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Active Site Dynamics of Xylene Hydroxylation by Cytochrome P-450 As Revealed by Kinetic Deuterium Isotope Effects

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Abstract: The cytochrome P-450 catalyzed hydroxylation of *o*- and *p*-xylene and five deuterated derivatives of each has been investigated using phenobarbital-induced rat liver microsomes. All possible monohydroxylation products were observed but benzylic hydroxylation predominated strongly (88–96%). H/D discrimination was strongest when both isotopes were located on the same methyl group, less when they were located in different methyl groups on the same xylene molecule, and least when they were located in methyl groups on different molecules. Benzylic hydroxylation is subject to a large intrinsic (intramolecular) deuterium isotope effect (CH_3/CD_3 = 7.5–9.5), comprised of a large primary component (5.3–7.8) and a large *normal* α-secondary component (1.09–1.19). These isotope effects suggest a transition state for benzylic H-abstraction that is linear and symmetrical with substantial rehybridization toward planarity at the benzylic carbon and little residual C–H bond order remaining. In contrast aromatic hydroxylation of *o*- and *p*-xylene shows a small *inverse* α-seconcdary isotope effect (0.83–0.94). The $^{D}(V/K)$ isotope effect observed for benzylic hydroxylation in intermolecular competitions (ca. 1.9–2.3 for d_0/d_6 substrate mixtures) is substantially reduced by commitment to catalysis, with $C_f = (k_H + k_r)k_{-1}$ = 3.6 for *p*-xylene and 5.9 for *o*-xylene. These results suggest a dynamic picture of catalysis with the following relative rates: methyl group rotation > substrate re-orientation within the Michaelis complex (i.e. isotopically sensitive branching to different products) > product formation (i.e. commitment to catalysis) > substrate dissociation prior to hydroxylation.

Introduction

Compared to aromatic or aliphatic positions, benzylic positions in molecules often show heightened chemical reactivity. This is particularly true with respect to oxidative attach by cytochrome P-450 enzymes on xenobiotic substrates. Examples include toluene and substituted toluenes,[1-3] xylene isomers,[4,5] methylated polycyclic aromatic hydrocarbon carcinogens,[6,7] antioxidants such as BHT (butylated hydroxytoluene),[8] and natural products such as psoralens,[9] safrole,[10] and even morphine.[11] P-450 catalyzed hydroxylations at sp^3-hybridized carbon atoms are thought to involve abstraction of a hydrogen atom by the oxoiron group of the P-450 enzyme, followed by transfer of the resultant OH group

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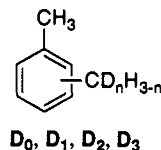
from the heme iron to the transient carbon radical.[12,13] Thus the special reactivity of benzylic positions in enzymatic hydroxylation might arise from the special resonance stabilization of benzylic radicals and corresponding transition states leading to their formation.

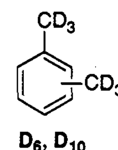
In previous work we sought to explore this mechanism by means of kinetic deuterium isotope effects (KDIEs) on the benzylic hydroxylation of toluene.[2] In particular, we had hoped to observe secondary KDIEs that might support the involvement of a planar resonance-stabilized benzylic radical intermediate. We studied a series of deuterated toluenes (d_0, d_1, d_2, d_3, d_5, and d_8) as substrates and observed the effects of deuteration on product yields, product ratios, and product deuterium content both as single substrates (i.e. intramolecular H/D competition) and as mixtures of two substrates (i.e. intermolecular H/Dcompetition conditions). Stepwise deuteration of the benzylic methyl group was found to retard benzylic hydroxylation relative to aromatic hydroxylation, indicating that deuterium induced metabolic switching or isotopically sensitive branching was occurring. Analysis of the benzyl alcohol produced from $PhCH_2D$ and (in separate experiments) $PhCD_2H$ showed that there was a large *intrinsic* KDIE on the product-forming step (i.e. hydrogen abstraction). However, intermolecular competition experiments with $PhCH_3/PhCD_3$ mixtures showed significantly reduced IEs, suggesting that toluene was a sticky substrate and that the large intrinsic isotope effect was suppressed by a high commitment to catalysis.

To avoid the sticky problem, and to have some hope of being able to determine the secondary KDIEs associated with benzylic hydroxylation as mentioned above, we turned to dimethylbenzenes (i.e. *o*- and *p*-xylene) so that CH_3/CD_3 isotope effects could be determined *intra*molecularly. In this manuscript we describe our studies of the P-450 catalyzed hydroxylation of *o*- and *p*-xylene and five isotopic variants of each employing both mixed-substrate formats yielding intermolecular IEs and single-substrate formats yielding intramolecular IEs.

Chart I

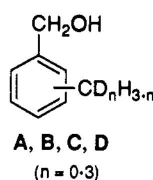
Substrates

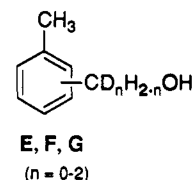




D_0, D_1, D_2, D_3 D_6, D_{10}

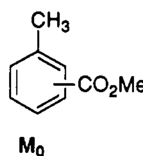
Metabolites

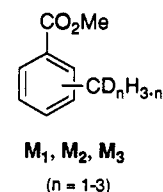




A, B, C, D (n = 0-3) E, F, G (n = 0-2)

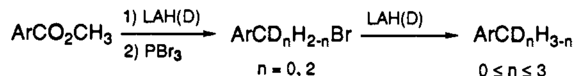
Ester Derivatives

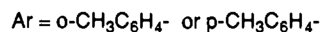




M_0 M_1, M_2, M_3 (n = 1-3)

Scheme I





Experimental Procedures

Substrates. *o*-Xylene and *p*-xylene were obtained from Aldrich and were filtered through active silica gel before use. Deuterated xylenes D_1–D_3 (see Chart I), both ortho and para isomers, were synthesized according to Scheme I using methods described previously.[14,15] *o*-Xylene-d_6 and *p*-xylene-d_6 were synthesized analogously starting from dimethyl phthalate or terephthalate, respective. *o*-Xylene-d_{10} and *p*-xylene-d_{10} were obtained from Merck Isotopes. The deuterium content of each xylene substrate was determined by electron impact mass spectrometry at an ionizing energy of 12 eV.[16] The observed isotopic purities (mol·% d_n) for the series D_1–D_3, and D_{10} were 99.2, 98.8, 98.1, 97.2, and 81.5 for the *o*-xylenes and 99.5, 98.9, 98.5, 96.8, and 90.6 for the *p*-xylenes. The majority of the excess H in D_{10} was present as the d_9 species, and ^{1}H-NMR analysis of the neat liquids showed it was present on the ring and not on the methyl groups.

