Intrinsic Isotope Effects Suggest That the Reaction Coordinate Symmetry for the Cytochrome P-450 Catalyzed Hydroxylation of Octane Is Isozyme Independent

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The mechanism of the ω-hydroxylation of octane by three catalytically distinct, purified forms of cytochrome P-450, namely, P-450$_b$, P-450$_c$, and P-450$_{LM2}$, was investigated by using deuterium isotope effects. The deuterium isotope effects associated with the ω-hydroxylation of octane-*1,1,1*-$^2H_3$, octane-*1,8*-$^2H_2$, and octane-*1,1,8,8*-$^2H_4$ by all three isozymes were determined. From these data the intrinsic isotope effects were calculated and separated into their primary and secondary components. The primary intrinsic isotope effect for the reaction ranged from 7.69 to 9.18 while the secondary intrinsic isotope effect ranged from 1.13 to 1.25. Neither the primary nor secondary isotope effect values were statistically different for any of the isozymes investigated. These data are consistent with a symmetrical transition state for a mechanism involving initial hydrogen atom abstraction followed by hydroxyl radical recombination which is essentially independent of the specific isozyme catalyzing the reaction. It is concluded that (1) in general the porphyrin-$[FeO]^{3+}$ complex behaves as a source of a triplet-like oxygen atom, (2) the regioselectivity for the site of oxidation is dictated by the apoprotein of the specific isozyme of cytochrome P-450 catalyzing the reaction, and (3) the maximum primary intrinsic isotope effect for any cytochrome P-450 catalyzed oxidation of a carbon center is about 9, assuming no tunneling effects.

Cytochrome P-450 is a family of isozymes having high chemical reactivity (e.g. they will oxidize hydrocarbons), broad and overlapping substrate specificity, and a high degree of stereoselectivity. It may appear incongrous that a group of highly reactive enzymes which lack substrate specificity can still elicit a high degree of stereoselectivity. These properties may be rationalized by postulating that a catalytically reactive species (porphyrin-$[FeO]^{3+}$), common to all isozymes of the cytochrome P-450 family,[1] largely controls the basic mechanism of any given reaction, e.g. direct C-H insertion versus initial hydrogen abstraction, while the apoprotein provides the architecture for the active site of the various isozymes and largely controls both the regioselectivity and stereoselectivity of the reaction being considered. While the molecular mechanisms by which cytochrome(s) P-450 oxidize substrate have been the subject of intense investigation,[2,3] the question of whether the reactivity of the porphyrin-$[FeO]^{3+}$ species is constant from isozyme to isozyme remains unanswered. It is conceptually possible that the binding of the substrate to the enzyme could alter the stability of the $[FeO]^{3+}$ complex and hence its reactivity. One approach to addressing this problem is to determine and compare intrinsic deuterium isotope effects associated with a given reaction catalyzed by different isozymic forms of P-450. If the reactivity of the porphyrin-$[FeO]^{3+}$ species is conserved, the magnitudes of the isotope effects should be similar if not identical.

Recently, we reported a technique for determining the intrinsic deuterium isotope effect associated with the cytochrome P-450$_b$ catalyzed oxidation of the terminal carbon of octane,[4,5] a substrate known to exhibit a significant isotope effect.[6] The technique was based on isotopically sensitive branching from the C-1 position to other positions in the molecule. The enzyme mechanism was further elucidated by separation of the observed isotope effect into its primary and secondary components.[7,8] In this report we extend these studies to include two other purified and well-characterized cytochrome P-450's, namely, cytochrome P-450$_c$ and cytochrome P-450$_{LM2}$. These isozymes represent the major 3-methylcholanthrene- and phenobarbital-inducible forms of cytochrome P-450 in rat and rabbit, respectively. They were also chosen because they represent two readily obtainable, well-studied, distinct genetic subfamilies of cytochrome P-450.[9] While P-450$_b$ and P-450$_{LM2}$ belong to the same genetic subfamily, they are obtained from different species and possess different substrate specificities, stereoselectivities, and regioselectivities.[10] These differences presumably reflect significant differences in the spatial characteristics of the amino acid residues present in their active sites and or differences in the reactivity of the porphyrin-$[FeO]^{3+}$ prosthetic group. This report details the mechanism of oxidation of octane by these three isozymes of cytochrome P-450 and addresses the question of conservation of reactivity.

