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Intramolecular Determination of Substituent Effects in Hydroxylations Catalyzed by Cytochrome P-450

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SUMMARY

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Hydroxylation of monosubstituted 1,3-diphenylpropanes by rat liver microsomes leads predominantly to a mixture of the isomeric benzylic alcohols at positions 1 and 3. Quantitation of the relative concentrations of these alcohols can be achieved by combined gas chromatography-mass fragmentography. *p*-Methyl, *p*-trifluoromethyl, and *p*-fluoro substituents yield product distributions which clearly indicate an electrophilic mechanism for the hydroxylation reaction.

INTRODUCTION

Oxidative metabolism of hydrocarbons mediated by cytochrome P-450 is commonly thought to involve an electrophilic species of enzymatically activated oxygen (1). Although the exact nature of the oxidizing species is not well understood, several studies of various types of hydroxylation reactions have provided strong evidence for an electrophilic mechanism. Aliphatic hydroxylation is known to proceed with substantial retention of configuration for a variety of substrates (2-4), and aromatic hydroxylation of monosubstituted benzenes yields mixtures of product phenols with low ratios of *meta* to *para* isomers (5-7). Both retention of configuration in aliphatic hydroxylation and low values of *meta* to *para* ratios in aromatic hydroxylation are considered classical features of electrophilic reaction mechanisms (8).

It would seem natural as a consequence of these studies to investigate substituent effects on the rates of aromatic side chain oxidations catalyzed by P-450. Only one such study has appeared, however, and the conclusion drawn by the author (9) was that the K_m and V_{max} of the reactions investigated had little or no dependence on electronic differences in the substituents. More recent studies on the kinetics of substrate oxidation by P-450 suggest an explanation for the observed lack of substituent effects. Although some controversy exists as to which individual step in the over-all reaction cycle of P-450 is rate-determining, or whether a single rate-determining step actually exists, it is now clear that the interaction of the activated oxygen species with the substrate is often fast relative to the prior events of substrate binding and cytochrome reduction (10-12). With this observation in mind, it is apparent that substituents might significantly alter the rate of the individual step of substrate oxi-

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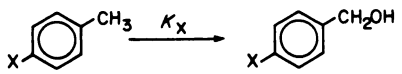
dation without substantially affecting the over-all enzymatic velocity.

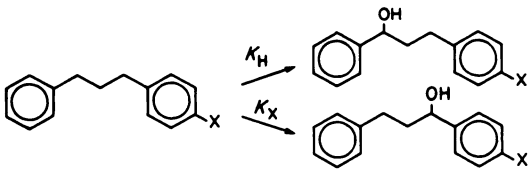
A conventional study of substituent effects in P-450-catalyzed hydroxylations might involve measuring the rate of the following reaction for several substrates with different substituents, e.g.,



In addition to the previously mentioned possibility that the over-all enzymatic velocity might not reflect differences in the rate of the hydroxylation step, substituents are likely to change the affinity of each of the substrates for the enzyme. The common notion that lipid solubility is functionally related to the apparent K_m of oxidations catalyzed by P-450 may be an expression of this effect (9).

One direct method for examining the effect of substituents on the individual step of substrate oxidation is intramolecular competition for hydroxylation sites. For this kind of experiment, an analysis of product distribution replaces the measurement of enzymatic velocities as the primary observation. The ideal substrate for such a study has two sites which have very similar steric properties but different electronic environments. These two sites then compete for the active oxygen intermediate to produce isomeric alcohols. Since only one molecule is involved, differences in binding due to different substituents do not influence the final results.

We have designed a series of substituted 1,3-diphenylpropanes which meet the requirements of substrates to be used for intramolecular competition experiments. The reactions studied are



The ratio of the individual rates, k_H/k_X, is directly given by the ratio of the product alcohols (13), and by measuring such ratios for several substituents, a direct determination of the substituent effect can be achieved without measuring enzymatic velocities. After considering several factors, such as steric bulk, side reactions, and possible substrate ligation to the iron of the P-450 heme group, the trifluoromethyl, methyl, and fluoro groups were chosen as reasonable substituents. This paper presents our initial results with substituted 1,3-diphenylpropanes and liver microsomes as a system for examining substituent effects in P-450-catalyzed hydroxylations.

MATERIALS AND METHODS

Microsomes. The liver microsomal fraction from adult male Sprague-Dawley rats fasted for 12 hr prior to decapitation was isolated according to Mazel (14). Pellets were washed once in 50 mM phosphate buffer, pH 7.4, reisolated, and stored in stoppered plastic vials layered with 2 ml of the same buffer at −20°. Protein was determined by a modified Lowry method (15).

