# Substrate Specificity for the Epoxidation of Terpenoids and Active Site Topology of House Fly Cytochrome P450 6A1

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Heterologous expression in *Escherichia coli*, purification, and reconstitution of house fly P450 6A1 and NADPH-cytochrome P450 reductase were used to study the metabolism of terpenoids. In addition to the epoxidation of cyclodiene insecticides demonstrated previously [Andersen et al. (1994) *Biochemistry* **33**, 2171−2177], this cytochrome P450 was shown to epoxidize a variety of terpenoids such as farnesyl, geranyl, and neryl methyl esters, juvenile hormones I and III, and farnesal but not farnesol or farnesoic acid. P450 6A1 reconstituted with NADPH-cytochrome P450 reductase and phosphatidylcholine did not metabolize α-pinene, limonene, or the insect growth regulators hydroprene and methoprene. The four geometric isomers of methyl farnesoate were metabolized predominantly to the 10,11-epoxides, but also to the 6,7-epoxides and to the diepoxides. The 10,11-epoxide of methyl (2*E*,6*E*)-farnesoate was produced in a 3:1 ratio of the (10*S*) and (10*R*) enantiomers. Monoepoxides of methyl farnesoate were metabolized efficiently to the diepoxides. Methyl farnesoate epoxidation was strongly inhibited by a bulky substituted imidazole. The active site topology of P450 6A1 was studied by the reaction of the enzyme with phenyldiazene to form a phenyl−iron complex. Ferricyanide-induced *in situ* migration of the phenyl group showed formation of the *N*-phenylprotoporphyrinporphyrin IX adducts in a 17:25:33:24 ratio of the $N_B$:$N_A$:$N_C$:$N_D$ isomers. These experiments suggest that metabolism of xenobiotics by this P450, constitutively overexpressed in insecticide-resistant strains of the house fly, is not severely limited by stereochemically constrained access to the active site.

## Introduction

*CYP6A1* is a cytochrome P450 (P450)[1] gene that is constitutively overexpressed in some insecticide-resistant strains of the house fly, *Musca domestica* (*1*, *2*). When produced in *Escherichia coli* and reconstituted with NADPH-cytochrome P450 reductase, CYP6A1 (P450 6A1) carries out the epoxidation of the cyclodiene insecticides aldrin and heptachlor (*3*). We felt that a more extensive study of the substrate specificity of P450 6A1 would contribute to our understanding of insecticide resistance and metabolic detoxification in insects and extend our understanding of P450 substrate specificity in general.

Insecticide-resistant strains which overexpress the *CYP6A1* gene are cross-resistant to synthetic structural analogs of juvenile hormone (*4*), some of which are commercially produced as insect growth regulators. Furthermore, several studies have reported the oxidative metabolism of juvenile hormone I [methyl (2*E*,6*E*,10*Z*)-10,11-epoxy-7-ethyl-3,11-dimethyl-2,6-tridecadienoate] and of insect growth regulators in insecticide-resistant strains of the house fly, in addition to the well-described hydrolytic pathways involving juvenile hormone esterase and epoxide hydrolase (*5*). It was therefore possible that P450 6A1 would metabolize terpenoid compounds related to juvenile hormone (Figure 1). Interest in these types of compounds, long considered to be highly selective to insects, has increased following the reports that insect juvenile hormone III and farnesol activate the heterodimeric complex between the retinoid X receptor and an orphan receptor (FXR) from mammals (*6*) and that the juvenile hormone analog methoprene and its metabolite methoprene acid specifically bind and activate the retinoid X receptor (*7*).

We have used a reconstituted system of P450 6A1, NADPH-cytochrome P450 reductase and phospholipid to characterize the regio- and stereoselectivity of the epoxidation reactions of a number of terpenoids. We show here that P450 6A1 metabolizes juvenile hormones I and III to their diepoxides. The sequential epoxidations of methyl farnesoate and related compounds by P450 6A1 have also allowed us to assess the features of the substrate binding site of this enzyme by examining the amount and type of products formed as a function of chain length, geometric isomerism, and substitution pattern. The regio- and stereochemistries of olefin epoxidation observed in this study indicate that the vicinity of the heme active site is quite spacious and can accommodate a variety of substrate geometries and orientations.

## Experimental Procedures

**Enzyme Preparation.** P450 6A1 was expressed in *E. coli* as described previously (*3*) and purified from the membrane fraction by a combination of hydrophobic interaction chromatography on ω-aminooctyl agarose and chromatography on

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[1] Abbreviations: P450, cytochrome P450; P450 6A1, CYP6A1; BSTFA, *N*,*O*-*bis*(trimethylsilyl)trifluoroacetamide; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DTT, dithiothreitol; EI-MS, electron impact mass spectrometry; MOPS, 4-morpholinepropanesulfonic acid; PCR, polymerase chain reaction; PMSF, *p*-phenylmethanesulfonyl fluoride.

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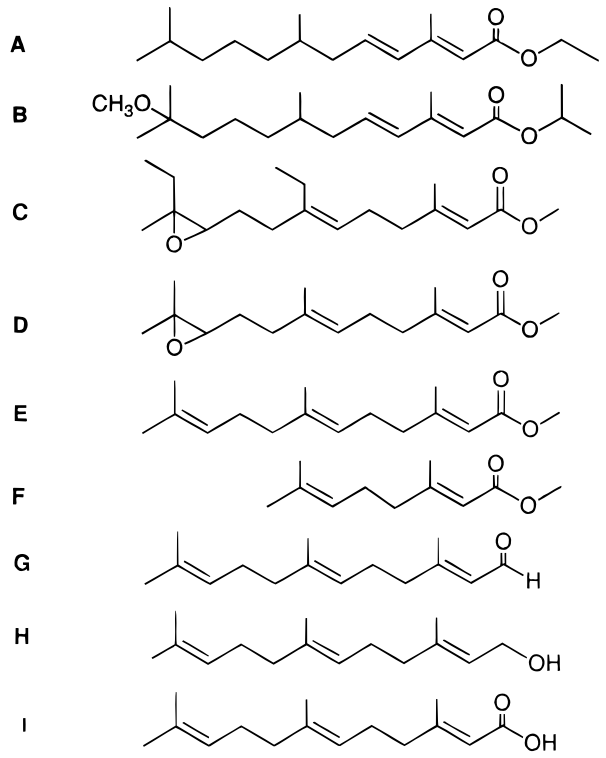


**Figure 1.** Terpenoid substrates tested as substrates with P450 6A1: (A) hydroprene; (B) methoprene; (C) juvenile hormone I; (D) juvenile hormone III; (E) methyl (2*E*,6*E*)-farnesoate; (F) methyl geranate; (G) (2*E*,6*E*)-farnesal; (H) (2*E*,6*E*)-farnesol; (I) (2*E*,6*E*)-farnesoic acid.