Table I. Ratio of C-2 to C-1 Hydroxylation and Observed Isotope Effect for Various Isozymes of Cytochrome P-450

isozyme	2-octanol[a]/1-octanol[a]	isotope effect
P-450$_b$	22.8 ± 1.5 (4)[b]	11.77 ± 0.19[c]
P-450$_c$	>50 (3)	12.08 ± 0.93
P-450$_{LM2}$	38.6 ± 4.3 (3)	11.54 ± 0.54

[a] 3-Octanol also appeared to be formed as a minor product by the three isozymes but could only readily be measured from the P-450$_b$ incubations. The ratio of 1-octanol:2-octanol:3-octanol was found to be 1:23:7 by gas chromatography. With a branched reaction pathway the only effect that formation of 3-octanol could have on the formation of 1-octanol would be to further unmask the intrinsic isotope effect. Therefore, accurate measurement of 3-octanol formation from the other two isozymes was not pursued. [b] The numbers after the ± sign are the standard deviations while the numbers in the parentheses are the number of determinations. [c] The datum is taken from Jones et al.[4] and is corrected (Jones et al.[5]).

Results

Cytochrome P-450$_b$, P-450$_c$, and P-450$_{LM2}$ were purified to apparent electrophoretic homogeneity (Figure 3). The

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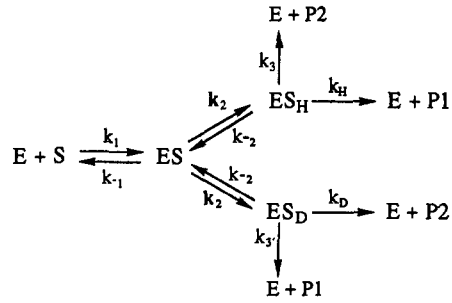

Figure 1. Kinetic model for isotopically sensitive branched reaction pathways. The model assumes that there is no isotope effect associated with binding (i.e., a single rate constant, k_2, describes the fractionation of ES to ES_H and ES_D and a single rate constant, k_{-2}, describes the formation of ES from ES_H and ES_D) and that product formation is irreversible.

overall turnover of octane by the various isozymes of P-450 occurs in the order P-450_b > P-450_{LM2} ≫ P-450_c under the incubation conditions employed. In addition, the products of the oxidation by the various isozymes revealed a marked regioselectivity. The rate of hydroxylation is a least 20-fold faster at the C-2 position than at the C-1 position for cytochrome P-450_b, better than 30-fold faster for cytochrome P-450_{LM2}, and greater than 50-fold faster for cytochrome P-450_c (Table I). These ratios were determined by mass spectral analysis of the trimethylsilyl ether derivative of the respective alcohols and are corrected for the differences in mass spectral fragmentation patterns by the use of standard ratios as previously described.[4,5] The large difference in rates for oxidation at the two positions ensures that if a significant isotope effect is operative in the C-1 hydroxylation of octane, then the observed isotope effect will approach the intrinsic isotope effect. This can be seen by evaluation of eq 1 adapted from Jones et al.[4] to describe the model in Figure 1.

$$(k_H/k_D)_{obsd} = \frac{k_H/k_D + k_H/(k_{-2} + k_3)}{1 + k_H/(k_{-2} + k_3')} \quad (1)$$

