Hypersensitive Mechanistic Probe Studies of Cytochrome P450-Catalyzed Hydroxylation Reactions. Implications for the Cationic Pathway

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Abstract: Details of the mechanism of cytochrome P450-catalyzed hydroxylation reactions were investigated by oxidation of *trans*-2-phenyl-1-alkylcyclopropanes (alkyl = methyl (**1**), ethyl (**2**), 1-propyl (**3**), 1-methylethyl (**4**)) and *trans*-2-(4-(trifluoromethyl)phenyl)-1-alkylcyclopropanes (alkyl = methyl (**5**), ethyl (**6**)). The syntheses of **3** and **6** and their possible oxidation products are reported. Oxidation of the probes with the cytochrome P450 isozyme CYP2B1 gave unrearranged cyclopropylcarbinols as major products and small amounts of ring-opened alcohol products in all cases except for **4**. Phenolic products also were produced from substrates **1**−**4**. The maximum lifetimes of putative radical intermediates were less than 1 ps, and the results with substrate **4** require that no intermediate was formed. The results were analyzed in the context of recent mechanistic proposals for cytochrome P450-catalyzed hydroxylations. Oxidation of a "radical" component in the transition state of an insertion reaction to produce a cation is inconsistent with the results. The results also provide little support for a new alternative mechanism for hydroxylation, the agostic complex model (Collman, J. P.; Chien, A. S.; Eberspacher, T. A.; Brauman, J. I. *J. Am. Chem. Soc.* **1998**, *120*, 425). Formation of "cationic" rearrangement products via solvolysis of first-formed protonated alcohol products produced by insertion of the "OH^+" moiety from iron-complexed hydrogen peroxide also is not supported by the results. The most consistent mechanistic description is the recently reported multistate reactivity paradigm (Shaik, S.; Filatov, M.; Schroder, D.; Schwarz, H. *Chem. Eur. J.* **1998**, *4*, 193).

Introduction

The cytochrome P450 (P450) enzymes are broadly distributed in plants, animals, and bacteria.[1−3] These enzymes are capable of catalyzing a variety of reactions, the most energetically difficult of which is the hydroxylation of unactivated C−H positions at ambient temperatures. The active sites of P450 enzymes are characterized by a protoporphyrin IX−iron complex (heme) with thiolate from a cysteine of the enzyme serving as the fifth ligand to iron. Considering the invariant structure of the active sites of the P450 enzymes, it is reasonable to believe that a common mechanism exists for hydrocarbon hydroxylation reactions catalyzed by these enzymes and that substrate specificity and regio- and stereoselectivity results from the tertiary protein structures. Considerable information concerning the timing of events in P450 hydroxylations, such as substrate and oxygen binding, is known, but the mechanism of the hydroxylation step is not resolved.

For the past two decades, the consensus mechanism for P450-catalyzed hydroxylation involved production of an iron−oxo species that reacted with hydrocarbons in a two-step reaction, hydrogen abstraction by the iron−oxo to give an alkyl radical that subsequently displaced hydroxy from iron in a homolytic substitution.[4−7] Recent studies with hypersensitive radical probe/clock substrates, however, indicate that the "lifetime" of a radical in P450 hydroxylation is too short (<100 fs) for a true intermediate, implicate an insertion process, and further suggest that cationic species can be produced in P450 hydroxylations.[8] Details of the studies that led to the various conclusions are in the Discussion. As evidence militating against a true radical intermediate in P450 hydroxylations has appeared, so also have mechanistic alternatives to the abstraction−rebound pathway. For example, Shestakov and Shilov discussed the possibility that a five-coordinate carbon species might explain isomerization results in P450 hydroxylations.[9] Collman, Brauman, and co-workers have recently proposed an "agostic alternative" to the oxygen rebound mechanism and demonstrated that hydroxylation reactions by iron−oxo species can be inhibited by both hydrogen and methane.[10] Shaik, Schwarz, and co-workers have presented an insightful analysis of the P450

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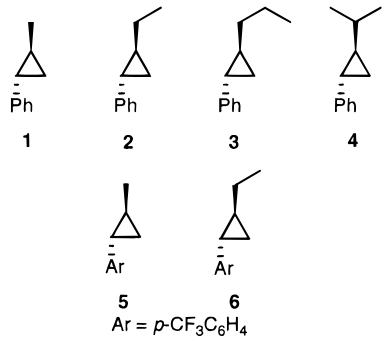


Figure 1. Probes employed in P450-catalyzed hydroxylations.

hydroxylation reaction in terms of a multistate reactivity paradigm that permits stepwise processes in competition with concerted processes and relates P450 hydroxylations to reactions of metal oxenoid cations.[11]

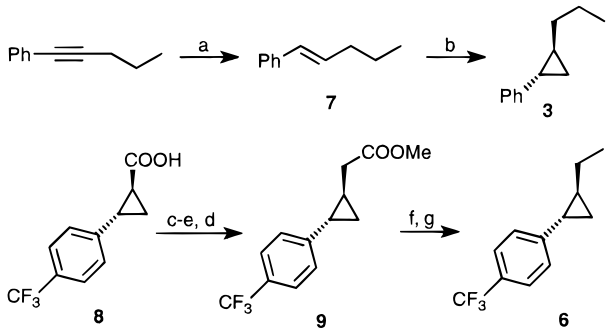
The consensus abstraction−rebound mechanism and most of the mechanistic alternatives begin from the premise that an iron−oxo species is the active oxidant in P450, but it is also possible that a hydroperoxo−iron or an iron−hydrogen peroxide complex is the active oxidant in P450 (see the Discussion). An understanding of the details of the pathway for formation of a cationic species in P450 hydroxylation appears to be critical for descriptions of both the mechanism of hydroxylation and the active oxidant. In an attempt to study this aspect of P450 hydroxylations, we have employed two sets of "radical probes" with varying alkyl substitution at the probe center in P450 hydroxylations. The study was designed specifically to test for the possibility that cationic rearrangement products are formed by oxidation of "radicals" in transition states for insertion reactions; large changes in product distributions would be expected to follow changes in the radical oxidation potentials. Our results are not consistent with radical oxidation events as the sources of cationic species and are most in line with predictions of the multistate reaction model described by Shaik, Schwarz, and co-workers.[11]

Results

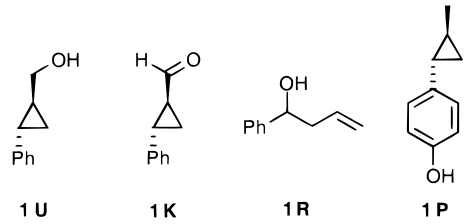
Probes and Oxidation Products. Oxidations of two series of probes (Figure 1) are compared in this work. One series (**1**−**4**) contained the *trans*-2-phenylcyclopropyl moiety. The parent for this series, methyl-containing probe **1**, has been used previously in studies of hydroxylations by P450,[12,13] chloroperoxidase,[14,15] soluble methane monooxygenase (sMMO),[16] and an alkane monooxygenase.[17] The ethyl- and isopropyl-containing probes **2** and **4** were used with equivocal results in a study of chloroperoxidase.[15] Probes **5** and **6** contain the *trans*-2-(*p*-(trifluoromethyl)phenyl)cyclopropyl moiety. Both enantiomers of probe **5** were used in a study of P450 hydroxylations.[18]

A variety of oxidation products can be formed in the P450-catalyzed hydroxylations. Hydroxylation at the cyclopropylcarbinyl position gives an unrearranged alcohol product which can be oxidized to a carbonyl compound. Functionalization of the cyclopropylcarbinyl position with a radical or cationic ring opening ultimately gives a ring-opened alcohol product. The phenyl-containing probes **1**−**4** also can be oxidized to phenolic products, but phenols have not been observed with (trifluoromethyl)phenyl-containing probes **5** and **6**. For simplicity, we have labeled the oxidation products consistently with the number corresponding to the probe from which they derive and a letter indicating the type of oxidation product. Thus, the products from each probe are unrearranged alcohol **U**, aldehyde or ketone product **K**, rearranged alcohol **R**, and phenol **P** as shown below for probe **1**.

Scheme 1[a]



[a] Key: (a) LAH, THF, Reflux; (b) (i) Et_2Zn, CH_2I_2, CH_2Cl_2, −30 °C; (ii) *m*-CPBA, CH_2Cl_2, rt; (c) Oxalyl Chloride, DMF, Benzene, rt; (d) CH_2N_2, CH_2Cl_2, Ether, rt; (e), Ag_2O, $Na_2S_2O_3$, Dioxane, Water, 75 °C; (f) LAH, THF, 0 °C; (g) (i) Et_3N, MsCl, THF, −30 °C; (ii) $LiEt_3BH$, −78 °C to rt.



