Evidence for a hydrogen abstraction mechanism in P_{450}-catalyzed *N*-dealkylations†

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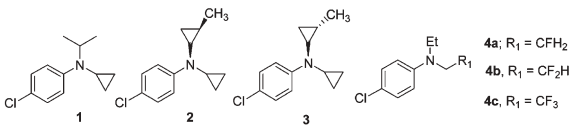
The experimental evidence presented in this manuscript suggest against the widely accepted single electron/proton transfer mechanism for P_{450} catalyzed *N*-dealkylations and provides strong support for a hydrogen atom abstraction mechanism.

Cytochrome P_{450} is a family of heme monooxygenases found in most organisms including bacteria, plants, and animals. These enzymes are responsible for the oxidative metabolism and detoxification of xenobiotics and a number of important biochemical transformations. Although, the molecular mechanism of P_{450} catalyzed reactions have been studied extensively, some aspects of the mechanism still remains highly controversial.[1]

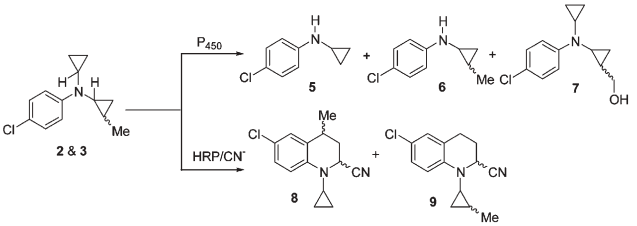
P_{450}-catalyzed *N*-dealkylations are widely believed to occur through a single electron/proton transfer mechanism (SET/H^+; Scheme 1). The direct hydrogen atom transfer mechanism (HAT; Scheme 1) has been ruled out based on the evidence derived from the studies with chemical model systems,[1] mechanistically well characterized heme enzymes such as horseradish peroxidase (HRP),[1] kinetic isotope effects,[1] redox potential correlations,[2] and irreversible enzyme inactivation by radical clock substrates.[1–4] However, several recent studies have provided experimental evidence in support of a HAT mechanism for P_{450}-catalyzed *N*-dealkylations.[5,6]

In a recent study, we have shown that the P_{450} catalyzed *N*-dealkylation of radical probe (**1**) proceeds *via* a mechanism in which the C_α–Hs of both cyclopropyl and alkyl substituents are removed at isotopically sensitive steps without producing detectable amounts of cyclopropyl ring opened products.[6] Based on this and other evidence we proposed that the nitrogen cation radical is not an intermediate in P_{450} catalyzed *N*-dealkylation of **1** and that the reaction may proceed through a HAT mechanism.[6] However, while our findings strongly support this proposal, they are also consistent with a SET mechanism, provided that the relative rate of C_α-deprotonation of the nitrogen cation radical is much faster than the rate of cyclopropyl ring opening. In order to test this possibility, we have carried out a comparative study of the product distributions of HRP, an accepted SET model, and P_{450} catalyzed *N*-dealkylation of a series of radical probes, [*N*-(alkyl)cyclopropyl-*N*-cyclopropyl-*p*-chloroaniline **2**–**3**], and a series of C_α-acidity probes, [*N*-ethyl-*N*-2-fluoroethyl-*p*-chloroaniline **4a**–**c**]. The results of these studies in conjunction with our previous findings demonstrate that the HAT, but not the SET/H^+ mechanism could be operative in the P_{450} catalyzed *N*-dealkylations.

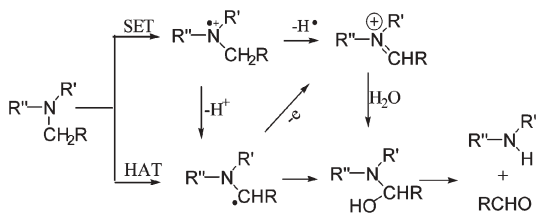


Incubation of *N*-cyclopropyl-*N*-(*cis*-2-methyl)cyclopropyl-*p*-chloroaniline (**2**), with phenobarbital-induced rat liver microsomal P_{450} (PB) or purified CYP2B1 gave a major product, which is tentatively identified as the methyl hydroxylated product **7**, a minor product, *N*-(*cis*-2-methyl)cyclopropyl-*p*-chloroaniline (**6**), and a trace amount of *N*-cyclopropyl-*p*-chloroaniline (**5**) (Scheme 2; Table 1). In contrast, to the preferential hydroxylation of the *cis* isomer, the *trans* isomer **3** produced *N*-cyclopropyl-*p*-chloroaniline (**5**) and *N*-(*trans*-2-methyl)cyclopropyl-*p*-chloroaniline (**6**) as the primary products with no detectable hydroxylation products under similar incubation conditions (Scheme 2; Table 1). The primary isotope effect determined from the partition ratio of **3** and the corresponding deuterated derivative, *N*-1-*d*-cyclopropyl-*N*-(*trans*-2-methyl-cyclopropyl)-*p*-chloroaniline (**3**-*d*) was 2.7 ± 0.3.