DEAE-Sepharose. *E. coli* (pI-13C) cells were harvested, lysed by sonication, and fractionated by centrifugation as described previously. P450 6A1 was solubilized from membranes by stirring with 0.5% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and the suspension was centrifuged at 100000*g*. The supernatant was loaded onto an $\omega$-aminooctyl agarose column equilibrated with 100 mM sodium phosphate buffer (pH 7.4) containing 0.2 mM dithiothreitol (DTT), 0.4% sodium cholate, and 20% glycerol (buffer A). The column was washed with buffer A, and P450 6A1 was eluted with buffer B [10 mM sodium phosphate buffer (pH 7.4), 0.2 mM DTT, 0.4% sodium cholate, 0.2% Emulgen 911, 20% glycerol]. The reddish fractions were pooled and loaded onto a 20-mL column of DEAE-Sepharose equilibrated with buffer C [10 mM Tris-HCl buffer (pH 7.6), 0.2 mM DTT, 0.1% Emulgen 911], eluted with a gradient of buffer C containing 0−0.3 M NaCl, and then dialyzed against buffer C. Detergents were removed prior to use by applying the purified P450 to a small DEAE-Sepharose column, washing with buffer D [10 mM Tris-HCl buffer (pH 7.6), 0.2 mM DTT, 20% glycerol] until the absorbance of the eluent at 280 nm was equal to that of the wash buffer, and eluting with buffer D containing 0.3 M NaCl. The specific content of P450 in these preparations was 12 nmol/mg of protein.

House fly NADPH-cytochrome P450 reductase (*8*) was also expressed in *E. coli* and purified by a combination of chromatography on DEAE-Sepharose followed by chromatography on phenyl-Sepharose. Membranes were collected from *E. coli* (pI-95), and proteins were solubilized with CHAPS as described previously (*3*). The solubilized material was loaded onto a DEAE-Sepharose column equilibrated with buffer E [25 mM Tris-HCl buffer (pH 7.4) containing 0.5 mM DTT, 0.1% Emulgen 911, 10% glycerol]. The column was washed with buffer A and eluted with a gradient of 0−0.4 M NaCl in buffer A. The yellow fractions were pooled, diluted 2-fold with buffer F [20 mM Tris-HCl buffer (pH 7.6), 0.5 mM DTT, 1.0 mM EDTA, 10% glycerol], and loaded onto a column of phenyl-Sepharose equilibrated with buffer F. The column was washed with buffer F and eluted with a gradient from 0% detergent to 0.8% cholate and 0.15% Emulgen 911 in buffer F. Detergents were removed from the purified material by chromatography on a small column of DEAE-Sepharose as described above for P450 6A1.

Cytochrome P450 was quantified by measurement of its dithionite-reduced vs reduced-CO bound difference spectrum by the method of Omura and Sato (*9*). Spectra were taken at 4 °C on a Perkin-Elmer Lambda 19 UV−visible spectrophotometer. Previous studies (*3*) have shown that the reduced CO complex of P450 6A1 is labile at higher temperatures. Reductase activity was quantified by measurement of the reduction of cytochrome *c* as described previously. Ligand binding was quantified by measurement of type I or type II difference spectra (for the ligands with an imidazole moiety) of oxidized cytochrome P450. The spectra were measured in 100 mM 4-morpholinepropanesulfonic acid (MOPS) buffer (pH 7.4) at 25 °C, and the ligands were added in ethanol. An equivalent amount of ethanol was added to the reference cuvette, and the maximal amount of ethanol did not exceed 1% of the total volume.

**Preparation of Substrates—Methyl (2*E*,6*E*)-Farnesoate, Methyl (2*Z*,6*E*)-Farnesoate, Methyl (2*E*,6*Z*)-Farnesoate and Methyl (2*Z*,6*Z*)-Farnesoate.** These compounds were synthesized via the Wittig reaction (*10*). Trimethyl phosphonoacetate (4.7 g) was added dropwise to 12 mL of dry dimethylformamide containing 1.5 g of NaH (60%) and stirred at room temperature for 30 min. *trans*-Geranylacetone (5 g) in 12 mL of dry dimethylformamide was addded dropwise and the resultant mixture was stirred at room temperature for 18 h. Water (200 mL) was added, and the aqueous mixture was extracted with hexane (2 × 100 mL). The organic extract was washed with water and brine and dried over anhydrous $Na_2SO_4$. Evaporation of the solvent yielded 3.1 g of a mixture of methyl (2*E*,6*E*)-farnesoate (42.5%) and methyl (2*Z*,6*E*)-farnesoate (22.5%) as indicated by gas chromatographic comparison with authentic standards. After chromatography on 5% water-deactivated Florisil (5% diethyl ether in hexane), the geometric isomers were separated by centrifugal planar chromatography on a Chromatotron apparatus using a 2-mm silica gel layer eluted with 2% diethyl ether in hexane at a flow rate of 6 mL/min. The identities of the products were determined by GC/MS. Methyl (2*E*,6*Z*)-farnesoate and methyl (2*Z*,6*Z* )-farnesoate were prepared in an identical manner using *cis*-geranylacetone in place of *trans*-geranylacetone. The yield in this case was 4.3 g of a mixture containing 52 (2*Z*,6*Z*) and 26% (2*Z*,6*E*). Each of the separated isomers was 97−99% pure as measured by GC/MS, on a Hewlett-Packard 5890 gas chromatograph containing a 15 m × 0.3 mm i.d. HP-1 fused silica column, coupled with a Hewlett-Packard 5970 mass-selective detector. The standard temperature program was a 1 min hold at 100 °C followed by an increase of 10 °C/ min to 250 °C. Under our GC conditions, the retention times ($t_R$) of the methyl farnesoates were 10.13, 9.85, 9.64, and 9.29 min, respectively, for the (2*E*,6*E*), (2*E*,6*Z*), (2*Z*,6*E*), and (2*Z*,6*Z*) isomers.The olefinic methyl esters were stored as neat liquids under nitrogen at −20 °C.

**Methyl Geranate and Methyl Nerate.** Active $MnO_2$ (10g) was added to 1.0 g of citral (65% geranial, 35% neral) in 125 mL of hexane and stirred at 0 °C for 30 min. The mixture was filtered to remove $MnO_2$, and the hexane evaporated. Acetic acid (0.3 g), 0.8 g of NaCN, and 10 g of $MnO_2$ in methanol were added to the residue, and the mixture was stirred at room temperature for 4 h. The mixture was filtered to remove $MnO_2$, and the methanol was evaporated. The residue was dissolved in ethyl ether and washed with water and brine to give 0.57 g of a 65:35 mixture of geranyl methyl ester and neryl methyl ester. The mixture was purified by flash chromatography on silica (10% ether in hexane), and the products were identified by GC/MS. Retention times for methyl geranate and methyl nerate were 4.43 and 3.85 min, respectively, under the conditions described above.

