Cytochrome P450-Dependent Transformations of 15*R*- and 15*S*-Hydroperoxyeicosatetraenoic Acids: Stereoselective Formation of Epoxy Alcohol Products†

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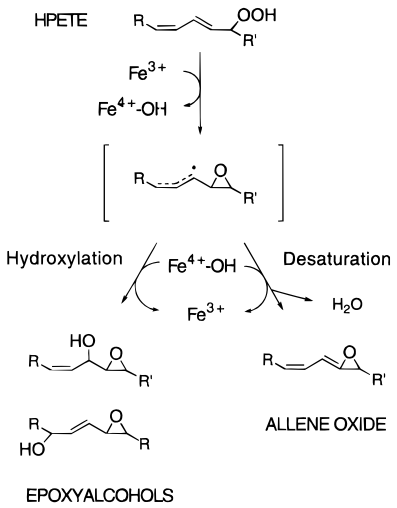
ABSTRACT: Although there are many reports of epoxy alcohol synthesis from lipoxygenase products (fatty acid hydroperoxides) in mammalian tissues, there are no well-defined examples of the stereoselective synthesis of individual epoxy alcohol diastereomers. An earlier report on the metabolism of 15*S*-hydroperoxyeicosatetraenoic acid (15*S*-HPETE) in rat liver microsomes suggested such a specific reaction [Weiss, R. H., et al. (1987) *Arch. Biochem. