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# Mechanism of Oxidative Amine Dealkylation of Substituted *N,N*-Dimethylanilines by Cytochrome P-450: Application of Isotope Effect Profiles

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**Abstract:** Isotope effect profiles were determined for the deprotonation of a series of para-substituted *N*-methyl-*N*-(trideuteriomethyl)aniline cation radicals by pyridine and for hydrogen atom abstraction from the corresponding neutral amines by the *tert*-butoxyl radical. The profiles model reaction steps in two mechanisms commonly proposed for the oxidative dealkylation of amines by cytochrome P-450. Isotope effect profiles were also determined for the P-450 oxidation of the same set of *N,N*-bis(dideuteriomethyl)anilines by purified CYP2B1, expressed CYP2B1, phenobarbital-induced microsomal P-450, expressed CYP4B1, expressed CYP1A2, and purified CYP102 (BM3). The profiles for all of the P-450 oxidations were found to be experimentally indistinguishable from the hydrogen atom abstraction profile, and distinctly different from the deprotonation profile. This agreement provides strong evidence that the P-450 oxidatively dealkylates the amines by a hydrogen atom abstraction mechanism. Furthermore, the P-450 isotope effect profiles indicate that the reaction mechanism is conserved in both mammalian and bacterial enzymes.

## Introduction

The oxidative dealkylation of amines can be catalyzed by a number of different enzymes including monoamine oxidase, horseradish peroxidase, hemoglobin, myoglobin, lactoperoxidase, chloroperoxidase, and the P-450 cytochromes.[1,2] Knowledge of the molecular mechanisms for the catalysis of *N*-dealkylation reactions is important to our understanding of disease states, such as Parkinson's disease, and in our understanding of the metabolism of xenobiotics and endogenous compounds. To date, it has proved difficult to establish definitive methods for elucidating the mechanisms of these important biotransformation reactions. Thus, these reactions provide an ongoing challenge to mechanistic enzymologists. We describe a new, general strategy for solving these problems and illustrate its application to the mechanism of P-450 oxidations.

Amine oxidations by cytochrome P-450 (abbreviated as O=Fe(IV)−P$^{\bullet+}$) have been proposed to proceed by either an electron/proton transfer mechanism (A) or a hydrogen atom abstraction mechanism (B).[3] The magnitudes of hydrogen/deuterium kinetic isotope effects for amines labeled at the carbon atom α to nitrogen have been previously used to distinguish these mechanisms.[2]

It was believed that the electron/proton transfer mechanism would show small isotope effects and that the hydrogen atom abstraction mechanism would show large isotope effects. On the basis of the fact that small isotope effects were observed for amine dealkylations by P-450, an electron/proton transfer mechanism was proposed for the reaction.[2] This mechanism

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(1) Silverman, R. B. In *Advances in Electron Transfer Chemistry*; Mariano, P. S., Ed.; JAI Press: Greenwich, CT, 1992; Vol. 2, p 177.
(2) Miwa, G. T.; Walsh, J. S.; Kedderis, G. L.; Hollenberg, P. F. *J. Biol. Chem.* **1983**, *258*, 14445.
(3) (a) *Cytochrome P-450*; Ortiz de Montellano, P. R., Ed.; Plenum Press: New York, 1986. (b) Guengerich, F. P.; Macdonald, T. L. *FASEB J.* **1990**, *4*, 2453. (c) Porter, T. D.; Coon, M. J. *J. Biol. Chem.* **1991**, *266*, 13469. (d) Hollenberg, P. F. *FASEB J.* **1992**, *6*, 686. (e) Guengerich, F. P.; Macdonald, T. L. In *Advances in Electron Transfer Chemistry*; Mariano, P. S., Ed.; JAI Press: Greenwich, CT, 1993; Vol. 3, p 191.

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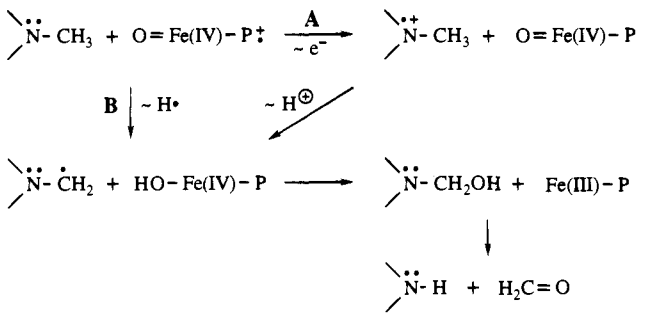
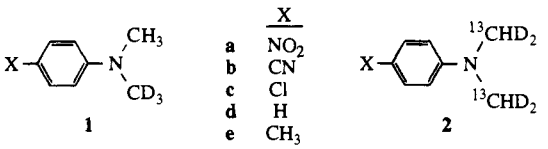


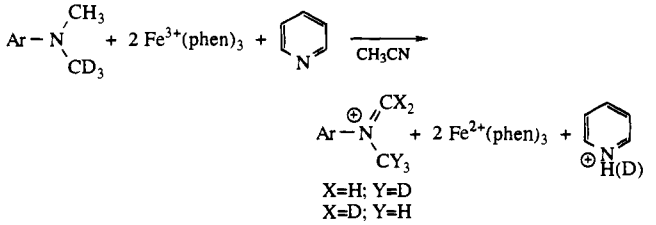
has been generally accepted for the past decade. Recently, direct measurements of isotope effects for the deprotonations of amine cation radicals and for hydrogen atom abstractions from amines showed that isotope effect magnitudes *cannot* distinguish the proposed P-450 mechanisms.[4] Consequently, evidence for the electron/proton transfer mechanism has seriously eroded.

We recently proposed a potentially more discriminating approach to this mechanistic problem.[4] It is also based on isotope effects, but uses them quite differently. The strategy is to compare *isotope effect profiles* for model deprotonation and hydrogen atom abstraction reactions to profiles from P-450 oxidations to determine which model reaction best fits the P-450 results. Using this approach, the assignment of reaction mechanism no longer rests on the magnitude of a single isotope effect, but rather on the response of the isotope effects to variations in chemical structure. Isotope effect profiles have been determined for the oxidation of a series of para-substituted *N,N*-dimethylanilines (**1** and **2**) by two model systems and four isoforms of P-450 in order to test this strategy. Our results are described herein.



## Results and Discussion

The deprotonation isotope effect profile for the cation radicals of **1a**–**e** was determined using pyridine as a base. The cation radicals were generated by *in situ* oxidation with $Fe^{3+}(1,10\text{-phenanthroline})_3(PF_6^-)_3$, a known outer-sphere one-electron oxidant.[5] Pyridine was chosen as a base because of its moderate basicity and because it is difficult to oxidize. The exact choice of base is not particularly crucial to our experimental strategy because we are primarily interested in the functional dependence of the isotope effects on the para substituents, i.e., the isotope effect profile, rather than in the exact magnitudes of the isotope effects.



The mechanism for the above reaction is well established[6] and is analogous to path A. As shown in Figure 1, it begins with one-electron oxidation of the amines by $Fe^{3+}(phen)_3$. The resulting cation radicals are then deprotonated by pyridine (present in large excess) at either the methyl or the trideuteriomethyl group, resulting in isotopic fractionation. The strongly reducing α-amino radicals[7] produced by deprotonation are oxidized to the product iminium ions by a second equivalent of $Fe^{3+}(phen)_3$.

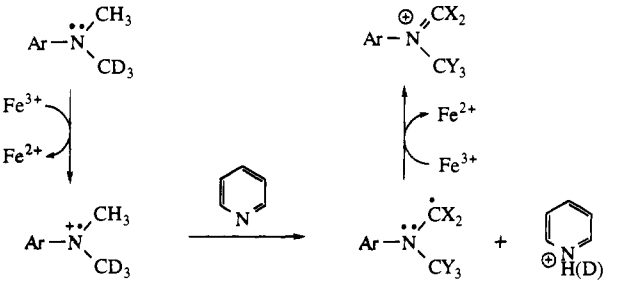


**Figure 1.** Mechanism for the reaction of para-substituted *N,N*-dimethylanilines with $Fe^{3+}(1,10\text{-phenanthroline})_3$ and pyridine.

