A Nonsynchronous Concerted Mechanism for Cytochrome P-450 Catalyzed Hydroxylation

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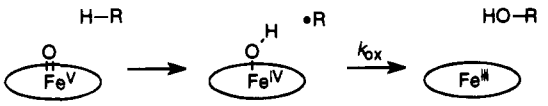
Abstract: Recent attempts to quantify the rate constant for radical capture in cytochrome P-450 hydroxylations employing substrates that are precursors to radicals that rearrange very rapidly have given widely differing apparent rate constants, suggesting that the consensus hydroxylation mechanism is incomplete or incorrect or that the probe substrates behave in an unexpected manner. We report cytochrome P-450 hydroxylations of a new, calibrated hypersensitive radical probe substrate, (*trans,trans*-2-*tert*-butoxy-3-phenylcyclopropyl)methane (**1a**), that permits discrimination between radical and cationic intermediates. Cytochrome P-450 oxidation of the methyl group in **1a** gave unrearranged and rearranged hydroxylation products. Most of the rearranged products derived from a cationic intermediate apparently produced during the course of the hydroxylation reaction; this unanticipated process is the origin of the confusing results obtained with other probes. The radical species in the hydroxylation reaction has a lifetime of only 70 fs; it is not a true intermediate but part of a reacting ensemble. The small amount of radical rearrangement occurs because the insertion reaction is nonsynchronous with C−H bond cleavage leading C−O bond formation. The short radical lifetime also requires that the oxygen atom is within bonding distance of carbon at the instant of hydrogen abstraction; that is, a "side-on" approach of oxygen to the C−H bond is suggested as opposed to a linear C−H−O array of a conventional abstraction.

The heme-containing cytochrome P-450 enzymes effect a wide range of oxidation reactions including the remarkably difficult ambient-temperature hydroxylation of unactivated C−H bonds in hydrocarbons. The consensus mechanism for hydroxylation involves hydrogen atom abstraction from substrate by a high-valent iron−oxo intermediate followed by a bimolecular homolytic substitution step in which the substrate-derived radical captures hydroxyl radical from iron, the so-called oxygen rebound step (Scheme 1).[2-4] This mechanism was deduced by the detection of rearranged products in several P-450 oxidations thus requiring formation of a radical or cationic intermediate and subsequent exclusion of a requisite cationic intermediate. For example, hydroxylation of 2,2,6,6-tetradeuteriocyclohexene gave a mixture of deuterated 2-cyclohexenol isomers,[5] but norcarane was hydroxylated at the cyclopropylcarbinyl position without formation of a rearranged product expected from a cationic intermediate.[6] In a seemingly conclusive mechanistic study, Ortiz de Montellano and Stearns reported that, whereas methylcyclopropane was hydroxylated without rearrangement, bicyclo[2.1.0]pentane was hydroxylated to give both unrearranged bicyclo[2.1.0]pentan-2-ol and the rearranged product 3-cyclopentenol.[7] The consensus P-450 hydroxylation mechanism is supported by primary kinetic isotope effects that indicate considerable C−H bond stretching in the rate-limiting transition state although such isotope effects might logically be consistent with any type of C−H functionalization reaction.

Scheme 1



Despite accumulated evidence for the P-450 mechanism, recent attempts to quantify the rate constant for the oxygen rebound step in hydroxylations (k_{ox} in Scheme 1) by a rat liver P-450 isozyme[7-10] gave widely varying results (1.4×10^{10} to 1.4×10^{13} s^{-1}). The lack of correlation between the amounts of rearranged products and the rate constants for rearrangements of the putative radicals formed from the probes[10] suggests a number of possible problems: (1) the rate constants for at least some of the radical rearrangements are grossly in error, (2) the clocks behaved in an unexpected manner, or (3) the mechanism in Scheme 1 is incorrect or incomplete. In regard to the second point, several possibilities exist, but it is noteworthy that all of the calibrated hypersensitive probes used in previous studies suffered from the common problem that the skeletal reorganizations for radical and cationic ring openings were the same. Thus, the origins of rearranged products actually were uncertain, and we have noted that the variable apparent k_{ox} values might reflect the intrusion of ionic rearrangements that cannot be discerned from radical rearrangements.[10]

We report here studies of cytochrome P-450 hydroxylation of a new hypersensitive probe that permits discrimination between radical and cationic intermediates, (*trans,trans*-2-*tert*-

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(1) (a) Wayne State University. (b) University of Michigan.

(2) *Cytochrome P-450 Structure, Mechanism and Biochemistry*; Ortiz de Montellano, P. R., Ed.; Plenum: New York, 1986.

(3) Guengerich, F. P.; Macdonald, T. L. *FASEB J.* **1990**, *4*, 2453−2459.

(4) Woggon, W.-D.; Fretz, H. In *Advances in Detailed Reaction Mechanisms*; Coxon, J. M., Ed.; JAI Press: Greenwich, CT, 1992; Vol. 2, pp 111−147.

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(6) White, R. E.; Groves, J. T.; McClusky, G. A. *Acta Biol. Med. Ger.* **1979**, *38*, 475−489.

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(8) Atkinson, J. K.; Ingold, K. U. *Biochemistry* **1993**, *32*, 9209−9214.

(9) Atkinson, J. K.; Hollenberg, P. F.; Ingold, K. U.; Johnson, C. C.; Le Tadic, M.-H.; Newcomb, M.; Putt, D. A. *Biochemistry* **1994**, *33*, 10630−10637.

(10) Newcomb, M.; Le Tadic, M.-H.; Putt, D. A.; Hollenberg, P. F. *J. Am. Chem. Soc.* **1995**, *117*, 3312−3313.