Xylene Metabolism. Procedures for preparation of liver microsomes from phenobarbital-pretreated rats, incubation of substrates, and isolation of metabolites were identical with those described in detail for toluene metabolism,[2,14] but two aspects of this deserve further comment here. First, induction of male Sprague-Dawley rats with phenobarbital at the high dosage used (100 mg/kg for 3 days) has been shown to yield microsomes whose cytochrome P-450 content is comprised ≥75% of isozymes CYP2B1 and CYP2B2.[17,18] These isozymes, which have different genes but differ by only a few amino acids out of 491, hydroxylate benzene,[1] carry out both aromatic and benzylic hydroxylation on toluene[1,19] as well as larger methyl aromatics such as 7,12-dimethylbenzanthracene,[17] and oxidize a variety of other organic compounds ranging from benzphetamine to testosterone. In our hands phenobarbital-induced rat liver microsomes have shown consistently high hydroxylase activity toward many simple aromatic compounds including mono-[20] and disubstituted[21] benzenes and *N,N*-dimethylbenzamides.[22,23] These observations argue that CYP2B1 and 2B2 are likely the major agents responsible for xylene oxidation in phenobarbital-induced microsomes, but they do not rule out possible contributions from other less abundant P-450 isoforms. However, for a minor isozyme to make a major contribution to xylene metabolism would require it to have an extraordinarily high turnover number compared to the 2B1 and 2B2 isozymes; while not impossible this seems rather unlikely.

Second, all our incubations used a single xylene concentration of 10.4 mM. Typical $K_{m,app}$ values for P-450 catalyzed oxidations of small relatively nonpolar organic compounds are in the range of 0.1–1.0 mM; true K_m values are probably even lower because the extraneous microsomal lipids act as a reservoir for hydrophobic substrates and skew $K_{m,app}$ upward. The use of a single high substrate concentration for all experiments assures that if two or more P-450 isozymes in our microsomes are contributing to xylene metabolism, their relative contributions will remain constant throughout, so that changes in product composition can be attributed solely to the effects of substrate deuteration. Finally, to minimize potential variation in enzymatic activity due to interanimal variability, microsomes from 20–30 PB-induced rats (180–200 g) were prepared in two large batches, pooled for homogeneity, aliquoted, and stored at −70 °C until used. Both our earlier work on toluene[2] and the work on xylenes described herein were conducted with the same two batches of microsomes. Throughout the course of all these studies both deuterated and nondeuterated toluenes and xylenes were metabolized (usually in triplicate parallel

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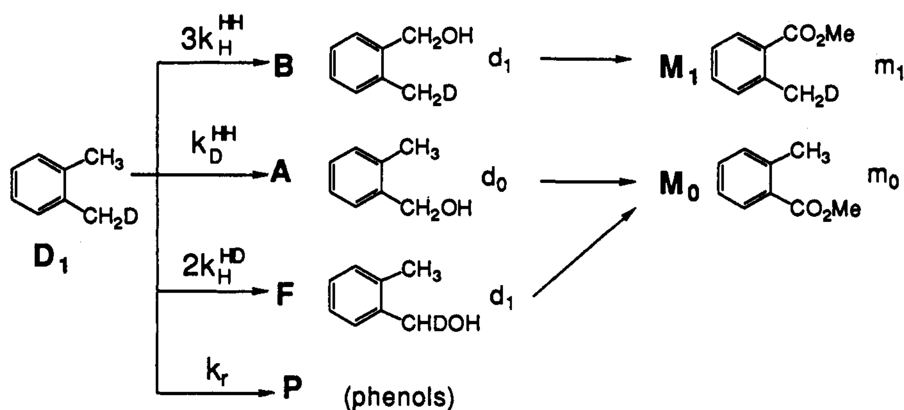
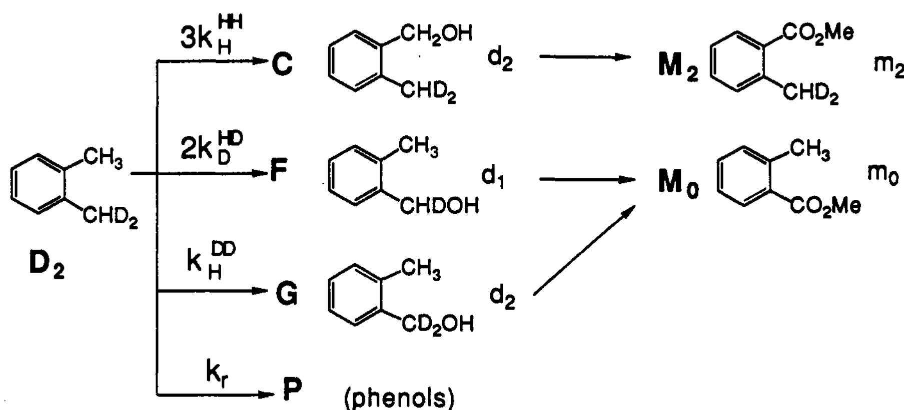
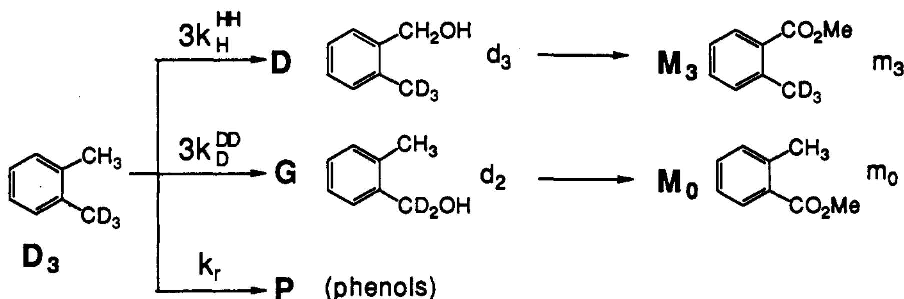
Scheme II



Scheme III



Scheme IV



runs) on at least 2–4 different occasions. Observed variations in product profiles were never more than ±4 mol·% and were usually closer to ±2 mol·%. Enzymatic results obtained from microsomes reflect a weighted average of the contribution of different P-450 isoforms present, but the nature of the induction used and the great internal consistency of the data obtained (see below) suggest that the mechanistic inferences drawn provide a fair characterization of the major PB-inducible forms of rat liver cytochrome P-450, i.e. CYP2B1/2B2.