The iminium ion products were hydrolyzed and the resulting secondary amines were derivatized with trifluoroacetic anhydride. The isotope effects were defined as the $d_3/d_0$ ratios of the trifluoroacetylated amines, which were measured by gas chromatography–mass spectrometry (GC–MS). The results are given in Table 1. It is seen that the isotope effects first increase and then decrease with increasing $pK_a$ of the cation radicals. This kind of bell-shaped isotope effect profile has been observed in numerous other proton transfer reactions,[8] including the deprotonation of amine cation radicals,[9] and is related to the varying extents of bond-making and bond-breaking in the transition states.[8]

To model mechanism B, the isotope effect profile for hydrogen atom abstraction from amines **1a**–**d** was determined using the *tert*-butoxyl radical ($^{\bullet}OBu^t$) as the hydrogen atom abstracting agent. This radical was chosen because it unambiguously abstracts hydrogen atoms from the α-carbon atoms of amines (vide infra).[10] The *tert*-butoxyl radical was generated by the dye-sensitized photoreduction of *N-tert*-butoxy-4-phenylpyridinium hexafluorophosphate, $\mathbf{3}^+$.

The mechanism for the reaction is shown in Figure 2 and follows literature precedence. One-electron reduction of $\mathbf{3}^+$ by the singlet excited state of the coumarin dye gives the *N-tert*-butoxy-4-phenylpyridinyl radical ($\mathbf{3}^{\bullet}$) and the dye cation radical.[11] *N*-Alkoxypyridinyl radicals are known to undergo rapid fragmentation to the corresponding pyridine and an alkoxy radical.[11] The *tert*-butoxyl radical then reacts with **1a**–**d** by hydrogen or deuterium atom abstraction from the α-methyl groups.[10] Finally, the resulting α-amino radicals are oxidized

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(5) (a) Fukuzumi, S.; Wong, C. L.; Kochi, J. K. *J. Am. Chem. Soc.* **1980**, *102*, 2928. (b) Schlesener, C. J.; Amatore, C.; Kochi, J. K. *J. Phys. Chem.* **1984**, *90*, 3747. (c) Schlesener, C. J.; Kochi, J. K. *J. Org. Chem.* **1984**, *49*, 3142.

(6) (a) Fukuzumi, S.; Kondo, Y.; Tanaka, T. *Chem. Lett.* **1982**, 1591. (b) Fukuzumi, S.; Kondo, Y.; Tanaka, T. *J. Chem. Soc., Perkin Trans. 2* **1984**, 673. (c) Fukuzumi, S.; Tokudo, Y.; Fujita, M. *J. Phys. Chem.* **1992**, *96*, 8413. (d) Fukuzumi, S.; Tokudo, Y.; Kitano, T.; Okamoto, T.; Otera, J. *J. Am. Chem. Soc.* **1993**, *115*, 8960.

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(8) (a) Bell, R. P. *The Proton in Chemistry*, 2nd ed.; Cornell University Press: Ithaca, NY, 1973; Chapter 12. (b) Melander, L.; Saunders, W. H., Jr. *Reaction Rates of Isotopic Molecules*; Wiley: New York, 1980; Chapter 5.

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**Table 1.** Isotope Effect Profiles for Deprotonation of the Cation Radicals of **1a–e** by Pyridine, for Hydrogen Atom Abstraction from Amines **1a–d** and **2a–d** by $^{\bullet}OBu^{t}$, and for Oxidative Dealkylation of Amines **2a–d** by Various Cytochrome P-450's

| | | isotope effect ($k_H/k_D$)[a] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| substituent X | $pK_a$[b] ($ArNMe_2^{\bullet+}$) | $ArNMe_2^{\bullet+}$ + pyridine | $ArNMe_2$ + $^{\bullet}OBu^{t}$ | CYP2B1 (purified) | CYP2B1 (expressed) | rat liver microsomes | CYP4B1 (expressed) | CYP1A2 (expressed) | CYP102 (purified) |
| $NO_2$ | 3 | 6.9(2) | 3.9(1)[c]<br>3.8(1)[d] | 4.1(2) | 3.9(2) | 4.1(1) | 4.0(1) | 3.8(2) | 4.0(2) |
| CN | 6 | 7.4(2) | 3.7(1)[c]<br>3.5(1)[d] | 3.7(1) | 3.7(1) | 3.8(1) | 3.6(1) | 3.5(1) | 3.4(1) |
| Cl | 9 | 8.8(1) | 2.9(2)[c]<br>2.7(1)[d] | 2.6(2) | 2.7(2) | 2.8(1) | 2.9(1) | 3.0(2) | 2.6(1) |
| H | 9 | 7.8(2) | 2.6(1)[c]<br>2.5(1)[d] | 2.4(1) | 2.5(1) | 2.5(1) | 2.7(1) | 2.7(1) | 2.5(1) |
| $CH_3$ | 12 | 6.2(1) | | | | | | | |

[a] All isotope effects are an average of at least three independent determinations. The error (standard deviations) in the last significant digit is given in parentheses. [b] In acetonitrile (ref 15). [c] Isotope effects determined with amines **1a–d**. [d] Isotope effects determined with amines **2a–d**.

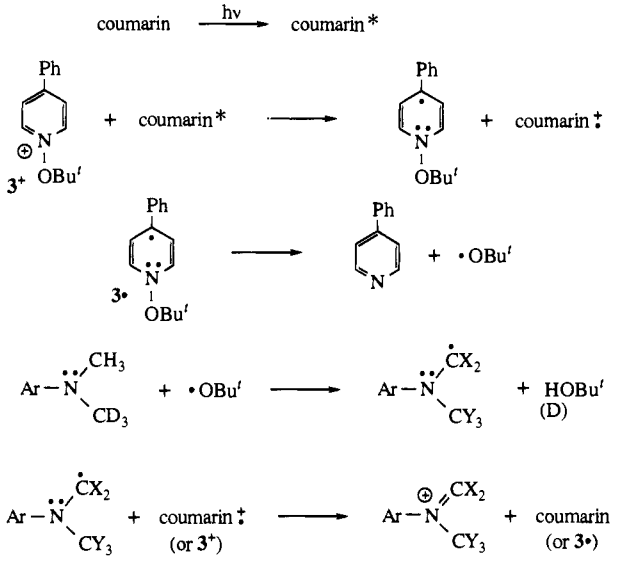
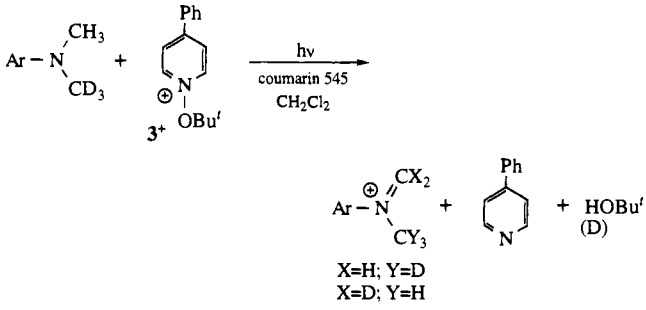



**Figure 2.** Mechanism for the coumarin-photosensitized reaction of para-substituted *N,N*-dimethylanilines with *N-tert*-butoxy-4-phenylpyridinium hexafluorophosphate.




to the product iminium ions by the dye cation radicals produced in the initial pyridinium cation reduction. Alternatively, the α-amino radicals might reduce **3**$^+$ to give the iminium ion products and **3**$^{\bullet}$, resulting in a chain reaction. The possibility of a chain reaction seems most likely for the *N,N*-dimethylanilines that do not have strongly electron-withdrawing substituents since their corresponding α-amino radicals should be better reducing agents. The fact that reaction efficiencies were found to increase as the para substituents became more electron-donating is consistent with this interpretation. Control experiments showed that no products were formed if either **3**$^+$ or the coumarin dye were omitted from the reaction. Isotope effects for the hydrogen atom abstractions (Table 1) were determined after hydrolysis of the iminium ion products as described above. The isotope effect determined for **1d** in this work is the same as that previously reported by using nanosecond transient absorption kinetic techniques, where the *tert*-butoxyl radical was generated by photolysis of di-*tert*-butyl peroxide.[4]