The syntheses of probes **1**, **2**, **4**, and **5** and several of their possible oxidation products were reported previously.[15,16,18] Probes **3** and **6** are new, and the synthetic routes to these compounds are shown in Scheme 1. Probe **3**, previously reported as a mixture of diastereomers from a carbenoid addition to 1-pentene,[19] was prepared as a pure diastereomer in two steps from commercially available 1-phenyl-1-pentyne. Lithium aluminum hydride (LAH) reduction by the method of Schwan[20] afforded selectively the *trans*-alkene **7**. Alkene **7** was cyclopropanated by diethylzinc and diiodomethane to afford **3**. Compound **3** was conveniently purified by treating the crude product mixture from the cyclopropanation reaction with 3-chloroperoxybenzoic acid, to epoxidize unreacted alkene, and chromatography. The synthesis of probe **6** started with carboxylic acid **8**, which was an intermediate in the synthesis of probe **5**.[18] Homologation of **8** by the Arndt−Eistert sequence[21] followed by esterification with diazomethane afforded the corresponding cyclopropylacetic acid derivative **9**. The acid resulting from the homologation sequence was esterified for purification purposes. Reduction of ester **9** with LAH to give

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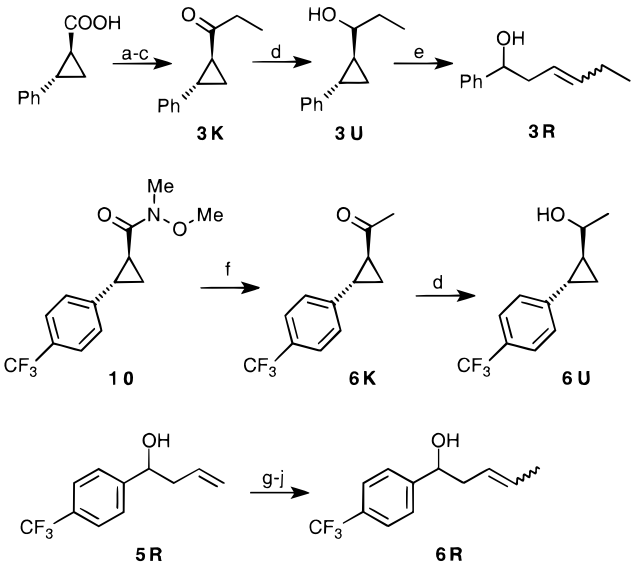
Scheme 2[a]



[a] Key: (a) Oxalyl Chloride, DMF, Benzene, rt; (b) MeONHMe·HCl, Pyridine, CH_2Cl_2, rt; (c) PrMgBr, Ether, 0 °C; (d) LAH, THF, 0 °C; (e) (i) Et_3N, MsCl, THF, −30 °C; (ii) Water, Reflux; (f) MeMgBr, Ether, 0 °C; (g) TBSCl, Imidazole, CH_2Cl_2, rt; (h) OsO_4, $NaIO_4$, Ether, Water, rt; (i) Ph_3PEtBr, BuLi, Benzene, rt; (j) Bu_4NF, THF, rt.

the corresponding alcohol was followed by a two-step methanesulfonation/lithium triethylborohydride reduction sequence[22] that afforded probe **6**. We note that all probes used in this study were characterized by ^1H and ^{13}C NMR spectroscopy and HRMS and were demonstrated to be >98% pure by NMR spectroscopy and GC analysis.

The products from oxidation of probe **3** were synthesized from commercially available *trans*-2-phenyl-1-cyclopropanecarboxylic acid (Scheme 2). This acid was converted to an amide by the two-step procedure of Weinreb.[23] Addition of ethylmagnesium bromide to the Weinreb amide afforded ketone **3K** that was reduced to a 2:1 mixture of diastereomeric alcohols **3U**[24] with LAH. Methanesulfonation of **3U** followed by hydrolysis in refluxing water afforded the diastereomeric acyclic alcohols **3R**[25] in a 13:1 ratio.

The cyclic products from the oxidation of probe **6** were prepared from Weinreb amide **10** (Scheme 2), also an intermediate in the synthesis of probe **5**.[18] Addition of methylmagnesium bromide to **10** afforded ketone **6K**. Reduction of **6K** with LAH afforded an inseparable 1:1 mixture of diastereomeric alcohols **6U**. Attempts to convert alcohols **6U** to acyclic alcohols **6R** by formation and solvolysis of a mesylate failed; apparently the requisite sulfonate was not formed. Therefore, a more circuitous synthesis of **6R** was developed using **5R**,[18] itself a product from the P450 oxidation of probe **5**, as the starting material. The first step was to protect the hydroxyl group of **5R** as a silyl ether. Oxidation of the double bond to the aldehyde was achieved by treatment of the silyl ether with osmium tetroxide and sodium periodate. The resulting aldehyde was olefinated with the ylide derived from ethyltriphenylphosphonium bromide, and removal of the silyl group with tetrabutylammonium fluoride afforded acyclic alcohols **6R** as a 4:1 mixture of diastereomers.

The phenol **1P** was previously prepared independently in order to demonstrate its formation in enzyme-catalyzed hydroxylations of **1**.[16] This phenol displayed a strong molecular ion in its electron-impact mass spectrum and a significantly longer retention time than other oxidation products of **1** in GC analyses on Carbowax. The production of phenols competes with oxidations at the cyclopropylcarbinyl position of probes **1**−**4** but has little significance in regard to the mechanistic analysis of this work. Thus, phenolic products from P450-catalyzed oxidations of probes **2**−**4** were not synthesized but were identified in the product mixtures from the enzyme oxidations by their GC retention times and mass spectra.

Enzyme-Catalyzed Oxidations. In this work, probes **2**−**4** and **6** were oxidized by the phenobarbital-induced rat liver isozyme CYP2B1.[26] This enzyme is known to be a relatively nonselective oxidation catalyst and has been employed widely in mechanistic studies, including previous studies of probes **1** and **5**.[12,18] The hepatic P450 enzymes require a reductase enzyme which delivers reducing equivalents from NADPH to the substrate−enzyme−oxygen complex and to the subsequently formed substrate−enzyme−superoxide complex. The reductase used in this study was expressed in *E. coli*.[27]

Oxidations were conducted with reconstituted systems consisting of the P450 and reductase enzymes in a 1:2 molar ratio, NADPH, and dilauroylphosphatidylcholine. These oxidations were performed under the same conditions as previously used for the "low concentration" oxidations of probe **5**.[18] Specifically, a solution of approximately 1.2 μmol of substrate in 10 μL of methanol was added to the enzyme mixture, and the reactions were initiated by addition of NADPH. Reactions were terminated after 30 min, and the products were extracted into methylene chloride and analyzed by GC and GC−MS.

Either four or eight P450-catalyzed oxidations of each probe were conducted, and complete results are given in the Supporting Information. Table 1 contains a summary of those hydroxylation reactions as well as the results previously reported for **1** and **5**.[12,18,28] Oxidations of probes **1**−**4** gave unrearranged alcohols **U** and phenols **P**. Rearranged alcohol products **R** were observed for **1**−**3**, but product **4R** from hydroxylation of **4** was not present at the limit of our detection capabilities (ca. 1% relative to **4U**). Some oxidation of **2U** and **3U** occurred to give **2K** and **3K**, respectively. Because oxidation to the carboxylic acid occurs when **1** reacts with chloroperoxidase,[14,15] we have previously searched for this product in the form of its methyl ester (after diazomethane treatment of a product mixture) as well as for product **1K** but did not detect them.[28] In a similar manner, the carboxylic acid from probe **5** was shown not to be formed in P450 hydroxylations.[18] The (trifluoromethyl)phenyl probes **5** and **6** gave results similar to those for **1** and **2** with the exception that phenolic products were not formed. Although hydroxylation reactions might occur at positions other than the cyclopropylcarbinyl center and the phenyl ring, such oxidation reactions were apparently quite minor as deduced from the observation that no unidentified products with GC retention times similar to those of products **U**, **K**, and **R** were present in more than 2% relative to other products. Favored oxidation of the cyclopropylcarbinyl position is consistent with the fact that

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(28) The results for probe **1** are from reactions with microsomes from livers of phenobarbital-treated rats. The major isozyme induced by this treatment is CYP2B1. In unpublished work, virtually identical ratios of unrearranged to rearranged product from probe **1** oxidations by the isozyme CYP2B1 with expressed reductase were observed. Toy, P. H.; Choi, S.-Y.; Newcomb, M. Unpublished results.

Table 1. Results from CYP2B1 Hydroxylation of Probes

substrate	yield of products (nmol)				C/A[a]	% conv[b]	% rec[c]	turnover[d]
	acyclic alcohol	cyclic alcohol	ketone	phenol				
1[e]	60.0	224.0		149.0	4.2			
2	9.5	228.4	9.3	15.8	25.0	17.0	62.0	454
3	2.8	137.8	7.1	19.3	51.7	10.7	69.3	290
4	<0.5	126.7	NA[f]	15.2	>100.0	9.8	79.1	236
5[g]	34.1	128.9	6.4[h]	NA	4.0	13.0		280
6	3.4	78.6	24.3	NA	30.2	8.5	72.3	218

[a] Observed ratio of cyclic to acyclic products. [b] Percent conversion of substrate. [c] Percent recovery of substrate. [d] Number of enzyme turnovers. [e] Data from ref 12. [f] NA = not applicable. [g] Data from ref 18. [h] Aldehyde formed.

Table 2. Recovery and Stability Control Reactions for Oxidation Products

substrate	amount (nmol)	relative percent yields		% yield admixture[c]
		recovery[a]	stability[b]	
2U	740	97	89 (12)[d]	
2K	649	95	109	
2R	518	109	81	
2R	11			118
3U	85	96	31 (44)[e]	
3K	12	88	27	
3R	79	96	0	
3R	20			65
4U	477	91	88	
4R	431	95	43	
4R	17			86
6U	608	105	71 (30)[f]	
6K	351	99	94	
6R	52	98	0	
6R	13			38

[a] NADPH was omitted from the system. [b] The enzyme oxidation system was fully competent. [c] The parent probe and test substrate were subjected to oxidation by the fully competent enzyme system; the percent yields are based on the initial amount of test compound **U** corrected by subtraction of the expected amount of this product from oxidation of the probe. [d] Yield of ketone **2K**. [e] Yield of ketone **3K**. [f] Yield of ketone **6K**.

the cyclopropyl group slightly weakens a C−H bond on an adjacent carbon.[29]

Under the conditions used in this work, approximately 10% of the probe substrate was converted to oxidation products. The recoveries of unreacted probes averaged approximately 70%, resulting in over 80% mass balance in most cases, a very high recovery given that a small amount of relatively volatile probe evaporates when the product mixture is concentrated for GC analysis. An interesting and expected trend apparent from Table 1 is that there was a decrease in the efficiency of the enzyme hydroxylations that correlates with the size of the probes. Nevertheless, the oxidation products were formed in sufficient quantities such that accurate quantitation was possible.