Metabolite Analysis. The xylene metabolites were analyzed with use of the two-stage approach outlined in Schemes II–IV. In the first stage all hydroxylated metabolites were silylated and analyzed by GC/MS to determine the relative amounts of regioisomeric hydroxylation products. Product identification was based on comparisons of retention times and mass spectra (70 eV) to those of authentic standards and quantitation of metabolites was based on integration of total ion chromatograms. The total deuterium content of each metabolite was determined by GC/MS with selected ion monitoring of a 6–8 amu window encompassing the masses of the M – 15 ions of the silylated metabolites. Metabolites of the deuterated xylenes showed only M – 15 ions, indicating that these arose only by fragmentation of the silyl moiety and not by loss of methyl groups from the aromatic ring.

Hydroxylation of xylene $\mathbf{D_1}$ can lead to two isomeric d_1 benzylic alcohols (viz. **B** vs **F** in Scheme II), and a similar situation obtains for xylene $\mathbf{D_2}$ (Scheme III). Since isomerically deuterated compounds are indistinguishable chromatoghraphically, the metabolites were oxidized, esterified, and re-analyzed as described below.

Oxidation and Esterification of Benzylic Alcohol Metabolites. A solution of chromic acid (Jones' reagent) was prepared by dissolving 26.7 g of CrO_3 with stirring and cooling in 50 mL of H_2O, adding cautiously with stirring 23 mL concentrated H_2SO_4, and diluting to 100 mL with water. The alcohol to be oxidized (usually with accompanying phenolic metabolites) was dissolved in 1 mL of acetone, 0.5 mL of Jones reagent was added, and the mixture was stirred overnight at 20–25 °C. The liquid phase was decanted, the residue washed with acetone (2 × 1 mL), the combined liquid phases diluted with 2 vol of water and extracted with ether (3 × 1 mL), and the latter evaporated to dryness under vacuum. The residue obtained was dissolved in methanol (0.5–1.0 mL) and treated with ethereal CH_2N_2 until a yellow color persisted for 15 min, after which the solution was concentrated under vacuum to ca. 0.1 mL in preparation for GC/MS analysis.

Mass Spectral Data Handling. Mass spectral data relevant to deuterium analyses were analyzed by using the adaptation of Brauman's matrix algebraic procedure that we have used and described previously;[16] an expanded dicussion of this method has recently been given by Korzekwa et al.[24] This yields the mole fraction of each deuterated isotopomer ($d_0...d_n$) comprising each chromatographically separated oxidation product.

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Table I. Deuterium-Induced Metabolic Switching in Cytochrome P-450 Catalyzed Hydroxylation of *o*-Xylene[a]

o-xylene substrate	products (mol·%)[b]			product ratios		
	2-MBA	3,4-DMP	2,3-DMP	3,4-DMP/2,3-DMP	benzylic OH/phenols	CH_3 group/ring
D_0	87.5 ± 2.0	10.5 ± 1.7	2.0 ± 0.4	5.25	7.06	3.53
D_1	84.2 ± 2.6	13.2 ± 2.4	2.6 ± 0.2	5.07	5.33	3.24
D_2	80.4 ± 2.5	16.5 ± 2.2	3.1 ± 0.4	5.32	4.10	3.14
D_3	78.3 ± 1.5	18.0 ± 1.4	3.7 ± 0.3	4.86	3.60	3.28
D_6	48.4 ± 2.3	43.0 ± 2.5	8.6 ± 0.2	5.00	0.94	*c*
D_{10}	45.3 ± 3.1	46.9 ± 3.4	7.8 ± 0.4	6.01	0.83	*c*

[a] Values reported are mean ± standard deviation (n = 3 separate experiments). [b] 2-MBA, 2-methylbenzyl alcohol; 3,4-DMP, 3,4-dimethylphenol; 2,3-DMP, 2,3-dimethylphenol. [c] Undefined as D_6 and D_{10} have no CH_3 groups.

Table II. Deuterium-Induced Metabolic Switching in Cytochrome P-450 Catalyzed Hydroxylation of *p*-Xylene[a]

p-xylene substrate	products (mol %)[b]		product ratios	
	4-MBA	2,5-DMP	benzylic OH/phenols	CH_3 group/ring
D_0	95.6 ± 0.9	4.4 ± 0.9	21.7	10.8
D_1	93.4 ± 0.3	6.6 ± 0.3	14.2	8.5
D_2	92.9 ± 0.5	7.1 ± 0.5	13.1	9.0
D_3	87.0 ± 0.4	13.0 ± 0.4	6.7	5.9
D_6	69.3 ± 0.6	30.7 ± 0.6	2.26	*c*
D_{10}	62.9 ± 1.0	37.1 ± 1.0	1.7	*c*

[a] Values reported are mean ± standard deviation (n = 3 separate experiments). [b] 4-MBA, 4-methylbenzyl alcohol; 2,5-DMP, 2,5-dimethylphenol. [c] Undefined as D_6 and D_{10} have no CH_3 groups.

Intra- and Intermolecular Isotope Effects. We performed two types of experiments with a number of differently-deuterated forms of *o*- and *p*-xylene. In some experiments, although only a single isotopic form of substrate was incubated with the microsomes, two types of effects were observed (by comparison to the unlabeled substrate studied in a separate experiment), namely, metabolic switching (a change in product ratios) and isotope discrimination during hydroxylation (e.g. Tables I–IV). These two types of "isotope effects" are distinct from the more familiar ^{D}V or $^{D}(V/K)$ isotope effects on enzymatic reactions,[25,26] and to interpret them quantitatively requires a comprehensive kinetic model (e.g. eqs 1–12 below). Since isotope discrimination of this type takes place *only* during the product-forming step it is not susceptible to suppression by the kinetics of other steps in turnover, which frequently cause ^{D}V and $^{D}(V/K)$ to be less than the intrinsic isotope effect associated with the chemical steps of catalysis. Thus, when corrected for metabolic switching, intramolecular isotope effects approach the intrinsic isotope effect for the chemical step of catalysis.