Figure 2 assumes a hydrogen atom transfer mechanism for the reaction between the substituted *N,N*-dimethylanilines and $^{\bullet}OBu^{t}$. It is possible, however, that the mechanism could instead involve a two-step, electron transfer/proton transfer process. We have previously suggested that this latter mechanism is unlikely based upon several lines of evidence.[4] The most crucial evidence was based on a comparison of the free energies of activation for the reaction of the substituted *N,N*-dimethylanilines with $^{\bullet}OBu^{t}$ ($\Delta G^{\ddagger}$) to the free energies of electron transfer from the *N,N*-dimethylanilines to $^{\bullet}OBu^{t}$ ($\Delta G^{\circ}_{et}$). In general, if $\Delta G^{\ddagger} < \Delta G^{\circ}_{et}$, then an electron transfer mechanism is thermodynamically excluded. On the other hand, if $\Delta G^{\ddagger} > \Delta G^{\circ}_{et}$, then an electron transfer is thermodynamically permitted, although not necessarily kinetically permitted.[12] Our previous analysis relied on an estimated reduction potential for $^{\bullet}OBu^{t}$ of ca. +0.5 V vs SCE in $CH_3CN$.[13] The estimated reduction potential has since been substantially revised. The currently accepted value is ca. −0.3 V vs SCE in $CH_3CN$.[14] For this reason we reanalyze the problem here. The current analysis also explicitly takes into account the fact that the redox potentials and the rate constant measurements were made in different solvents.

The activation free energies for reaction of the substituted *N,N*-dimethylanilines with $^{\bullet}OBu^{t}$ have been reported from measurements in low dielectric solvents ($\epsilon \approx 2$).[4] In contrast, the redox potentials for both $^{\bullet}OBu^{t}$ and $ArNMe_2$ are reported in acetonitrile ($\epsilon = 36$).[14,15] Therefore, in order to properly apply the electron transfer mechanism test, it is necessary to approximate the free energy of electron transfer in a nonpolar solvent from that in a polar solvent. Figure 3 shows two ways to do this. The quantity of interest, $\Delta G^{\circ}_{et}(CIP,\epsilon\approx2)$, is the free energy of electron transfer to form a contact ion pair (CIP) in a solvent with $\epsilon \approx 2$ relative to "free" $ArNMe_2$ and $^{\bullet}OBu^{t}$. This analysis makes the reasonable approximation that the free energy

(12) For a discussion on the estimation of electron transfer activation energies to assess reaction mechanism, see: Eberson, L. *Electron Transfer Reactions in Organic Chemistry;* Springer-Verlag: New York, 1987, Chapter 4.

(13) (a) Eberson, L. *Acta Chem. Scand. B* **1984**, *38*, 439. (b) Wayner, D. D. M. In *CRC Handbook of Organic Photochemistry*; Scaiano, J. C., Ed.; CRC Press: Boca Raton, FL, 1989; Vol. II, Chapter 18.

(14) On the basis of more reliable thermochemical data, the oxidation potential for the *tert*-butoxide anion in acetonitrile has been revised from the original estimate.[13] A detailed analysis will be published elsewhere: Workentin, M. S.; Maran, F.; Wayner, D. D. M. *J. Am. Chem. Soc.* **1995**, *117*, 2120. We thank Lennart Eberson (University of Lund) and Daniel Wayner (National Research Council, Canada) for a very helpful exchange of correspondence on this matter.

(15) Parker, V. D.; Tilset, M. *J. Am. Chem. Soc.* **1991**, *113*, 8778.

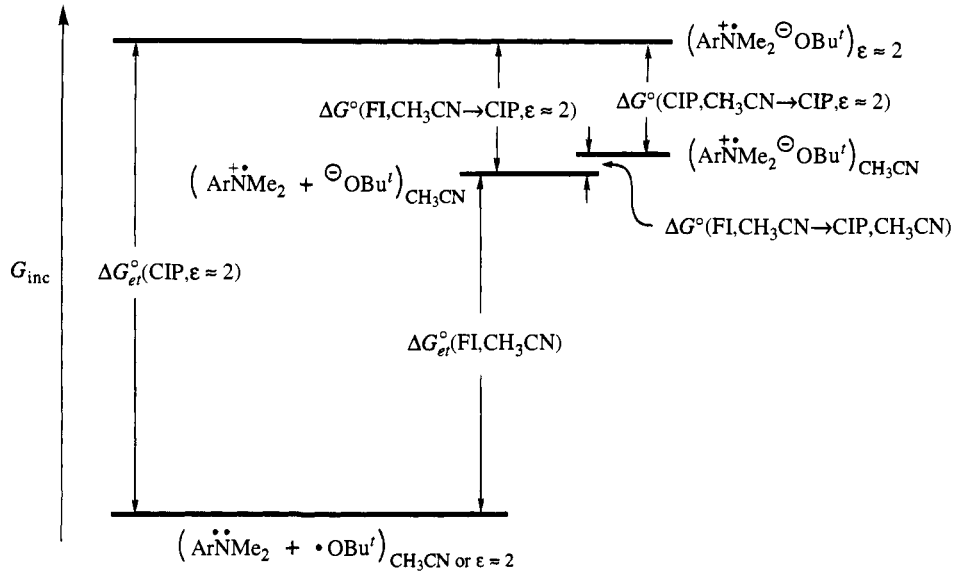



**Figure 3.** Energy scheme to estimate the free energy of electron transfer from $ArNMe_2$ to $^{\bullet}OBu^t$ in a solvent with $\epsilon \approx 2$.

of transfer of $ArNMe_2$ and $^{\bullet}OBu^t$ from $CH_3CN$ to a nonpolar organic solvent is negligible.[16] On the basis of the thermodynamic quantities in Figure 3, $\Delta G°_{et}(CIP,\epsilon\approx2)$ can be estimated from either eq 1 or eq 2. Both equations contain the term $\Delta G°_{et}(FI,CH_3CN)$ which is equal to $E_{ox}(ArNMe_2) - E_{red}(^{\bullet}OBu^t)$ in $CH_3CN$, i.e., the free energy of electron transfer to form free ions (FI) in $CH_3CN$. The second term in eq 1, $\Delta G°(FI,CH_3CN \rightarrow CIP,\epsilon\approx2)$, is the free energy difference between FI in $CH_3CN$ and a CIP in a solvent with $\epsilon \approx 2$. This has been recently estimated by Gould and co-workers for several organic ion radical pairs to be ca. +0.3 V.[17] $\Delta G°(FI,CH_3CN \rightarrow CIP,\epsilon\approx2)$ can also be approximated by the sum of the last two terms in eq 2, $\Delta G°(FI,CH_3CN \rightarrow CIP,CH_3CN) + \Delta G°(CIP,CH_3CN \rightarrow CIP,\epsilon\approx2)$. These are, respectively, the free energy differences between FI and a CIP in $CH_3CN$ and between a CIP in $CH_3CN$ and a CIP in a solvent with $\epsilon \approx 2$. The first term, $\Delta G°(FI,CH_3CN \rightarrow CIP,CH_3CN)$, has recently been estimated by Gould and co-workers to be ca. +0.05 V.[18] The second term, $\Delta G°(CIP,CH_3CN \rightarrow CIP,\epsilon\approx2)$, can be approximated by the semiempirical equation of Weller, eq 3,[19] which gives a value of +0.2 V for $\epsilon_1 = 36$ and $\epsilon_2 = 2$. Thus, eq 2 gives a total correction to $\Delta G°_{et}(FI,CH_3CN)$ of +0.25 V, i.e., essentially the same as that estimated from eq 1, +0.3 V. This finally results in eq 4.