**Mono- and Diepoxides of Methyl Farnesoate.** The epoxides were synthesized by *m*-chloroperoxybenzoic acid oxidation (*11*, *12*). A 1.2 molar excess of *m*-chloroperoxybenzoic acid was added to 75 mg each of methyl (2*E*,6*E*)- and (2*Z*,6*E*)-farnesoate in 4 mL of methylene chloride. The reaction was stirred on ice for 30 min and stopped by adding 8 mL of 5%

aqueous sodium bicarbonate. The organic phase was collected, washed with water, and dried over anhydrous sodium sulfate. Silica gel thin-layer chromatography developed with benzene/ethyl acetate (85:15) showed the presence of products corresponding to unreacted methyl farnesoate, its 10,11-epoxide, its 6,7-epoxide, and its 6,7,10,11-diepoxide. The epoxides were preparatively separated by silica gel TLC using the same solvent system, and the products were eluted from silica gel with ethyl acetate. The purities of these isolated substrates, measured by GC/MS, were as follows: methyl (2*Z*)-6,7-epoxyfarnesoate 95% ($t_R$ 10.79 min); methyl (2*Z*)-10,11,-epoxyfarnesoate 99% ($t_R$ 10.74 min); methyl (2*E*)-6,7-epoxyfarnesoate 85% ($t_R$ 11.28 min); methyl (2*E*)-10,11-epoxyfarnesoate 96% ($t_R$ 11.17 min). The epoxides were stored in ethanol at −20 °C.

**Determination of the Absolute Configuration of 10,11-Epoxides of Methyl (2*E*,6*E*)-Farnesoate.** Optically pure juvenile hormone III [methyl (2*E*,6*E* )(10*R* )-10,11-epoxyfarnesoate] was prepared by incubating excised corpora allata from 5-day-old adult mated female *Diploptera punctata* in TC199 medium containing 50 $\mu$M methionine (*13*). Thirty pairs of corpora allata were incubated at 30 °C for 24 h. The incubation medium was then extracted with five volumes of isooctane. The isooctane extract was dried over anhydrous $Na_2SO_4$ and concentrated under a stream of nitrogen. Juvenile hormone III was identified by comparison of its mass spectrum and retention time with an authentic sample of racemic hormone.

Derivatives of the racemic 10,11-epoxide of methyl (2*E*,6*E*)-farnesoate, of the reaction product with P450 6A1, or of the product of cultured corpora allata were prepared as follows. The epoxides were first converted to the methoxyhydrin by acid methanolysis (*14*). The conversion to methoxyhydrin was quantitative as indicated by GC/MS (EI-MS *m*/*z* 266, 225, 193, 135, 73). The methoxyhydrins were reacted with (*S* )-(+)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride in pyridine at 60 °C for 1 h. The diastereomeric esters were then analyzed by capillary gas chromatography on a 15 m × 0.32 mm i.d. DB-5 fused silica capillary column. The temperature program was a 1-min hold at 170 °C followed by an increase of 5 °C/ min to 300 °C. The identity of the esters ($t_R$ 21.80 and 21.91 min) was verified by GC/MS (70 eV EI-MS *m*/*z* 248, 189, 135, 73).

**Enzymatic Reactions.** Enzymatic assays were performed in siliconized glass tubes containing 10 $\mu$g of dilaurylphosphatidylcholine, 1.2 mM $NADP^+$, 100 mM glucose 6-phosphate, 0.5 unit of glucose-6-phosphate dehydrogenase, 0.03 nmol of NADPH-cytochrome P450 reductase, and 0.017 nmol of P450 6A1 in 100 mM MOPS buffer. Nonionic detergent concentration remaining from the purification procedure was below the spectrophotometric detection limit. The reactions were started by the addition of substrate in 1−2 $\mu$L of ethanol and brief vortexing. The total volume of the reactions was 200 $\mu$L. After 20−30 min, the reactions were stopped and the products extracted with isooctane.

Products of enzymatic reactions were analyzed by GC/MS on a HP-1 fused silica column as described above, and product quantitation was done by integrating the peak areas in the total ion chromatogram. The relative responses to methyl farnesoate and its mono- and diepoxides were determined using authentic standards of these compounds.

**Formation and Analysis of *N*-Phenylprotoporphyrin IX Isomers.** The formation of an iron−phenyl complex of P450 6A1 and the subsequent formation and HPLC analysis of *N*-phenylprotoporphyrin IX adducts were adapted from the procedures outlined by Tuck et al. (*15*). Briefly, 5 nmol of P450 6A1 in 10 mM sodium phosphate buffer (pH 7.4) containing 1 mM DTT, 0.4% sodium cholate, 0.2% Emulgen 911, and 20% glycerol was reacted with 0.8 mM phenyldiazene for 15 min at 15 °C. The solution was oxidized with 1 mM potassium ferricyanide for 2 min at 15 °C. The mixture was added to 5 mL of 5% aqueous sulfuric acid and kept at 4 °C for 2−4 h. The porphyrin adducts were extracted with dichloromethane, the solvent was removed, and the residue was dissolved in HPLC solvent A (6:4:1 methanol/water/acetic acid). HPLC separation of the *N*-phenylprotoporphyrin IX isomers was done on a Supelco LC-18DB column (5 $\mu$m, 25 × 0.46 cm), with a 30-min isocratic elution with 70% A/30% B, where solvent B was 10:1 methanol/acetic acid. This was followed by a 1-min gradient to 100% B and isocratic elution at 100% B for 19 min. The flow rate was 1.5 mL/min, and absorbance of the eluant was monitored at 416 nm with a Perkin-Elmer LC95 spectrophotometer. Fresh stock solutions of potassium ferricyanide and phenyldiazene (prepared by adding 2.5 $\mu$L of phenyldiazenecarboxylate azo ester to 200 $\mu$L of fresh 1 N NaOH) were stored on ice. The procedure was verified by preparing the adducts of horse heart myoglobin under the same conditions.

**Table 1. Metabolism of Terpenoid Substrates by P450 6A1**

| | products (nmol of product)$^{-1}$ (nmol of P450)$^{-1}$ min$^{-1}$ [b] | | |
|---|---|---|---|
| substrate[a] | 10,11-epoxide | 6,7-epoxide | other |
| hydroprene | | | nd[c] |
| methoprene | | | nd |
| juvenile hormone I | | 0.96 ± 0.08 | 1.33 ± 0.21[d] |
| juvenile hormone III | | 4.18 ± 0.19 | 1.83 ± 0.25[d] |
| methyl (2*E*,6*E*)-farnesoate | 2.84 ± 0.20 | 0.54 ± 0.03 | 0.71 ± 0.18[e] |
| methyl geranate[f] | | 3.10 ± 0.15 | nd |
| (2*E*,6*E*)-farnesal | 1.65 ± 0.30 | 0.89 ± 0.02 | nd |
| (2*E*,6*E*)-farnesol | nd | nd | nd |
| (2*E*,6*E*)-farnesoic acid | nd | nd | nd |
| α-pinene | | | nd |
| limonene | | | nd |

[a] All substrates at nominal concentration of 50 $\mu$M. [b] (nmol of product formed)$^{-1}$ (nmol of P450)$^{-1}$ (min$^{-1}$ ± SE (mean of three determinations). Time of incubation 20 min. [c] nd, no product detected. Approximate limit of detection is 0.1% conversion to product or a rate of 0.03. [d] Unidentified product. [e] 6,7,10,11-Diepoxide product. [f] 65:35 mixture of geranyl and neryl methyl esters.

## Results

**Metabolism of Terpenoids by P450 6A1/Reductase Reconstituted System.** The insect growth regulators methoprene and hydroprene were not metabolized by P450 6A1 in our reconstituted system (Table 1). Both compounds are characterized by a pair of double bonds conjugated to an alkyl ester. However, the insect juvenile hormones that these terpenoids were designed to mimic were metabolized to the diepoxides at their unconjugated 6,7-double bond.