The second type of experiment we performed involves allowing a mixture of two isotopic forms of a single substrate to compete for metabolism. For an enzyme with a *linear* kinetic mechanism this would yield an ordinary $^{D}(V/K)$ isotope effect for formation of the single reaction product measured. When a branched pathway obtains, as is often the case with P-450 catalyzed reactions, several alternate products can arise from a single Michaelis complex via parallel first-order processes; therefore several different "isotope effects" can be defined depending upon which product or combinations of products are considered (see eqs 13–16, Table VII and associated text for examples). While these isotope effects are not identical with the $^{D}(V/K)$ isotope effects associated with enzymes having linear kinetic mechanisms yielding single products, they have similar properties, i.e. they are affected by the kinetics of all steps up to and including the first irreversible step of catalysis (i.e. the product-forming step), and thus often show reduced values compared to the intrinsic isotope effect (≈intramolecular isotope effect) with substrates that are "sticky" or committed to catalysis. Thus comparisons of intra- vs intermolecularly determined isotope effects on P-450 catalyzed reactions gives insights to the relative kinetics of substrate exchange vs product formation.

Deconvolution of Observed Isotope Effects. Hydroxylation at a benzylic methyl group is subject to both primary and secondary KDIEs. Depending on whether hydrogen or deuterium is being transferred, and on whether the "bystander" positions contain hydrogen or deuterium, it is possible to define three primary and four secondary KDIEs.[16]

$$P_1 = (k_H^{HH}/k_D^{HH}),\quad P_2 = (k_H^{HD}/k_D^{HD}),\quad P_3 = (k_H^{DD}/k_D^{DD})$$

$$S_1 = (k_H^{HH}/k_H^{HD}),\quad S_2 = (k_H^{HD}/k_H^{DD}),\quad S_3 = (k_D^{HH}/k_D^{HD}),\quad S_4 = (k_D^{HD}/k_D^{DD})$$

Unfortunately, because of the symmetry and rapid rotation of methyl groups neither P_1–P_3 nor S_1–S_4 can be observed directly. Analysis of how deuteration at *one* site in a molecule affects the relative chemical reactivity at *each* site in the molecule requires the integration of information on metabolite ratios, the total deuterium content of each metabolite, and the deuterium distribution within each metabolite. This was accomplished by using eqs 1–12 in conjunction with Schemes II–IV. In devising this model it was assumed that all metabolites of a given substrate can be formed within a single enzyme active site by parallel first-order processes (i.e. a branched pathway) as suggested by Schemes II–IV and as described previously for toluene metabolism; in fact one of the goals of this research was to test this hypothesis, and as discussed below, our observations on metabolic switching strongly support this assumption.

$$6k_H^{HH} = (1.0 - k_r) \tag{1}$$

$$3k_H^{HH} = \mathbf{b} = \mathbf{m}_1 \tag{2}$$

$$2k_H^{HD} = \mathbf{f} = (\mathbf{d}_1 - \mathbf{m}_1) = (\mathbf{m}_0 - \mathbf{d}_0) \tag{3}$$

$$k_D^{HH} = \mathbf{a} = \mathbf{d}_0 \tag{4}$$

$$3k_H^{HH} + 2k_H^{HD} + k_D^{HH} = (1.0 - k_r) \tag{5}$$

$$3k_H^{HH} = \mathbf{c} = \mathbf{m}_2 \tag{6}$$

$$2k_D^{HD} = \mathbf{f} = \mathbf{d}_1 \tag{7}$$

$$2k_H^{DD} = \mathbf{g} = (\mathbf{d}_2 - \mathbf{m}_2) = (\mathbf{m}_0 - \mathbf{d}_1) \tag{8}$$

$$3k_H^{HH} + 2k_D^{HD} + k_H^{DD} = (1.0 - k_r) \tag{9}$$

$$3k_H^{HH} = \mathbf{d} = \mathbf{d}_3 = \mathrm{m}_3 \tag{10}$$

$$3k_D^{DD} = \mathbf{g} = \mathbf{d}_2 = \mathbf{m}_0 \tag{11}$$

$$3k_H^{HH} + 3k_D^{DD} = (1.0 - k_r) \tag{12}$$

For xylene $\mathbf{D}_0$, normalized total metabolite formation can be described by eq 1, in which k_r represents all possible ring hydroxylations, k_H^{HH} represents the reactivity of one of the six equivalent benzylic C–H bonds, and the constant 1.0 represents the total reactivity of the molecule. For xylene $\mathbf{D}_1$, normalized total metabolite formation (see Scheme II) is described by eq 5, while the mole fractions of products **A**, **B**, and **F** (represented by the lower case letters **a**, **b**, and f, respectively) are related to the rate constants for each distinct benzylic hydroxylation through eqs 2–4. Analogous relationships obtain for xylenes $\mathbf{D}_2$ and $\mathbf{D}_3$ as indicated in Schemes III (eqs 6–9) and IV (eqs 10–12). Finally, because the degree to which metabolic switching from benzylic to ring hydroxylation varies among substrates $\mathbf{D}_0$–$\mathbf{D}_3$, the normalized k values for benzylic hydroxylations must be further scaled (multiplied) by the factor $(1 - \mathbf{p}_0)/(1 - \mathbf{p}_n)$, where n is the number of deuterium atoms in the substrate and $\mathbf{p}$ is the mole fraction of phenolic products observed for substrate $\mathbf{D}_n$.

Results and Discussion

Both *o*-xylene and *p*-xylene were found to be excellent substrates for PB-microsomal cytochrome P-450. Product analysis revealed that for both substrates all possible regioisomeric monohydrox-

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ylation products were formed. As will become apparent, differences in metabolism between *o*-xylene and *p*-xylene are small and quantitative rather than qualitative. In addition, there is considerable similarity between the metabolic and kinetic effects of stepwise deuteration of the xylenes and those observed for stepwise deuteration of toluene.[2,14]

Hydroxylation of Deuterated Xylenes as Single Substrates. Deuterated xylenes D_0–D_{10} were incubated separately with microsomes and the products analyzed by GC/MS. The effects of stepwise deuteration on the regioselectivity of product formation are summarized in Tables I and II, while the deuterium contents of the benzylic hydroxylation products are given in Table III. Quantitative analysis of these data with eqs 1–12 (cf. Schemes II–IV) provides information on the relative susceptibility of each chemically unique position in each substrate toward enzymatic hydroxylation. These "relative positional reactivities" are summarized in Figure 1.