$$\Delta G°_{et}(CIP,\epsilon\approx2) = \Delta G°_{et}(FI,CH_3CN) + \Delta G°(FI,CH_3CN \rightarrow CIP,\epsilon\approx2) \quad (1)$$

$$\Delta G°_{et}(CIP,\epsilon\approx2) = \Delta G°_{et}(FI,CH_3CN) + \Delta G°(FI,CH_3CN \rightarrow CIP,CH_3CN) + \Delta G°(CIP,CH_3CN \rightarrow CIP,\epsilon\approx2) \quad (2)$$

$$\Delta G°(CIP,CH_3CN \rightarrow CIP,\epsilon\approx2) \approx (0.75\ eV)\left(\frac{\epsilon_1 - 1}{2\epsilon_1 + 1} - \frac{\epsilon_2 - 1}{2\epsilon_2 + 1}\right) \quad (3)$$

Using the revised reduction potential for $^{\bullet}OBu^t$ of −0.3 V vs SCE in $CH_3CN$[14] and the oxidation potentials for $ArNMe_2$ in

$$\Delta G°_{et}(CIP,\epsilon\approx2) \approx \Delta E_{ox}(ArNMe_2) - \Delta E_{red}(^{\bullet}OBu^t) + 0.3\ V \quad (4)$$

$CH_3CN$,[15] $\Delta G°_{et}(CIP,\epsilon\approx2)$ for each amine can be calculated from eq 4. The results are given in Table 2. The table also contains the activation free energies reported for reaction of the substituted *N,N*-dimethylanilines with $^{\bullet}OBu^t$.[4] The results clearly show that $\Delta G^{\ddagger}(\epsilon\approx2) \ll \Delta G°_{et}(CIP,\epsilon\approx2)$ for all of the amines. These data definitively exclude an electron transfer mechanism for the reactions of $ArNMe_2$ with $^{\bullet}OBu^t$ in nonpolar solvents. The large differences between $\Delta G^{\ddagger}$ and $\Delta G°_{et}$ make improbable an electron transfer mechanism even in relatively polar, organic solvents. For example, we have recently shown that $\Delta G^{\ddagger}$ for the reaction of $PhNMe_2$ with $^{\bullet}OBu^t$ is rather insensitive to solvent polarity.[4] Furthermore, as discussed above, $\Delta G°_{et}$ can only be decreased by ~0.3 V or 7 kcal/mol on going from a nonpolar solvent to a polar solvent like $CH_3CN$. Thus, $\Delta G°_{et}$ in a polar organic solvent would still exceed $\Delta G^{\ddagger}$ by >18 kcal/mol, excluding an electron transfer mechanism.

For the coumarin-photosensitized reactions of amines **1a−d** with $\mathbf{3}^+$, we also considered the possibility that the amines might be oxidized to their cation radicals by intermediate dye cation radicals. The amine cation radicals might then be deprotonated by neutral $ArNMe_2$ to give α-amino radicals that are subsequently oxidized to the iminium ion products. If this mechanism is operating, it would have to be in competition with the hydrogen atom abstraction mechanism shown in Figure 2 in order to account for the formation of 4-phenylpyridine and $HOBu^t$. The measured isotope effects would then be a composite average of the two mechanisms. Several pieces of experimental evidence argue against oxidation of $ArNMe_2$ by the dye cation radicals. First, authentic one-electron oxidation of amine **1d** by $Fe^{3+}(phen)_3$ without any added base gives an isotope effect for formation of PhNHMe of 5.2 ± 0.1, which is much larger than the isotope effect measured for the reaction of **1d** with $\mathbf{3}^+$ ($k_H/k_D = 2.6 \pm 0.1$). Importantly, previous work has shown that the reaction of **1d** with $^{\bullet}OBu^t$ has an isotope effect of 2.5 ± 0.2, i.e., indistinguishable from that of the coumarin-sensitized reaction of **1d** with $\mathbf{3}^+$. These data rule out any significant oxidation of **1d** by the dye cation radicals. Second, simultaneous hydrogen atom and electron/proton transfer mechanisms require that the iminium ion/$HOBu^t$ product ratio be greater than 1. This is because iminium ions are formed

(16) Cf., Parker, A. J. *Chem. Rev.* **1969**, *69*, 1.
(17) Gould, I. R.; Noukakis, D.; Gomez-Jahn, L.; Young, R. H.; Goodman, J. L.; Farid, S. *Chem. Phys.* **1993**, *176*, 439.
(18) Gould, I. R.; Young, R. H.; Moody, R. E.; Farid, S. *J. Phys. Chem.* **1991**, *95*, 2068.
(19) Weller, A. *Z. Phys. Chem. Neue Folge* **1982**, *133*, 93.

**Table 2.** Comparison of $\Delta G°_{et}$ and $\Delta G^{‡}$ for $ArNMe_2$ + $^{•}OBu^{t}$

| substituted *N,N*-dimethylaniline | $E_{ox}$[a] (V) (SCE) | $\Delta G°_{et}$(CIP,$\epsilon\approx2$)[b] (kcal/mol) | $\Delta G^{‡}(\epsilon\approx2)$[c] (kcal/mol) |
|---|---|---|---|
| *p*-CN | 1.049 | 38 | 6.7 |
| *p*-Cl | 0.789 | 32 | 6.4 |
| *p*-H | 0.767 | 31 | 6.3 |

[a] In acetonitrile (ref 15). [b] Calculated using eq 4 in text. [c] Reference 4.

by both mechanisms whereas $HOBu^{t}$ is formed only by hydrogen atom abstraction. Experimentally, the product ratio was found to be 1.0 ± 0.1, excluding any significant one-electron oxidation of **1d** by the dye cation radical. Since **1d** is clearly not oxidized by the dye cation radical, it seemed unlikely that the other amines (**1a−c**) would be as well since they are more difficult to oxidize. Consistent with this expectation, the reactions of **1a−c** with $\mathbf{3}^{+}$ all had iminium ion/$HOBu^{t}$ product ratios of 1 within experimental error, consistent with a pure hydrogen atom abstraction mechanism.

Returning to the isotope effect profiles, it can be seen from the data in Table 1 that they are markedly different for cation radical deprotonation and hydrogen atom abstraction. In particular, the deprotonation profile increases and then decreases as the electron-donating ability of the para substituents increases, as is expected for a proton transfer reaction. In contrast, the hydrogen atom abstraction profile shows a small, steady decrease in the isotope effects. These differences clearly demonstrate that isotope effect profiles can serve as sensitive probes for distinguishing the P-450 amine dealkylation mechanisms.

The P-450 isotope effect profiles were determined with amines **2a−d**, which are preferred to amines **1a−d** because the latter can suffer from isotope effect masking due to slow exchange of the $CH_3/CD_3$ groups in the active site of the enzyme.[4] The $^{13}C$ labels in **2a−d** were incorporated purely for analytical reasons. The isotope effects for the P-450 demethylations of **2a−d** require that the formaldehyde-$d_2/d_1$ product ratios be determined by GC−MS after conversion to their dimedone adducts. Unfortunately, formaldehyde-$d_0$ is frequently a contaminant in many P-450 samples, and this prohibits accurate quantitation of formaldehyde-$d_1$ due to interference from the M + 1 ion of the formaldehyde-$d_0$ dimedone adduct. The $^{13}C$ label moves the molecular ion of the formaldehyde-$^{13}C$-$d_1$ adduct one mass unit higher and thus avoids interference from the M + 1 ion of the formaldehyde-$d_0$ adduct. The isotope effects are defined as 2 times the $d_2/d_1$ dimedone adduct ratios which statistically corrects for the number of H vs D in amines **2a−d**.