Juvenile hormone III ($t_R$ 11.17 min) and its homolog juvenile hormone I ($t_R$ 12.81 min), possessing ethyl branches at positions 6 and 11, were each metabolized to two products. The earlier eluting peak in each case was the 6,7,10,11-diepoxide (Table 1) ($t_R$ 12.16 and 13.83 min, respectively) (the hormones are themselves 10,11-epoxides). For juvenile hormone I, the mass spectrum of the diepoxide showed characteristic ion fragments at *m*/*z* 125, 114, 109, 107, 95, and 82. The identity of the later eluting peak ($t_R$ 14.22 and 12.79 min, respectively) obtained with juvenile hormones I and III was not rigorously determined. Relative to the diepoxide, it was produced in smaller amounts with juvenile hormone III than with juvenile hormone I (Table 1). This product did not result from thermal rearrangement of the 6,7,10,11-diepoxide in the gas chromatograph, because injection of the synthetic diepoxide did not result in its formation. The metabolite did not form a trimethylsilyl derivative on reaction with *N*,*O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA), and its infrared spectrum showed no indication of hydroxylation or carbonyl formation. With

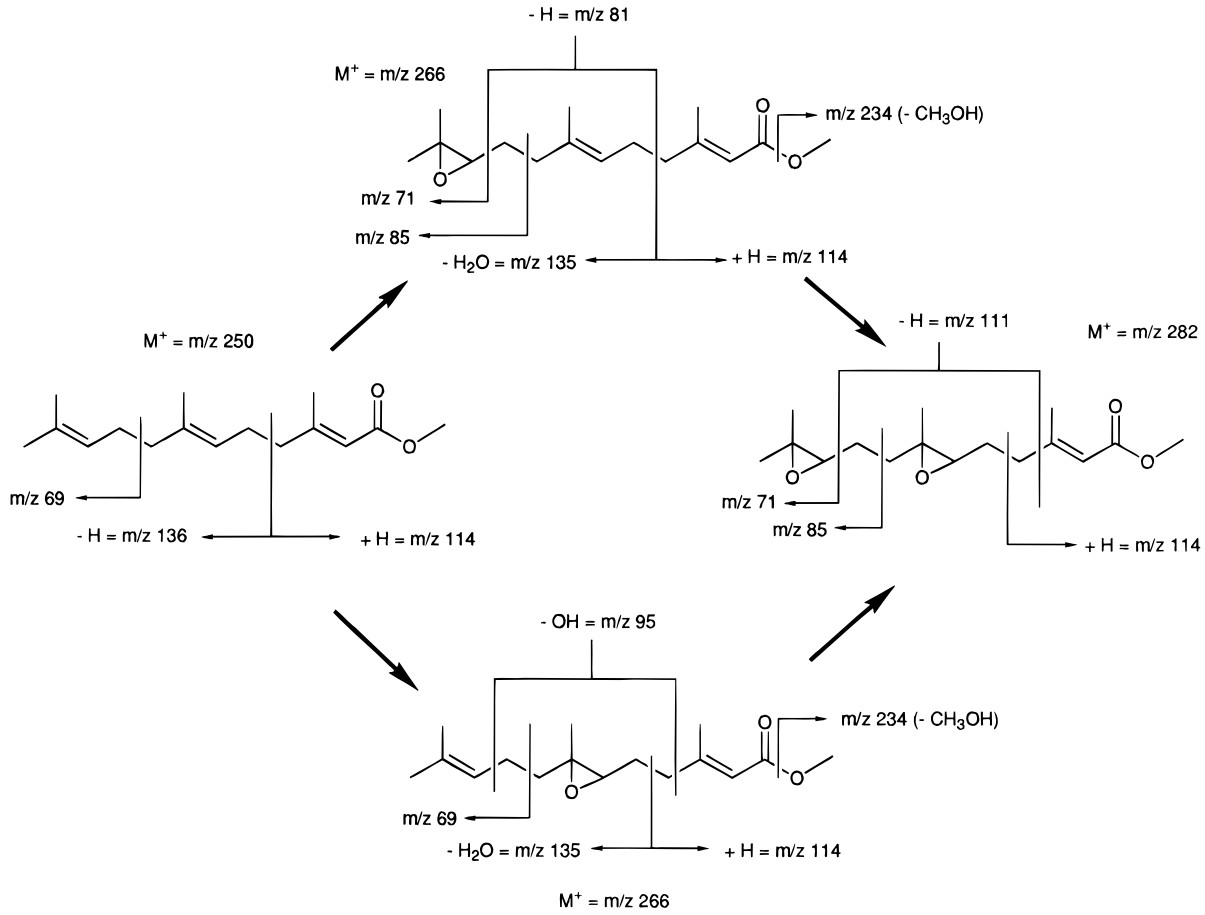


**Figure 2.** Conversion of methyl (2*E*,6*E*)-farnesoate to its mono- and diepoxides by P450 6A1. Molecular ions and fragmentation pattern observed by mass spectrometry are indicated for each compound.

juvenile hormone III, the product gave a mass spectrum with a molecular ion at *m*/*z* 282, a base ion at *m*/*z* 165, and other prominent ions at *m*/*z* 71, 85, and 114. The fragments at *m*/*z* 71 and 85 were attributed to cleavages α and *β* to the 10,11-epoxy group, which indicate that this moiety was intact. The fragment at *m*/*z* 114 showed that no modification of the 2,3-unsaturation occurred. The infrared spectrum of the unknown compound was nearly identical to that of the 6,7,10,11-diepoxide, suggesting that the product may be a rearranged diepoxide. The product seen with juvenile hormone I showed ions at *m*/*z* 85, 99, and 179 that were homologous to the fragments at *m*/*z* 71, 85, and 165, respectively, in the juvenile hormone III product and were indicative of the ethyl side chains.

The sesquiterpenoid aldehyde (2*E*,6*E*)-farnesal was metabolized to the 6,7-and 10,11-monoepoxide products ($t_R$ 10.69 and 10.63 min) in a 1:2 ratio (Table 1). Unlike the aldehyde, the sesquiterpenoid alcohol (2*E*,6*E*)-farnesol was not metabolized by P450 6A1, nor was the corresponding carboxylic acid, farnesoic acid (Table 1).

**Products from Methyl Farnesoate and Its Relatives.** Gas chromatographic analysis showed that methyl (2*E*,6*E*)-farnesoate was converted to three detectable products by P450 6A1 (Table 1, Figure 2). The mass spectrum of each contained an ion at *m*/*z* 114 ($CH_2C(CH_3)$═$CHC(OH)OCH_3^+$) that is a characteristic fragment in EI-MS spectra of farnesyl methyl esters (*16*). The major product showed prominent ions at *m*/*z* 71 and 85 which are indicative of cleavage α and *β* to a 10,11-epoxy group in the farnesyl skeleton and suggested that this compound was the 10,11-epoxide of methyl farnesoate. Chromatographic and spectral comparison with an authentic sample of methyl (2*E*,6*E* )-10,11-epoxyfarnesoate verified this assignment. The optically pure (10*R*) form of this compound is insect juvenile hormone III.