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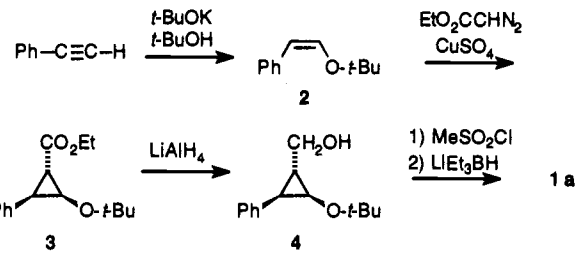
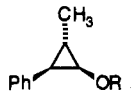
Scheme 2



butoxy-3-phenylcyclopropyl)methane (**1a**). The predominant



1a: R = *t*-Bu
1b: R = Me
1c: R = H

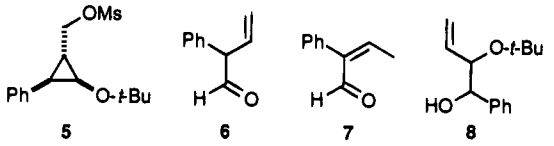
product from oxidation of the methyl group in **1a** was the unrearranged alcohol product, but both cationic and radical derived rearrangement products also were formed. The origin of the confusing results from previous hypersensitive probe studies is seen to arise from two pathways, radical and cationic, to the same rearrangement products. In addition, the lifetime of the "radical" is confirmed to be less than 0.1 ps, which is too short a time for formation of a true intermediate; it can only be a component in a reacting ensemble produced in a nonsynchronous concerted insertion process, with hydrogen abstraction preceding C–O collapse, but with no local energy minimum. Further, the extremely short lifetime of the radical moiety apparently requires that the hydrogen abstraction component of the reaction involves an organization of atoms wherein the C–H–O bond angle is small, inconsistent with linear or nearly linear C–H–X arrays in conventional hydrogen abstraction processes. The structure of the reacting ensemble might be an important feature differentiating enzyme-catalyzed hydroxylations from reactions of their chemical models.

Results

Characterization of Probe Reactions. We recently demonstrated that a methylcyclopropyl probe architecture containing both a phenyl group and an alkoxyl group not only maintained the hypersensitive radical reactivity desired for enzyme mechanistic studies but also permitted high discrimination between radical and cationic intermediates.[11] The corresponding probe substrate (**1b**) was not useful in P-450 hydroxylation studies, however, because the major product produced was the normethyl alcohol **1c** presumed to be formed by hydroxylation of the methoxy group in **1b** and subsequent hydrolysis of the resulting hemiacetal. Replacement of the methoxy group in **1b** with a *tert*-butoxy group was an obvious alternative.

The synthesis of the desired system generally followed a conventional sequence of reactions (Scheme 2). Addition of *tert*-butyl alcohol to phenylacetylene proceeded in high yield to give *cis*-β-*tert*-butoxystyrene (**2**) virtually free of the *trans* isomer; this reaction was based on the known addition of *tert*-butyl alcohol to acetylene.[12] Reaction of this alkene with ethyl diazoacetate gave ester **3**, which was reduced to alcohol **4**. Conversion of this alcohol to the corresponding mesylate (**5**) at low temperature followed by reaction with $LiEt_3BH$ gave, after purification, the desired probe **1a**. All of the isolated intermediates were characterized by NMR spectroscopy. Probe **1a** and alcohol **4** had appropriate molecular ions in high-resolution mass spectrometry. The stereochemistry of ester **3** was established by NOE experiments.

The expected high discrimination for a cationic intermediate in the new probe system was readily confirmed. Production of mesylate **5** in THF followed by addition of water gave known



5 **6** **7** **8**

aldehyde **6**[13] that, upon standing, isomerized to the conjugated isomer **7**. This reaction is the same as that previously conducted with the methoxy analog system; the same behavior and the same aldehyde products were previously found.[11] Also similar to the behavior of the analogous methoxy system, to the limit of detection by GC–mass spectral analysis, none of the alcohol products **8** (see below), the ultimate products formed by cationic cleavage toward the phenyl group, was produced. The GC–mass spectral detection limit was established to be >1000:1 by successful analysis of mixtures of authentic samples of the products in this ratio.

In order to study the radical chemistry of the new system, the PTOC (acronym for ((2-thioxopyridinyl)-*N*-oxy)carbonyl) ester derivative **9** was prepared.[14] PTOC esters react in radical chain reactions in which a number of different types of radicals successfully propagate the chain by addition to the thione moiety.[15] Thus, they can be employed in indirect kinetic studies wherein the reactive hydrogen atom donors thiophenol and benzeneselenol serve as calibrated trapping agents.[16,17]

Radical chain reactions of **9** in the presence of thiophenol (2.0 M, 34 °C) or benzeneselenol (0.43 and 0.91 M, 37 °C) gave products **11H** and **12H** in a 47:1 ratio, respectively, along with minor amounts (0.06, 0.2, and 0.4%) of cyclopropane **1a** (Scheme 3). The rate constants for ring opening of radical **10•** can be calculated by making the standard assumption of indirect kinetics that the rate constants for reaction of the hydrogen atom transfer trapping agents with radical **10•** are the same as those for reactions with the parent radical, cyclopropylcarbinyl.[18] From the results for the PhSeH reactions at 37 °C,[19] one calculates rate constants of 6×10^{11} s^{-1} (**10•** → **11•**) and 1.2×10^{10} s^{-1} (**10•** → **12•**). For the reaction conducted with PhSH at 34 °C,[20] one calculates approximate rate constants of 5×10^{11} and 1×10^{10} s^{-1}, respectively, for the two ring openings.

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(12) van Daalen, J. J.; Kraak, A.; Arens, J. F. *Recl. Trav. Chim. Pays-Bas* **1961**, *80*, 810–818.

(13) Barbot, F.; Miginiac, P. *Synth. Commun.* **1983**, 651–654.

(14) The requisite acid for preparation of PTOC ester **9**, (*trans,trans*-2-*tert*-butoxy-3-phenylcyclopropyl)acetic acid, was synthesized by Swern oxidation of alcohol **4** to give the aldehyde, methylenation of this aldehyde with the methylene Wittig reagent, hydroboration–oxidation of this alkene with 9-BBN, Swern oxidation of the homologated alcohol to the aldehyde, and oxidation of this aldehyde to the desired acid with $AgNO_3$/KOH. Reaction of this acid with oxalyl chloride followed by the sodium salt of *N*-hydroxypyridine-2-thione gave PTOC ester **9**. All intermediates and PTOC ester **9** had appropriate NMR spectra. The acid precursor to **9** had an appropriate molecular ion in high-resolution mass spectrometry. Full details of the preparation and reactions of PTOC ester **9** will be submitted for publication.

(15) Barton, D. H. R.; Crich, D.; Motherwell, W. B. *Tetrahedron* **1985**, *41*, 3901–3924.

(16) Newcomb, M. Glenn, A. G. *J. Am. Chem. Soc.* **1989**, *111*, 275–277.

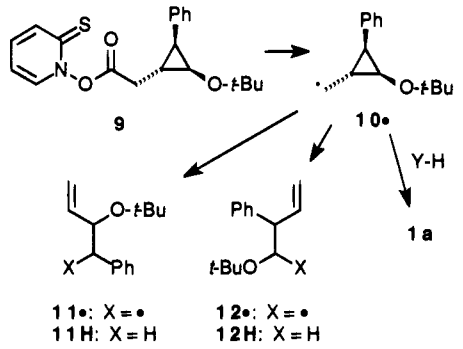
(17) Newcomb, M.; Manek, M. B. *J. Am. Chem. Soc.* **1990**, *112*, 9662–9663.