The P-450 isotope effect profiles were measured with six P-450 systems: (1) purified CYP2B1(rat),[20] (2) expressed CYP2B1(rat),[21] (3) phenobarbital-induced rat liver microsomes that principally contain CYP2B1,[22] (4) expressed CYP4B1-(rabbit),[21] (5) expressed CYP1A2(human),[21] and (6) purified CYP102(bacterial).[23] These enzymes cover four different P-450 families[24] and include P-450 from three mammalian sources as well as from a bacterium. It is clear from the data in Table 1 that the isotope effect profiles are independent of the source of P-450. More importantly, they reveal that the P-450 profiles are remarkably similar to that for hydrogen atom abstraction by $^{•}OBu^{t}$. In fact, the isotope effects for the two reactions are nearly the same for any given substrate, as are the substituent effects on the isotope effects! *This provides powerful evidence that P-450 reacts with all of the substituted N,N-dimethylanilines by a hydrogen atom abstraction mechanism.*

Since the isotope effects for hydrogen atom abstractions by $^{•}OBu^{t}$ and for the P-450 oxidations used different amines (**1a−d** vs **2a−d**), it seemed important to determine if the close agreement between the isotope effects was simply a fortuitous consequence of different secondary isotope effect contributions to the measured isotope effects. This was tested by measuring the isotope effects for hydrogen atom abstraction from amines **2a−d** by $^{•}OBu^{t}$. The results are shown in Table 1 and reveal that the isotope effects for hydrogen atom abstraction from amines **1a−d** are virtually the same as those for **2a−d**, which requires rather small secondary isotope effects.[25]

The nearly identical isotope effects for the reactions of the substituted *N,N*-dimethylanilines with $^{•}OBu^{t}$ and with P-450 suggest that the two reactive species have similar hydrogen atom abstracting abilities. As we have pointed out earlier,[4] this may not be so surprising. On the basis of the primary and secondary isotope effects observed for the $C_1$ hydroxylation of *n*-octane by P-450, Trager and co-workers have argued that the reaction involves a symmetric transition state structure for hydrogen atom transfer, which implies that the H−C bond being broken and the H−O being made have similar energies.[26] The H−$C_1$ bond dissociation energy for *n*-octane is ca. 101 kcal/mol.[27] The H−O bond dissociation energy in $HOBu^{t}$ is quite similar, 105 kcal/mol.[28] Therefore, the similar isotope effects for the reactions with $^{•}OBu^{t}$ and with P-450 appear to be quite reasonable assuming, of course, *that P-450 reacts by a hydrogen atom abstraction mechanism.* It will be interesting to see how general this correspondence turns out to be.

At this point it is important to ask whether the P-450 isotope effect profiles for amines **2a−d** can be accommodated by an electron/proton transfer mechanism. Perhaps, but the mechanism requires several unlikely features. The first relates to the nature of the base that is needed to account for the observed isotope effects. Note that the isotope effects for the cation radical deprotonations by pyridine in $CH_3CN$ show a maximum at a cation radical p$K_a$ of ~9. Although the P-450 isotope effect profiles show no maximum, it is clear from the trend in the data that the maximum would occur for a cation radical with p$K_a$ < 3. The shift of the isotope effect maximum to a lower

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(22) The isotope effects reported here for reaction of **2a** and **2b** with phenobarbital-induced P-450 microsomes differ slightly from those measured earlier using the corresponding amines that did not contain $^{13}C$.[4] We believe the isotope effects measured using **2a** and **2b** are more accurate for the reasons discussed in the text.

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(25) The primary ($z_1$) and secondary isotope effects ($z_2$) for the reaction of the para-substituted *N,N*-dimethylanilines with $^{•}OBu^{t}$ can be estimated from the experimental isotope effects for reaction with amines **1** and **2** from the following relations: $z_2 = [k_H/k_D(\mathbf{1})/k_H/k_D(\mathbf{2})]^{1/3}$ and $z_1 = [k_H/k_D(\mathbf{2})]z_2$. The calculated $z_2$ values fall in the range 1.01−1.02, although the uncertainties in these estimates are rather large, ca. 0.06.

(26) Jones, J. P.; Rettie, A. E.; Trager, W. F. *J. Med. Chem.* **1990**, *33*, 1242.

(27) The bond dissociation energy for the $C_1$−H bond of *n*-octane is assumed to be equal to the $C_1$−H bond energy in *n*-heptane, which can be obtained from the heats of formation of the *n*-heptyl radical, the hydrogen atom, and *n*-heptane: 4, 52.1, and −44.8 kcal/mol, respectively. Lias, S. G.; Bartmess, J. E.; Liebman, J. F.; Holmes, J. L.; Levin, R. D.; Mallard, W. G. *J. Phys. Chem. Ref. Data* **1988**, *17*, Suppl. No. 1.

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$pK_a$ suggests that the putative base in P-450 is >6 $pK_a$ units less basic than pyridine. Such a weak base would not be expected to efficiently deprotonate the amine cation radicals.[15] Secondly, the shallow dependence of the P-450 isotope effect profiles on the $pK_a$ of the amine cation radicals is difficult to explain by a proton transfer mechanism; the isotope effects are simply too insensitive to the large differences in cation radical $pK_a$'s. Perhaps the weak dependence on $pK_a$ could be explained by assuming that the enzyme somehow compresses the $\Delta pK_a$'s of the cation radicals. This might occur, for example, if a group in the active site preferentially stabilized the more reactive cation radicals via complex formation. Although none of these possibilities can be completely excluded at the present time, it is important to emphasize that there is no compelling reason to invoke such *ad hoc* hypotheses when the P-450 isotope effect profile agrees so well with the hydrogen atom abstraction profile.

In summary, several conclusions can be drawn from this work. First, isotope effect profiles provide compelling evidence in support of a hydrogen atom abstraction mechanism for the P-450 oxidation of substituted *N,N*-dimethylanilines. Second, the similarities of the isotope effect profiles across different P-450 families suggests a common reaction mechanism. Third, the profiles provide a sensitive tool for elucidating the mechanism of other amine oxidations by P-450, and also oxidations by other hemoproteins (e.g., chloroperoxidase and horseradish peroxidase). Fourth, the quantitative similarities between the P-450 isotope effects and the hydrogen atom abstraction isotope effects with $^{\bullet}OBu^t$ suggest possible applications for the chemical modeling of P-450 reactivity.

More work will be necessary, of course, to test the generality of the P-450 hydrogen atom abstraction mechanism for amine oxidations. For example, the reactions of P-450 with dihydropyridines[29] and cyclopropylamines[30] have been previously rationalized by electron transfer mechanisms. It remains to be established whether electron transfer mechanisms are required for these reactions, or if they can be better explained by unified hydrogen atom abstraction mechanisms.

## Experimental Section

**General Procedures.** Unless otherwise stated, all reactions were done under an atmosphere of dry nitrogen. Anhydrous diethyl ether and tetrahydrofuran were distilled from sodium benzophenone ketyl under nitrogen. Nitromethane was distilled from calcium hydride. Acetonitrile was distilled under nitrogen successively from aluminum trichloride, potassium permanganate/lithium carbonate, potassium bisulfite, calcium hydride, and phosphorus pentoxide and then stored under nitrogen.[31] Paraformaldehyde-$^{13}C$-$d_2$ (99+ atom % $^{13}C$) was obtained from Cambridge Isotopes from remaining MSD Isotope stock. $\beta$-Nicotinamide adenine dinucleotide phosphate reduced form (NADPH) and Chelex 100 were obtained from Sigma and used as received. Coumarin 545 was obtained from Exciton and used as received. *N*-Methyl-*p*-toluidine was obtained from Kodak, vacuum distilled (79 °C, 4.2 mmHg), and stored under nitrogen. *N*-Methyl-*N*-(trideuteriomethyl)aniline, 4-chloro-*N*-methyl-*N*-(trideuteriomethyl)aniline, 4-cyano-*N*-methyl-*N*-(trideuteriomethyl)aniline and 4-nitro-*N*-methyl-*N*-(trideuteriomethyl)aniline, were prepared as previously described.[4] Tris(1,10-phenanthroline)iron(III) tris(hexafluorophosphate) was prepared by a literature method.[32] All other chemicals were obtained from Aldrich Chemical and used as received. Inert atmosphere manipulations were conducted under nitrogen in a Vacuum Atmospheres HE-43-2 DRI-Lab glovebox equipped with a Vacuum Atmospheres HE-493 DRI-TRAIN. Elemental analyses were performed by Quantitative Technologies, Whitehouse, NJ.