The second product peak, less prominent than the 10,11-epoxide and with a slightly longer retention time, showed a base ion at *m*/*z* 69 in its EI-MS and showed ions at *m*/*z* 95 and 135. These spectral features are indicative of a lack of modification of the terminal prenyl unit of the farnesyl skeleton and would be consistent with the 6,7-epoxide of methyl (2*E*,6*E*)-farnesoate (Table 1). Comparison with an authentic standard verified this assignment. The third peak showed an apparent molecular ion at *m*/*z* 282 with major fragment ions at *m*/*z* 71, 85, 93, 108, and 111. This molecular mass and fragmentation pattern are suggestive of the 6,7,10,11-diepoxide of methyl (2*E*,6*E*)-farnesoate, and a sample of synthetic diepoxide was used to verify this assignment (Table 1). The (6*S*,7*S*,10*R*)-enantiomer of this compound is the major product secreted by the corpora allata, and may be the active juvenile hormone, in a number of species of higher Diptera (*17*).

It is clear from these data that methyl farnesoate is converted to its 6,7,10,11-diepoxide through a monoepoxide intermediate (Figure 2). The major intermediate, based on its abundance in the reaction mixtures is methyl (2*E*,6*E*) 10,11-epoxyfarnesoate (Table 1). When the conversion of methyl (2*E*,6*E*)-farnesoate to mono- and diepoxide was examined as a function of substrate concentration, it was found that the amount of diepoxide in the total product increased with increasing substrate concentration (Figure 3).

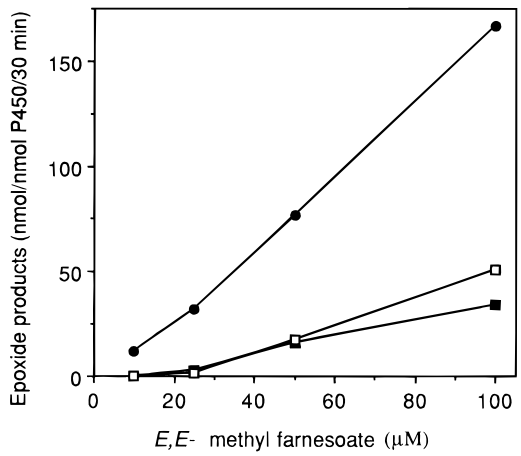


**Figure 3.** Production of mono- and diepoxide from methyl (2*E*,6*E*)-farnesoate by P450 6A1 in relation to the concentration of substrate: (■) 6,7-monoepoxide; (●) 10,11-monoepoxide; (□) diepoxide. The P450 6A1 concentration was 85 nM, and the reaction products were measured after 30 min. Each point is the mean of two separate experiments.

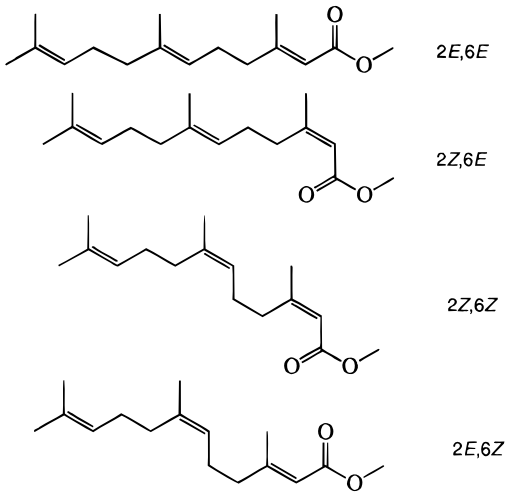


**Figure 4.** Structures of the four geometric isomers of methyl farnesoate.

The $C_{10}$ monoterpenoid homologs of methyl farnesoate were also converted to monoepoxides by P450 6A1. A 65:35 mixture of the methyl esters of neric (2*Z*) and geranic (2*E*) acids was converted by P450 6A1 to a mixture of products having abundant ions in the EI mass spectrum at *m*/*z* 59, 81, 85, and 112 that were consistent with the 6,7-epoxides of these two geometric isomers (Table 1). Comparison with a synthetic mixture of the two epoxides verified the identity of the products ($t_R$ 5.04 and 5.66 min, respectively).

**Reaction of P450 6A1 with the Geometric Isomers of Methyl Farnesoate.** The metabolism of the four geometric isomers of methyl farnesoate by the reconstituted system of P450 6A1 and NADPH-cytochrome P450 reductase were examined to determine the amount of epoxide formation for each (Figure 4). Incubation with P450 6A1 resulted in the formation of detectable levels of monoepoxide and diepoxide with each of the isomers (Table 2). The overall reaction rates were similar, but the distribution of products between the 6,7- and 10,11-epoxides varied somewhat. The 6,7- and 10,11-epoxides of methyl (2*Z*,6*E*)-farnesoate did not separate well on the columns used in this study, but the mass spectrum of the monoepoxide indicated that the product was predominantly the 10,11-epoxide. While methyl (2*Z*,6*Z*)-farnesoate is also converted mainly to 10,11-epoxide, methyl (2*E*,6*Z*)-farnesoate is converted to a 1.5:1 ratio of the 10,11- and 6,7-monoepoxides (Table 2).

**Table 2. Conversion of Geometric Isomers of Methyl Farnesoate to Epoxide Products by P450 6A1[a]**

| substrate isomer | products (nmol of product)$^{-1}$ (nmol of P450)$^{-1}$ min$^{-1}$ | | |
|---|---|---|---|
| | 10,11-epoxide | 6,7-epoxide | diepoxide |
| (2*E*,6*E*) | 2.84 ± 0.20[b] | 0.54 ± 0.03 | 0.71 ± 0.18 |
| (2*Z*,6*E*) | 1.76 ± 0.30[c] | *c* | 0.24 ± 0.04 |
| (2*Z*,6*Z*) | 1.93 ± 0.20 | 0.45 ± 0.04 | 0.43 ± 0.05 |
| (2*E*,6*Z*) | 2.83 ± 0.10 | 1.81 ± 0.07 | 0.65 ± 0.08 |

[a] The four geometric isomers of methyl farnesoate at a nominal concentration of 50 μM were incubated with 85 nM P450 6A1 for 20 min, and the products were analyzed by GC/MS. [b] Mean of three determinations ± SE. [c] 10,11- and 6,7-epoxides were not separated on GC but mass spectrum indicated mainly 10,11-epoxide.

**Table 3. Conversion of Methyl Farnesoate Epoxides to Diepoxides by P450 6A1[a]**

| substrate | diepoxide[a] |
|---|---|
| methyl (2*E*,6*E* )-6,7-epoxyfarnesoate | 41.7 ± 8.2[b] |
| methyl (2*E*,6*E* )-10,11-epoxyfarnesoate | 14.0 ± 2.9[c] |
| methyl (2*Z*,6*E* )-6,7-epoxyfarnesoate | 17.0 ± 0.3 |
| methyl (2*Z*,6*E* )-10,11-epoxyfarnesoate | 11.8 ± 0.7 |

[a] The four geometric isomers of methyl farnesoate at a nominal concentration of 250 μM were incubated with 85 nM P450 6A1 for 20 min, and the products were analyzed by GC/MS. [b] (nmol of product formed)$^{-1}$ (nmol of P450)$^{-1}$ min$^{-1}$. [c] Mean of three determinations ± SE. [d] In these experiments, conversion of methyl (2*E*,6*E* )-10,11-epoxyfarnesoate to the unknown metabolite (see Table 1) was less than 2%. The other substrates were not metabolized to metabolites other than the diepoxide.