(18) Newcomb, M. *Tetrahedron* **1993**, *49*, 1151–1176.

(19) Newcomb, M.; Varick, T. R.; Ha, C.; Manek, M. B.; Yue, X. *J. Am. Chem. Soc.* **1992**, *114*, 8158–8163.

(20) Franz, J. A.; Bushaw, B. A.; Alnajjar, M. S. *J. Am. Chem. Soc.* **1989**, *111*, 268–275.

Scheme 3



The 4 orders of magnitude acceleration of the ring opening by the phenyl group in **10•** over that of the parent cyclopropylcarbinyl radical is similar to that previously found for other phenyl-substituted cyclopropylcarbinyl radicals.[17,21,22] Thus, probe **1a** maintains hypersensitive radical reactivity and has the desired high discrimination factor for radical and cationic species.

Enzymatic Hydroxylations. Authentic samples of the expected products of enzymatic hydroxylations of probe **1a** at the methyl position were prepared to permit unambiguous identifications by GC–mass spectrometry and for use in control reactions. Unrearranged alcohol **4** was available from the synthetic sequence leading to probe **1a**. Both diastereomers of alcohol **8**, the ultimate product from ring opening toward the phenyl-substituted carbon of the probe, were prepared by reaction of the zinc chloride derivative of *tert*-butyl allyl ether with benzaldehyde according to the methods of Evans[23] and of Still.[24]

In the hydroxylation reaction of the methyl group in **1a**, ring opening toward the *tert*-butoxy-substituted carbon will initially give a hemiacetal that hydrolyzes to the β,γ-unsaturated aldehyde **6**. As noted above, an authentic sample of **6** was prepared, but a control reaction of **6** showed that, in the buffer system used for the enzyme hydroxylations, it rearranged rapidly to the α,β-unsaturated aldehyde **7**.

In addition to permitting identification and quantitation of enzyme hydroxylation products, the independent synthesis of the ultimate products from oxidation of the methyl group of **1a** permitted important control reactions both for the enzyme oxidations (see below) and for the GC analyses. For example, alcohol **4** gave a single peak with an appropriate mass spectrum in GC–mass spectral analyses with the injector temperature at or below 160 °C. When the injector was above 200 °C, however, authentic **4** decomposed partially to give aldehyde **7** and a new species assigned as 2-*tert*-butoxy-1-phenyl-1,3-butadiene on the basis of the GC retention time and mass spectrum.

Enzymatic hydroxylations of probe **1a** were conducted with microsomal cytochrome P-450 from livers of rats treated with phenobarbital and with reconstituted purified rat P-450 isozyme CYP2B1.[25] The products and yields as determined by GC–mass spectrometry are given in Table 1. The good agreement between the microsome and purified isozyme reactions was expected because CYP2B1 is the predominant isozyme expressed in rat livers by phenobarbital treatment.

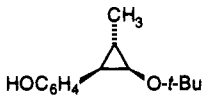
Table 1. Products from Cytochrome P-450 Oxidations of Probe **1a**[a]

enzyme	time (min)	**4**	**7**	**8a**	**8b**	**13a**	**13b**	% yield[b]
microsomes	30	122.7	37.1	4.78	1.17	92	8.4	2.72
CYP2B1[c]	30	53.7	5.41	1.85	0.46	56	7.6	1.28
CYP2B1[d]	10	60.0	6.46	2.14	0.49	73	3.9	1.47
	20	67.2	7.53	2.62	0.58	77	6.6	1.65
	30	84.7	9.67	3.04	0.73	100	8.3	2.11

[a] Absolute yields in nmol as determined by GC–mass spectrometry from oxidations at 37 °C of 2 mg of substrate **1a** with 5 nmol of microsomal cytochrome P-450 or 0.6 nmol of CYP2B1 reconstituted with 1.2 nmol of reductase. The two diastereomers of **8** are designated as **8a,b**. Compounds **13a,b** are tentatively assigned as phenols. [b] Total % yield of oxidation products. [c] Isozyme batch A. [d] Isozyme batch B.

For the products produced by oxidation of the methyl group in **1a**, the unrearranged alcohol **4** was by far the most prevalent, but products from ring opening toward the *tert*-butoxy-substituted carbon (**7**) and toward the phenyl-substituted carbon (**8**) also were formed. Given the high discrimination of the probe substrate, the formation of both **7** and **8** in the amounts observed requires that both radical and cationic routes to rearrangement were followed.

Two other products from hydroxylation of **1a** also were produced, one of these in substantial amounts. Although authentic samples of these two products were not prepared, the GC retention times and mass spectral fragmentation patterns permitted tentative assignments of these products as phenols **13** formed by oxidation of the aromatic ring. Specifically, the



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GC retention times of these products on a Carbowax column were considerably longer than those of alcohols **4** and **8** in a manner reminiscent of the behavior seen for the oxidation products of the *trans*-2-phenylmethylcyclopropane.[8,9] More importantly, these products had similar mass spectra, and the major isomer displayed a strong molecular ion. The latter behavior is typical of phenols whereas the mass spectra of both isomers of **8** and the analogous ring-opened homoallylic alcohol from hydroxylation of *trans*-2-phenylmethylcyclopropane display weak or absent molecular ions due to facile dehydration. We tentatively assign the major isomer **13a** as the *p*-hydroxy species and the minor isomer **13b** as the *o*-hydroxy species.

As indicated in Table 1, only a small amount of probe **1a** was oxidized. In reactions with the microsomes, product recoveries are difficult and low overall yields (ca. 60–70%) of products are often obtained. However, from oxidations with the reconstituted isozyme, product recoveries are much better. In practice, unreacted probe **1a** typically was recovered in 96% yield from the isozyme reactions.

A series of control reactions demonstrated that oxidation products **4**, **7**, and **8** were recovered in high yields and also showed that they were not subject to secondary reactions under the conditions of our experiments. In independent control reactions, authentic samples of these products, in the approximate amounts observed from the oxidations of **1a**, and a test substrate were subjected to CYP2B1 hydroxylation reactions. The observation of hydroxylation of the test substrate guaranteed that actual hydroxylation conditions were obtained.