$^1H$ and $^{13}C$ NMR spectra were recorded with a General Electric/Nicolet QE-300 spectrometer. Proton chemical shifts ($\delta$) are reported in parts per million (ppm) downfield from tetramethylsilane or in parts per million relative to the singlet at 7.24 ppm for the residual $CHCl_3$ in the chloroform-*d* or the multiplet at 1.93 ppm for the residual $CHD_2CN$ in the acetonitrile-$d_3$. Proton−proton coupling constants are reported in hertz (Hz) and reflect assumed first-order behavior. Splitting patterns are designated as s, singlet; d, doublet; t, triplet; q, quartet; p, pentet; m, multiplet; and br, broad. Carbon chemical shifts are reported in parts per million relative to internal acetonitrile-$d_3$ (117.61 and 0.60 ppm). Gas chromatography was performed on a Hewlett-Packard 5890 Series II gas chromatograph equipped with a HP 7673A automatic sampler on a 30 m DX3 capillary column from J&W Scientific. The gas chromatograph was interfaced to an Everex 286/16 computer with a HP 7673A controller. Mass spectral analyses were performed on a GC−MS system made up of a Hewlett-Packard 5890 Series II gas chromatograph (with a 30 m or a 10 m DX3 capillary column) which was interfaced to a Hewlett-Packard 5970 Series mass selective detector equipped with a Hewlett-Packard 6212C variable power supply. UV−vis spectra were recorded with a Beckman DU-65 spectrophotometer or a Hewlett-Packard 8452A diode array spectrophotometer. A Beckman L8-55 ultracentrifuge equipped with a type 60TI rotor was used for high-speed centrifugation.

**Preparation of 4-Methyl-*N*-methyl-*N*-(trideuteriomethyl)aniline.** A 25 mL round-bottomed flask equipped with a Teflon-coated magnetic stir bar and a reflux condenser was charged with *N*-methyl-*p*-toluidine (0.3099 g, 2.56 mmol), tetra-*n*-butylammonium iodide (0.0814 g, 0.22 mmol), potassium hydroxide (0.454 g, 8.09 mmol), benzene (4 mL), and water (0.5 mL). After 10 min, iodomethane-$d_3$ (99.5+ atom % D; 0.180 mL) was added dropwise over 2 min. After stirring at room temperature for 20 min, the reaction mixture was heated to 90 °C for 21 h. After cooling to room temperature, the organic layer was taken up in 50 mL of diethyl ether which was washed successively with 10 mL portions of water, saturated sodium carbonate, and brine, and was dried over anhydrous sodium sulfate. Filtration and solvent removal *in vacuo* gave an oil (0.277 g). Chromatography on silica gel using 90:10 hexane/diethyl ether as an eluant gave, after solvent removal, a colorless oil (0.229 g, 65%). $^1H$ NMR ($CDCl_3$): $\delta$ 7.07 (d, $J$ = 8.41 Hz, 1.96 H), 7.00 (d, $J$ = 8.48, 1.96 H), 2.92 (s, 3.01 H), 2.28 (s, 3.06 H).

**Preparation of *N,N*-Bis(dideuteriomethyl-$^{13}C$)aniline.**[4] A glass tube charged with paraformaldehyde-$^{13}C$-$d_2$ (99+ atom % $^{13}C$; 0.101 g, 3.05 mmol) was heated to 200 °C under a stream of argon and the evolved gas collected in sulfuric acid (3 M, 5 mL). After all the paraformaldehyde had evaporated, the heating bath was removed and the flask containing the formaldehyde/sulfuric acid solution was cooled in an ice−water bath and placed under nitrogen. Then a slurry of aniline (0.105 g, 1.127 mmol) and sodium borohydride (0.360 g, 9.51 mmol) in tetrahydrofuran (10 mL) was added dropwise over 10 min. The reaction mixture was allowed to warm to room temperature and stirred for 10 min. After recooling in an ice−water bath, the reaction mixture was made basic by dropwise addition of a sodium hydroxide solution (40%, 3 mL). The organic layer was separated and the aqueous layer was extracted with diethyl ether (10 mL). The combined organic layers were washed with water and then brine and dried over anhydrous sodium sulfate. Filtration and solvent removal *in vacuo* gave a clear liquid (0.111 g). Chromatography on silica gel using 90:10 pentane/diethyl ether as an eluant gave after solvent removal a clear liquid (0.103 g, 72%). $^1H$ NMR ($CD_3CN$): $\delta$ 7.20 (t, $J$ = 7.27 Hz, 2.02 H), 6.74 (d, $J$ = 8.07 Hz, 1.97 H), 6.66 (t, $J$ = 7.22 Hz, 0.98 H), 2.85 (dm, $J$ = 134.04 Hz, 2.02 H).

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**Preparation of 4-Chloro-*N*,*N*-bis(dideuteriomethyl-$^{13}C$)aniline.** This compound was prepared as described above except 4-chloroaniline was used and the product was chromatographed on silica gel using 40:60 diethyl ether/pentane as an eluant (87% yield). $^1$H NMR ($CDCl_3$): $\delta$ 7.17 (d, $J$ = 8.39, 1.93 H), 6.64 (d, $J$ = 6.89, 1.93 H), 2.89 (d, $J$ = 134.86, 2.14 H).

**Preparation of 4-Cyano-*N*,*N*-bis(dideuteriomethyl-$^{13}C$)aniline.** This compound was prepared as described above except 4-aminobenzonitrile was used and the product was chromatographed on silica gel using 50:50 diethyl ether/chloroform as an eluant (95% yield). $^1$H NMR ($CDCl_3$): $\delta$ 7.46 (d, $J$ = 8.91 Hz, 2.01 H), 6.63 (d, $J$ = 8.86 Hz, 1.99 H), 2.99 (dd, $J$ = 137.25 Hz, $J$ = 1.7 Hz, 2.0 H).

**Preparation of 4-Nitro-*N*,*N*-bis(dideuteriomethyl-$^{13}C$)aniline.** This substance was prepared as described above except 4-nitroaniline was used and the product was chromatographed on silica gel using 50:50 diethyl ether/chloroform as an eluant (98% yield). $^1$H NMR ($CDCl_3$): $\delta$ 8.12 (d, $J$ = 9.42 Hz, 1.97 H), 6.59 (d, $J$ = 9.43 Hz, 2.02 H), 3.07 (dm, $J$ = 137.93 Hz, 2.01 H).

**Preparation of 4-Phenyl-*N*-*tert*-butoxypyridinium hexafluorophosphate.**[33] A two-necked 100 mL round-bottomed flask was charged with 4-phenylpyridine *N*-oxide (3.517 g, 20.5 mmol), dry nitromethane (3 mL), *tert*-butylbromide (1.902 g, 13.88 mmol), and a Teflon-coated stir bar. After cooling in an ice−water bath, a solution of silver hexafluorophosphate (3.60 g, 14.2 mmol) in nitromethane (10 mL) was slowly added to the reaction mixture over 90 min. The reaction mixture was stirred for 4 h, allowed to warm to room temperature, and filtered. The filtrate was washed with water (4 × 50 mL) and dried over anhydrous sodium sulfate. After filtration, diethyl ether (ca. 400 mL) was added to the filtrate, resulting in the formation of a white powder which was isolated by filtration and vacuum dried (0.01 mmHg, 25 °C; 2.89 g, 56%). A portion (1.07 g) of the solid was taken up in 50 mL of dichloromethane containing 0.35 g of poly(4-vinylpyridine). After 15 min, the mixture was filtered and the filtrate was washed with water (3 × 50 mL) and dried over anhydrous sodium sulfate. After filtration, 300 mL of diethyl ether was added to the filtrate which produced a white powder that was washed with diethyl ether (50 mL) and vacuum dried (0.01 mmHg, 25 °C; 0.365 g, 34%). Anal. Calcd for $C_{15}H_{18}NOPF_6$: C, 48.27; H, 4.86; N, 3.75. Found: C, 48.08; H, 4.74; N, 3.66. $^1$H NMR ($CD_3CN$): $\delta$ 8.74 (d, $J$ = 5.37 Hz, 1.94 H), 8.27 (d, $J$ = 5.4 Hz, 1.94 H), 7.93 (m, 2.0 H), 7.66 (m, 3.03 H), 1.5 (s, 9.1 H). $^{13}$C NMR ($CD_3CN$): $\delta$ 157.07, 143.79, 134.04, 132.87, 130.28, 128.66, 126.06, 95.16, 26.24.