This equation describes the effect of branched pathways on the observed isotope effect. From eq 1 it can be seen that the observed isotope effect $[(k_H/k_D)_{obsd}]$ deviates from the intrinsic isotope effect (k_H/k_D) as a function of the term $k_H/(k_{-2} + k_3)$. Because the rate of debinding (k_{-2}) appears in the denominator, any finite value it might have can only effect the relationship between the observed isotope effect and the intrinsic isotope effect by making the observed isotope effect more closely approach the intrinsic isotope effect. If k_{-2} is zero (the worst possible case in terms of its effect on masking), then the rate of the branching pathway, k_3, will be the sole determinant in modifying the magnitude of the observed isotope effect. As k_3, the rate of C-2 oxidation, becomes large, k_H/k_3 approaches zero and the observed isotope effect approaches the intrinsic isotope effect. Since the rate k_3 has been shown to be at least 20-fold faster than the rate k_H, the ratio k_H/k_3 is at most 0.05. With this information, eq 1 can be rewritten as eq 2, and the maximum possible value

$$(k_H/k_D)_{obsd} = \frac{k_H/k_D + 0.05}{1.05} \quad (2)$$

for the intrinsic isotope effect (P-450_b) can be calculated to be 12.3 as compared to the observed intramolecular isotope effect of 11.77 ± 0.19 (Table I). If the intrinsic isotope effects are calculated for P-450_c and P-450_{LM2} with eq 2 and the appropriate k_H/k_3 ratio (Table I) and the average value for all three isozymes determined, it is found to be 12.15. The average experimentally observed intramolecular isotope effect is found to be 11.80 ± 0.55. Thus, within experimental error, the observed isotope effects are not different from each other (visual inspection of the standard deviation, Table I) and are essentially equal to the intrinsic isotope effect for the ω-hydroxylation of octane by these isozymes.

Table II. Observed Isotope Effects for the Oxidation of Octane-*1,8*-$^2H_2$ and Octane-*1,1*,8,8-$^2H_4$ by Purified Cytochrome P-450's

isozyme	substrate	isotope effect
P-450_b[a]	[1,8-H_2]octane	16.21 ± 0.68[b]
P-450_b[a]	[1,1,8,8-$^2H_4$]octane	4.21 ± 0.02[b]
P-450_c	[1,8-$^2H_2$]octane	12.29 ± 1.09
P-450_c	[1,1,8,8-$^2H_4$]octane	3.86 ± 0.33
P-450_{LM2}	[1,8-$^2H_2$]octane	15.66 ± 0.38
P-450_{LM2}	[1,1,8,8-$^2H_4$]octane	3.99 ± 0.27

[a] Data are taken from Jones et al.[4,5,7,8] [b] The numbers after the ± sign are the standard deviations.

Table III. Separated Primary and Secondary Isotope Effects for the Major Purified Cytochrome P-450's as Calculated from the Ratios of $^2H_3/^2H_4$ or $^2H_3/^2H_2$.

	primary isotope effect		secondary isotope effect	
isozyme	$^2H_3/^2H_4$	$^2H_3/^2H_2$	$^2H_3/^2H_4$	$^2H_3/^2H_2$
P-450_b[a]	9.10 ± 0.03[b]	9.18 ± 0.28	1.137 ± 0.003	1.134 ± 0.034
P-450_c	8.96 ± 0.17	7.69 ± 0.60	1.161 ± 0.02	1.253 ± 0.098
P-450_{LM2}	8.91 ± 0.12	9.02 ± 0.32	1.138 ± 0.016	1.131 ± 0.040

[a] Data are taken from Jones et al.[7,8] [b] The numbers after the ± sign are the standard deviations.

To determine the secondary isotope effects, the observed isotope effects for the ω-hydroxylation of [1,8-$^2H_2$]octane and [1,1,8,8-$^2H_4$]octane were measured (Table II). Ideally, the observed isotope effects should closely approximate the intrinsic isotope effects for these reactions. This is necessary since determination of the primary and secondary isotope effects from eqs 3 and 4, shown below, requires knowledge of the intrinsic isotope effect.