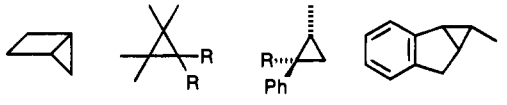
(21) Newcomb, M.; Johnson, C. C.; Manek, M. B.; Varick, T. R. *J. Am. Chem. Soc.* **1992**, *114*, 10915–10921.
(22) Martin-Esker, A. A.; Johnson, C. C.; Horner, J. H.; Newcomb, M. *J. Am. Chem. Soc.* **1995**, *116*, 9174–9181.
(23) Evans, D. A.; Andrews, G. C.; Buckwalter, B. *J. Am. Chem. Soc.* **1974**, *96*, 5560–5561.
(24) Still, W. C.; Macdonald, T. L. *J. Am. Chem. Soc.* **1974**, *96*, 5561–5563.
(25) Nelson, D. R.; Kamataki, T.; Waxman, D. J.; Guengerich, F. P.; Estabrook, R. W.; Feyereisen, R.; Gonzalez, F. J.; Coon, M. J.; Gunsalus, I. C.; Gotoh, O.; Okuda, K.; Nebert, D. W. *DNA. Cell Biol.* **1993**, *12*, 1–51.

From these reactions, products **4**, **7**, and **8** were recovered in 91−98% yield. A critically important point is that no isomerization of unrearranged alcohol **4** could be observed to the limit of detection by GC−mass spectrometry; thus, products **7** and **8** must be primary products from the enzyme-catalyzed hydroxylation of **1a**.

Discussion

The results from oxidation of probe substrate **1a** clarify the disparate kinetic results found with other hypersensitive radicals probes and appear to provide a mechanistic picture consistent with all previous results. In addition, they suggest an unusual mode of reaction for P-450 hydroxylation that might be dissimilar to the pathways of hydroxylations by model compounds.

Previous Hypersensitive Probe Results. When only qualitative results from probe studies of P-450 hydroxylations were available, there was little reason to question the mechanism of Scheme 1. The report by Ortiz de Montellano and Stearns[7] on the oxidation of bicyclo[2.1.0]pentane (**14**), where both unrearranged and rearranged alcohol products were produced, suggested that the rate constant for the oxygen rebound step in Scheme 1 (k_{ox}) could be measured by "timing" the rearrangement of the bicyclo[2.1.0]pent-2-yl radical. Soon after the rearrangement of this radical was established as a "clock" reaction,[26,27] however, Atkinson and Ingold reported the results of a series of P-450 hydroxylations of substrates that were precursors to calibrated radical clocks.[8] Mixtures of rearranged and unrearranged products were found for the five probes **14**−**18**, but the values for k_{ox} calculated from these results varied by nearly 3 orders of magnitude.[8]



14 **15**: R = H **16**: R = CH_3 **17**: R = H **18**: R = Ph **19**

The disparate k_{ox} values from **14**−**18** were rationalized by noting that (1) the radical from **14** was secondary and might be trapped slowly (giving a small k_{ox} value) and (2) the kinetics of rearrangement of the radicals from **17** and **18** might be reduced by the enzyme constraining the phenyl groups such that they overlapped poorly with the breaking cyclopropyl C−C bond (leading ultimately to apparently large k_{ox} values).[8] However, the radical from **14** is better characterized as a "cyclobutyl" type radical and was found to react faster, not slower, than a primary alkyl radical in the only S_H2 reactions measured.[26] Similarly, the idea that the enzyme slowed rearrangements of the radicals from **17** and **18** was not supported by studies of ring openings of a series of structurally constrained aryl-substituted cyclopropylcarbinyl radicals; the structural constraints preventing optimal overlap of the aryl π-system with the breaking C−C bonds were found to have essentially no effect on the kinetics of rearrangement.[22] Later, a detailed study of P-450 hydroxylations of both enantiomers of substrate **17** showed that similar product distributions were obtained from each which, in the context of the enzyme "slowing" the radical rearrangement, would require the unlikely situation wherein the chiral enzyme exerted almost exactly the same influence on both enantiomeric radicals from **17**.[9] Finally, the constrained substrate **19** was hydroxylated by P-450 to give a mixture of products which suggested a k_{ox} value of 1.4×10^{13} s^{-1}.[10] For the series of substrates **14**−**19**, the apparent k_{ox} range for CYP2B1 is about 3 orders of magnitude or the activation free energies span about 4 kcal/mol, a very unlikely premise for a reaction that displays zero activation free energy in the fastest case.

As noted in the introductory comments, probes **14**−**19** suffer from the common limitation that a cyclopropylcarbinyl cationic species will give the same structural rearrangement as a cyclopropylcarbinyl radical species. Accordingly, the observation of rearranged products does not necessarily inform one of the mode of rearrangement. This shortcoming, which is addressed in the design of probe **1a**, was recognized several years ago by White, Groves, and McClusky, who employed norcarane, bicyclo[4.1.0]heptane, as a substrate in P-450 oxidations;[6] different rearrangement pathways exist for the bicyclo[4.1.0]hept-2-yl radical and cation, but unfortunately, only unrearranged products were formed in the P-450 hydroxylations. Another probe that might have differentiated between radical and cationic species is *trans*-1,2-dimethylcyclopropane, the cyclopropylcarbinyl radical from which has a slight preference for opening to the primary radical product,[27] but again, P-450 hydroxylation of this substrate did not give rearranged products.[8,28,29]

A seemingly unquestionable result from the P-450 hydroxylations of probe **1a** is that rearrangements occurred from both radical and cationic species. This provides a simple explanation for the highly variable values of k_{ox} found in previous hypersensitive probe studies. It seems likely that the radical clocks were not functioning as desired. Specifically, in most cases, a portion of the rearranged products apparently derived from cationic intermediates. With the presumption that rearrangement occurred only from radicals, the total amount of rearrangement products from the two processes resulted in apparent lifetimes of radical intermediates that were too long, *i.e.* the value of k_{ox} was calculated to be smaller than it actually was.

Mode of Cation Formation. The above discussion does not address the origin of the cationic rearrangement products. Several possibilities for this exist including (1) requisite cation formation during the hydroxylation reaction, (2) production of a cationic species during the hydroxylation reaction in competition with one or more other processes, (3) formation of a cationic species after the hydroxylation reaction but before enzyme release of product, and (4) cation generation from alcohol products after release. The results from the present and previous probe studies permit one to limit these choices considerably, but a final mechanistic conclusion cannot be made.

The last possibility is easily demonstrated not to be the case. In all previous studies with hypersensitive probes in which rearranged alcohol products were formed, the unrearranged alcohols were shown to be stable to the buffer conditions.[7−10] This behavior was also demonstrated in the present work for alcohol **4**.

Previously, we suggested that an isomerization of alcohol product might occur after hydroxylation but before product release by the enzyme,[10] but this conjecture was naive. Alcohols are competitive inhibitors of P-450,[30] and a recently reported crystal structure of cytochrome P-450(cam) bound to its natural product, *exo*-5-hydroxycamphor, revealed that the product alcohol is held in a position nearly identical to that of the

(26) Newcomb, M.; Manek, M. B.; Glenn, A. G. *J. Am. Chem. Soc.* **1991**, *113*, 949−958.