Benzylic hydroxylation of *o*-xylene is more extensive (88 mol·%, Table I) than that of toluene (68 mol·%) but less extensive than that of *p*-xylene (96 mol·%, Table II). With *o*-xylene 4-hydroxylation exceeds 3-hydroxylation by a factor of 5.25 (Table I). These trends no doubt reflect mainly statistical and steric factors, i.e. two methyl groups in xylene vs one in toluene, and increasing steric hindrance of aromatic hydroxylation on going from toluene to *o*-xylene to *p*-xylene. Stepwise introduction of deuterium into *one* methyl group of *o*-xylene leads to small incremental decreases in net benzylic hydroxylation with corresponding increases in ring hydroxylation. Such changes in product profile are referred to as metabolic switching or isotopically sensitive branching and reflect the operation of a kinetic isotope effect on benzylic hydroxylation. In contrast, complete deuteration of both methyl groups results in extensive switching from benzylic to ring hydroxylation, and this effect is perceptibly enhanced when the aromatic protons are also deuterated. Similar shifts in product profile accompany the stepwise deuteration of *p*-xylene; their smaller size reflects the intrinsically lowered reactivity of the aromatic vs methyl positions in *p*-xylene-d_0 vs *o*-xylene-d_0 (due, probably, to steric factors).

Three additional observations support the concept of a branched pathway in which several different metabolites can emanate from a single enzyme–substrate complex as shown in Schemes II–IV (and as demonstrated previously for toluene).[2,19,27] First, although deuteration shifts metabolism away from benzylic hydroxylation toward phenol formation, the 3,4-DMP/2,3-DMP ratio from *o*-xylene remains constant at around 5.25 (Table I). Second, in *o*-xylene, the ratio of reactivity at the nondeuterated methyl group to that at the aromatic ring also remains constant as deuterium is introduced into the other methyl group (Table I, last column). For *p*-xylene the latter index is essentially constant as the first two deuteriums are introduced but decreases by 46% upon introduction of the third deuterium (Table II). We believe this and other evidence to be discussed below indicates that substrate reorientation within the active site is simply less facile with *p*-xylene than with *o*-xylene.

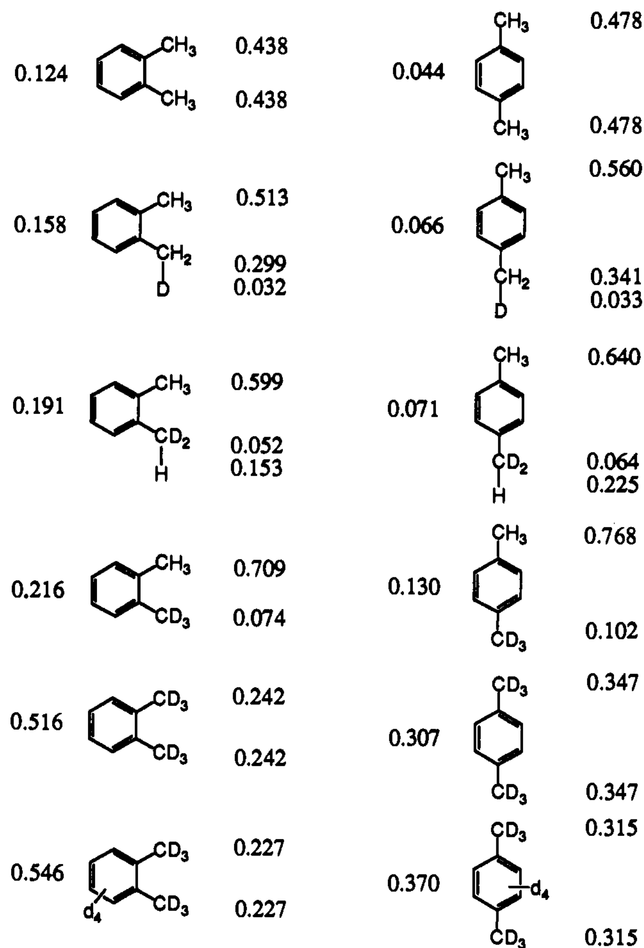

Figure 1. Relative positional reactivity of deuterated xylenes toward microsomal hydroxylation.

Further insight into isotopically sensitive branching during xylene metabolism can be gained from analysis of Figure 1 and Table IV. Following down either column of Figure 1 it is apparent that the reactivity "lost" upon introduction of D into a methyl group is actually redistributed almost statistically to other C–H bonds in the same methyl group, to the other methyl group, and to the aromatic ring. As Table IV emphasizes, with increasing deuteration the reactivity ratio CH_3/CD_nH_{3-n} increases more rapidly for *o*-xylene than for *p*-xylene, again suggesting that switching between methyl groups in the latter is not as facile as with the former. This is reasonable since methyl–methyl interchange within the Michaelis complex would require much more molecular motion and would be correspondingly slower or less probable with *p*-xylene than with *o*-xylene.

The CH_3/CD_3 values in Table IV are apparent intramolecular kinetic deuterium isotope effects (KDIEs) for benzylic hydroxylation. Their magnitude is in the range typically observed for

Table III. Isotopic Composition of Benzylic Oxidation Products from Deuterated Xylenes

substrate	alcohol (mole fraction)[a]		ester (mole fraction)[b]	
D_1	d_0	d_1	m_0	m_1
ortho	0.0386 ± 0.0066	0.9614 ± 0.0066	0.3917 ± 0.0110	0.6083 ± 0.0024
para	0.0358 ± 0.0024	0.9642 ± 0.0024	0.4006 ± 0.0007	0.5994 ± 0.0007
D_2	d_1	d_2	m_0	m_2
ortho	0.0647 ± 0.0028	0.9310 ± 0.0028	0.2511 ± 0.0024	0.7338 ± 0.0043
para	0.0689 ± 0.0043	0.9270 ± 0.0053	0.3090 ± 0.0496	0.6859 ± 0.0484
D_3	d_2	d_3	m_0	m_3
ortho	0.0791 ± 0.0024	0.9169 ± 0.0022	0.0923 ± 0.0017	0.8733 ± 0.0014
para	0.1277 ± 0.0077	0.8662 ± 0.0057	0.1158 ± 0.0013	0.8686 ± 0.0077

[a] Deuterium content of the methylbenzyl alcohol metabolites whose relative yields are reported in Tables I and II. [b] Deuterium content remaining after oxidation and esterification of the benzylic alcohol. See Schemes II–IV and text for further explanation.