A series of control experiments was performed to determine the recoverability and stability of the products to the oxidation conditions (Table 2). In the first group of experiments, a stock solution of substrate was prepared and equal portions of this solution were added to three reaction mixtures, one lacking enzymes, one lacking NADPH, and one completely competent oxidizing mixture. The reactions were treated in the same manner as the probe oxidation reactions. Following the reaction period, equal amounts of a stock solution containing the hydrocarbon standard were added to each mixture, and the reactions were worked up and analyzed by GC. The GC results from the buffer mixtures were used for calibration, and the relative amount of oxidation product to standard was defined as 100% for these reactions. The yields of the other two reactions relative to the calibration reaction are given in Table 2.

The reactions conducted without NADPH were recovery experiments. The high yields in these experiments demonstrate that in all cases the enzyme mixture does not retain the products. The reactions conducted with fully competent enzyme systems are stability experiments. Cyclic oxidation products from probes **2**, **4**, and **6** were returned in high yields even though a portion of the cyclic alcohols **2U** and **6U** was oxidized to the corresponding ketone. The total recovery from cyclic product **3U** was reduced (75% recovery of **3U** + **3K**), and ketone **3K** was clearly unstable, suggesting that the origin of reduced yield for **3U** involved oxidation to **3K** and further conversion to unknown products. Given the excellent stabilities of the other secondary probe oxidation products (alcohols **2U** and **6U** and ketones **2K** and **6K**), it is tempting to ascribe the instability of the oxidation products from **3** to the presence of the propyl group, but it is not apparent to us why this should be the case.

As one might expect on the basis of the presence of double bonds, the rearranged alcohol products **R** were generally unstable in the presence of the fully competent system. Ring-opened alcohols **3R** and **6R** were completely destroyed, and somewhat more than half of product **4R** was lost, whereas only about 20% of acyclic alcohol product **2R** was lost in the enzyme oxidation.

The stability test was quite severe for two reasons: (1) the test substrates were added at the beginning of the reactions rather than formed as products throughout the reactions, and (2) no substrate other than the oxidation product being tested was present. The latter point is important because the P450 and/or reductase enzymes are destroyed during the course of the reactions[13] and the high concentrations of probe substrate relative to oxidation products might provide some protection for the oxidation products by competitive inhibition. Therefore, we designed a potentially milder stability test for the rearranged oxidation products. In the admixture stability test, the probe substrate mixed with the corresponding rearranged alcohol product **R** (in a small amount consistent with the amount of oxidation observed in the probe studies) was subjected to the enzymatic oxidation. The objective was to determine whether rearranged oxidation product **R** would survive when a competent oxidation reaction was in progress. The yields of rearranged products were determined by GC and then corrected for the amount of product **R** that was expected to be observed in the enzyme oxidation of the probe (i.e., the amount observed in Table 1 studies). In the admixture test, the yield of product **2R**, which was formed in the enzyme oxidation of **2** (see Table 1), exceeded that originally added, suggesting good stability in this more realistic test. Products **3R** and **6R**, which were consumed completely in the severe stability test, were returned in about half of the initial amounts added (65% and 38%, respectively). Product **4R**, which was not observed in the

(29) Walton, J. C. *Magn. Reson. Chem.* **1987**, *25*, 998−1000.

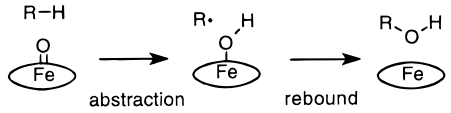


Figure 2. Hydrogen abstraction−oxygen rebound mechanism for P450-catalyzed hydroxylation.

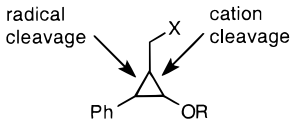
oxidation of probe **4** (see Table 1), was returned in quite good yield from the admixture test, consistent with the partial stability found in the more severe test. In the Discussion, we will argue that the absence of **4R** from oxidation of probe **4** is one of the most important observations of this work, and we consider the stability results with **4R** to be a clear indication that the absence of detectable amounts of **4R** from oxidations of **4** requires that very little, if any, rearranged product was formed from probe **4**.

In one sense, even the relatively mild admixture test should be considered as a "worst case scenario" because the average concentrations of the products in the probe oxidation reactions are only half of the final concentrations. Further, the initial concentrations of the oxidation products being tested in both stability tests were greater than the final concentrations of these products detected in the probe oxidation reactions, and the final concentrations of products in all cases were 10% or less than those of the probe substrates. The partial consumption of oxidation products in the **3** and **6** series equivocates the absolute quantitation of studies with these two probes, and we attempt to address this in the Discussion. However, we conclude that quantitation problems are relatively minor, especially for systems **2** and **4**. In earlier studies, the same conclusion was reached for P450-catalyzed hydroxylations of probes **1** and **5**.[12,18]

Discussion

Mechanistic Descriptions of P450-Catalyzed Hydroxylations. The mechanism of P450-catalyzed hydroxylation that held a consensus position for nearly two decades is shown in Figure 2.[4−7] An iron−oxo species similar to compound I of peroxidase is the oxidant. This species is highly electrophilic, described either as an Fe^{V}═O or an Fe^{IV}═O/porphyrin radical cation. The iron−oxo abstracts hydrogen from an unactivated C−H to give an alkyl radical that subsequently reacts with the iron−hydroxy species in a homolytic substitution reaction termed the oxygen rebound step. This mechanism was deduced from the results of probe studies that gave rearranged products consistent with a radical intermediate and was permissively supported by the observation that kinetic isotope effects were found in P450-catalyzed oxidations of deuterated substrates.

The series of studies that dispute the abstraction−rebound mechanism had the initial objective of "timing" the oxygen rebound step via the use of calibrated radical clocks. A wide range of "radical" lifetimes were found,[12,13,30−32] consistent with free energies of activation for oxygen rebound ranging from 0 to 4 kcal/mol. Although enzymes can easily affect overall activation barriers by more than 4 kcal/mol, the important points here are the zero activation energy found in some cases and the necessity that a relatively large barrier for collapse of a radical pair would have to be imparted in other cases by steric effects that have been demonstrated to be too small to hinder free tumbling of probe **1** in the active site.[12] An eventual conclusion from the cumulative observations with calibrated radical clocks was that an unidentified process could be contributing to the amount of rearranged products obtained from the probe/clocks. Up to that point, all of the probes that gave some rearranged products in P450-catalyzed hydroxylations had the deficiency that the same skeletal reorganization would occur for both radical and cationic rearrangements, and the possibility existed that the unidentified process involved a cationic species. We developed a probe/clock design (**11**) that maintained the ultrafast radical rearrangement necessary for enzyme studies but gave distinct products from radical and cationic rearrangements.[33,34] P450-catalyzed hydroxylation of probe **12** gave



11: X = radical or cation **12**

products from both types of rearrangements, and the radical lifetime from studies of hydroxylation of **12** was only 70 fs.[8] Such a lifetime is too short for a true intermediate, the lifetime for an intermediate calculated from conventional transition state theory must exceed 150 fs, and requires that the radical can only be a component of the transition structure for a concerted, albeit nonsynchronous, insertion process.

The picture that emerges from studies with **12** is that the exceptionally fast ring openings of the radicals from hypersensitive probes (rate constants of about 1×10^{11} s^{-1})[21] are minor processes in competition with collapse of the transition structure that gives unrearranged product (rate constant of ca. 1×10^{13} s^{-1}) and cationic rearrangement products are produced by some competing pathway. But this view leads to new questions. How do "cationic" rearrangements occur during the hydroxylation reactions, and what are the implications of these reactions in regard to the nature of the oxidant?

Recent mechanistic proposals contain competitive processes that might explain the above results. We envisioned two possible routes to cations shown in Figure 3.[8] In (A), an electron-transfer process would compete with collapse in the transition state of the hydroxylation reaction. In (B), the first-formed product in the hydroxylation step is a protonated alcohol subject to a solvolytic-type rearrangement that competes with deprotonation. The former process could be cast in the context of the iron−oxo as the reactive intermediate, whereas the latter seems to require that the actual oxidant is iron-complexed hydrogen peroxide that inserts the OH^{+} moiety. The recent mechanistic description by Collman, Brauman, and co-workers[10] offers an alternative explanation in the context of the iron−oxo as oxidant; in this model, an agostic complex involving an iron interaction with the C−H bond could rearrange in a manner expected for cations before reductive elimination of alcohol product occurred ((C) in Figure 3).

The mechanistic description by Shaik, Schwarz, and co-workers[11] is a new paradigm for P450 hydroxylations that relates them to reactions of simple metal oxenoid complexes. In this model, a competition exists between two reaction manifolds, a high-spin (HS) manifold that is effectively "spin-unpaired" and a low-spin (LS) manifold that is effectively "spin-paired". A portion of this complex model is shown in (D) in Figure 3. The

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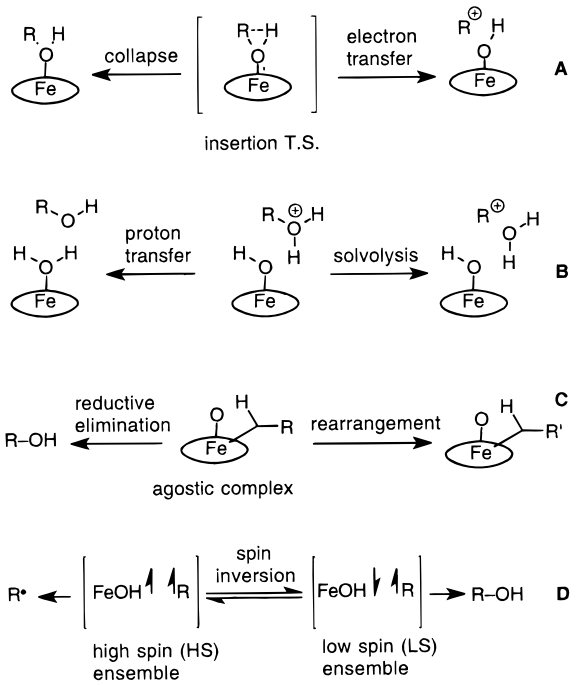

Figure 3. Possible competitions in P450 hydroxylations. In (A), an electron transfer competes with collapse of a transition structure. In (B), solvolysis and deprotonation of a first-formed protonated alcohol product compete. In (C), rearrangement of an iron-bound species competes with reductive elimination. In (D), a high-spin ensemble can react to give a radical by H atom abstraction or experience a state crossing to a low-spin ensemble that can react by insertion. See the text for details and references.

reaction starts in the HS surface and either crosses to the LS surface via spin inversion or remains on the HS surface. If crossing to the LS surface occurs, insertion is possible and stereochemical information is conserved. If, however, the reaction remains on the HS surface, insertion is not possible, and stereochemical information would be lost (or probe rearrangement would occur) due to the formation of a relatively long-lived radical intermediate. A key point in this model is that the probability of spin inversion, or state crossing from the HS to LS surface, is a function of the position of the state crossing point on the reaction coordinate and should be probe dependent.