**Determination of the Absolute Configuration of the 10,11-Epoxidation Catalyzed by P450 6A1.** A comparison of the reaction product of P450 6A1 with optically pure juvenile hormone III obtained by culture of corpora allata from the cockroach *Diploptera punctata* and with racemic synthetic methyl (2*E*,6*E*)-10,11-epoxyfarnesoate allowed us to determine the absolute configuration of the 10,11-epoxide product from methyl (2*E*,6*E*)-farnesoate. These epoxides were converted to methoxyhydrins containing a free hydroxyl group at C10. The methoxyhydrins were then reacted with a chiral reagent, and the resulting diastereomers were separated by capillary gas chromatography. The derivative prepared from the synthetic racemic methyl (2*E*,6*E*)-10,11-epoxyfarnesoate showed two peaks with near-identical areas separated by 0.1 min. A derivative produced from authentic (10*R*) juvenile hormone III obtained from *D. punctata* corpora allata gland cultures produced a single peak coincident with the more slowly eluting peak of the racemic material. The derivative of the reaction product from P450 6A1 contained a ratio of ~3:1 in favor of the more rapidly eluting form. P450 6A1 therefore converts methyl (2*E*,6*E*)-farnesoate predominantly to the antipode (10*S*) of natural juvenile hormone III (10*R*) (*18, 19*).

**Conversion of Monoepoxides of Methyl Farnesoate to Diepoxides.** The monoepoxides of methyl (2*E*,6*E*)- and (2*Z*,6*E*)-farnesoate were compared as substrates of P450 6A1 for conversion to their diepoxides in the reconstituted system. Conversion of the monoepoxides to diepoxides occurred regardless of the position of the epoxide moiety or the geometry of the substrate at the C2 double bond (Table 3). With the (2*E*,6*E*) isomer, epoxidation of the 10,11-double bond (6,7-epoxide as a substrate) occurred at a substantially higher rate than at the 6,7-double bond (10,11-epoxide as a sub-

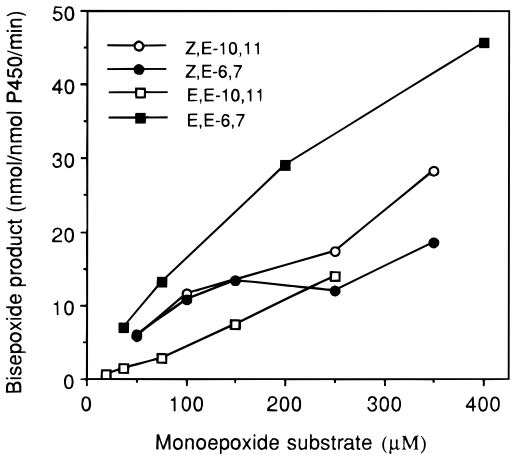


**Figure 5.** Concentration dependence of the epoxidation of methyl (6*E*)-epoxyfarnesoates to their respective diepoxides.

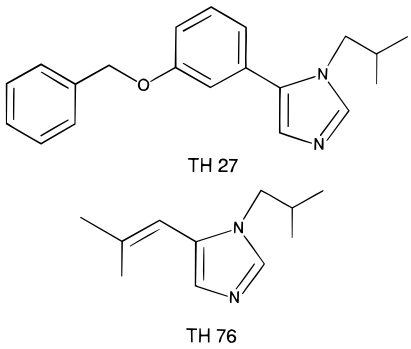


**Figure 6.** Structures of substituted imidazole inhibitors of P450 6A1.

**Table 4. Interaction of P450 6A1 with Imidazole Inhibitors**

| compound | $IC_{50}$ ($\mu$M)[a] | $K_s$ ($\mu$M)[b] |
|---|---|---|
| TH27 | 2.3 ± 0.2 | 0.08 ± 0.01 |
| TH76 | 55.7 ± 11.2 | 2.3 ± 0.1 |

[a] Concentration for 50% inhibition ± SE of methyl (2*E*,6*E* )-farnesoate epoxidation. Substrate concentration 50 $\mu$M; P450 concentration 85 nM. [b] Spectral dissociation constant ± SE calculated from type II difference spectra. P450 concentration 50 nM.

strate), while with the (2*Z*,6*E*) isomers, both the 6,7- and 10,11-monoepoxides were epoxidized at similar rates (Table 3). The reaction rates for the monoepoxides continued to increase at substrate concentrations above 300 $\mu$M, at which point the incubation mixtures became visibly cloudy (Figure 5). Although small amounts of a second product were observed in the metabolism of the 10,11-epoxide of methyl (2*E*,6*E*)-farnesoate (see metabolism of JH III above), this product was not seen with the other three monoepoxides tested here.

**Inhibition of P450 6A1.** The ability of two substituted imidazole inhibitors of juvenile hormone biosynthesis to inhibit P450 6A1 was examined. The imidazole compound TH27 (Figure 6) is a very potent inhibitor of the methyl farnesoate epoxidase from the corpora allata of the cockroach *D. punctata*, with an $IC_{50}$ of ~10 nM (*20*). We found here that this compound is also an effective inhibitor of P450 6A1 giving a concentration for 50% inhibition of activity ($IC_{50}$) of ~2 $\mu$M in the conversion of methyl (2*E*,6*E*)-farnesoate to products (Table 4). A second compound, TH76, has an imidazole moiety with a 1-isobutyl subsituent like TH27 but the 5-(benzyloxy)-phenyl moiety is replaced with an isobutenyl substituent (Figure 6). This inhibitor was ~25-fold less effective than

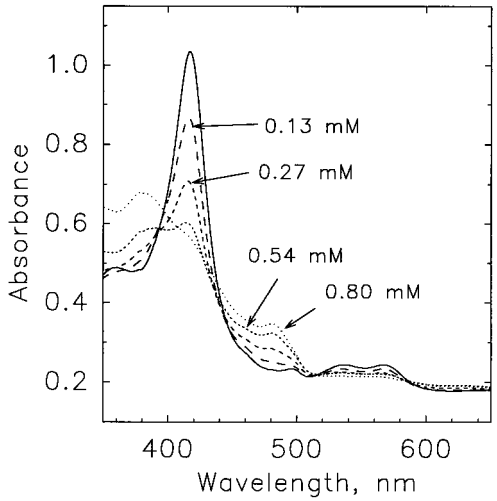


**Figure 7.** Absorption spectrum of P450 6A1 after reaction with phenyldiazene. P450 6A1 (0.5 nmol) was titrated with phenyldiazene at a final concentration of 0.13, 0.27, 0.54, and 0.80 mM. The decrease in the heme Soret band at 416 nm corresponds to the formation of the phenyl−iron complex which absorbs at 481 nm.