$$\frac{[1,1,1\text{-}^2H_3]\text{octane}}{[1,1,8,8\text{-}^2H_4]\text{octane}} = \frac{2(k_H^{HH}/k_D^{DD})}{k_H^{DD}/k_D^{HD}} = 2S^3 \quad (3)$$

$$\frac{[1,1,1\text{-}^2H_3]\text{octane}}{[1,1,8,8\text{-}^2H_2]\text{octane}} = \frac{k_H^{HH}/k_D^{DD}}{2(k_H^{HD}/k_D^{HH})} = \tfrac{1}{2}S^3 \quad (4)$$

The isotope effects for the ω-hydroxylation of [1,8-$^2H_2$]- and [1,1,8,8-$^2H_4$]octane by P-450_c and P-450_{LM2} were determined as previously described for P-450_b[7,8,11] and are given in Table III. With eqs 3 and 4 the observed isotope effects for P-450_c and P-450_{LM2} were separated into their primary and secondary components.

Primary isotope effects ranged from 7.69 to 9.18, while secondary isotope effects had values between 1.13 and 1.25 (Table III). The range of isotope effects in Table III reflects the two separate isotope effects that are calculated by using two different equations, eqs 3 and 4. The theoretical primary isotope effect has already been determined to be maximally 8.58 at 300 K.[12]

Since fractionation factors cannot be determined for a reaction forming a radical product, the equilibrium secondary isotope effect, and thus the maximum secondary kinetic effect, can only be determined theoretically. In order to compare experimental and theoretical values for

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Table IV. Calculated Equilibrium Isotope Effects for the Equilibrium between Ethane and Ethyl Radical

temp, K	equilibrium isotope effect[a]	temp, K	equilibrium isotope effect[a]
100	2.15	310	1.20
200	1.40	400	1.13
300	1.21	900	1.02

[a] Calculation based on one deuterium atom on the α-carbon.

the intrinsic secondary isotope effects, molecular orbital calculations were carried out by using the MNDO formalism. Ethane was used as a model for octane, and ethyl radical was used as a model for the primary octyl radical (the product of hydrogen atom abstraction from the C-1 position). The calculated equilibrium secondary isotope effect (1.20 at 37 °C, Table IV) represents the maximum value a secondary kinetic isotope effect can achieve at physiological temperature if the effect of tunneling is neglected.[13]

Discussion

The observed intramolecular effects for all three isozymes are not significantly different (Table II) and are essentially equal to the intrinsic isotope effect for the reaction (eq 2). These results suggest that the structure of the apoprotein has no effect on either the basic mechanism of the reaction or even the symmetry of the reaction coordinate but is primarily responsible for the observed differences in regioselectivity (Table I). However, since the observed isotope effect is a combination of both primary and secondary isotope effects, it is possible that while two enzymes give identical observed intramolecular isotope effects the relative contributions of their primary and secondary components may differ. Such a situation could mask any isozyme-dependent mechanistic differences such as a difference in reaction symmetry coordinates. Consequently, a better indicator for the degree of conservation of the reactivity of the porphyrin-$[FeO]^{3+}$ species for the three isozymes is a direct comparison of the primary and secondary isotope effects associated with each reaction. The separated primary and secondary isotope effects are presented in Table III. It is apparent that within experimental error each of the isozymes give rise to the same primary and secondary isotope effect. These data provide additional evidence that indeed the three different isozymes operate by the same mechanism with identical reaction coordinate symmetry. The magnitude of the isotope effects associated with the hydroxylation of octane is consistent with the mechanism that was first described by Groves et al.[14] for the hydroxylation of norbornane and involves initial abstraction of a hydrogen atom by a triplet-like oxygen atom in the porphyrin-$[FeO]^{3+}$ complex to form the octyl radical and a porphyrin-$[FeO]^{3+}$-stabilized hydroxy radical (Figure 2a). This is followed by recombination of hydroxy radical with octyl radical to form product. By comparison a direct-insertion mechanism would involve a singletlike oxygen atom and a much smaller isotope effect (Figure 2b). This is true since the transition state for attack by a singlet-like oxygen atom would involve more bending vibrational modes and less stretching modes than hydrogen atom abstraction. The theoretical value for direct insertion has been calculated to be 1.65.[12] To compare the experimentally determined primary and secondary isotope effect values with what