Implications of Probe Results. The two series of probes employed in the present work provide some insight to the details of the P450-catalyzed hydroxylation mechanism. A single isozyme of P450 was employed, so the results are not complicated by minor processes ascribed to reactions of other enzymes. The enzyme turnover was reasonably high, and the slight decrease in turnover with increasing bulk of the probes is consistent with expected steric effects in binding the substrates.

Because the enzyme system is reconstituted in liposomes, the experiments were designed such that small amounts of substrate were present in order to limit disruption of the enzyme complex. We have previously observed *smaller* enzyme turnover numbers when the concentrations of substrate were increased.[18] The small substrate concentrations and good turnover numbers result in oxidation of about 10% of the probe substrates, and secondary oxidation reactions occur. The severe control reactions showed that ring-opened products **3R** and **6R** are unstable in the absence of substrate, but the more realistic admixture control reactions indicated that about half of these materials would survive the reactions. The unrearranged products **U** largely survived the control reactions although some oxidation to aldehyde/ketone products **K** occurred, and the rearranged alcohol product **4R** from the isopropyl-containing probe **4** was relatively stable even to the severe stability test. Thus, the exact ratios of rearranged to unrearranged oxidation products might not be known for some of the probes, but good approximate ratios can be calculated. In the following discussion, we use the "worst case" calculations of product ratios in that we estimate that the ratios of unrearranged to rearranged oxidation products (C/A) are *smaller* than those observed in the probe oxidation studies. Specifically, for cases where instability of rearranged product was indicated in the control reactions, we have calculated C/A ratios that are adjusted for the amounts of materials lost in the control reactions, and accordingly, these are lower limits of the actual C/A ratios.

The rate constants for rearrangements of putative intermediates are necessary for quantitative discussions. A 2-aryl-substituted cyclopropylcarbinyl cation has no "rate constant" for rearrangement because it is not a true intermediate. Wiberg has demonstrated experimentally and computationally that the actual species is a composite of a cyclopropylcarbinyl cation and a homoallyl cation and, importantly, that production of these aryl-substituted cations in solvolytic reactions of 3-substituted cyclobutyl tosylates resulted in exclusive formation of the homoallyl products.[35] Therefore, although no rate constant is available, one knows that a "cyclopropylcarbinyl cation" produced from probe **1** or **5** must proceed to ring-opened product. The Wiberg results are for primary cyclopropylcarbinyl systems, and alkyl substitution will stabilize the cationic center. Given that an unsubstituted primary "cyclopropylcarbinyl cation" is not an intermediate, that aryl substitution strongly distorts the species toward the homoallyl resonance structure,[35] and that a tertiary carbocation is less stable than a secondary aryl-substituted carbocation as judged by gas-phase hydride ion affinities,[36] we assume that the same situation holds for all other cases of interest here; any cation formed from a probe will result in a rearranged product.

The rate constant for ring opening of the radical derived from probe **1** is known.[21] Using the same method for measuring the ring-opening rate constants, indirect competition kinetics employing benzeneselenol trapping, we have determined the rate constants for ring opening of the cyclopropylcarbinyl radicals derived from probes **2** and **4**;[37] these radicals open somewhat less rapidly than that from **1**. Ring opening of the cyclopropylcarbinyl radical derived from probe **5** has also been determined by the same method;[38] this radical opens about 3 times as fast as that from **1** at ambient temperature due to the stabilizing effect of the CF_3 group. We assume that the radical derived from **3** will ring open with a rate constant equal to that for the radical from **2**, and we estimate the rate constant for ring opening of the radical derived from **6** from the measured value for that from **5** and the observed relative reactivity of the radicals from **1** and **2**.

The minimum value for the ratio of unrearranged to rearranged products and the rate constants at 37 °C, the temperature of the P450 studies, for the radicals derived from each probe are collected in Table 3. Using these values, we compute the minimum value for the oxygen rebound rate constant (k_{ox}) that

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Table 3. Limiting Values for P450 Hydroxylations

probe	C/A[a]	k_R[b] (s^{-1})	k_{ox}[c] (s^{-1})	τ[d] (ps)
1	4.3	3.0×10^{11}	1.3×10^{12}	0.8
2	25	1.5×10^{11}	4×10^{12}	0.3
3	26	(1.5×10^{11})[e]	4×10^{12}	0.3
4	>100	1.2×10^{11}	$>1.2 \times 10^{13}$	<0.08
5	4	9×10^{11}	4×10^{12}	0.3
6	12	(4×10^{11})[f]	5×10^{12}	0.2

[a] Minimum ratio of unrearranged to rearranged product; see the text. [b] Rate constant for ring opening of radicals at 37 °C; see the text. [c] Minimum oxygen rebound rate constant. [d] Maximum radical lifetime. [e] Rate constant assumed to be equal to that for the radical from **2**. [f] Estimated rate constant.

would be necessary if radical ring opening was the only pathway to rearranged products and the corresponding radical lifetime ($\tau = 1/k_{ox}$).

The results in Table 3 in isolation from other results might be rationalized in terms of an extremely fast rebound rate constant if one assumes that the results from **1** and **4** are anomalous. Two obvious problems with this conclusion are (1) the *minimum* value for the oxygen rebound at which the results appear to converge (4×10^{12} s^{-1}) is nearly as fast as collapse of a transition state as calculated from conventional transition state theory (6×10^{12} s^{-1}) and (2) as judged from the product stability studies, the results with probes **1** and **4** should be among the most reliable. In addition, other probes with the same P450 isozyme have given apparent rebound rate constants ranging from 2×10^{10} to 1.5×10^{13} s^{-1}.[8,12,13,30−32] Thus, if radical rearrangements were the only pathway possible for ring-opened products, then the rate constant for the oxygen rebound step would be highly variable which is, in our opinion, unreasonable for a process that must have virtually no activation energy in many cases. We emphasize that the results with the isopropyl-containing probe **4** are especially noteworthy because the products were demonstrated to be relatively stable. The apparent oxygen rebound rate constant for **4** is a minimum value limit based on the failure to detect any rearranged product, but this minimum value exceeds the theoretical maximum predicted from conventional transition state theory and is quite close to the value obtained from probe **12**, which permitted separation of the radical- and cation-derived rearrangement products.

The possible explanation that some steric effects of the enzyme have slowed the rearrangement rates of all of the radicals derived from probes **1**−**6** (and those from other probes that gave subpicosecond radical lifetimes) by 2 or more orders of magnitude and the oxygen rebound rate constant is actually approximately 2×10^{10} s^{-1} (the smallest apparent rebound rate constant yet found) has, in our opinion, reached the point of an absurdity. Another especially damaging fact in regard to this steric constraint argument is the observation that isotopic substitution at the methyl position in probe **1** resulted in metabolic switching between methyl and phenyl oxidation which requires that the probe tumbled freely in the active site of the enzyme;[12] that is, no steric constraints on probe **1** were apparent with the isozyme used in this work.

Another rationalization of the results in the context of radical reactions, involving selective trapping of an equilibrating system of ring-opened and cyclic radicals, is not possible for several reasons. The equilibrium constants for ring opening of the radicals derived from **1**−**6** and other aryl-substituted cyclopropane systems[21] are about 10^{13}, which would require that trapping of cyclic radicals is about 15 orders of magnitude faster than trapping of ring-opened radicals; i.e., if the cyclic radical collapsed with no energy barrier, the acyclic radical would have a 20 kcal/mol barrier. More to the point, the observed substrate turnover velocities for **1**−**6** (ca. 0.2 substrate/enzyme per second) are 1−2 orders of magnitude greater than the velocities of the *cyclization* reactions of the acyclic radicals for these systems (rate constants on the order of 10^{-2} s^{-1}). Thus, the ring-opened radicals are *demonstrably kinetically incompetent* as intermediates for formation of cyclic products.