HRP catalyzed oxidation of **2** or **3**, in the presence of CN^-, exclusively produced the 2-methylcyclopropyl ring opened radical cyclized cyanide adduct, **8**,[7] with no detectable amount of the corresponding *N*-cyclopropyl ring opened product **9** based on GC-MS, ^{1}H-, ^{13}C- and 2D-NMR analyses[8] (see Supporting Information†).[9] However, examination of the P_{450} incubates of **2** & **3** revealed that no detectable amounts of **8** or **9** were produced,



Scheme 2 Product distribution of cytochrome P_{450} and HRP catalyzed *N*-dealkylations of *N*-cyclopropyl-*N*-(2-methyl)cyclopropyl-*p*-chloroanilines (**2** & **3**).



Scheme 1 Postulated mechanisms of P_{450} catalyzed *N*-dealkylation of amines.

† Electronic supplementary information (ESI) available: GC-MS chromatograms and experimental details. See http://www.rsc.org/suppdata/cc/b4/b412221f/

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Table 1 Product distributions of rat liver microsomal and CYP2B1-catalyzed *N*-dealkylation of *N*-cyclopropyl-*N*-(2-methyl) cyclopropyl-*p*-chloroanilines (**2** & **3**): (**5**), *N*-cyclopropyl-*p*-chloroaniline; (**6**), *N*-(2-methyl)cyclopropyl-*p*-chloroaniline; and (**7**), 2-Me-hydroxylated product

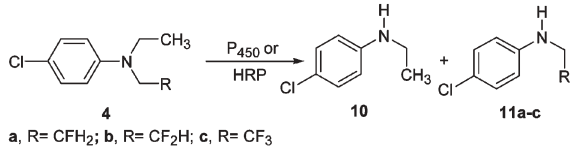
	Microsomal P_{450}[a]			CYP2B1[a]		
	5	**6**	**7**	**5**	**6**	**7**
2	4 (1)	18 (1)	77 (1)	6 (1)	16 (1)	78 (1)
3	8 (2)	92 (2)	ND[b]	7 (2)	93 (2)	ND

[a] Each entry is an average of at least three independent determinations. The standard deviation for the last significant figures are given in parentheses. [b] ND, not detected.

under similar incubation conditions demonstrating that, while HRP reactions proceed exclusively through a cyclopropyl ring opening pathway, P_{450} reactions proceed through a pathway that does not involve the opening of the cyclopropyl rings. On the other hand, C_α–Hs of the cyclopropyl rings in P_{450} catalyzed *N*-dealkylation of **2** & **3** are removed at isotopically sensitive steps along the catalytic pathway.

P_{450} catalyzed *N*-dealkylations of **2** & **3**, should have preferentially produced the 2-methylcyclopropyl ring opened/cleaved products, if a nitrogen cation radical is a transient intermediate, based on the product distributions of HRP catalyzed oxidation of **2** & **3**. In contrast, as shown in Table 1, P_{450} catalyzed reactions preferentially produces the *N*-cyclopropyl cleaved product **6**. Although, the steric constraints of the active site residues may be responsible for these differences, the high steric tolerance of the P_{450} active site[10] toward structurally diverse substrates rules out this possibility. Similarly, since the acidity of the C_α–H should not be significantly affected by the presence of the methyl group on the cyclopropyl ring, the difference in product distribution could not be due to the difference in acidities of the C_α–H. Therefore, these findings strongly suggest that the nitrogen cation radical could not be an intermediate in P_{450}-catalyzed *N*-dealkylation of **2** & **3**, which is in excellent agreement with our previous results.[6]