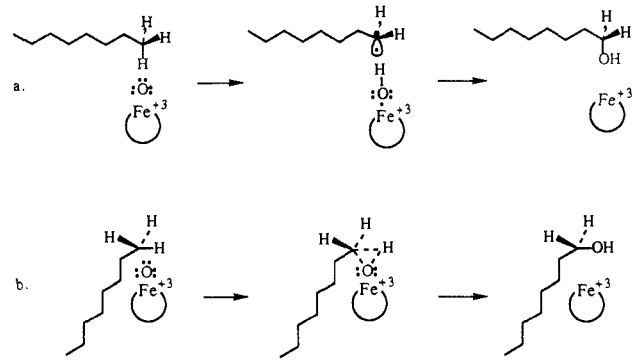

Figure 2. (a) Hydrogen atom abstraction–hydroxy radical recombination mechanism; (b) direct oxygen-insertion mechanism.

would be expected theoretically we have used MNDO calculations on the ethyl–ethyl radical pair as a model. This method has been shown to reproduce these effects in an accurate fashion[15] and should do particularly well in this instance, since the product is not a charged species and thus would not be subjected to large solvation effects. From the theoretical calculations (Table IV) the secondary kinetic isotope effect for a symmetrical reaction coordinate at 37 °C should be approximately 1.10 (mean value between 1 and 1.2). On average, the observed secondary isotope effect (Table III) is somewhat larger as is the primary isotope effect. The discrepancy between the maximum theoretical values and the experimentally determined values may indicate that either the model chosen for the theoretical calculations is not ideal and/or tunneling contributes to the rate of these reactions. However, whatever the reason for the discrepancy, the magnitude of the primary and secondary isotope effects can only be reconciled with a symmetrical reaction coordinate for the oxidation of octane by all three of these enzymes.

This conclusion has several significant ramifications. According to the Melander–Westheimer principle,[16,17] which is based on the Hammond postulate,[18,19] the magnitude of a primary isotope effect in a hydrogen-transfer reaction varies with the symmetry of the transition state and is maximal for a symmetrical reaction coordinate. This means that the generation of a primary carbon radical sets the limit on the magnitude, barring tunneling effects, of a primary isotope effect that can be observed for any cytochrome P-450 catalyzed reaction that involves initial attack at a carbon center. Thus, any cytochrome P-450 generated radical intermediate, of either higher or lower energy than a primary carbon radical, will lead to a smaller intrinsic primary deuterium isotope effect. The magnitude and invariance of the calculated primary isotope effect for the cytochrome P-450 catalyzed ω-hydroxylation of octane not only establishes hydrogen-atom abstraction as the initiating event in the reaction but implies that the porphyrin-$[FeO]^{3+}$-bound reactive oxygen atom must be triplet-like in nature and that its reactivity is conserved from isozyme to isozyme. The latter conclusion further implies that all cytochrome P-450 reactions can be expected to proceed by stepwise radical mechanisms. Indeed,

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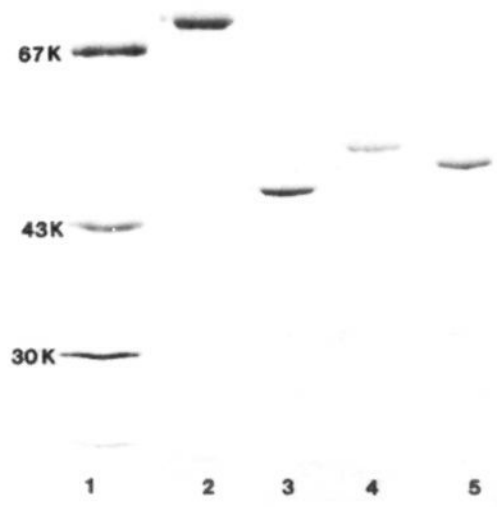

Figure 3. SDS-polyacrylamide gel, 10%, of purified enzyme preparations: (1) molecular weight standards, (2) cytochrome P-450 reductase (1.5 mg), (3) cytochrome P-450_{LM2} (1.0 mg), (4) cytochrome P-450_c (0.75 mg), (5) cytochrome P-450_b (1.0 mg).

this is exactly what has been found experimentally for a number of cytochrome P-450 catalyzed oxidative reactions in addition to aliphatic hydroxylation[3] where unambiguous data is available. Thus, singlet-like mechanisms, e.g. direct insertion across a carbon–hydrogen bond, need not be considered.