We believe the combined evidence is compelling that radical *intermediates* are not formed and some process other than radical ring opening in competition with rebound contributes to the amounts of rearranged products. The objective of studying the series of probes discussed here was to attempt to evaluate the possibility that an electron-transfer process, an oxidation of the radical in the transition state to a cation, competes with collapse to product. Such a pathway seems possible in light of the apparent alkyl radical oxidation reactions that occur with, for example, the P450-related prostacyclin and thromboxane synthetase enzymes,[39] and it has been proposed both in the "simple" view of P450 hydroxylation as an insertion with a competing process[8] and as one possible reaction in the more complex mechanistic picture presented by Shaik et al.[11]

The results strongly suggest that cation formation by radical oxidation is not important for the series of probes **1**−**4** and the pair **5** and **6**. An increase in methyl substitution at a radical center results in a decrease in the oxidation potential. Given that the radical does not exist as an intermediate, an electron-transfer reaction would have to be an outer-sphere process, and the distances between the radical and the iron species that oxidizes it must be similar in all cases because there is too little time for atomic translation of more than a few tenths of an angstrom. Therefore, the amount of cation formation should track with the oxidation potentials. As noted above, the 2-aryl-substituted cyclopropylcarbinyl cations that would be formed will rearrange. If we assume an essentially constant, small amount of radical ring opening in the TS of the insertion process as a background reaction, the results found here clearly are going in the wrong direction for the oxidation model. One sees less rearrangement with more highly substituted systems, and ultimately, the tertiary radical precursor **4**, progenitor to the radical that should be oxidized most efficiently, apparently reacts with no cation formation. In a qualitative sense, related results were reported by Ingold and Bowry, who found no rearranged products from P450-catalyzed hydroxylation of isopropylcyclopropane, the substrate among their probe set most likely to give a cation.[31] This inconsistency of the electron-transfer hypothesis was previously noted in a comparison of the results from probe **5** with those from probe **1**,[18] and we believe the results of the present study effectively exclude this mechanism.

The agostic complex alternative presented by Collman et al.[10] also appears not to be supported by the results of the present study. In that model, cationic-type rearrangements would occur for the iron complex in competition with reductive elimination that gives alcohol. One might expect that both the energetic preference for more highly substituted cations and the steric compression resulting from increased alkyl substitution would favor more rearrangement for the more highly substituted system in contradistinction to the observations for **1**−**4**. It is conceivable, however, that the steric demand from addition of methyl groups at the reactive center results in a large increase in the rate of the reductive elimination from the complex, which would reduce the lifetime of the complex and the amount of rearrangement. In that case, one might compare the results from **1**

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to those from **5** and the results from **2** to those from **6**. Both pairs would have similar steric compression effects, and less rearrangement should be observed for **5** and **6** due to the trifluoromethyl group that strongly destabilizes a cation. This was not the observed trend.

The agostic complex alternative might be in conflict with the overall steric demands presented by various probes. The complex involves an iron−carbon bond, and it is not clear that the large probes can approach an iron atom held in a porphyrin ring. This problem seems to be especially severe in the case of oxidation of (2,2-diphenylcyclopropyl)methane, a known substrate for P450,[13] which apparently would have to form a complex with one phenyl group protruding into the porphyrin.

The Shaik/Schwarz mixed electronic state paradigm presents a number of pathways for rearrangements,[11] but one important point must be appreciated at the outset. The amount of stereochemical or structural rearrangement observed with a given probe can be controlled by the efficiency of intersystem crossing (spin inversion) processes and, as such, will not necessarily track with the kinetics of rearrangement of a radical or cationic intermediate. If the reacting complex fails to cross from the HS surface to the LS surface, then "insertion" is not possible, and a radical intermediate would be produced. In addition, with the very fast rates of rearrangement of the radicals corresponding to the probes used in this study, some rearrangement could occur even when the reacting ensemble crosses to the LS surface; this portion of the model is effectively the same picture one has for rearrangement occurring in competition with collapse in an insertion process.

For a meaningful analysis of our results in the context of the mixed state model, one can assume that the extent of rearrangement that occurs on the LS surface will be nearly the same for each of the probes **1**−**4**, an assumption based on the observation that the rate constants for ring opening of the cyclopropylcarbinyl radicals corresponding to **1**, **2**, and **4** are in a ratio of 1.0:0.5:0.4. Accordingly, because no rearranged product was observed with probe **4**, the "background" rearrangement on the LS surface is effectively undetectable, and all rearranged products would be ascribed to reactions occurring on the HS surface.

The probability of state crossing is linked to the position of the HS/LS crossing point on the reaction coordinate, and this point will be a function of the donor property of the reaction center, with a better electron donor center having an earlier position and, thus, a higher probability of crossing. In this context, the probe results of the present study are consistent with the two-state reactivity model. The increasing donor properties of the reactive center in moving from primary probe **1** to the secondary (**2** and **3**) and tertiary (**4**) probes result in increasingly earlier and more efficient HS to LS crossings, with the tertiary probe **4** ultimately crossing completely. The results with the CF_3-substituted probes **5** and **6** also fit this model, not only because the amount of rearrangement for the secondary probe **6** was less than that with primary probe **5** but also because the effect of the remote CF_3 group should be to reduce slightly the donor abilities at the reactive center and disfavor HS to LS crossing, giving slightly more rearrangement for **5** and **6** relative to **1** and **2**, respectively, as is observed.

The Shaik/Schwarz model presents several competing processes that could result in rearranged products which might be considered a weakness, but it is the mechanistic description most in line with our results. Other tests of this model that attempt to alter the probability of spin inversion by heavy atom effects in the probes or the presence of external magnetic fields, as suggested by the authors,[11] are definitely warranted.

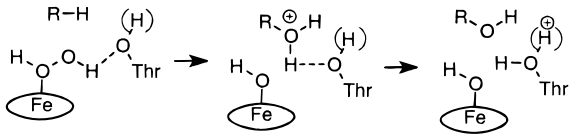

Figure 4. "Normal" reaction sequence in the protonated alcohol model. Threonine in its neutral or alcoholate form serves as a base to activate iron-complexed hydrogen peroxide, and insertion of OH^+ is followed by rapid deprotonation.

The electron-transfer, agostic complex, and multistate models are cast in terms of an iron−oxo species as the oxidant in P450. It is possible, however, that the actual oxidant in P450 might be iron-complexed hydrogen peroxide, a precursor to the iron−oxo species. Oxidation by an iron−hydrogen peroxide complex would be similar to oxidation by protonated hydrogen peroxide, a reaction demonstrated by Olah decades ago,[40] and supported computationally much more recently.[41] Some evidence for this possibility exists in addition to previous probe results; Pratt concluded that a hydrogen peroxide complex was the oxidant in P450-catalyzed oxidations on the basis of the observation that H_2O_2-shunted P450 reacted differently from P450 activated by iodosobenzene.[42]

If an iron-complexed hydrogen peroxide was the oxidant in P450 hydroxylations, then the first-formed product would be a protonated alcohol from insertion of the elements of OH^+ into a C−H σ bond, and solvolysis of this protonated alcohol to give a cation would compete with a deprotonation that gives neutral alcohol product. The "normal" sequence is represented in Figure 4, where, for the sake of speculation below, we show deprotonation by threonine, which is known to be highly conserved in the active sites of P450 enzymes[43] and, in the case of cytochrome $P450_{cam}$, is located near the heme.[44]

The formation of rearrangement products in P450 hydroxylations of the probes studied here and previously is consistent with a "protonated alcohol" model in some ways but not in others. Most importantly, some type of cationic species is implicated from the results with probe **12** discussed earlier where radical and cationic rearrangement products differ, and both were observed in P450 oxidations.[8] A protonated alcohol with aryl substitution on the cyclopropane ring would be expected to undergo an internally assisted solvolysis reaction leading to ring-opened product as shown below for **13**. Cyclopropane-based probes lacking the aryl group would be expected to be less reactive toward the solvolysis, and little or no ring opening occurs with such probes with the exception of the sterically strained probes **14**.[13,31] Finally, the observation that bicyclo[2.1.0]pentane (**15**) suffers partial rearrangement upon P450 hydroxylation[30,31] is consistent with the protonated alcohol model in that the bicyclo[2.1.0]pent-2-yl system is known to be especially reactive in solvolytic reactions.[45]

Nevertheless, the results of the present study do not lend support to this model. On one hand, the cation-stabilizing effects of alkyl groups in the series of probes **1**−**4** should have

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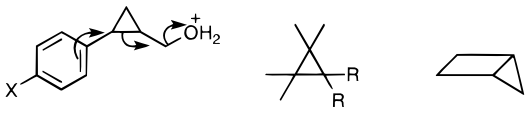

13 **14:** R = H or Me **15**

given more cationic rearrangement for more highly substituted systems; i.e., probe **4** should have given the most rearrangement, not the least. In a similar manner, the effect of the electron-withdrawing CF_3 group in probes **5** and **6** should be to disfavor the aryl-assisted solvolysis reaction shown in **13**, and less rearrangement should have been observed for **5** relative to **1** and for **6** relative to **2** and **3**.

A weak rationalization for the results in the context of the protonated alcohol model would note that, unlike the radical oxidation model with its outer-sphere electron-transfer event competing with collapse to product, the deprotonation reaction that would compete with solvolysis in the protonated alcohol model is a bond-forming event and will be subject to steric and (with the requisite short lifetimes) orientational effects. We offer as speculation the possibilities that methyl substitution at the cyclopropylcarbinyl positions in the probes could increase the kinetic acidity by favorable positioning of the protonated alcohol moiety with respect to the base that deprotonates it (Figure 4) or disfavor the solvolytic process by a similar orientational biasing. In either case, increased substitution at the reactive center would lead to less cationic rearrangement. On balance, we consider the present and past probe results to be inconclusive at best regarding the formation of a protonated alcohol product formed by insertion of the elements of OH^+ from a hydrogen peroxide complex.