(27) Bowry, V. W.; Lusztyk, J.; Ingold, K. U. *J. Am. Chem. Soc.* **1991**, *113*, 5687−5698.

(28) Bowry, V. W.; Ingold, K. U. *J. Am. Chem. Soc.* **1991**, *113*, 5699−5707.

(29) It is important to note that the claim that *cis*- and *trans*-1,2-dimethylcyclopropane give rearranged hydroxylation products upon P-450 oxidation (ref 28) was subsequently corrected by the same group (ref 8).

(30) Ortiz de Montellano, P. R.; Reich, N. O. In *Cytochrome P-450 Structure, Mechanism and Biochemistry*; Ortiz de Montellano, P. R., Ed.; Plenum: New York, 1986; pp 273−314.

substrate before oxidation.[31] It is most reasonable to assume that, for all hypersensitive probe studies, the unrearranged alcohol products could access the active site of P-450 after product release. Our observation here and previously[9,10] that the unrearranged alcohols do not isomerize when present during a P-450 oxidation of a test substrate strongly suggests that hydroxylation to the alcohol did not precede rearrangement.

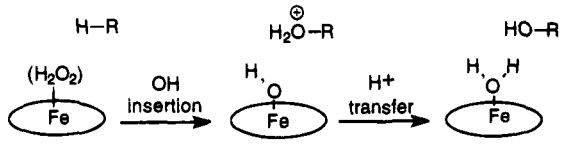
A *requisite* cationic intermediate in P-450 hydroxylation can be excluded for the same reasons it has been excluded for more than a decade. The fact that simple cyclopropanes can be oxidized at the cyclopropylcarbinyl position without rearrangement[6-8,28] requires that cationic species were not formed in these particular reactions. For more than two decades, it has been known that the cyclopropylcarbinyl cation does not exist as a discrete intermediate but rather is a resonance form contributing to the "cyclopropylcarbinyl–homoallyl" cation,[32] and this conclusion remains consistent with recent *ab initio* computations.[33] Requisite cation formation in hydroxylation of probes such as norcarane,[6] methylcyclopropane,[7] and other simple polymethylcyclopropanes[8,28] should have resulted in some rearranged alcohol product, and one might expect that the hypersensitive probes, with powerful cation-stabilizing substituents on the cyclopropyl ring carbons, would extensively rearrange.

By elimination, we conclude that the cationic species was formed *during* the hydroxylation in competition with one or more other processes. We can imagine two possibilities for the competing processes: (1) a radical species formed by hydrogen abstraction is subsequently oxidized to a cationic species in competition with collapse that gives an alcohol; (2) the hydroxylation reaction gives a protonated alcohol as the first formed species, the deprotonation of this species competes with loss of water (solvolysis) to give the cation.

The aryl substituents in probes **17–19**, both the aryl group and the ether in **1a**, and the 1-methyl substituent in radicals from probes **15** and **16** might have an effect on the oxidation potential of a cyclopropylcarbinyl radical. It would appear even more likely, however, that the substituents in the hypersensitive probes would have a large effect on solvolysis reactions. For the aryl-substituted probes and **1a**, anchimeric assistance in a solvolysis step is likely, probes **15** and **16** would give tertiary carbocations upon rearrangement, and the high reactivity toward solvolysis of the bicyclic system in probe **14** is well documented.[34] In addition, if radical oxidation occurred, one must question why P-450 hydroxylation of isopropylcyclopropane gave no detectable rearranged products when probes **14–16** did;[8] the tertiary radical formed in this case almost certainly has a lower oxidation potential than the primary radicals from probes **15–19** and the "cyclobutyl" radical from **14**.

One might favor the solvolysis of a first formed protonated alcohol species as the likely origin of cationic species in P-450 hydroxylations, but such a process would require an almost heretical premise. The only reasonable method for producing a protonated alcohol product appears to be insertion of OH into the C–H bond before water is lost from the iron–hydrogen peroxide complex. Such a sequence would give an iron–hydroxide species and the protonated alcohol which should rapidly experience proton transfer to give the neutral product and water ligated to iron (Scheme 4). However, it is generally

Scheme 4



assumed that P-450 oxidation involves loss of water first to give an iron–oxo intermediate that effects the oxidation reaction. Although no iron–oxo intermediate has been detected in a P-450 oxidation reaction, this species is closely related to the known iron–oxo species formed in peroxidase decomposition of hydrogen peroxide (so-called compound 1) and to intermediates produced in P-450 model reactions.[35]

The results from probe studies to date limit the possibilities for but cannot provide the details of cation formation. Studies with related species might do so. The lifetime of the radical in the hydroxylation of **1a** is very short (see below). If one assumes that similarly short-lived radicals are produced in all P-450 hydroxylations, then the major pathway for rearrangement in most cases involves cationic species. Accordingly, a comparison of the amounts of rearrangement upon P-450 hydroxylation of a series of aryl-substituted cyclopropylmethane probes with (1) the solvolysis behavior of an analogous series of cyclopropylmethanol derivatives and (2) the (calculated) oxidation potentials of analogous cyclopropylcarbinyl radicals might be informative.

The Concerted, Nonsynchronous Hydroxylation Mechanism. Irrespective of the timing of water expulsion in a P-450 oxidation, whether "O" or "OH" is inserted into the C–H bond, or whether cationic species are produced by oxidation of a radical or solvolysis, the lifetime of the radical species produced in the hydroxylation of **1a** can be calculated as long as we assume that the enzyme does not influence the rate of radical rearrangement. From the ratio of rearranged alcohols **8** to the sum of unrearranged alcohol **4** plus cation-derived product **7** and using the measured rate constant for rearrangement of radical **10•**, one calculates a rate constant for capture of the radical of 1.5×10^{13} s^{-1}. That is, the lifetime of the radical is only about 70 fs. This lifetime is similar to that found in hydroxylation of constrained probe **19**.[10,36] The extremely short lifetime of the radical has broad-reaching ramifications.

First, a lifetime of less than 100 fs requires that the radical was not a true intermediate but, instead, was only a component of a reacting ensemble (or transition structure). Simply stated, there can be no local energy minimum for the radical (and hence, by definition, no intermediate) because there is no activation energy for the capture process which occurs at the rate of a single Fe–O bond vibration.[37] This assertion might at first seem to be counterintuitive because some of the radical was diverted to rearranged product, but it is correct. One simply has a situation wherein a zero activation energy process (collapse to unrearranged product) and a process with a small activation energy (rearrangement) compete.