Table IV. Effects of Stepwise Deuteration of One Methyl Group in Xylene on Relative Reactivity toward Hydroxylation on the Aromatic Ring, the Undeuterated Methyl Group, and the Deuterated Methyl Group

	reactivity ratio			
	CH_3/CD_nH_{3-n}		CD_nH_{3-n}/ring	
substrate	ortho	para	ortho	para
$\mathbf{D_0}$	(1.0)	(1.0)	3.53	10.86
$\mathbf{D_1}$	1.54	1.49	1.46	5.67
$\mathbf{D_2}$	2.92	2.21	1.07	4.07
$\mathbf{D_3}$	9.58	7.53	0.34	0.78

similar P-450 catalyzed oxidations.[28-33] Assuming that the *intrinsic* KDIEs for benzylic hydroxylation of *o*-xylene and *p*-xylene are the same, the lower *observed* effect with *p*-xylene is likely a result of commitment to catalysis caused by product formation being more facile than switching between the CH_3 and CD_3 groups (regardless of whether switching occurs by substrate dissociation/reassociation or by substrate reorientation within the active site). The CD_nH_{3-n}/ring reactivity ratio data in Table IV show that with stepwise deuteration, switching to the ring is actually more facile with *p*-xylene than with *o*-xylene by a factor of (10.86/0.78)/(3.53/0.34) = 1.34, despite the fact that ring hydroxylation is intrinsically less favorable (relative to benzylic hydroxylation) with *p*-xylene than with *o*-xylene. This, too, is consistent with switching from one methyl to the other being significantly easier with *o*-xylene than *p*-xylene.

Finally, comparison of the data for ring hydroxylation of the $\mathbf{D_6}$ vs $\mathbf{D_{10}}$ substrates in Tables I and II indicates that there are KDIEs on this process of (43.0 + 8.6)/(46.9 + 7.8) = 0.943 for *o*-xylene and (30.7/37.1) = 0.827 for *p*-xylene. The small size and inverse nature of these effects are consistent with expectations for attack of an enzymatic FeO group on an sp^2 carbon and with observations of KDIEs with other aromatic and olefinic substrates for cytochrome P-450.[34,35]

Primary and Secondary KDIEs on Benzylic Hydroxylation. Analysis of the data in Tables I–III for substrates $\mathbf{D_0}$–$\mathbf{D_3}$ with eqs 1–12 gives the relative rate constants listed in Table V. These differ from the relative positional reactivities in Figure 1 only in being normalized to (i.e. divided by) the observed value of k_H^{HH} for the particular substrate and statistically corrected for the number of identical hydrogens or deuteriums. These values can be combined as shown in the middle part of Table V to calculate values for the primary (P_1–P_3) and secondary (S_1–S_4) KDIEs for benzylic hydroxylation of xylenes by cytochrome P-450. Five of the six P values thus obtained are in the range of 5.3–7.2 and six of the eight S values are in the range 1.09–1.25. These values are typical of fully developed primary and α-secondary deuterium isotope effects and are similar in magnitude but statistically more precise than corresponding values observed in our previous study with toluene.[2] Only three of the fourteen values lie outside these ranges, and these three also have unusually large relative standard deviations. In the case of P_3 and S_2 for *p*-xylene, the error is directly attributable to experimental

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Table V. Relative Rate Constants and KDIEs for Benzylic Hydroxylation of *o*- and *p*-Xylene

	o-xylene	*p*-xylene
rate constants[a]		
k_H^{HH}	1.000 ± 0.0058	1.000 ± 0.0580
k_H^{HD}	0.8703 ± 0.0166	0.921 ± 0.0070
k_H^{DD}	0.7621 ± 0.0122	1.0503 ± 0.2292[b]
k_D^{HH}	0.1903 ± 0.0326	0.1792 ± 0.0120
k_D^{HD}	0.1325 ± 0.0057	0.1505 ± 0.0143
k_D^{DD}	0.1058 ± 0.0021	0.1333 ± 0.0018
Isotope effects[a]		
P_1 (k_H^{HH}/k_D^{HH})	5.25 ± 0.16	5.58 ± 0.49
P_2 (k_H^{HD}/k_D^{HD})	6.57 ± 0.16	6.06 ± 0.58
P_3 (k_H^{DD}/k_D^{DD})	7.20 ± 0.18	7.87 ± 1.72[b]
S_1 (k_H^{HH}/k_H^{HD})	1.149 ± 0.023	1.095 ± 0.064
S_2 (k_H^{HD}/k_H^{DD})	1.142 ± 0.028	0.869 ± 0.190[b]
S_3 (k_D^{HH}/k_D^{HD})	1.436 ± 0.254	1.190 ± 0.138
S_4 (k_D^{HD}/k_D^{DD})	1.252 ± 0.059	1.129 ± 0.108
CH_3/CD_3(obs)[c]	9.58	7.53
$P_1S_3S_4$(calc)	9.45 ± 1.74	7.50 ± 1.30
$S_1S_2P_3$(calc)	9.43 ± 0.39	7.49 ± 2.35

[a] Results presented are means ± standard deviation for three separate experiments. [b] See text regarding experimental errors. [c] See Table IV.

difficulties in CG/MS analysis of the ester derivatives of the metabolites of *p*-xylene-d_2 (see Table III), which introduced considerable uncertainty to the value of k_H^{DD} for *p*-xylene. Overall, however, considering the number of enzymatic, chemical, analytical, and mathematical manipulations involved in generating the KDIE values reported in Table V, the level of agreement of the final values, both among themselves and with theoretical expectations, is very satisfying.