The mechanistic probe results require some type of competition, and the above models present competing processes in the product-forming reactions. An alternative possibility that must now be considered is that a competition exists between oxidants. From comparisons of the product mixtures in oxidations by a truncated version of the P450 isozyme 2B4 and its mutant lacking the highly conserved threonine in the active site, Vaz, Coon, and co-workers deduced that multiple oxidant forms were possible.[46] In quite recent work, the same group showed that, for two P450 isozymes (2B4 and 2E1), the ratios of hydroxylation to epoxidation products observed in oxidations of alkenes changed in proceeding from the wild-type active site isozymes to mutants in which active site threonine was replaced with alanine.[47] This observation suggests not only that multiple oxidant forms are possible but also that two distinct electrophilic oxidants exist in the natural course of P450 oxidation reactions, possibly a hydroperoxo−iron complex and the iron−oxo species.[47] The possibility that multiple oxidant forms can exist definitely confuses the mechanistic issue for P450 hydroxylation. One could readily rationalize all probe results in the context of the "two oxidants" model because, if two oxidants actually exist, one does not know how the probes should behave with either of the oxidants in isolation. P450 2B4 is known to oxidize the hypersensitive probe **1**,[13] and it seems apparent that comparisons of probe oxidations by the wild-type and mutant enzymes would be enlightening. Such studies might address not only the two oxidants model of Vaz and Coon but also the "protonated alcohol" model in which iron-complexed hydrogen peroxide is the oxidant.

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Conclusion

The probe results reported here, combined with previous results, seem to leave little doubt that the hydrogen abstraction−oxygen rebound mechanism for cytochrome P450 hydroxylation is not complete. The mixtures of unrearranged and rearranged alcohol products and the widely disparate values of the oxygen rebound rate constants found with many hypersensitive probes require that some competition exists in P450 hydroxylations, either in the product-forming processes from a single oxidizing species or from reactions of two oxidants. Accepting that the two oxidants model cannot be evaluated from extant probe results, the probe results of the present study seem to be best in line with predictions of the multiple-state paradigm recently presented by Shaik, Schwarz, and co-workers.[11] New tests of the various recent hypotheses are in order, but we believe that one of the most important advances in understanding the mechanisms of P450-catalyzed hydroxylations that can be made at this time is an unequivocal demonstration of the active oxidant.[48,49]

Experimental Section

General Methods. Commercially available reagents were purchased from either the Sigma or Aldrich Chemical Co. and were used as received. All moisture sensitive reactions were carried out in flame-dried glassware under a nitrogen atmosphere. Tetrahydrofuran (THF) and diethyl ether were distilled under a nitrogen atmosphere from sodium and benzophenone ketyl. Methylene chloride was distilled under a nitrogen atmosphere from phosphorus pentoxide. Benzene was distilled under a nitrogen atmosphere from calcium hydride. Dimethyl sulfoxide (DMSO) and dimethyl formamide (DMF) were distilled in vacuo from calcium hydride.

NMR spectra were acquired on a Varian Gemini 300 spectrometer. Gas chromatography analyses were performed using flame ionization detection on a Varian 3400 chromatograph (15 m × 0.54 mm bonded phase Carbowax column, Alltech). Gas chromatography−mass spectral (GC−MS) analyses were performed using a Hewlett-Packard Model 5890 GC interfaced to a Hewlett-Packard Model 5971 mass selective detector (30 m × 0.25 mm capillary bonded phase Carbowax column, Alltech). High-resolution mass spectral analyses were performed by the Central Instrumentation Facility at Wayne State University (Detroit, MI). Melting points were determined using a Unimelt capillary melting point apparatus (Thomas-Hoover) and are uncorrected. Radial chromatography was performed on a Chromatotron Model 7294T (Harrison Research Corp.) using plates coated with TLC grade silica gel (Merck) with gypsum binder and fluorescent indicator.

Enzymatic oxidation reactions were performed in reconstituted systems using the purified isozyme CYP2B1 and expressed reductase at 37 °C in 50 mM potassium phosphate buffer, pH 7.4, containing 1 mM desferrioxamine and 0.075 M KCl, with a total reaction volume of 2 mL. A mixture of 0.6 nmol of CYP2B1 and 1.2 nmol of reductase in buffer was allowed to stand on ice for 5 min. A suspension of DLPC (0.96 nmol) in buffer was sonicated. The enzyme and micelle mixtures were combined, and this mixture was allowed to stand on ice for 5 min. The mixture was diluted with buffer, and the substrate was added as a solution in methanol (10 μL). The reaction mixture was incubated for 2 min at 37 °C, and the reaction was initiated by the addition of NADPH (final concentration of 1.2 mM). The reaction mixture was shaken gently at 37 °C for 30 min and then extracted with methylene chloride (3 × 2 mL). The combined organic phase was dried over

(48) We note that a spectroscopic detection of an iron−oxo in P450 probably will *not* provide such an unequivocal demonstration. Recent studies of microperoxidase oxidations indicate that hydroxylic solvent can react with an iron−oxo species, the reverse of formation of an iron−oxo from an iron−hydrogen peroxide complex, on the time scale of oxidation reactions. See ref 49.

(49) Osman, A. M.; Boeren, S.; Boersma, M. G.; Veeger, C.; Rietjens, I. M. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 4295−4299. Osman, A. M.; Koerts, J.; Boersma, M. G.; Boeren, S.; Veeger, C.; Rietjens, I. M. *Eur. J. Biochem.* **1996**, *240*, 232−238.

$MgSO_4$ and filtered through a plug of glass wool. An internal standard was added, and the solution was concentrated under a slow stream of nitrogen at room temperature to a final volume of approximately 0.2 mL. Quantitation of the product ratios was performed using gas chromatography with flame ionization detection, and product identities were determined by comparison with authentic samples using selected ion monitoring (SIM) GC−MS analysis. Phenolic products from **2**−**4** were identified by their mass spectra and GC retention times.

(*trans*-2-Phenylcyclopropyl)propane (3). A solution of *trans*-1-phenyl-1-pentene[20] (**7**) (1.90 g, 13.00 mmol) in CH_2Cl_2 (150 mL) was cooled to −30 °C. To this was added a solution of Et_2Zn (1.0 M in hexanes, 65.0 mL, 65.0 mmol). The reaction mixture was stirred for 5 min, and CH_2I_2 (10.50 mL, 130.3 mmol) was added dropwise over 30 min. The reaction mixture was allowed to warm slowly to room temperature and was stirred for 16 h. The resulting suspension was poured into a saturated, aqueous NH_4Cl solution (300 mL) and extracted with CH_2Cl_2 (3 × 200 mL). The combined organic phase was washed with brine (200 mL), dried over $MgSO_4$, filtered, and concentrated in vacuo at 0 °C.

The crude product was dissolved in CH_2Cl_2 (100 mL), and 3-chloroperoxybenzoic acid (2.00 g, 11.59 mmol) was added. The reaction mixture was stirred at room temperature for 6 h and then washed sequentially with 15% aqueous NaOH solution (200 mL), water (200 mL), and brine (200 mL). The organic layer was separated, dried over $MgSO_4$, filtered, and concentrated in vacuo at 0 °C. The crude product was purified by radial chromatography (pentane) to afford **3** (0.98 g, 6.12 mmol, 47%) as a clear, colorless oil. ^{1}H NMR ($CDCl_3$): δ 0.72−0.79 (1H, m), 0.88 (1H, dt, J_1 = 8.4 Hz, J_2 = 4.8 Hz), 0.94 (3H, t, J = 7.2 Hz), 0.97−1.08 (1H, m), 1.32−1.53 (4H, m), 1.60 (1H, dt, J_1 = 8.4 Hz, J_2 = 4.8 Hz), 7.02−7.27 (5H, m). ^{13}C NMR ($CDCl_3$): δ 13.9, 16.0, 22.5, 23.2, 23.5, 36.5, 125.1, 125.6 (2C), 128.2 (2C), 144.1. HRMS: calcd for $C_{12}H_{16}$, 160.1252; found, 160.1248.

1-(*trans*-2-Phenylcyclopropyl)propanone (3K). A solution of *trans*-2-phenyl-1-cyclopropanecarboxylic acid (8.00 g, 49.3 mmol) and DMF (5 drops) in benzene (100 mL) under a nitrogen atmosphere was cooled with a water bath as oxalyl chloride (8.60 mL, 98.6 mmol) was added dropwise. The reaction mixture was stirred for 1 h, and volatiles were removed in vacuo. The resulting crude carbonyl chloride was dissolved in CH_2Cl_2 (200 mL) under a nitrogen atmosphere and cooled to 0 °C. *N*,*O*-Dimethylhydroxylamine hydrochloride (5.30 g, 54.3 mmol) and pyridine (8.80 mL, 108.8 mmol) were added sequentially, and the resulting solution was stirred at room temperature for 2 h. The reaction mixture was washed sequentially with 10% aqueous HCl solution (150 mL), saturated aqueous $NaHCO_3$ solution (150 mL), and brine (150 mL). The organic layer was dried over $MgSO_4$, filtered, and concentrated in vacuo. The crude product was chromatographed on silica gel (40% ethyl acetate in hexanes) to afford *N*-methoxy-*N*-methyl-*trans*-2-phenylcyclopropanecarboxamide (9.25 g, 45.1 mmol, 91%) as a clear, colorless oil.

The amide (9.25 g, 45.1 mmol) was dissolved in ether (200 mL) and cooled to 0 °C under a nitrogen atmosphere. To this was added a solution of ethylmagnesium bromide (3.0 M in ether, 20.0 mL, 60.0 mmol). After 2 h, the reaction mixture was quenched by carefully pouring it into saturated, aqueous NH_4Cl solution (200 mL). The mixture was extracted with ether (3 × 100 mL). The combined organic phase was washed sequentially with water (150 mL) and brine (150 mL), dried over $MgSO_4$, filtered, and concentrated in vacuo. The crude product was chromatographed on silica gel (15% ethyl acetate in hexanes) to afford **3K**[24] (5.95 g, 34.1 mmol, 76%) as a clear, colorless oil. ^{1}H NMR ($CDCl_3$): δ 1.12 (3H, t, J = 7.5 Hz), 1.36 (1H, ddd, J_1 = 8.4 Hz, J_2 = 6.6 Hz, J_3 = 4.2 Hz), 1.68 (1H, ddd, J_1 = 9.3 Hz, J_2 = 5.4 Hz, J_3 = 4.5 Hz), 2.20 (1H, ddd, J_1 = 8.1 Hz, J_2 = 5.4 Hz, J_3 = 3.9 Hz), 2.52 (1H, ddd, J_1 = 9.3 Hz, J_2 = 6.6 Hz, J_3 = 4.2 Hz), 2.64 (2H, q, J = 7.2 Hz), 7.09−7.32 (5H, m). ^{13}C NMR ($CDCl_3$): δ 7.9, 18.8, 28.7, 31.9, 37.1, 126.0 (2C), 126.4, 128.5 (2C), 140.5, 209.4. HRMS: calcd for $C_{12}H_{14}O$, 174.1045; found, 174.1047.