Standard assay and incubation conditions. Assay media contained the following in a total volume of 4.7 ml: 50 mM phosphate buffer (pH 7.4), 0.30 mM NADP (Sigma), 2 units of Sigma type V glucose 6-phosphate dehydrogenase, 8 mM glucose 6-phosphate (Sigma), 5 mM $MgCl_2$, 0.002% (by weight) Tween 80, and 1 mM substrate unless otherwise noted. The final protein concentration was 1 mg/ml. Reactions were carried out in 25-ml Erlenmeyer flasks, and the assay media were incubated for 5 min at 37° prior to the addition of microsomes to start the reaction. After incubation in a Dubnoff shaking incubator with air as the gas phase for 15 min, the reaction was stopped by the addition of 1 ml of 0.25 M zinc sulfate and the resulting suspension was cooled on ice. The contents of each flask were then transferred to 15-ml glass-stoppered centrifuge tubes, and 2 ml of benzene were added. Extractions were carried out with a rocking mechanical extractor for 20 min, after which the contents of each tube were centrifuged, and the benzene layer was drawn off and placed in 4-ml glass vials. The benzene extract was then taken to dryness with a stream of dry nitrogen in a heated sand

bath, and the residue was redissolved in 0.1 ml of benzene for chromatography.

Gas chromatography. Routine chromatograms were run at 150° on a Varian 200 gas chromatograph equipped with flame ionization detectors. A 2 m × 2 mm (inner diameter) glass column packed with 3% OV-225 on 100–120 mesh Gas-Chrom Q (Applied Science) was used with a flow rate of 30 ml/min. Peak areas for kinetic experiments were quantitated with an Omni Scribe recorder equipped with an electronic integrator (Houston Instruments) against *o*-phenethylbenzyl alcohol (Aldrich) as an internal standard.

Mass fragmentography. Mass fragmentograms were measured on a Finnigan 3300 mass spectrometer at a source current of 700 μamp and accelerating voltage of 70 eV. A 1 m × 2 mm (inner diameter) glass column packed with 3% OV-225 on 100–120 mesh Gas-Chrom Q was used with a flow rate of 15 ml/min. Mass fragmentograms were run at a column temperature of 160° for the fluoro- and trifluoromethyl-substituted compounds and 180° for the methyl-substituted compounds.

The ratios of alcohols formed by enzymatic hydroxylation at either position 1 or 3 of the substituted 1,3-diphenylpropanes were quantitated by measuring the relative intensities of their respective α cleavage products in the mass fragmentogram. Figure 1, for example, shows the two isomeric alcohols formed by benzylic hydroxylation of 1-(4-methylphenyl)-3-phenylpropane, as well as the ions formed by α cleavage of these isomeric alcohols. The latter were the ion species used to quantitate products.

Calibration curves were obtained by measuring the ratio of the two α cleavage ions in a series of analytical standards where the mole fraction of the isomeric alcohols was systematically varied. For each substituent, standards were prepared which spanned the range of the expected mole fraction of alcohols produced by the enzyme. Figure 2 gives a sample calibration curve for the isomeric benzylic alcohols of 1-(4-methylphenyl)-3-phenylpropane, shown in Fig. 1. The ratio of 121/107 is seen to be a linear function of the mole fraction of the alcohols. Appropriate calibrations were run with each experiment.

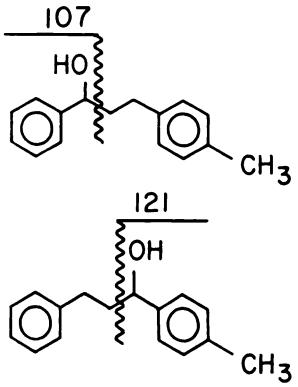


FIG. 1. *Structures of isomeric benzylic alcohols formed by hydroxylation of 1-(4-methyl)-3-phenylpropane*

The α cleavage fragment used to quantitate these alcohols by mass fragmentography is indicated for each isomer and in each case was the base peak of the mass spectrum.

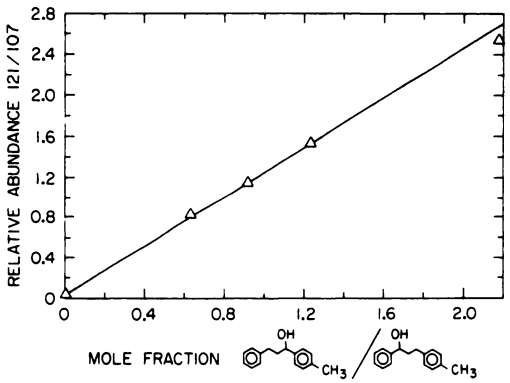


FIG. 2. *Calibration curve relating mole fraction of isomeric alcohols to relative abundance of their α cleavage fragments*

Synthetic standards were prepared and mixed in the relative concentrations indicated in the figure. The relative abundance of the α cleavage products was then determined by mass fragmentography to obtain a calibration curve.