Accordingly, the hydroxylation reaction must be a concerted insertion as opposed to a discrete abstraction reaction followed by a discrete collapse reaction. This again follows from definition; if no intermediate is produced in a reaction, then the reaction is concerted.

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(35) See the discussions in refs 2–4.

(36) The intent of the study in ref 10 was to use a species which would give a radical that had an unambiguous rate constant for rearrangement, but the effect might have been to give an alcohol product resistant to cationic rearrangement due to the structural constraints of the system.

(37) *Infrared Band Handbook, Supplements 3 and 4*; Szymanski, H. A., Ed.; Plenum: New York, 1966; p 232.

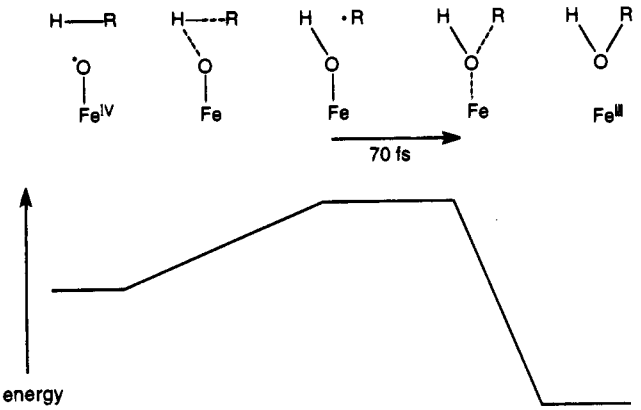

Figure 1. Sequence of nuclear motions in cytochrome P-450 hydroxylation. The "iron−oxo" species is arbitrarily formulated as an Fe(IV)−oxyl radical.

Despite the fact that the hydroxylation process is concerted, the short but finite lifetime of the radical indicates that nuclear motion is nonsynchronous. Abstraction of hydrogen from carbon must precede collapse of the C−O bond as shown schematically in Figure 1. Upon consideration, such nonsynchronous behavior is what would be expected for this insertion reaction. C−H bond vibrational rate constants are 4−6 times as fast as those of Fe−O bonds.[37] Effectively, vibrations are the dynamic components isolated as nuclear motions in the insertion.

Finally, the short "radical" lifetime suggests that the organization of the reacting ensemble is unlike that of a typical hydrogen atom abstraction reaction. Hydrogen atom abstractions from carbon occur with linear or nearly linear C−H−X arrays,[38] and the distance between the O and C atoms in a linear transition structure for hydrogen abstraction by an alkoxy radical is about 2.5 Å,[39,40] or greater than the length of a C−O bond by about 1 Å. A lifetime of about 100 fs is too short for translation over this distance but is consistent with the velocities of simple bond stretches or rotational processes.[41] Rather than a linear organization for the abstraction, a more appropriate model might involve a "side-on" approach of the oxygen atom to the C−H bond in which the oxygen atom is within bonding distance to carbon at the instant of hydrogen abstraction. It is interesting to note that a "side-on" approach of oxygen to the C−H bond might also be suggested by the very similar positions relative to the enzyme of the substrate (camphor) and product (*exo*-5-hydroxycamphor) in the respective crystal structures of cytochrome P-450(cam).[31,42]

Conclusions

The cyclopropyl probe substrate **1a** has been shown to be useful for discriminating between radical and cationic species, and the rate constant for rearrangement of the radical produced by hydrogen abstraction from the cyclopropylmethyl group in **1a** (i.e., radical **10**•) has been calibrated at 37 °C. Cytochrome P-450 hydroxylation of probe **1a** has clarified the confusing results regarding the rate constants for oxygen rebound determined with other hypersensitive probes. Both radical and cationic species are produced during the hydroxylation reaction, but in previous studies, the same rearranged products were formed from each species. Cation formation occurs during the hydroxylation reaction, but the details of this process remain speculative. The lifetime of the radical is extremely short, *ca.* 70 fs, which requires that the radical was a part of a reacting ensemble as opposed to a true intermediate. The hydroxylation reaction is thus a concerted insertion process, but nuclear motion is nonsynchronous with C−H bond breaking preceding C−O bond formation. From the short lifetime of the radical, we infer that hydrogen atom abstraction does not occur with a linear C−H−O organization but via a "side-on" approach of oxygen to the C−H bond.

In biochemical studies, probe **1a** and other probes of similar design should prove useful in mechanistic studies of other oxidizing enzymes such as the methane monooxygenases and monoamine oxidase. In the continuing efforts of chemists to emulate the remarkable enzyme-catalyzed hydroxylation of unactivated C−H positions, the product distributions from oxidation of probe **1a** should prove useful for mechanistic comparisons of enzymes and their mimics.

Experimental Section

General. Reagents were purchased from Aldrich Chemical Co. and used as received. All air and moisture sensitive reactions were performed in flame-dried glassware under an atmosphere of N_2. THF and ether were distilled from sodium and benzophenone ketyl. CH_2Cl_2 was distilled from P_2O_5. Benzene and hexanes were distilled from CaH_2. NMR spectra were obtained at 300 MHz (^{1}H) and 75 MHz (^{13}C). Analytical GC was accomplished on a Hewlett-Packard model 5890 GC interfaced to an HP model 5971 mass selective detector. A variety of columns were employed; superior resolutions were obtained on bonded phase Carbowax columns (15 m, 0.25 mm). Radial chromatography was performed on a Chromatotron model 7294T (Harrison Research Corp.) using plates coated with 2 mm of TLC grade silica gel (Merck) with gypsum binder and fluorescent indicator. Melting points were determined on a Thomas Hoover capillary melting point apparatus and are uncorrected. High-resolution mass spectral analyses were performed by the Central Instrument Facility at Wayne State University.

***cis*-β-*tert*-Butoxystyrene (2)** was prepared by reaction of potassium *tert*-butoxide with 0.5 equiv of phenylacetylene in dry DMF under N_2 at 135−140 °C for 4 days (80%) and by reaction of 0.4 equiv of potassium *tert*-butoxide with phenylacetylene in 5.4 equiv of *tert*-butyl alcohol at reflux ($CaCl_2$ drying tower) for 6 days (73%). The reaction mixtures were treated with water and extracted with ether. The ethereal layers were dried ($MgSO_4$) and concentrated. Residual phenylacetylene was distilled at reduced pressure to leave a residue of crude product which was a 96:4 mixture of *cis* and *trans* isomers and contained no other impurities by NMR spectroscopy. The crude mixture was used in the next step. ^{1}H NMR: δ 1.40 (s, 9 H), 5.32 (d, J = 7.2 Hz, 1 H), 6.50 (d, J = 7.2 Hz, 1 H), 7.15−7.18 (m, 1 H), 7.25−7.45 (m, 2 H), 7.65−7.75 (m, 2 H). ^{13}C NMR: δ 28.1, 105.7, 125.4, 128.1 (4 carbons), 137, 141.0.