**Isotopic Compositions of 2a−d.** GC−MS data on the amines were collected in the selected ion monitoring mode at an ionization voltage of 7.0 eV. At this ionization voltage the M − 1 contributions for each of the undeuteriated *N*,*N*-dimethylanilines was less than 0.5% of the $M^+$ peak. The deuterium isotopic composition percentages (standard deviation in the last significant digit) summarized below are an average of at least three independent determinations.

| X | % $d_4$ | % $d_3$ | % $d_2$ |
|---|---|---|---|
| H | 90.65(9) | 7.85(4) | 1.48(5) |
| Cl | 91.27(5) | 8.21(4) | 0.51(1) |
| CN | 91.74(4) | 7.92(4) | 0.33(1) |
| $NO_2$ | 91.74(4) | 7.86(8) | 0.40(9) |

Amines **2a−d** were all prepared from the same batch of paraformaldehyde-$^{13}C$-$d_2$. The $^{13}$C content was therefore determined in only one of the amines (**2d**) by integration of the $^1$H NMR multiplet centered at ca. 2.95 ppm (corresponding to $^{12}$C-$^1$H) relative to the multiplets centered at ca. 3.2 and ca. 2.75 ppm (corresponding to $^{13}$C-$^1$H). The percentage of $^{13}$C was found to be 99.3%.

**Isotope Effects for the Enzymatic Incubations of 2a−d with Phenobarbital-Induced Rat Liver Microsomes.** The microsomes[34] were removed from storage in a −80 °C freezer, thawed, homogenized in a Potter-Elvehjen tissue homogenizer using cold phosphate buffer (100 mM, pH 7.4), and centrifuged (100000*g*, 1 h, 5 °C). A suitably diluted microsomal preparation was reduced by adding several milligrams of solid sodium dithionite and was then saturated with carbon monoxide by bubbling the gas into the solution for ca. 1 min. The microsomal P-450 content was determined by the method of Omura and Sato[35] from the difference in visible absorption spectra between 450 and 490 nm.

Scintillation vials were charged with substrates (2 mol in 50 $\mu$L of solution in acetone), microsomal P-450 (10−12 nmol), and NADPH (2 $\mu$mol) in phosphate buffer (0.1 M, pH 7.4) to give a total volume of 2 mL. The mixtures were incubated at 37 °C for 30 min in a Dubnoff metabolic shaking incubator. The incubations were terminated by adding sodium hydroxide solution (100 $\mu$L, 40%) and were derivatized with 100 $\mu$L of a 0.2 M dimedone solution in 0.2 M sodium hydroxide. The mixture was shaken and allowed to sit at room temperature for 30 min. Then hydrochloric acid (4 M) was added dropwise until the mixture became acidic. The enzymatic mixture was extracted with dichloromethane (3 × 10 mL) and dried over anhydrous sodium sulfate and the solvent removed *in vacuo*. The residue was taken up in 50 $\mu$L of dichloromethane and analyzed by GC−MS as described below. The isotope effects are summarized in Table 1.

**GC−MS Analysis of the Enzymatic Products of 2a−d.** The dimedone adducts of formaldehyde-$^{13}C$ formed in the incubations of **2a−d** were analyzed by GC−MS at 70 eV using the selected ion monitoring mode. At this ionization voltage the M − 1 peak for the dimedone adduct of formaldehyde-$d_0$ was 0.16% of the $M^+$ peak. The abundances at *m*/*z* of 292, 293, 294, 295, and 296 were analyzed for the mole fraction of $d_0$, $^{13}C$-$d_1$, and $^{13}C$-$d_2$ by the method described by Brauman.[36] The $d_2/d_1$ ratios were corrected for the small amount of incomplete deuterium incorporation as described previously.[4] The relative abundances of M − 1, M + 1, and M + 2 were determined for the dimedone adduct of formaldehyde-$d_0$ before each set of analysis. A control oxidation which was not derivatized with dimedone did not give peaks with *m*/*z* 291−297 under the analysis conditions. In a second control experiment, a 2.42:1 mixture of formaldehyde-$d_2/d_1$ was added to the enzymatic incubations containing undeuteriated dimethylanilines. The incubations were terminated, derivatized, and analyzed by GC−MS as described above. The average ratio of the $d_2/d_1$ dimedone adducts from all of the incubations was 2.43(5).

**Isotope Effects for the Enzymatic Incubations of 2a−d with Purified Reconstituted CYP2B1.** Incubations were run at the National Cancer Institute using purified CYP2B1[20] (100 pmol), purified cytochrome P-450 reductase[20] (200 pmol), dilaurylphosphatidylcholine (10 $\mu$g), NADPH (1.0 $\mu$mol), and substrate (1−2 $\mu$mol in 10 $\mu$L of acetone) in 1 mL of potassium phosphate buffer (50 mM, pH 7.4). Incubations were carried out at 37 °C in a shaking water bath for 30 min. The incubations were terminated, derivatized, and analyzed by GC−MS as described above. The isotope effects are summarized in Table 1.

**Isotope Effects for the Enzymatic Incubations of 2a−d with Expressed CYP2B1.** Incubations were run at the National Cancer Institute using expressed CYP2B1[21] (ca. 70 pmol, washed with 20 $\mu$M desferal[37] and resuspended in buffer), NADPH (1 $\mu$mol), and substrate (1−2 $\mu$mol in 10 $\mu$L of acetone) in 1 mL of potassium phosphate buffer (50 mM, pH 7.4, Chelex washed[37]). Incubations were carried out at 37 °C in a shaking water bath for 30 min. The incubations were terminated, derivatized, and analyzed by GC−MS as described above. The isotope effects are summarized in Table 1.

**Isotope Effects for the Enzymatic Incubations of 2a−d with Expressed CYP4B1 and CYP1A2.** Incubations were run at the National Cancer Institute using expressed CYP4B1[21] (ca. 200 pmol) and NADPH (1 $\mu$mol) or using expressed CYP1A2[21] (85 pmol) and substrate (1−2 $\mu$mol in 10 $\mu$L of acetone) in 1 mL of potassium phosphate buffer (50 mM, pH 7.4, Chelex washed[37]). Incubations were carried out at 37 °C in a shaking water bath for 30 min. The incubations were terminated, derivatized, and analyzed by GC−MS as described above. The isotope effects are summarized in Table 1.

**Isotope Effects for the Enzymatic Incubations of 2a−d with Purified CYP102.** The enzymatic incubations contained purified CYP102 (BM3)[20] (2.8 nmol) and substrate (1 $\mu$mol in 10 $\mu$L of acetone) in 1 mL of potassium phosphate buffer (100 mM, pH 7.4). The incubation mixtures were preincubated at 30 °C for 5 min, and NADPH

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(1 μmol) was added to initiate the reactions. The mixtures were then incubated for 30 min at 30 °C in a water-bath shaker. The incubations were terminated, derivatized, and analyzed by GC–MS as described above. The isotope effects are summarized in Table 1.

**Isotope Effects for the Reactions of 1a–e with Tris(1,10-phenanthroline)iron(III) Tris(hexafluorophosphate) and Pyridine.** All glassware was acid washed and oven dried. Under nitrogen purge, a septum-capped test tube was charged with dry pyridine (0.078 g, 0.996 mmol), tris(1,10-phenanthroline)iron(III) tris(hexafluorophosphate) (0.0103 g, 0.001 mmol), 1.5 mL of acetonitrile, and a Teflon-coated stir bar. To the resulting blue solution, one of the amines **1a–e** (0.05 mmol) in 0.5 mL of acetonitrile was added dropwise via syringe. During the addition the solution turned from blue to red. After the addition was complete, the reaction mixture was stirred at room temperature for 10 min. The contents of the tube were then taken up in diethyl ether (50 mL), washed with water, saturated sodium bicarbonate, and brine, and dried over anhydrous sodium sulfate. The solvent was removed via rotary evaporation and the residue taken up in dry diethyl ether (1 mL), derivatized with 0.2 mL of trifluoroacetic anhydride, and allowed to sit for 10 min. The excess trifluoroacetic anhydride and the solvent were removed by rotary evaporation. The residue was then taken up in diethyl ether (10 mL) and ca. 0.2 g of poly(4-vinylpyridine). After 1 h, the mixture was filtered and the solvent removed from the filtrate by rotary evaporation. The residue was taken up in 0.1 mL of diethyl ether and analyzed by GC–MS as described below. The isotope effects are summarized in Table 1. Oxidations were separately analyzed by GC before derivatization to determine if any para-substituted anilines could be detected. In all cases, they amounted to <1% of the para-substituted *N*-methylanilines that were formed.