One final test of cohesiveness and internal consistency in both the method and the data is summarized in the bottom three lines of Table V. Recall that using only the $\mathbf{D_3}$ substrate it is possible to measure an intramolecular CH_3/CD_3 competitive KDIE directly. For *o*- and *p*-xylene the observed values were 9.58 and 7.53, respectively (Table IV). Using the rate constants in the top section of Table V one can also calculate the expected combined effect of one primary and two secondary KDIEs, i.e. $k_H^{HH}/k_D^{DD} = P_1S_3S_4$ or $S_1S_2P_3$. The last two lines of Table V show that for both *o*- and *p*-xylene there is extremely good agreement between the values observed with use of only the $\mathbf{D_3}$ substrates and those calculated with data from all three substrates (i.e. $\mathbf{D_1}$, $\mathbf{D_2}$, and $\mathbf{D_3}$). This excellent agreement lends additional confidence to both the model and the data. This comparison also serves to highlight one other interesting feature of the approach used, namely, that to the extent that less facile switching may have caused the observed intramolecular CH_3/CD_3 isotope effect for *p*-xylene to be lower than that for *o*-xylene, the same effect also operates on *p*-xylene-d_1 and -d_2, data from which are necessary for calculating the combined effect of one primary and two secondary KDIEs as $P_1S_3S_4$ or $S_1S_2P_3$.

The commonly accepted mechanism for hydroxylation by cytochrome P-450 involves abstraction of an aliphatic hydrogen from an sp^3-hybridized carbon by an enzymatic oxoiron group, followed by C–O bond formation via transfer of OH from iron to carbon. Classically, the abstraction step should give rise to a KDIE of around 7 if the transition state is linear and symmetrical, and this is what we observe (Table V). Benzylic hydroxylation is particularly favorable among hydroxylation processes, presumably because overlap of the reacting C–H σ bond with the aromatic π system weakens the σ bond and helps stabilize radical-like transition states and intermediates. Maximum resonance stabilization at a benzylic center requires sp^2 hybridization at the benzylic carbon, and a change from sp^3 to sp^2 geometry should generate a normal secondary KDIE of at least 1.15–1.20, which is also as observed (Table V). These results can be contrasted

Table VI. Intermolecular Isotope Discrimination in Xylene Oxidation by Cytochrome P-450

	Isotopic Composition (mole fraction)			
	substrates	products		
species	*o*-xylene	2-MBA	3,4-DMP	2,3-DMP
d_0	0.361	0.348 ± 0.007	0.061 ± 0.001	0.013 ± 0.001
d_5	0.018	0.274 ± 0.005	0.009 ± 0.001	0.001 ± 0.001
d_6	0.621	0.000	0.240 ± 0.001	0.048 ± 0.001
	p-xylene	4-MBA	2,5-DMP	
d_0	0.516	0.554 ± 0.006	0.055 ± 0.001	
d_5	0.015	0.269 ± 0.005	0.003 ± 0.001	
d_6	0.469	0.000	0.111 ± 0.001	

to those for the free radical chlorination and bromination of toluene which show large primary but only small secondary KDIEs, suggesting that in these transition states there is little rehybridization toward planarity at the benzylic carbon.[16] For benzylic hydroxylation of toluene by P-450 a large overall KDIE was obvious and although the statistical significance of individual S values was low, the results pointed to both P and S being large and normal.

With the xylenes the large P values point to a transition state that is linear and symmetrical with greatly reduced C–H bond order, while the large S values suggest considerable rehybridization toward sp^2 character at the benzylic carbon and, presumably, considerable resonance stabilization of the nascent benzylic radical, which calculations indicate to be planar.[36] Interestingly the 1-hydroxylation of *n*-octane shows an α-secondary DIE of 1.09–1.14 (along with a primary DIE of 7.6–9.2), even though benzylic stabilization is not possible.[28] Planar or nearly-planar radical intermediates in both cases would be consistent with observations of only partial retention of configuration during P-450 catalyzed 1-hydroxylation of *n*-octane[37] and benzylic hydroxylation of ethylbenzene.[29]

Although both benzylic and non-benzylic methyl groups may appear to undergo P-450 hydroxylation via transition states with similar overall structures as suggested by isotope effect criteria, their *relative* reactivities are very different. Whereas benzylic hydroxylation accounts for 85–95% of the total metabolism with the xylenes and 67% with toluene, hydroxylation at the methyl termini of *n*-octane accounts for only 2–5% of products, the majority being derived via hydroxylation at C-2 and C-3.[28]

Intermolecular KDIEs. Intermolecular competition experiments with mixtures of labeled and unlabeled substrates provide another independent way to approach measurement of isotope effects. However, isotope effects of this kind are subject to kinetic suppression by "commitment to catalysis" when the dissociation of unchanged substrate from the active site is slow relative to forward progress through an irreversible step (i.e. when the substrate is "sticky"). Thus by comparing intermolecular isotope effects to those measured by intramolecular approaches (e.g. Table V) one can gain information about the relative rates of the isotopically-insensitive vs isotopically-sensitive steps in turnover.[25,26]

Analysis of incubations of mixtures of D_0 and D_6 xylenes led to the product ratio and isotopic competition data shown in Table VI. Equations 13–17, derived previously for intermolecular competitive hydroxylation of deuterated toluenes,[2] were used with the data of Table VI to extract KDIEs on four observed parameters, i.e. benzylic hydroxylation (Q_1), phenol formation (Q_2), the benzylic alcohol/phenol ratio (Q_3), and total product formation (Q_4). Table VII compares the results for the xylenes to those determined earlier for toluene, revealing again considerable similarity in their behavior.

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Table VII. Relative Kinetics and Isotope Effects for P-450 Oxidation of Toluene, *o*-Xylene, and *p*-Xylene Based on Scheme V

parameter	toluene[a]	*o*-xylene[b]	*p*-xylene[b]
Q_1	3.6	2.25	1.93
Q_2	0.56	0.439	0.453
Q_3	6.29	5.12	4.27
Q_4	1.23	1.31	1.49
k_H	(1.0)	(1.0)	(1.0)
k_D	0.159	0.196	0.234
k_r	0.53	0.213	0.099
k_{-1}	0.68	0.205	0.300
C_f[c]	2.25	5.91	3.66
CH_3/CD_3	6.93[d] 8.67[e]	9.52	7.53

[a] Data from ref 2. [b] Calculated from data of Table VI. [c] $C_f = (k_H + k_r)k_{-1}$; see text. [d] Calculated from intramolecular B/P ratios for toluene-d_0/-d_3. [e] Calculated from intramolecular B/P ratios for toluene-d_5/-d_8.