1-(*trans*-2-Phenylcyclopropyl)propanol (3U). To a solution of **3K** (4.38 g, 25.1 mmol) in THF (150 mL) at 0 °C under a nitrogen atmosphere was added $LiAlH_4$ (1.20 g, 31.6 mmol). The mixture was stirred under a nitrogen atmosphere for 2 h, and the reaction was quenched by the sequential addition of water (1.20 mL), 15% aqueous NaOH solution (1.20 mL) and water (3.60 mL). The resulting suspension was stirred at room temperature for 1 h and then filtered. The filtrate was concentrated in vacuo, and the resulting crude product was chromatographed on silica gel (20% ethyl acetate in hexanes) to afford the two diastereomers of **3U** as clear, colorless oils.

3Ua (2.20 g, 12.5 mmol, 50%). ^{1}H NMR ($CDCl_3$): δ 0.93−0.99 (2H, m), 1.04 (3H, t, J = 7.5 Hz), 1.22−1.31 (1H, m), 1.61−1.76 (2H, m), 1.91 (1H, dt, J_1 = 8.4 Hz, J_2 = 5.7 Hz), 2.11 (1H, br s), 3.11 (1H, dt, J_1 = 7.2 Hz, J_2 = 6.0 Hz), 7.10−7.31 (5H, m). ^{13}C NMR ($CDCl_3$): δ 10.1, 13.7, 20.6, 29.2, 30.0, 76.8, 125.5, 125.9 (2C), 128.3 (2C), 142.9. HRMS: calcd for $C_{12}H_{16}O$, 176.1201; found, 176.1202.

3Ub (1.20 g, 6.81 mmol, 27%). ^{1}H NMR ($CDCl_3$): δ 0.95−1.07 (2H, m), 1.04 (3H, t, J = 7.5 Hz), 1.23−1.31 (1H, m), 1.72 (2H, dt, J_1 = 13.8 Hz, J_2 = 6.9 Hz), 1.85 (1H, dt, J_1 = 9.0 Hz, J_2 = 5.1 Hz), 2.22 (1H, br s), 3.13 (1H, dt, J_1 = 7.8 Hz, J_2 = 6.3 Hz), 7.09−7.32 (5H, m). ^{13}C NMR ($CDCl_3$): δ 10.2, 13.2, 21.2, 29.4, 30.3, 77.0, 125.6, 125.8 (2C), 128.4 (2C), 142.5. HRMS: calcd for $C_{12}H_{16}O$, 176.1201; found, 176.1194.

(*E*)- and (*Z*)-1-Phenyl-3-hexen-1-ol (3R). A solution of **3Ua** (0.80 g, 4.54 mmol) in THF (20 mL) was cooled to −30 °C under a nitrogen atmosphere. To this were added sequentially triethylamine (1.20 mL, 8.61 mmol) and methanesulfonyl chloride (0.40 mL, 5.17 mmol). After 1 h, water (15 mL) was added, and the reaction mixture was heated at reflux for 2 h. The reaction mixture was cooled to room temperature and extracted with ether (3 × 25 mL). The combined organic phase was washed sequentially with saturated, aqueous $NaHCO_3$ solution (50 mL) and brine (50 mL), dried over $MgSO_4$, and filtered. Concentration of the filtrate in vacuo gave crude product that was purified by radial chromatography (15% ethyl acetate in hexanes) to afford **3R**[25] (0.26 g, 1.48 mmol, 33%) as a clear, colorless oil. GC analysis showed this to be a 13:1 mixture of isomers.

Mixture of isomers. ^{1}H NMR ($CDCl_3$): δ 0.92−1.03 (3H, m), 2.02−2.11 (2H, m), 2.39−2.50 (2H, m), 2.41 (1H, s), 4.64−4.59 (1H, m), 5.36−5.46 (1H, m), 5.54−5.67 (1H, m), 7.27−7.36 (5H, m).

Major isomer. ^{13}C NMR ($CDCl_3$): δ 13.8, 25.7, 42.7, 73.6, 124.6, 125.9 (2C), 127.3, 128.3 (2C), 136.4, 144.2. MS: calcd for $C_{12}H_{16}O$, 176; $(M)^+$ found at m/z = 176 for each isomer.

Methyl *trans*-2-(4-(Trifluoromethyl)phenyl)cyclopropylacetate (9). A solution of **8**[18] (1.69 g, 7.34 mmol) and DMF (2 drops) in benzene (50 mL) under a nitrogen atmosphere was cooled with a water bath as oxalyl chloride (1.3 mL, 14.9 mmol) was added. The reaction mixture was stirred for 2 h, and volatiles were removed in vacuo. The crude carbonyl chloride was dissolved in CH_2Cl_2 (25 mL), and the resulting mixture was added slowly to a solution of CH_2N_2 in ether, prepared from Diazald (10 gm, 46.7 mmol). The reaction mixture was stirred at room temperature for 16 h and then concentrated in vacuo. The resulting diazoketone was chromatographed on silica gel (20% ethyl acetate in hexanes).

The diazoketone was dissolved in dioxane (30 mL), and the solution was added portionwise to a suspension of Ag_2O (3.00 g, 12.9 mmol) and $Na_2S_2O_3$ (2.04 g, 12.9 mmol) in water (100 mL) at 75 °C. The resulting suspension was stirred for 2 h, then cooled to room temperature, and filtered through Celite. The filtrate was made basic (NaOH) and washed with ether (2 × 100 mL). The aqueous layer was acidified (concentrated HCl) and extracted with ether (2 × 100 mL). The combined organic layer was washed with brine (100 mL), dried over $MgSO_4$, filtered, and concentrated in vacuo.

The resulting acid was dissolved in CH_2Cl_2 (25 mL) and added slowly to a solution of CH_2N_2 in ether, prepared from Diazald (10 gm, 46.7 mmol). The mixture was stirred at room temperature for 16 h and then concentrated in vacuo. The crude product was purified by radial chromatography (10% ethyl acetate in hexanes) to afford **9** (1.04 gm, 4.04 mmol, 55% combined yield) as a clear, colorless oil. ^{1}H NMR ($CDCl_3$): δ 0.94 (1H, dt, J_1 = 9.0 Hz, J_2 = 5.4 Hz), 1.05 (1H, dt, J_1 = 8.4 Hz, J_2 = 5.4 Hz), 1.37−1.48 (1H, m), 1.81 (1H, dt, J_1 = 9.0 Hz, J_2 = 4.8 Hz), 2.42 (2H, d, J = 7.2 Hz), 3.70 (3H, s), 7.17 (2H, d, J = 8.1 Hz), 7.49 (2H, d, J = 8.1 Hz). ^{13}C NMR ($CDCl_3$): δ 15.8, 19.1, 22.8, 38.5, 51.6, 124.3 (q, J_{C-F} = 270.3 Hz), 125.1 (2C, q, J_{C-F} = 3.4 Hz), 126.1 (2C), 127.8 (q, J_{C-F} = 33.1 Hz), 146.8, 172.8. HRMS: calcd for $C_{13}H_{13}F_3O_2$, 258.0868; found, 258.0871.

(*trans*-2-(4-(Trifluoromethyl)phenyl)cyclopropyl)ethane (6). To a solution of **9** (0.95 g, 3.68 mmol) in THF (25 mL) at 0 °C under a nitrogen atmosphere was added $LiAlH_4$ (0.15 g, 3.95 mmol). The mixture was stirred under a nitrogen atmosphere for 2 h, and the reaction was quenched by the sequential addition of water (0.15 mL), 15% aqueous NaOH solution (0.15 mL), and water (0.45 mL). The resulting suspension was stirred at room temperature for 1 h and then filtered. The filtrate was concentrated in vacuo, and the resulting crude product was purified by radial chromatography (30% ethyl acetate in hexanes) to afford the corresponding alcohol (0.67 g, 2.91 mmol, 79%) as a clear, colorless oil. ^{1}H NMR ($CDCl_3$): δ 0.89−1.04 (2H, m), 1.11−1.22 (1H, m), 1.46 (1H, br s), 1.61−1.77 (3H, m), 3.77 (2H, t, J = 6.3 Hz), 7.12 (2H, J = 8.4 Hz), 7.49 (2H, J = 8.4 Hz). ^{13}C NMR ($CDCl_3$): δ 16.3, 21.1, 22.7, 27.1, 62.5, 124.4 (q, J_{C-F} = 270.2 Hz), 125.1 (2C, q, J_{C-F} = 33 Hz), 125.7 (2C), 127.7 (q, J_{C-F} = 33.1 Hz), 147.8. HRMS: calcd for $C_{12}H_{13}F_3O$, 230.0918; found, 230.0916.

