone (150 mg, 10%).

NMR Analysis of Metabolites. Microsomal incubations were conducted as described above and contained 5 μmol of substrate **2a** in a final volume of 10 mL. Incubations using both [1′-^{14}C]-**2a** and [1′-^{13}C]-**2a** were also conducted similarly and contained 2.5 μmol of each isotopically labeled probe. After 90 min (>80% oxidation of **2a** as determined by HPLC), the incubation was quenched with 15% $ZnSO_4$ (2 mL), and the microsomal protein was sedimented by centrifugation. The supernatant (~10 mL) was transferred by Pasteur pipet to a separate vial in which it was concentrated in vacuo to ca. 500 μL. The concentrate was added to a 5 mm NMR tube containing 50 μL of D_2O, and the ^{1}H-decoupled ^{13}C NMR spectrum was obtained by a Bruker 500 MHz NMR at a spectral frequency of 125.8 MHz (NS = 64 250). Spectra of authentic standards of **11** and **12** were obtained similarly. **11** ^{13}C NMR (125.8 MHz, D_2O): δ 13.54, 79.21. **12** ^{13}C NMR (125.8 MHz, D_2O): δ 8.16, 27.00, 179.94.

***N*-Methylacetanilide.** Acetyl chloride (450 μL, 6.3 mmol) was added dropwise to an ice-cooled stirred solution of Kugelrohr-distilled *N*-methylaniline (850 μL, 7.8 mmol) and triethylamine (1.1 mL, 7.9 mmol) in CH_2Cl_2 (10 mL). After 10 min at 0 °C, the solution was stirred at room temperature for 3 h. The reaction was quenched with 1 N HCl (20 mL), extracted with CH_2Cl_2 (5 × 5 mL), dried ($MgSO_4$), and concentrated in vacuo. The crude solid was recrystallized from pentane to afford the title compound (938.8 mg, 99%) as white crystals. mp 98−100 °C. TLC (EtOAc:hexanes, 1:1) R_f = 0.32. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.87 (s, 3H), 3.27 (s, 3H), 7.19 (d, J = 7.4, 2H), 7.34 (t, J = 7.4, 1H), 7.42 (t, J = 7.5, 2H). ^{13}C NMR (100.6 MHz, $CDCl_3$): δ 22.59, 37.33, 121.71, 127.26, 127.88, 129.90, 170.74. GC t_R = 10.7 min. EIMS: $[M]^+$ = 149.

***N*-(1′-Methyl)cyclopropyl-*N*-methylaniline (2b).[64]** Ethylmagnesium bromide (3.0 M in Et_2O, 3.4 mL, 10 mmol) was added dropwise to a

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vigorously stirred solution of *N*-methylacetanilide (500 mg, 3.4 mmol) and titanium isopropoxide (1.3 mL, 4.4 mmol) in THF (20 mL). The solution was heated at 65 °C for 30 min and then stirred at room temperature for 24 h. After quenching the reaction with saturated NH_4Cl (15 mL) and removing the THF under reduced pressure, we diluted the crude solution with 1 N HCl (15 mL), extracted it with Et_2O (5 × 5 mL), dried it ($MgSO_4$), and concentrated it in vacuo. The crude oil was purified by flash chromatography (2.4% Et_2O in pentane) to afford **16** (171 mg, 31%) as a colorless oil. TLC (pentane:Et_2O, 40:1) $R_f = 0.66$. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.71 (s, 2H), 0.86 (s, 2H), 1.25 (s, 3H), 2.93 (s, 3H), 6.70 (t, $J = 7.2$ Hz, 1H), 6.90 (d, $J = 8.3$ Hz, 2H), 7.23 (t, $J = 8.1$ Hz, 2H). ^{13}C NMR (100.6 MHz, $CDCl_3$): δ 17.04, 17.73, 36.62, 37.93, 113.40, 116.51, 129.06, 148.86. GC t_R = 13.0 min. EIMS: $[M]^+ = 161$.

***N*-Methylpropionanilide (14).** Propionic anhydride (600 μL, 4.7 mmol) was added dropwise to a solution of Kugelrohr-distilled *N*-methylaniline (500 μL, 4.6 mmol) and 4-(dimethylamino)pyridine (573 mg, 4.7 mmol) in CH_2Cl_2 (10 mL) and stirred at 25 °C for 24 h. The organic solution was washed with 1 N HCl (4 × 10 mL), dried ($MgSO_4$), and concentrated in vacuo. The crude oil was purified by flash chromatography (20% EtOAc in hexanes) to afford **14** (731 mg, 97%) as a white crystalline solid. mp 57−59 °C. TLC (hexanes:EtOAc, 4:1) $R_f = 0.32$. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.05 (t, $J = 7.4$ Hz, 3H), 2.09 (q, $J = 7.1$ Hz, 2H), 3.27 (s, 3H), 7.19 (d, $J = 7.3$ Hz, 2H), 7.34 (t, $J = 7.3$ Hz, 1H), 7.42 (t, $J = 7.5$ Hz, 2H). ^{13}C NMR (100.6 MHz, $CDCl_3$): δ 9.85, 27.64, 37.44, 127.43, 127.82, 129.85, 144.38, 174.07. GC t_R = 11.7 min. EIMS: $[M]^+ = 163$.

1-Ethoxycyclopropanol.[65] (1-Ethoxycyclopropyl)oxy-trimethylsilane (3 mL, 14.9 mmol) was added all at once to MeOH (10 mL), and the solution was stirred at room temperature for 21 h. The title compound (1.4 g, 92%) was isolated from the reaction solution by rotary evaporation as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.90 (dm, 4H), 1.18 (t, $J = 7.1$ Hz, 3H), 3.74 (q, $J = 7.1$ Hz, 2H), 4.58 (br s, 1H). ^{1}H NMR (400 MHz, D_2O): δ ppm 0.90 (dm, 4H), 1.14 (t, $J = 7.1$ Hz, 3H), 3.71 (q, $J = 7.1$ Hz, 2H). ^{13}C NMR (100.6 MHz, $CDCl_3$): δ 14.26, 15.48, 62.09, 85.57.

Cyclopropanone Hydrate (11).[66] A solution of 1-ethoxycyclopropanol (80 mg, 780 μmol) in 400 μL of KH_2PO_4 buffer (0.1 M, pH 7.4) and 50 μL of D_2O was heated at 100 °C for 10 min in a 5 mm NMR tube to afford **11** in quantitative yield. The conversion of the hemiketal to **11** was observable by using ^{1}H NMR to monitor the disappearance of the two doublets from the methylene protons of 1-ethoxycyclopropanol and the concomitant appearance of a singlet for the methylene protons of **11**. ^{1}H NMR (500 MHz, D_2O): δ 0.89 (s, 4H). ^{13}C NMR (125.8 MHz, D_2O): δ 13.80, 79.41.

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Supporting Information Available: HPLC chromatogram of an incubation of [1′-^{13}C, 1′-^{14}C]-**2a** with PB microsomes and ^{13}C NMR spectra of microsomal incubations with [1′-^{13}C]-**2a**, controls, and standards (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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