In summary, it may be concluded that in general cytochrome(s) P-450 behaves as a source of a triplet-like oxygen atom which dictates the mechanism by which the reaction will proceed while the regioselectivity and stereoselectivity for that reaction depends upon the specific isozyme of cytochrome P-450 used for catalysis. Work on other cytochrome P-450 catalyzed reactions (N-dealkylation, aromatic hydroxylation) in order to test this hypothesis is currently in progress.

Experimental Section

Materials. BSTFA and acetonitrile were obtained from Pierce Chemical Co., diethyl ether was obtained from J. T. Baker, and pentane was purchased from Burdick and Jackson. All other organic chemicals were purchased from Aldrich Chemical Co., while all biochemicals were obtained from Pharmacia or Whatman.

Instrumentation. GCMS analysis of the product alcohols was performed on a VG 7070H mass spectrometer in the selected ion recording mode, interfaced to an HP 5710A gas chromatograph fitted with a J & W capillary column. Difference spectra were determined with an HP 8451A UV spectrophotometer.

Synthesis. All selectively deuterated substrates, octane-*1,1,1-*$^2H_3$, octane-*1,8-*$^2H_2$, and octane-*1,1,8,8-*$^2H_4$, were synthesized according to published procedures.[4]

Enzyme Purification. Cytochrome P-450_b and cytochrome P-450 reductase (Figure 3) were purified as previously described[4] to yield specific contents of 13.0 nmol holoenzyme/mg of protein for the former enzyme and 10.5 nmol holoenzyme/mg of protein for the latter. Purification of P-450_c was performed in a fashion similar to that of Ryan et al.,[20] with minor modifications to the procedure as follows. Twenty male Long–Evans rats (60–70 g) were injected once ip with 3-methylcholanthrene (40 mg/kg) on three consecutive days and sacrificed on the fourth day. Microsomes were prepared by differential centrifugation and diluted to 10 mg/mL protein in 100 mM phosphate buffer, pH 7.25, 30% glycerol, 1 mM edetic acid (EDTA), 1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF) (buffer A) and solubilized by the addition of recrystallized sodium cholate to a final concentration of 2% w/v. An 8–15% polyethylene glycol 8000 precipitate was dissolved in 10 mM potassium phosphate buffer containing 20% glycerol, 0.1 mM EDTA, 0.1 mM DTT, 0.1 mM PMSF, 0.5% cholate, and 0.2% Lubrol PX (buffer B) and loaded onto a Whatman DE 52 column equilibrated with buffer B. The column was washed with 4 column volumes of equilibration buffer, then an 800-mL, 20–80 mM potassium chloride gradient was used to elute the P-450_c fraction. The fractions containing P-450_c were concentrated 20-fold with an Amicon filter, diluted with 20% glycerol, and applied to a fresh Whatman DE 53 column, which was equilibrated, washed, and eluted as above except that a gradient from 20 to 200 mM potassium chloride in a total volume of 2 L was employed. Fractions judged homogeneous on 10% SDS-polyacrylamide gels[21] were combined and dialyzed overnight against buffer containing 10 mM potassium phosphate, pH 6.5, 20% glycerol, 0.2% Lubrol PX, and 0.1 mM EDTA (buffer C). This sample was applied to a CM-Sepharose CL-6B column equilibrated with buffer C. The cation exchanger was washed extensively with equilibration buffer minus Lubrol PX, and P-450_c was eluted by increasing the phosphate concentration to 300 mM. Homogeneous P-450_c was obtained essentially free from detergent with a specific content of 13.9 nmol holoenzyme/mg protein (Figure 3). P-450_{LM2} was isolated from the hepatic microsomes of phenobarbital-induced rabbits by the method of Haugen and Coon,[22] with minor modifications. Briefly, the microsomes were solubilized as described above for P-450_c, and an 8–12% polyethylene glycol 8000 precipitation of cholate-solubilized microsomes was applied to a Whatman DE 52 column as described.[20] The unbound fraction containing P-450_{LM2} was loaded directly onto a hydroxyapatite column equilibrated with buffer C, adjusted to pH 7.4. The column was washed with equilibration buffer which had the phosphate concentration increased sequentially to 40, 90, and 150 mM. Essentially homogeneous P-450_{LM2} was eluted with the 90 mM wash. Minor contaminants were removed following adsorption of the protein to CM-Sepharose CL-6B after dialysis against buffer C. Bound P-450 was washed extensively with the equilibration buffer minus Lubrol PX (ca. 20 column volumes) and eluted in buffer which had the phosphate concentration increased to 200 mM. Homogeneous P-450_{LM2} was obtained with a specific content of 14.7 nmol holoenzyme/mg protein (Figure 3).