**Table 5. HPLC Elution Times and Ratios of *N*-Phenylprotoporphyrin Regioisomers Formed after Reaction of Myoglobin or P450 6A1 with Phenyldiazene**

| myoglobin[a] | | | P450 6A1[b] | | |
|---|---|---|---|---|---|
| isomer | elution time (min) | % of total | isomer | elution time (min) | % of total |
| $N_B$ | 19.5 ± 0.2 | 13.4 ± 2.2 | $N_B$ | 19.9 ± 0.4 | 17.1 ± 1.9 |
| $N_A$ | 21.5 ± 0.3 | 11.7 ± 3.2 | $N_A$ | 22.1 ± 0.5 | 25.1 ± 2.8 |
| $N_C$ | 27.1 ± 0.4 | 38.3 ± 2.5 | $N_C$ | 28.1 ± 0.9 | 33.3 ± 4.1 |
| $N_D$ | 30.5 ± 1.0 | 36.5 ± 2.9 | $N_D$ | 30.9 ± 1.0 | 24.4 ± 4.5 |

[a] Mean ± SD of three determinations. [b] Mean ± SD of four determinations.

TH27. Both of these inhibitors produced type II difference spectra when incubated with P450 6A1, with a peak at 432.0 nm and a trough at 411.5 nm. The spectral dissociation constants were ~25-fold lower than the $IC_{50}$ values for inhibition of the catalytic reaction under the conditions described in Table 4.

**Active Site Topology of P450 6A1.** P450 6A1 was titrated with phenyldiazene to form a phenyl−iron complex. The absorbance spectra showed that the loss of the Soret band at 416 nm was accompanied by the appearance of a band at 481 nm, with a saturation at 0.8 mM phenyldiazene (Figure 7). Treatment with ferricyanide caused the 481-nm band to disappear, indicating complete shift of the phenyl group to the pyrrole nitrogens. Extraction and HPLC separation of the *N*-phenylprotoporphyrin IX adducts showed that all four isomers were formed with P450 6A1. Validation of the procedure and identification of the isomers by HPLC was done by comparison to standards obtained in a similar fashion by reaction of phenyldiazene with horse heart myoglobin. Table 5 shows that the adducts ($N_B$:$N_A$:$N_C$:$N_D$ isomers as eluted by HPLC) were formed in P450 6A1 in a 17:25:33:24 ratio. This indicated that the space over the pyrrole rings of the active site heme is not selectively occluded in P450 6A1 and is only slightly more accessible over pyrrole ring C.

## Discussion

The oxidative metabolism of juvenile hormone I suggested to occur in Diptera (*21*) in addition to the more

commonly seen juvenile hormone esterase and epoxide hydrolase pathways was shown to be a 6,7-epoxidation in house fly microsomes (*22*, *23*). Epoxidation of juvenile hormone I to the diepoxide was inducible by phenobarbital and was elevated in insecticide-resistant strains (*23*). Furthermore, juvenile hormone I was shown to inhibit aldrin epoxidation and to produce a type I binding spectrum in microsomes of phenobarbital-induced resistant flies (*24*). The metabolism of juvenile hormone I to its diepoxide by P450 6A1, a major phenobarbital-inducible P450 of insecticide-resistant flies (*25*), is entirely consistent with these observations.

The presence in both adults and larvae of the house fly of phenobarbital-inducible microsomal P450 enzyme(s) capable of metabolizing the insect growth regulators methoprene and hydroprene was first shown by Terriere and Yu (*26*). These authors also reported that these compounds were modest inhibitors of heptachlor epoxidation and that hydroprene and methoprene metabolism was higher in microsomes from two insecticide-resistant strains. Interestingly, phenobarbital treatment did not induce the oxidative metabolism of four closely related insect growth regulators (*27*). *O*-Demethylation was shown to be the principal oxidative pathway of methoprene *in vivo* and in microsomes (*22*, *28*), whereas epoxidation at the 4,5-unsaturation was the major NADPH-dependent reaction for hydroprene (*29*). Neither of the two synthetic insect growth regulators was converted to detectable products by the P450 6A1/NADPH-cytochrome P450 reductase reconstituted system (Table 1). Methoprene and hydroprene are not unsaturated at the 6- or 10-positions, but rather have a 2,4-conjugated system. The lack of any epoxidized products suggests that an unsaturation at the 4-position may be too distant from the $\omega$-terminus to be brought into proximity with the active site or that the conjugated dienoate has inherently less chemical reactivity at that position. Interestingly, the *O*-methyl group located at the 11-position of the growth regulator methoprene is not attacked by P450 6A1 under our experimental conditions, despite occupying a position close to the 10,11-unsaturation of methyl farnesoate. Furthermore, there is no hydroxylation of hydroprene at positions homologous to the 10,11-unsaturation of methyl farnesoate. Hydroprene and methoprene were also not metabolized[2] in a reconstituted system of P450 6A1, NADPH-cytochrome P450 reductase, and house fly cytochrome $b_5$. Thus, the reported epoxidation of hydroprene and *O*-demethylation of methoprene by house fly microsomes is due to a different microsomal form of cytochrome P450.

Methyl farnesoate isomers are sequentially converted by P450 6A1 to either 6,7- or 10,11-monoepoxides and then to 6,7,10,11-diepoxide (Table 1, Figure 2). All four possible geometric isomers of methyl farnesoate were epoxidized, demonstrating that the binding pocket is spacious enough to accommodate the numerous configurations that would be represented by these isomers (Table 2). The (2*E*,6*E*), (2Z,6*E*), and (2*Z*,6*Z*) isomers of methyl farnesoate are converted predominantly to the 10,11-epoxide, indicating that in its most favorable orientation the substrate has its $\omega$-terminus held in the vicinity of the heme iron. For the (2*E*,6*Z*) isomer, the orientation allowing $\omega$-epoxidation is still predominant but to a lesser degree than for the other isomers. As the concentration of the substrate methyl (2*E*,6*E*)-farnesoate is increased, the amount of diepoxide produced also increases (Figure 3). The rate of increase in diepoxide formed is consistent with the release of monoepoxide from the enzyme followed by competition with methyl farnesoate for binding.

Methyl farnesoate monoepoxides derived from the (2*E*,6*E*) and (2*Z*,6*E*) isomers are converted efficiently to diepoxides regardless of the position of the substrate epoxy group. Epoxidation of the internal 6,7-double bond of the 10,11-epoxide of methyl (2*E*,6*E*)-farnesoate occurs at a lower rate than epoxidation of the 10,11-double bond of the 6,7-epoxide (Table 3). This indicates again that the most favorable substrate orientation has the $\omega$-terminus in the vicinity of the heme. However, variation in the geometry of the conjugated 2,3-double bond from *E* to *Z* appears to reduce the access of the 10,11-double bond to the activated oxygen (Table 3). The metabolism of methyl farnesoate shows that a substrate can be metabolized at different positions by the same P450 protein. CYP2C11 and CYP2C3 metabolize benzphetamine at three positions and several such examples have been described for xenobiotic-metabolizing P450 proteins (*30*).