$$Q_1 = \frac{B_H}{B_D}\frac{[S_D]}{[S_H]} = \frac{k_H}{k_D}Z \tag{13}$$

$$Q_2 = \frac{P_H}{P_D}\frac{[S_D]}{[S_H]} = Z \tag{14}$$

$$Q_3 = \frac{B_H/P_H}{B_D/P_D} = \frac{Q_1}{Q_2} = \frac{k_H}{k_D} = {}^{D}k \tag{15}$$

$$Q_4 = \frac{\Sigma_H}{\Sigma_D}\frac{[S_D]}{[S_H]} = \frac{k_H + k_r}{k_D + k_r}Z \tag{16}$$

$$Z = \frac{k_{-1} + k_D + k_r}{k_{-1} + k_H + k_r} \tag{17}$$

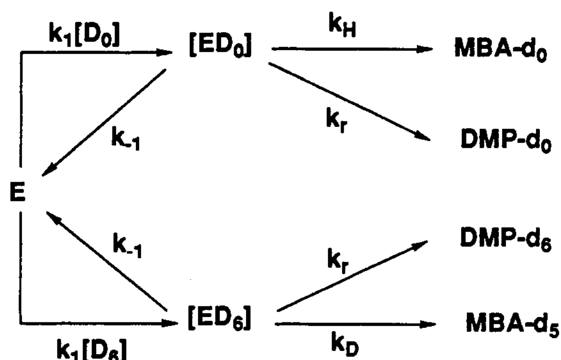
As indicated in Table VII, the intermolecular isotope effects for benzylic hydroxylation (Q_1) are greatly reduced compared to the CH_3/CD_3 isotope effects determined intramolecularly. Since the Q_1 calculation considers only the mole fractions of labeled vs unlabeled benzylic alcohol derived from a given substrate, it does not take into account that the effect of substrate deuteration is expressed in part through metabolic switching leading to an altered product ratio within the deuterated substrate (viz. Figure 1). In contrast the isotope effect Q_3, which is based on the benzylic alcohol/phenol product ratio, and thus takes metabolic switching into account, is much larger than Q_1. Thus as noted by Jones et al.,[27,38] metabolic switching has the effect of "unmasking" the true or intrinsic isotope effect.

Another consequence of metablic switching from benzylic to aromatic hydroxylation is that Q_2, the isotope effect on phenol formation, appears large and inverse. This apparent isotope effect arises as a kinetic artifact of metabolic switching and has no significance regarding the chemical mechanism of aromatic hydroxylation. In contrast the KDIEs for ring deuteration measured by intramolecular H/D competition (see above) are small and inverse as expected and thus probably do have significance for the mechanism of aromatic hydroxylation.

Even with the unmasking effect of metabolic switching taken into account, Q_4, the isotope effect on total product formation from D_0 vs D_6 is very much less than the observed intramolecular CH_3/CD_3 isotope effect observed with D_3 alone. This difference probably represents kinetic suppression of the intrinsic isotope effect by forward commitment to catalysis, which in the context of Scheme V is defined as $C_f = (k_H + k_r)/k_{-1}$. Solving eqs 13–17 using data from Table VI yields the relative rate constants and commitment factors given in Table VII. For both xylenes and toluene, $k_H > k_{-1}$ and C_f is large, indicating that for a given nondeuterated substrate in the active site of cytochrome P-450

(38) Jones, J. P.; Korzekwa, K. R.; Rettie, A. E.; Trager, W. F. *J. Am. Chem. Soc.* **1986**, *108*, 7074–7078.

Scheme V



there is indeed a greater probability of undergoing catalysis than of dissociating back into solution. On the other hand, deuteration retards benzylic hydroxylation without affecting k_r or k_{-1} such that $k_H > k_d \approx k_{-1}$. Assuming that there is no isotope effect on substrate binding, isotopically different substrate molecules enter the active site of an enzyme in proportion to their concentrations (see Scheme V). However, if bound substrate molecules do not dissociate easily and often prior to irreversible catalysis, the isotopic composition of the product will tend to resemble that of the substrate and the apparent isotope effect will be quite small, even if there is a large intrinsic isotope effect for the catalytic step; this is the essence of forward commitment to catalysis. When $k_{-1} < k_H$ and branching is not an option ($k_r = 0$), the isotope effect will cause $[ES_D]$ to accumulate relative to $[ES_H]$ such that $[ES_D]/[ES_H] > k_1[S_D]/k_1[S_H]$. The increase in $[ES_D]$ will offset the slower turnover of S_D and diminish the *observed* isotope effect on product formation. Increasing either k_{-1} or k_r or both provides a way to dissipate the relative accumulation of $[ES_D]$ and allows the *observed* isotope effect on product formation to approach k_H/k_D.

Conclusions

Stepwise deuteration of one and then both methyl groups in *o*- and *p*-xylene leads to stepwise decreases in benzylic hydroxylation at the deuterated methyl and to proportional increases in hydroxylation on the undeuterated methyl group and the aromatic ring as a result of isotopically sensitive branching within the xylene•P-450 Michaelis complex. Benzylic hydroxylation is subject to a large intrinsic deuterium isotope effect (CH_3/CD_3 = 7.5–9.5), comprised of a large primary component (5.3–7.8) and a large normal α-secondary component (1.09–1.19). In contrast a small *inverse* α-secondary isotope effect (0.83–0.94) was observed for aromatic hydroxylation of both xylenes, consistent with an addition reaction at an sp^2-hybridized carbon. The isotope effects for benzylic hydroxylation suggest a transition state for H-abstraction which is linear and symmetrical with substantial rehybridization toward planarity at the benzylic carbon and little residual C–H bond order remaining. The ${}^D(V/K)$ isotope effect observed for benzylic hydroxylation in intermolecular competitions (ca. 1.9–2.3 for $\mathbf{D_0}$ vs $\mathbf{D_6}$) is substantially reduced by commitment to catalysis, with $C_f = (k_H + k_r)/k_{-1}$ = 3.6–5.9. H/D discrimination is greatest when the competing H and D are in the same methyl group, less when they are in different methyls on the same molecule, and least when they are in methyl groups on different molecules. This suggests a dynamic picture with the following relative reaction rates: methyl group rotation > substrate reorientation *within the Michaelis complex* (i.e. isotopically sensitive branching to different products) > product formation (i.e. commitment to catalysis) > substrate dissociation to effect H/D exchange prior to hydroxylation.

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