Synthesis of substrates. Substrates were synthesized according to the scheme indicated in Fig. 3. The appropriate phenylmagnesium bromides were allowed to react with hydrocinnamaldehyde, and the resulting alcohols were dehydrated to the styrene derivatives, which were in turn reduced to the hydrocarbon with 10% pal-

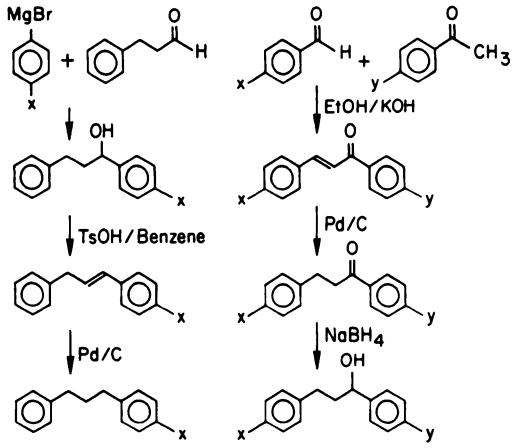


FIG. 3. *Synthetic pathways used to prepare substrates and analytical standards*

Substrates were prepared as described in MATERIALS AND METHODS and shown on the left-hand side of the figure. The synthesis of the analytical standards is indicated on the right-hand side.

ladium on carbon in an atmospheric hydrogenation unit. Distillation afforded substituted 1,3-diphenylpropanes which were colorless liquids and showed in each case one spot by thin-layer chromatography on silica gel GF with benzene and no impurities by gas chromatography on OV-225; average yield, 25%.

$C_{15}H_{16}$ (X = H)

Calculated: C 91.78, H 8.22
Found: C 91.52, H 8.25

$C_{15}H_{15}F$ (X = F)

Calculated: C 84.08, H 7.06
Found: C 83.87, H 7.14

$C_{16}H_{15}F_3$ ($X = CF_3$)

Calculated: C 72.71, H 5.72
Found: C 72.62, H 5.83

$C_{16}H_{18}$ ($X = CH_3$)

Calculated: C 91.37, H 8.63
Found: C 90.99, H 8.75

Synthesis of analytical standards. Analytical standards were synthesized according to the scheme indicated in Fig. 3. The appropriate *para*-substituted benzaldehydes and *para*-substituted acetophenones were condensed to give crystalline chalcone derivatives. These were reduced to the crystalline ketones with palladium on carbon, and the ketones were subsequently reduced to the standard alcohols with $NaBH_4$ in ethanol. Chromatography on OV-225 showed each alcohol to be free of both ketone and chalcone precursors.

RESULTS AND DISCUSSION

The time course for hydroxylation of the parent compound, 1,3-diphenylpropane, is given in Fig. 4. The reaction is linear for at least 15 min, with a specific activity of 2 nmoles of product per milligram of protein per minute, which is a typical value for microsomal hydroxylations (16).

For the results of the competition experiment to be meaningful, it was necessary to show that there was no interconversion of the enzymatically formed alcohols subsequent to their formation. If equilibration had occurred, the ratio of alcohols would reflect only their relative thermodynamic stability and not the kinetics of the hydroxylation reaction. Since all of the benzylic alcohols and ketones of the trifluoromethyl substrate were readily separable on OV-225, these compounds were used for the major control experiments. Each compound in Fig. 5 was separately incubated with our standard microsomal system at a concentration of 1 mM. The products of each reaction were then examined for each of the four other compounds by gas chromatography. The parent compound gave rise to both alcohols and small amounts of

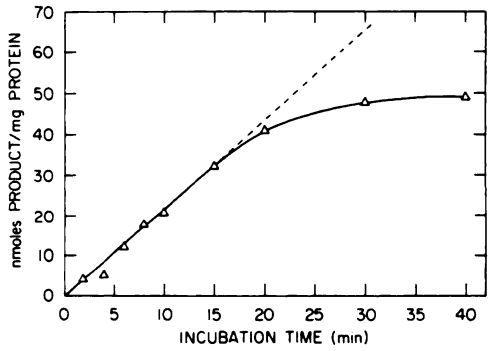


FIG. 4. *Time course of hydroxylation of 1,3-diphenylpropane by rat liver microsomes*

Points are single determinations quantitated by gas chromatography against *o*-phenethylbenzyl alcohol as an internal standard as described in MATERIALS AND METHODS.