Reaction Conditions. For reactions catalyzed by P-450_b and P-450_{LM2}, 1 nmol of purified P-450, 1 nmol of P-450 reductase, 0.6 μmol of NADPH, 0.5 nmol of phosphatidylcholine, and 2.4 nmol of substrate in a final volume of 2 mL at pH 8.2 were incubated for 10 min as previously described.[4] For reactions catalyzed by P-450_c, P-450 reductase, hemoprotein, and dilaurylphosphatidylcholine concentrations were doubled.

Analysis. The relative amounts of 1- and 2-octanol formed by each enzyme preparation were determined by capillary gas chromatographic/mass spectral analysis of the $(M - CH_3)^+$ fragment of the trimethylsilyl ether derivatives of each of the alcohols. Ion intensity ratios of the $(M - CH_3)^+$ fragments were converted to relative amounts of the two products by comparison to a standard curve generated with authentic metabolites. Isotope effect determinations were also performed with capillary gas chromatography/mass spectrometry as published previously.[4,5] Controls were run to determine the product profile obtained from octane in the presence of each of the three isozymes studied. Since an intramolecular design was used for the experiments, i.e. single substrate, there are only two potential products which could interfere with the isotope effect determination. These are octanal and octanoic acid. Since these two products can only arise as a consequence of further oxidation of the primary alcohol, we examined the product profile for each isozyme after incubation with 0.1 mM 1-octanol and analysis by gas chromatography. When 1-octanol was incubated with P-450_b, 5% was converted to octanal and a trace of octanoic acid. With P-450_{LM2} and P-450_c the yields of octanal were 7% and 0%, respectively, while those of octanoic acid were about 2% and 0%. Since 1-octanol is only a minor product of octane metabolism by all three isozymes, its conversion to octanol in the presence of a huge excess of substrate (octane) would be trivial at best and thus could not introduce significant error into the determination of the isotope effect.

MNDO Calculations. MNDO calculations were carried out on ethane and ethyl radical (models for octane and primary octyl radical, respectively) using MOPAC (4th ed.), a package of semiempirical programs available through the Quantum Chemical Program Exchange, Department of Chemistry, Indiana University,

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Bloomington, IN, 47405. The calculations were preformed with UHF wave functions and the geometries were optimized by using a combination of the Broyden–Fletcher–Goldfarb–Shanno optimizer and Bartel's method. The mass-weighted Hessian matrix was used to calculate the vibrational frequencies. All $3n - 6$ vibrational frequencies were used to calculate the equilibrium isotope effects.[12,15]

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Registry No. P-450, 9035-51-2; $^2H_2$, 7782-39-0; ω-hydroxylase, 9059-16-9; octane, 111-65-9.