Ethyl *trans*, *trans*-2-*tert*-Butoxy-3-phenylcyclopropanecarboxylate (3). A mixture of crude *cis*-β-*tert*-butoxystyrene (4.5 g, 25.6 mmol) and 0.42 g of anhydrous $CuSO_4$ in 10 mL of benzene was heated at 75 °C as a solution of 5.4 mL (51.2 mmol) of ethyl diazoacetate in 20 mL of benzene was added dropwise over 2 h. The mixture was heated at reflux for an additional 2 h and allowed to cool to room temperature. Stirring was continued for 12 h. The reaction mixture was treated with water, and the resulting phases were separated. The aqueous layer was extracted with ether, and the combined organic phases were extracted with water and saturated aqueous NaCl solution and dried ($MgSO_4$). Concentration of the organic phase at reduced pressure gave a residue which contained a mixture of **3** and ethyl *cis*,*cis*-2-*tert*-butoxy-3-phenylcyclopropanecarboxylate. Separation of the mixture by column chromatography (silica gel, ethyl acetate−hexanes) gave 3.4 g (51%) of **3** as an oil. The structure of **3** was confirmed by NOE experiments; key observations were an NOE for the signal at δ 2.13 upon irradiation of the aryl region and an NOE for the signal at δ 2.59 upon irradiation of the signal at δ 3.87. ^{1}H NMR: δ 1.15 (s, 9 H), 1.27 (t, J = 7 Hz, 3 H), 2.13 (dd, J = 6.2, 3 Hz, 1 H), 2.59 (dd, J = 7, 6.2 Hz, 1 H), 3.87

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(dd, $J = 7$, 3 Hz, 1 H), 4.16 (q, $J = 7$ Hz, 2 H), 7.18−7.32 (m, 5 H). ^{13}C NMR: δ 14.3, 27.7, 30.1, 32.0, 59.2, 60.7, 75.8, 126.2, 127.8, 128.3, 136.1, 172.6. MS: *m/z* (relative intensity) 206 (16), 188 (48), 178 (16), 177 (100), 161 (12), 149 (15), 133 (39), 132 (18), 131 (70), 115 (19), 104 (15), 103 (23), 91 (7), 77 (14), 57 (97). HRMS: calcd for $C_{12}H_{14}O_3$ $(M - 56)^+$, 206.0943; found, 206.0938.

(*trans*, *trans*-2-*tert*-Butoxy-3-phenylcyclopropyl)methanol (4). A mixture of ester **3** (3.4 g, 13 mmol) and $LiAlH_4$ (1.0 g, 26 mmol) in 50 mL of ether was heated at reflux for 12 h. The mixture was cooled to 0 °C, and excess hydride reagent was quenched by sequential addition of 1 mL of water, 1 mL of 15% aqueous NaOH solution, and 3 mL of water. The ethereal solution was filtered, and the solid residue was triturated with ether. The combined organic solutions were washed with saturated aqueous NaCl solution and dried ($MgSO_4$). Solvent was removed at reduced pressure, and the residue was purified by column chromatography (silica gel, ethyl acetate−hexanes) to give 2.67 g (95%) of **4** as a white solid. Mp: 65 °C. ^{1}H NMR: δ 1.05 (s, 9 H), 1.64−1.72 (m, 1 H), 1.87 (t, $J = 6.6$ Hz, 1 H), 3.46 (dd, $J = 6.6$, 3.3 Hz, 1 H), 3.61−3.75 (m, 2 H), 7.14−7.29 (m, 5 H). ^{13}C NMR: δ 27.7, 28.2, 29.1, 56.7, 64.2, 75.1, 125.3, 127.6, 128.0, 138.4. MS: *m/z* (relative intensity) 220 (M^+, 2), 189 (5), 164 (16), 146 (45), 133 (71), 117 (73), 105 (12), 91 (30), 77 (12), 57 (100), 41 (34), 29 (15). HRMS: calcd for $C_{14}H_{20}O_2$, 220.1463; found, 220.1458.

***trans*, *trans*-2-*tert*-Butoxy-3-phenylmethylcyclopropane (1a).** Alcohol **4** (0.2 g, 0.9 mmol) was converted to its mesylate by reaction with 77 μL of freshly distilled methanesulfonyl chloride (1 mmol) and Et_3N (190 μL, 1.36 mmol) in 10 mL of dry THF at −20 °C for 1 h. The temperature was maintained at −20 °C as the mixture was treated with $LiEt_3BH$ (3.6 mL of a 1 M solution in THF, 3.6 mmol). The mixture was stirred at −20 °C for 2 h, at −10 °C for 1 h, and at room temperature for 12 h. Unreacted hydride reagent was quenched by slow addition of 2.2 mL of aqueous 3 N NaOH solution. The mixture was treated with 2.2 mL of 30% H_2O_2 (slow addition) and then heated at reflux for 1 h. After the mixture was cooled, the layers were separated and the aqueous layer was extracted three times with ether. The combined organic phases were washed with saturated aqueous NaCl solution, dried ($MgSO_4$), and concentrated at reduced pressure. Purification of the residue by radial chromatography (silica gel, ethyl acetate−hexanes) gave a 2:1 mixture of the desired **1a** and 4-*tert*-butoxy-3-phenyl-1-butene. The mixture was allowed to react with 78 mg of mcpba in 5 mL of CH_2Cl_2 for 12 h. The solution was washed with water and concentrated at reduced pressure. Radial chromatography (silica gel, ethyl acetate−hexanes) gave 100 mg (54%) of **1a** which solidified on standing. Mp: 33 °C. ^{1}H NMR: δ 1.06 (s, 9H), 1.17 (d, $J = 6$ Hz, 1 H), 1.25−1.31 (m, 1 H), 1.61 (t, $J = 6.3$ Hz, 1 H), 3.23 (dd, $J = 6.3$, 3.6 Hz, 1 H), 7.11−7.29 (m, 5 H). ^{13}C NMR: δ 16.9, 21.9, 27.8, 31.6, 59.9, 74.6, 124.9, 127.5, 127.9, 139.6. MS: *m/z* (relative intensity) 204 (M^+, 2), 148 (84), 133 (20), 146 (45), 133 (71), 131 (10), 117 (9), 115 (9), 105 (26), 91 (52), 78 (10), 57 (100). HRMS: calcd for $C_{14}H_{20}O$, 204.1514; found, 204.1510.