**Isotope Effects for the Reactions of 1a–d with Tris(1,10-phenanthroline)iron(III) Tris(hexafluorophosphate).** All glassware was acid washed and oven dried. Under nitrogen purge, a septum-capped test tube was charged with one of the amines **1a–d** (0.12 mmol), 1.5 mL of dry methylene chloride, and a Teflon-coated stir bar. To the resulting blue solution, tris(1,10-phenanthroline)iron(III) tris-(hexafluorophosphate) (0.0103 g, 0.001 mmol) and 0.5 mL of methylene chloride were added dropwise via syringe. During the addition the solution turned from blue to red. After the addition was complete, the reaction mixture was stirred at room temperature for 5 min. The volatiles were removed by rotary evaporation, and the residue was taken up in diethyl ether (20 mL), washed with water, saturated sodium bicarbonate, and brine, and dried over anhydrous sodium sulfate. The solvent was removed by rotary evaporation and the residue taken up in dry diethyl ether (1 mL), derivatized with 0.2 mL of trifluoroacetic anhydride, and allowed to sit for 10 min. The excess trifluoroacetic anhydride and the solvent were removed by rotary evaporation. The residue was then taken up in diethyl ether (10 mL) and ca. 0.2 g of poly(4-vinylpyridine). After 1 h, the mixture was filtered and the solvent removed from the filtrate by rotary evaporation. The residue was taken up in 0.2 mL of diethyl ether and analyzed by GC–MS as described below. The measured isotope effects for **1a–d** were 7.1(1), 6.2(1), 3.7(1), and 5.2(1), respectively.

**Isotope Effects for the Coumarin-Sensitized Reactions of 1a–d with 4-Phenyl-*N*-*tert*-butoxypyridinium Hexafluorophosphate.** A 1 cm Pyrex cuvette with a 10/30 ⅋ joint attached was charged with **1d** (12.28 mg, 0.0989 mmol), 4-phenyl-*N*-*tert*-butoxypyridinium hexafluorophosphate (4.3 mg, 0.0115 mmol), coumarin 545 (2.56 mg, 0.00683 mmol), and a Teflon-coated stir bar. In a dark room, deaerated methylene chloride was added to the cuvette (2 mL), argon was bubbled gently through the solution for 1 min, and the cuvette was capped. The cuvette was then placed in a homemade cuvette holder mounted on an optical bench consisting of an Oriel 66002 universal lamp housing equipped with an Oriel 6283 200 W Hg lamp connected to an Oriel 68805 power supply and mounted on an Oriel 11150 high-stability optical bench. Attached in series to the lamp housing was an Oriel 71430 shutter, an Oriel 6194 liquid filter filled with water, and a Schott Glass Technologies KV 500 longpass filter ($h\nu$ > 500 nm). The cuvette holder was placed 18 cm from the edge of the longpass filter, and the sample was irradiated with a power supply output of 100 W. Care was taken to eliminate stray light. The sample temperature was maintained at 25 °C by flowing water through a jacket in the cuvette holder using a Poly Science Series 900 constant temperature bath. After 40 min of photolysis, water (0.1 mL) was added to the reaction mixture, the methylene chloride layer was separated, and the volatiles were removed by rotary evaporation. The residue was taken up in dry diethyl ether (1 mL) and derivatized by adding 0.2 mL of trifluoroacetic anhydride. After 10 min, the excess trifluoroacetic anhydride and the solvent were removed by rotary evaporation, and the residue was taken up in diethyl ether (10 mL) and ca. 0.2 g of poly(4-vinylpyridine). After 1 h, the mixture was filtered and the solvent removed by rotary evaporation. The residue was taken up in 0.1 mL of diethyl ether and analyzed by GC–MS as described below. The photolyses with **1a**, **1b**, and **1c** were done in a manner similar to that above except that the photolysis times for the first two were 110 and 55 min, respectively. The isotope effects are summarized in Table 1.

Oxidations were separately analyzed by GC before derivatization to see if any para-substituted anilines could be detected; none were. In a control experiment, 4-phenyl-*N*-*tert*-butoxypyridinium hexafluorophosphate was omitted from the reaction mixture. No *N*-methylanilines could be detected by GC after photolysis. In a separate control experiment, a solution of amine **1d** (0.12 mmol) and coumarin 545 (0.007 mmol) in 2.0 mL of methylene chloride was stored in the dark at 25 °C for 1.5 h. Subsequent analysis by GC showed that no *N*-methylaniline was formed.

**GC–MS Analysis of the Products from the Reaction of Tris-(1,10-phenanthroline)iron(III) Tris(hexafluorophosphate) with 1a–e and the Coumarin-Sensitized Reaction of 4-Phenyl-*N*-*tert*-butoxypyridinium Hexafluorophosphate with 1a–d.** The trifluoroacetylated products were analyzed by GC–MS at 70 eV using the selected ion monitoring mode as described previously.[4] In each case, known mixtures of the trifluoroacetylated, para-substituted *N*-methylanilines and *N*-(trideuteriomethyl)anilines were analyzed and found to be within 3% of the known ratios. The measured isotope effects ($k_H/k_D$) measured were taken to be equal to the ratios of abundances $d_3/d_0$ after correction for incomplete deuterium incorporation as described previously.[4]

**Isotope Effects for the Coumarin-Sensitized Reaction of 4-Phenyl-*N*-*tert*-butoxypyridinium Hexafluorophosphate with 2a–d.** The reactions were run in a manner similar to that with **1a–d**. After photolysis, each reaction mixture was extracted with water (3 × 2.0 mL) and 0.2 M sodium hydroxide solution (1 × 2.0 mL). The combined aqueous extracts were treated with 40% sodium hydroxide (100 μL) followed by 200 μL of a 0.27 M dimedone solution in 0.2 M sodium hydroxide. The resulting solution was allowed to sit at room temperature for 15 min and was then made acidic with 4 M HCl solution. The solution was extracted with methylene chloride (3 × 5 mL) and the organic layer dried over anhydrous sodium sulfate. After filtration and solvent removal via rotary evaporation the residue was taken up in ca. 200 μL of methylene chloride and analyzed by GC–MS as described above for the enzymatic incubations of **2a–d**. The isotope effects are summarized in Table 1.

**Stoichiometries for the Coumarin-Sensitized Reactions of 4-Phenyl-*N*-*tert*-butoxypyridinium Hexafluorophosphate with Para-Substituted *N*,*N*-Dimethylanilines.** Photolyses were carried out using the undeuteriated, para-substituted *N*,*N*-dimethylanilines corresponding to **1a–d**. After photolysis, 0.1 mL of water was added to the reaction mixture, the methylene chloride layer was separated, and the volatiles were evaporated by rotary evaporation. The residue was taken up in ca. 15 mL of diethyl ether. The diethyl ether was then removed via rotary evaporation. The residue was taken up in ca. 2 mL of methylene chloride and analyzed by GC to determine the relative amounts of the para-substituted *N*-methylanilines and 4-phenylpyridine that were formed. The measured peak areas were corrected for differential response factors with authentic samples. The ratios of the para-substituted *N*-methylanilines to 4-phenylpyridine were 1.0(1), 1.0(2), 0.9(2), and 0.8(3), respectively, for X = H, Cl, CN, and $NO_2$.

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