In addition to binding different geometric isomers, the substrate-binding pocket of P450 6A1 is capable of accommodating methyl farnesoate analogs of varying chain length and with different functionalization at the acyl terminus. The enzyme epoxidizes methyl geranate (the monoterpene homolog of methyl farnesoate), juvenile hormone I [an 18-carbon analog of the 10,11-epoxide of methyl (2*E*,6*E*)-farnesoate] and the aldehyde analog of methyl (2*E*,6*E*)-farnesoate, suggesting that the distance between the carboxyl and $\omega$-termini is not critical and that the methyl ester functionality is not required for productive substrate binding (Table 1). No epoxides were seen with (2*E*,6*E*)-farnesol or farnesoic acid as a substrate, however, indicating that the increase in polarity of the alcohol or carboxylic acid over the ester or aldehyde may act to exclude the substrate from the binding pocket (Table 1).

The presence of a large 5-(benzyloxy)phenyl substituent in the imidazole inhibitor TH27 increased the efficacy of this compound by a factor of ~25 over TH76, which has a smaller 5-isobutenyl substituent. This suggests that either steric stabilization or a greater opportunity for hydrophobic binding near the active site heme contributes to the potency of this compound. Hydrophobic interactions of 1-alkylimidazoles with microsomal P450 have been well documented (*31*).

The degree of stereospecificity observed in P450-mediated epoxidation reactions has been examined in a number of instances. $P450_{BM-3}$ (CYP102) from the bacterium *Bacillus megaterium* will epoxidize styrene derivatives with degrees of stereospecificity varying from 40:60 (*S*:*R*) to 27:73 (*S*:*R*), whereas P450terp (CYP108) shows much higher degree of stereospecificity with the same set of styrene analogs (*32*). The crystal structures of these two enzymes have been determined, and the stereospecificity of the epoxidation reactions has been rationalized in part by steric constraints imposed by the geometry of the substrate-binding pocket (*33*, *34*). The pocket of $P450_{BM-3}$ is comparatively spacious, while the pocket of P450terp is constrained by impinging amino acid side chains. Stereoselectivity in the 10,11-epoxidation of methyl (2*E*,6*E*)-farnesoate by P450 6A1 is observed with a *S*:*R* ratio of ~3:1 at the 10-position. This degree of stereospecificity suggests that the binding pocket of P450 6A1 is spacious and allows multiple

[2] V. Guzov and R. Feyereisen, unpublished results.

orientations of the 10,11-unsaturation as opposed to the enzyme that synthesizes juvenile hormones (see below).

The complex resulting from the interaction of phenyldiazene with P450cam was shown by X-ray crystallography to be a $\sigma$-bonded phenyl−iron complex (*35*). Treating this complex with ferricyanide can induce the migration of the phenyl group from the iron to the porphyrin nitrogens, with the direction of the phenyl shift being controlled by the structure of the active site. Thus, the pattern of *N*-phenylprotoporphyrin IX isomers formed, specific for each P450 protein, provides information on the regions above the heme group that are occluded by the P450 protein residues (*36*). This technique revealed a relatively open active site above the heme in P450 6A1 (Table 5) or at least a relatively similar level of protein occlusion above each of the four pyrrole rings.

P450 6A1 and CYP102 ($P450_{BM-3}$) are the only P450 proteins studied so far in which the B pyrrole ring is exposed and in which all four rings are labeled to some extent. Biosynthetic P450s, such as CYP11A ($P450_{scc}$) and CYP51 (lanosterol 14α-demethylase), or the highly stereospecific bacterial CYP108 ($P450_{terp}$) form only a single *N*-phenylprotoporphyrin isomer under similar conditions. An attempt to correlate the ratio of isomers formed with the position of the P450 in a phylogenetic tree indicates that there is no discernible evolutionary constraint on the occlusion of the heme group.[3] Thus, relatively few amino acid changes may be needed to modify the topology of the active site, as suggested also by site-directed mutagenesis studies (*37*−*39*). However, only a dozen P450 proteins have been analyzed by this method to date, and the mutagenesis studies have been directed at highly conserved residues.

In summary, the lack of geometric specificity, the regiospecificity of epoxidation and enantioselectivity, and the ratio of formation of *N*-phenylporphyrin adducts suggest that the binding pocket of P450 6A1 is spacious and allows a number of different substrate orientations. Additionally, the accommodation of substrates with chain lengths shorter than methyl farnesoate, acceptance of the aldehyde as a substrate, and the epoxidation of nonoxygenated substrates such as cyclodiene insecticides suggest a lack of specific protein interactions with the methyl ester function. The increased efficacy of an imidazole inhibitor having a large hydrophobic substituent at the 5-position over one with a shorter substituent indicates that the enzyme possesses a large hydrophobic binding surface, possibly similar to that of the substrate access channel seen in the crystal structure of $P450_{BM-3}$ (*33*). It is likely that P450 6A1 can metabolize a large number of unrelated xenobiotics. Overexpression of this P450 in insecticide-resistant strains (*1, 2*) therefore may confer cross-resistance to several classes of insecticides. The fitness costs associated with this adaptation remain to be elucidated. Reduced fecundity and altered developmental rates in resistant strains might be related to the altered metabolism of endogenous substrates or to the overproduction of reactive oxygen species by a P450 whose broad substrate specificity does not allow tight coupling of substrate oxygenation and consumption of NADPH and molecular oxygen. This aspect of P450 6A1 biochemistry is currently under investigation.

In most insect species, juvenile hormone is the 10*R*,-11-epoxide of methyl-(2*E*,6*E*)-farnesoate (juvenile hormone III) or its homoisoprenoid congeners (*40*). In cockroaches and locusts, a microsomal cytochrome P450 enzyme is responsible for the epoxidation of methyl farnesoate in the corpora allata, the glands that secrete the hormone (*19, 41*). In higher Diptera (flies), however, the primary secreted product of the corpus allatum is reported to be the 6,7,10,11-diepoxide (*12, 42*). Does the metabolism of methyl farnesoate and juvenile hormone III to a diepoxide by house fly P450 6A1 provide a model for juvenile hormone biosynthesis in higher Diptera? It is not known if the two epoxidations occurring in the dipteran corpus allatum are catalyzed by a single enzyme or a pair of enzymes. A single glandular epoxidase would have to perform two regiochemically distinct epoxidations while maintaining absolute stereospecificity (*17*) in each. The chemistry of the sequential conversion of methyl farnesoate to mono- and diepoxide by P450 6A1 would be identical to the likely path taken by a single allatal epoxidase. However, P450 6A1 is not completely stereospecific and produces juvenile hormone III in a 3:1 ratio of the (10*S*) and (10*R*) enantiomers. Furthermore, Moshitsky and Applebaum (*43*) have claimed that the allatal epoxidase(s) of *Drosophila melanogaster* utilize farnesoic acid rather than methyl farnesoate as substrate for the epoxidations. Thus, the allatal epoxidase(s) of higher Diptera probably bind their sesquiterpenoid substrate in a manner significantly different from P450 6A1. The fate of juvenile hormone III and its diepoxide in Diptera has been shown to involve specific epoxide hydrolases (*44*).

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