The Synthesis and Antibacterial Activities of Quinolones Containing Five- and Six-Membered Heterocyclic Substituents at the 7-Position

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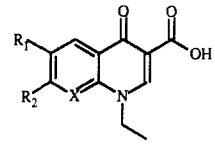
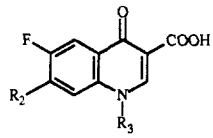
A series of 6-fluoro-7-substituted-1-ethyl-1,4-dihydro-4-oxoquinoline-3-carboxylic acids were prepared. The substituents at the 7-position included five- and six-membered heterocyclic rings such as oxazoline and oxazine as well as five-membered heteroaromatic rings such as oxazoles and imidazoles. The structure–activity relationships (SAR) of these compounds indicated that oxazole substituent containing a 2-methyl group had the greatest in vitro potency. The compounds showed greater in vitro antibacterial activity against Gram-positive organisms than against Gram-negative organisms.

Oxolinic acid (**1a**) and nalidixic acid (**1b**) are members of the quinolone class of orally active antibacterial agents.[1]



1a R_1,R_2=OCH_2O X=CH
1b R_1=H R_2=CH_3 X=N
1c R_1=H R_2=4-pyridyl X=CH

Among the members of this class of compounds is rosoxacin (**1c**), which contains a heteroaromatic substituent attached to the 7-position via a carbon–carbon bond.[2] This compound has been shown to have good oral antibacterial activity in animals.[3] The antibacterial activity of the quinolones against Gram-positive organisms was increased when a fluorine atom was introduced into the 6-position, leading to compounds such as norfloxacin (**1d**)[4a]



1d R_2=1-piperazinyl R_3=CH_2CH_3
1e R_2=1-piperazinyl R_3=cyclopropyl
1f R_2=4-methyl-1-piperazinyl R_3=CH_2CH_3
1g R_2=4-methyl-1-piperazinyl R_3=4-fluorophenyl

and more recently ciprofloxacin (**1e**),[4b] perfloxacin (**1f**),[4c] and difloxacin (**1g**).[4d] Besides rosoxacin, few compounds have been reported which contain substituents attached to the 7-position of the quinolone system via a carbon–carbon linkage.[5] Most of these compounds had weak antibacterial activity. Recently, Uno et al.[6] and Culbertson et al.[7] have reported on the synthesis and biological evaluation of some 7-(azole substituted)-quinolones and Nishimura and Matsumoto[8] have reported on the preparation and evaluation of some 7-(4-pyridyl)-1,8-naphthyridine-3-carboxylic acid antibacterial agents. These papers have prompted us to report on our investigations of replacing the traditional nitrogen atom at the 7-position of the quinolone ring system with a substituent attached through a carbon–carbon bond. This series of compounds containing heterocyclic rings attached via such a carbon–carbon linkage was used to probe the importance of the nitrogen at the 7-position for antibacterial activity. In this paper we wish to report on the synthesis and biological evaluation of the antibacterial activity of a series of 6-fluoro-1-ethylquinolones which contain five- and six-membered heterocyclic or heteroaromatic substituents at the 7-position having a C–C covalent bond.

Chemistry

All of the quinolone derivatives in this study were synthesized from a common intermediate, **9**. This intermediate was prepared by the synthetic route outlined in Scheme I. The commercially available 2-fluoroacetophenone (**2**) was nitrated with fuming HNO_3 and concentrated H_2SO_4 to give **3** in 79% yield. The ketone was protected by ketalization with an appropriate diol (the choice of diol for the protecting group will be discussed later). Nitroketal **4** was reduced with Raney nickel under a hydrogen atmosphere to give amine **5**. Condensation of **5** with diethyl (ethoxymethylene)malonate (EMME) gave enamine **6**. The quinolone ring system was obtained by thermal cyclization of **6** in Dowtherm A. When ethylene glycol was used for the protection of ketone **4** (R = H), a 50:50 mixture of **7** and **7a** was obtained during the cycli-

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