When the above reaction was conducted at −5 °C instead of −20 °C, 4-*tert*-butoxy-3-phenyl-1-butene was the major product. Purification by radial chromatography as above gave this product as an oil in 31% yield. ^{1}H NMR: δ 1.16 (s, 9H), 3.50−3.61 (m, 3 H), 5.05−5.15 (m, 2 H), 6.02−6.14 (m, 1 H), 7.21−7.35 (m, 5 H). ^{13}C NMR: δ 27.5, 50.3, 65.6, 72.8, 115.6, 126.3, 128.1, 128.2, 139.5, 142.2. MS: *m/z* (relative intensity), 204 (M^+, 2), 203 (3), 174 (18), 146 (9), 131 (17), 118 (53), 117 (35), 115 (18), 91 (18), 57 (100). HRMS: calcd for $C_{14}H_{20}O$, 204.1514; found, 204.1511.

2-*tert*-Butoxy-1-phenyl-3-buten-1-ol (8). Allyl *tert*-butyl ether was prepared by the method of Lawesson *et al.*[43] A solution of this ether (1 g, 8.8 mmol) in 10 mL of THF was added dropwise to a solution of 8.8 mmol of *sec*-butyllithium (7 mL of a 1.25 M solution in cyclohexane) in 40 mL of THF at −78 °C under nitrogen. Stirring was continued for 15 min. A solution of 1.2 g (1 equiv) of $ZnCl_2$ in THF was added to the −78 °C mixture.[23,24] After 30 min, 0.93 g (8.77 mmol) of freshly distilled benzaldehyde was added. The solution was allowed to warm to room temperature and was stirred for 24 h. The mixture was treated with saturated aqueous NH_4Cl solution, the phases were separated, and the aqueous phase was extracted three times with ether. The combined organic phases were washed with saturated aqueous NaCl solution, dried ($MgSO_4$), and concentrated at reduced pressure. Purification of the residue by column chromatography (silica gel, ethyl acetate−hexanes) gave the desired alcohol as a 3:1 mixture of *anti* (**8a**) and *syn* (**8b**) isomers (1.2 g, 57%). The ^{1}H NMR spectra were deduced from the mixture. ^{1}H NMR for **8a**: δ 1.21 (s, 9 H), 3.25 (s, 1 H), 3.99 (t, $J = 6.8$ Hz, 1 H), 4.37 (d, $J = 7.3$ Hz, 1 H), 5.04−5.08 (m, 2 H), 5.66−5.77 (m, 1 H), 7.26−7.34 (m, 5 H). ^{1}H NMR for **8b**: δ 1.14 (s, 9 H), 3.25 (s, 1 H), 4.09−4.14 (m, 1 H), 4.66 (d $J = 3.2$ Hz, 1 H), 5.13−5.24 (m, 2 H), 5.66−5.77 (m, 1 H), 7.26−7.34 (m, 5 H). MS for the mixture: *m/z* (relative intensity) 163 (6), 129 (4), 115 (22), 114 (23), 107 (100), 105 (13), 91 (4), 79 (28), 77 (22), 57 (91). HRMS for the mixture: calcd for $C_{10}H_{11}O_2$ $(M - 57)^+$, 163.0758; found, 163.0751.

(*Z*)-2-Phenyl-2-butenal (7). 4,4-Diethoxy-3-phenyl-1-butene was prepared by the method of Barbot *et al.*[44] As reported, hydrolysis of this acetal in formic acid−pentane by the method of Barbot and Miginiac[13] gave a crude mixture (6:1) of unconjugated (**6**) and conjugated (**7**) aldehyde that could not be purified by chromatography. The hydrolysis reaction[13] was repeated with the exception that the reaction was heated at 50 °C for 30 min to give a 10:1 mixture of the (*Z*) and (*E*) isomers of **7** in 55% yield. An ethereal solution of this mixture and a saturated aqueous $NaHCO_3$ solution was stirred vigorously for 1 h. After separation of the phases, the ethereal solution was dried ($MgSO_4$) and concentrated at reduced pressure to give (*Z*)-**7** as the only product detectable by NMR spectroscopy. ^{1}H NMR: δ 2.02 (d, $J = 7.3$ Hz, 3 H), 6.87 (q, $J = 4.5$ Hz, 1 H), 7.19−7.49 (m, 5 H), 9.62 (s, 1 H). ^{13}C NMR: δ 15.9, 127.9, 128.2, 129.5, 132.2, 145.0, 151.4, 193.6.

Enzyme Oxidations. Microsomes were prepared[45] from the livers of inbred Fischer 344 male rats (8−10 weeks old) which were fed rodent lab chow and water *ad libitum*. The rats were pretreated with phenobarbital (0.1% in the drinking water). The microsomes were suspended in 100 mM potassium phosphate buffer, pH 7.4, containing 1 mM EDTA and 20% glycerol and were stored at −70 °C. Reaction mixtures of 2.0 mL total volume in 5 mL round-bottomed flasks were prepared containing 5 nmol of microsomal cytochrome P-450 and *ca.* 2 mg (9.8 μmol) of substrate **1a** in 50 mM potassium phosphate buffer, pH 7.4, containing 1 mM desferrioxamine and 0.075 M KCl. The reaction mixtures were preincubated at 37 °C in a shaking water bath, and the reaction was initiated by the addition of 110 μL of 22 mM NADPH solution in buffer (the final concentration of NADPH was 1.2 mM). The reaction mixtures were incubated at 37 °C for varying lengths of time and terminated by placing the flasks on ice. Each reaction mixture was mixed with 2 mL of CH_2Cl_2. The resulting mixture was transferred to a test tube, stirred with a vortex stirrer for 1 min, and centrifuged at 2000 rpm for 10 min on a clinical centrifuge. The organic layer was removed, and the extraction procedure was repeated twice. The combined organic extracts were dried ($MgSO_4$), and a standard was added (10 μL of a solution containing 12 mg of hexadecane in 25 mL of hexane). The mixtures were concentrated under a gentle stream of nitrogen and analyzed by GC−MS. The identification of the oxidation products was achieved using the scan mode on the mass spectrometer. Quantitation was achieved by using the MS in selected ion monitoring (SIM) mode with response factors determined under the same SIM conditions.

For oxidations with isozyme CYP2B1, the purified[46] enzyme (0.6 nmol) was reconstituted with reductase[47] (1.2 nmol) and dilauroylcholine (96 nmol) in a total volume of 2 mL of buffer. The oxidations and analyses were conducted as described above.

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