A solution of the above alcohol (0.47 g, 2.04 mmol) in THF (20 mL) was cooled to −30 °C under a nitrogen atmosphere. To this were added sequentially triethylamine (0.60 mL, 4.30 mmol) and methanesulfonyl chloride (0.20 mL, 2.58 mmol). The mixture was stirred at −30 °C for 45 min and then cooled to −78 °C. A solution of $LiBEt_3H$ (1.0 M in THF, 10.0 mL, 10.0 mmol) was added dropwise. The mixture was allowed to warm slowly to room temperature and was stirred for 16 h. The reaction mixture was quenched by the successive addition of 15% aqueous NaOH solution (5 mL) and 30% H_2O_2 solution (5 mL). The resulting mixture was heated at reflux for 1 h and then cooled to room temperature. The organic layer was separated, and the aqueous layer was extracted with ether (3 × 25 mL). The combined organic phase was washed with brine (50 mL), dried over $MgSO_4$, filtered, and concentrated in vacuo at 0 °C. The crude product was purified by radial chromatography (pentane) to afford **6** (0.34 g, 1.59 mmol, 78%) as a clear, colorless oil. ^{1}H NMR ($CDCl_3$): δ 0.82−9.95 (2H, m), 1.01 (3H, t, J = 7.2 Hz), 1.02−1.11 (1H, m), 1.42 (2H, dt, J_1 = 14.4 Hz, J_2 = 6.9 Hz), 1.66 (1H, dt, J_1 = 9.0 Hz, J_2 = 4.8 Hz), 7.11 (2H, d, J = 8.1 Hz), 7.48 (2H, d, J = 8.1 Hz). ^{13}C NMR ($CDCl_3$): δ 13.3, 16.7, 23.0, 26.5, 27.4, 124.5 (q, J_{C-F} = 269.1 Hz), 125.5 (2C, q, J_{C-F} = 3.3 Hz), 125.7 (2C), 127.4 (q, J_{C-F} = 32.0 Hz), 148.6. HRMS: calcd for $C_{12}H_{13}F_3$, 214.0969; found, 214.0971.

1-(*trans*-2-(4-(Trifluoromethyl)phenyl)cyclopropyl)ethanone (6K). Amide **10**[18] (5.24 g, 19.2 mmol) was dissolved in ether (200 mL) and cooled to 0 °C under a nitrogen atmosphere. To this was added a solution of methylmagnesium bromide (3.0 M in ether, 8.0 mL, 24.0 mmol). After 2 h, the reaction mixture was quenched by carefully pouring it into a saturated, aqueous NH_4Cl solution (200 mL). This was extracted with ether (3 × 150 mL). The combined organic phase was washed sequentially with water (200 mL) and brine (200 mL), dried over $MgSO_4$, filtered and concentrated in vacuo. The crude product was chromatographed on silica gel (15% ethyl acetate in hexanes) to afford **6K** (4.02 g, 17.6 mmol, 92%) as a clear, colorless oil. ^{1}H NMR ($CDCl_3$): δ 1.34 (1H, ddd, J_1 = 8.1 Hz, J_2 = 6.6 Hz, J_3 = 4.5 Hz), 1.65 (1H, ddd, J_1 = 9.3 Hz, J_2 =5.4 Hz, J_3 = 4.5 Hz), 2.26 (1H, ddd, J_1 = 8.4 Hz, J_2 = 5.1 Hz, J_3 = 4.2 Hz), 2.26 (3H, s), 2.51 (1H, ddd, J_1 = 9.6 Hz, J_2 = 6.9 Hz, J_3 = 4.5 Hz), 7.14 (2H, d, J = 8.1 Hz), 7.47 (2H, d, J = 8.1 Hz). ^{13}C NMR ($CDCl_3$): δ 12.1, 28.1, 30.5, 32.7, 124.2 (q, J_{C-F} = 270.2 Hz), 125.3 (2C, q, J_{C-F} = 3.3 Hz), 126.2 (2C), 128.5 (q, J_{C-F} = 32.0 Hz), 144.6, 206.0. HRMS: calcd for $C_{12}H_{11}F_3O$, 228.0762; found, 228.0764.

1-(*trans*-2-(4-(Trifluoromethyl)phenyl)cyclopropyl)ethanol (6U). To a solution of **6K** (2.92 g, 12.8 mmol) in THF (100 mL) at 0 °C under a nitrogen atmosphere was added $LiAlH_4$ (0.50 g, 13.2 mmol). The mixture was stirred under a nitrogen atmosphere for 2 h, and the reaction was quenched by the sequential addition of water (0.50 mL), 15% aqueous NaOH solution (0.50 mL), and water (1.50 mL). The resulting suspension was stirred at room temperature for 1 h and then filtered. The filtrate was concentrated in vacuo, and the resulting crude product was chromatographed on silica gel (20% ethyl acetate in hexanes) to afford **6U** (2.93 g, 12.7 mmol, 99%) as a clear, colorless oil. ^{1}H NMR ($CDCl_3$): δ 0.95−1.13 (2H, m), 1.25−1.30 (1H, m), 1.33 (3H, d, J = 6.0 Hz), 1.60 (1H, br s), 1.82−1.99 (1H, m), 3.38−3.47 (1H, m), 7.13−7.17 (2H, m), 7.49 (2H, d, J = 7.8 Hz). MS: calcd for $C_{12}H_{13}F_3O$, 230; $(M)^+$ found at m/z = 230 for each isomer.

(*E*)- and (*Z*)-1-(4-(Trifluoromethyl)phenyl)-3-penten-1-ol (6R). A solution of **5R**[18] (4.76 g, 22.0 mmol), imidazole (3.00 g, 44.1 mmol), and *tert*-butyldimethylsilyl chloride (5.00 g, 33.2 mmol) in CH_2Cl_2 (150 mL) was stirred under a nitrogen atmosphere at room temperature for 24 h. The reaction mixture was diluted with additional CH_2Cl_2 (200 mL) and then washed sequentially with 10% aqueous HCl solution (200 mL), saturated, aqueous $NaHCO_3$ solution (200 mL), and brine (200 mL). The organic phase was dried over $MgSO_4$, filtered, and concentrated in vacuo. The crude product was chromatographed on silica gel (10% ethyl acetate in hexanes) to afford the silyl ether (5.70 g, 21.7 mmol, 99%) as a clear, colorless oil. ^{1}H NMR ($CDCl_3$): δ −0.12 (3H, s), 0.04 (3H, s), 0.89 (9H, s), 2.33−2.49 (2H, m), 4.74 (1H, t, J = 5.7 Hz), 4.96−5.03 (2H, m), 5.67−5.81 (1H, m), 7.41 (2H, d, J = 8.1 Hz), 7.56 (2H, d, J = 8.1 Hz). ^{13}C NMR ($CDCl_3$): δ −5.0, −4.8, 18.1, 25.7 (3C), 45.3, 74.4, 117.5, 125.0 (2C, q, J_{C-F} = 3.3 Hz), 126.1 (2C), 124.2 (q, J_{C-F} = 270.2 Hz), 129.2 (q, J_{C-F} = 32.0 Hz), 134.3, 149.1.

To a solution of the above silyl ether (1.91 g, 7.28 mmol) in ether (20 mL) was added an aqueous solution (20 mL) of $NaIO_4$ (17.50 g, 81.8 mmol) and OsO_4 (0.05 g, 0.19 mmol). The reaction mixture was stirred vigorously at room temperature for 24 h. After cooling, the suspension was filtered. The filtrate was extracted with ethyl acetate (3 × 50 mL), and the combined organic phase was washed with brine (75 mL), dried over $MgSO_4$, filtered, and concentrated in vacuo. The resulting aldehyde was used without purification. ^{1}H NMR ($CDCl_3$): δ −0.13 (3H, s), 0.57 (3H, s), 0.87 (9H, s), 2.64 (1H, ddd, J_1 = 16.2 Hz, J_2 = 4.5 Hz, J_3 = 1.8 Hz), 2.86 (1H, ddd, J_1 = 16.2 Hz, J_2 = 7.8 Hz, J_3 = 2.4 Hz), 5.28 (1H, dd, J_1 = 7.5 Hz, J_2 = 4.2 Hz), 7.46 (2H, d, J = 8.1 Hz), 7.60 (2H, d, J = 8.1 Hz), 9.78 (1H, t, J = 2.1 Hz).

To a suspension of ethyltriphenylphosphonium bromide (5.57 g, 15.0 mmol) in benzene (50 mL) under a nitrogen atmosphere was added a solution of BuLi (2.5 M in hexanes, 5 mL, 12.5 mmol). The mixture was stirred at room temperature for 15 min, and a solution of the above crude aldehyde in benzene (20 mL) was added via cannula. After 15 min, the reaction mixture was quenched by the addition of acetone (10 mL), and the resulting suspension was filtered through silica gel. The filtrate was concentrated in vacuo, and the crude product was filtered through silica gel (20% ethyl acetate in hexanes). The crude alkene was used without further purification.

The above alkene was dissolved in THF (20 mL), and a solution of Bu_4NF (1.0 M in THF, 10.0 mL, 10.0 mmol) was added. The reaction mixture was stirred at room temperature for 2 h and then poured into a saturated, aqueous NH_4Cl solution (50 mL). The resulting mixture was extracted with ether (3 × 75 mL), and the combined organic phase was washed sequentially with saturated, aqueous $NaHCO_3$ solution (75 mL) and brine (75 mL), dried over $MgSO_4$, filtered, and concentrated in vacuo. The crude product was purified by radial chromatography (10% ethyl acetate in hexanes) to afford **6R** (0.86 g, 3.74 mmol, 51% combined yield). GC analysis showed this to be a 4:1 mixture of isomers. ^{1}H NMR ($CDCl_3$) of the mixture: δ 1.58−1.71 (3H, m), 2.00 (1H, br s), 2.33−2.61 (2H, m), 4.72−4.81 (1H, m), 5.36−5.46 (1H, m), 5.59−5.75 (1H, m), 7.45−7.62 (4H, m). MS: calcd for $C_{12}H_{13}F_3O$, 230; $(M)^+$ found at m/z = 230 for each isomer.

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Supporting Information Available: Table of results from CYP2B1 hydroxylation of probes **2**−**4** and **6**, ^{1}H and ^{13}C NMR spectra of **3**, **6**, **9**, **3K**, **3Ua**, **3Ub**, **3R**, and **6K**, and ^{1}H NMR spectra of diastereomeric mixtures of **6U** and **6R** (19 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

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