In order to further test whether the acidities of C_α–Hs of *N*-substituents determine the product distributions of HRP or P_{450} catalyzed *N*-dealkylations under competitive conditions, we have synthesized and characterized a series of β-fluoro analogs of *N*, *N*-diethyl-*p*-chloroaniline (**4a–c**). Incubations of **4a–c** with P_{450} produced both *N*-ethyl-*p*-chloroaniline (**10**) and *N*-β-fluoroethyl-*p*-chloroaniline (**11a–c**) as the primary products (Scheme 3). The partition ratios were highly dependent on the number of fluorine atoms on the substrate and preferential formation of **11a–c** observed as the number of fluorine atoms on the *N*-ethyl substituent is increased as shown in Table 2. On the other hand, incubations of **4a–c** with HRP produced **10** as the major product,



Scheme 3 Product distribution of CYP2B1 and HRP catalyzed *N*-dealkylations of *N*-ethyl-*N*-(β-fluoro)ethyl-*p*-chloroaniline derivatives (**4a–c**).

Table 2 Product distributions of CYP2B1 and HRP catalyzed *N*-dealkylation of **4a–c**: (**10**), *N*-ethyl-*p*-chloroaniline; (**11**), *N*-β-fluoro-ethyl-*p*-chloroaniline

	CYP2B1[a]		HRP[a]	
	10	**11a–c**	**10**	**11a–c**
4a	68.2 (3)	31.8 (3)	77 (2)	23 (2)
4b	30.0 (3)	70.0 (3)	70 (2)	30 (2)
4c	7.9 (2)	92.1 (2)	94 (1)	6 (1)

[a] Each entry is an average of at least three independent determinations. The standard deviations for the last significant figures are given in parentheses.

which was increased up to 94% for the trifluoro derivative (**4c**). These results unequivocally demonstrate that the chemistries of the two systems are quite contrasting and distinct with respect to substrates **4a–c**.

The incremental fluorine substitution in **4a–c** increases the production of the *N*-fluoroethyl group cleaved product (**10**) in HRP catalyzed reactions, most likely due to the increased acidity of the C_α–Hs of the *N*-β-fluoroethyl group relative to the *N*-ethyl group of the SET intermediate, nitrogen cation radical. In contrast, the incremental fluorine substitution disfavours the *N*-β-fluoroethyl group cleavage in P_{450} reactions, suggesting that the acidities of the C_α–Hs are not an important determinant for P_{450}-catalyzed *N*-dealkylations. On the other hand, the decrease of the formation of **10** with the incremental fluorine substitution in P_{450}/**4a–c** reactions correlates well with the increase in C_α–H bond dissociation energies rather than with the acidities.[11]

The apparent similarity in the product distributions of monofluoro derivative **4a** in P_{450} and HRP reactions may be a consequence of the relative magnitudes of bond dissociation and C_α-radical stabilization energies of this particular derivative. For example, theoretical studies have shown that the C_α–H abstraction from the *N*-β-monofluoroethyl is favoured over *N*-ethyl due to the high relative stability of the β-fluoromethyl-C_α-radical in comparison to the β-methyl-C_α-radical.[11] Therefore, both C_α–H atom abstraction from the neutral substrate and C_α–H deprotonation of the cation radical intermediate could occur favourably from the *N*-β-monofluoroethyl group giving rise to the favourable cleavage of *N*-β-fluoroethyl group regardless of whether the SET/H^+ or HAT mechanisms are operative. Taken together, the above results suggest that while the partition ratios of HRP/**4a–c** reactions were determined by the relative acidities of the C_α–H of the *N*-substituents, partition ratios of P_{450}/**4a–c** reactions may be determined by the bond dissociation energies.

In conclusion, the lack of cyclopropyl ring opened or ring opened radical cyclized products from P_{450}/**2** & **3** reactions along with the product profiles of P_{450}/**4a–c** reactions suggest against a SET/H^+ transfer mechanism for P_{450} catalyzed *N*-dealkylations and provide strong support for the hydrogen atom abstraction mechanism.

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8 A stereoisomeric (*cis*/*trans*) mixture of **8** (85 : 15) with close GC retention times and identical GC-MS spectra were obtained (*see* Supporting Information).

9 This is consistent with our previous observation that the single electron oxidation of *N*-(2-methylcyclopropyl)aniline exclusively produces the products from the opening of the C1,2 bond of the cyclopropyl ring to generate a secondary radical (K. Wimalasena, H. B. Wickman and M. P. Mahindaratne, *Eur. J. Org. Chem.*, 2001, 3811).

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