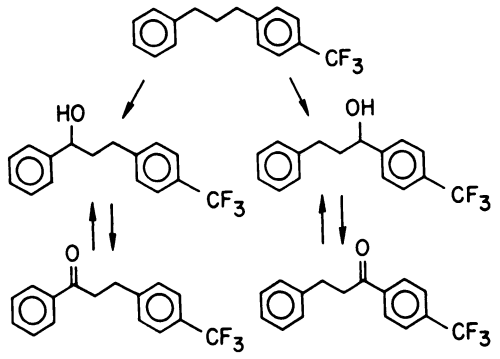


FIG. 5. *Metabolic pathways of 1-(4-trifluoromethylphenyl)-3-phenylpropane derivatives in rat liver microsomes*

Each of the five compounds indicated in the figure was separately incubated with rat liver microsomes as described in MATERIALS AND METHODS. Arrows indicate the reactions which we could detect by gas chromatography. In no case was interconversion of isomeric ketones or alcohols detected.

both ketones. Incubation of the alcohols did not lead to isomerization of either alcohol, nor was any back-reaction to the parent compound apparent. No dehydration of benzylic alcohols to the corresponding styrenes was observed. Many of the styrenes were available as synthetic precursors (Fig. 3) and were readily separated from all other compounds under the standard chromatographic conditions. Very minor (less than 5%) reduction of ketone to the corresponding alcohol or oxidation of alcohol to ketone was observed. It was concluded that the metabolically formed alcoholic products were not interconverted in this system.

To rule out possible complications arising from hydroxylation at the methyl substituent in 1-(4-methylphenyl)-3-phenylpropane, we synthesized the potential hydroxylation product. This compound, 4-(3-phenylpropyl)benzyl alcohol, was well separated from the two other isomeric benzylic alcohols by gas chromatography on OV-225 and therefore did not interfere with the measurement of the relative concentrations of the desired compounds by mass fragmentography. Other potential metabolites, such as ring-hydroxylated products, were not extracted by benzene and did not interfere with the assay procedures.

Table 1 presents the results of our competition experiments. The substrates for these experiments were designed so that pairs of compounds which were nearly isosteric could be examined. This was necessary since no information is currently available which describes the binding of these molecules to P-450,[3] and the possibility must therefore exist that steric factors play some role in the orientation of hydroxylation. These effects should be minimized, however, by examining the difference in the orientation of the hydroxylation reaction between substrates with substituents which are nearly isosteric but have different electronic effects. Both the fluorine-hydrogen pair and the methyl-trifluoromethyl pair fulfill these requirements as nearly as possible among the commonly available substituents used for this type of study.

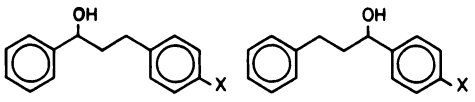
In examining the difference between the substituent effect of hydrogen vs. fluorine, or methyl vs. trifluoromethyl, it is apparent that hydroxylation is oriented away from electron-withdrawing groups and that this effect increases with the strength of the substituent effect. (CF_3 is a stronger electron-withdrawing group relative to CH_3 than F is relative to H.) This is clearly a demonstration of the electrophilic nature of hydroxylations catalyzed by P-450 and complements the previous studies on stereochemistry and selectivity. That CH_3, which possesses electron-donating properties, did not show any effect relative to hydrogen is probably best interpreted as an approximate balance of opposed steric and electronic effects. A substrate bearing a methyl group on one ring and a trifluoromethyl group on the other might help to resolve the relative importance of these two effects. We predict that hydroxylation would be favored at the benzylic position adjacent to the methyl-substituted benzene rather than at the benzylic position adjacent to the trifluoromethyl-substituted ring. The magnitude of the substituent effect in terms of a conventional ρ value is not obtainable, however, because of our limited data and the possible in-

[3] We have obtained difference spectra which indicate that these substrates all exhibit type I binding.

TABLE 1

Competitive hydroxylation of substituted diphenylpropanes by rat liver microsomes

The relative proportions of the alcoholic products were determined by mass fragmentography as described in MATERIALS AND METHODS.

Substituent	σ[a]	Products formed[b]		Ions measured
				
		%	%	
—F	0.06	70 ± 1	30	107, 125
—CH_3	−0.17	50 ± 1	50	107, 121
—CF_2	0.54	95 ± 3	5	107, 175

[a] σ constants for *para* substituents were obtained from ref. 8, p. 30.

[b] Percentage of total hydroxylated products at positions 1 and 3 of substituted diphenylpropanes.

volvement of multiple P-450s in these reactions. It is perhaps fair to conclude that the observed effects are lower than those which we should expect of reactions having fully developed carbonium ion character in the transition state. Nevertheless, the data presented in this paper demonstrate the importance of electronic substituent effects in hydroxylation reactions catalyzed by cytochrome P-450.

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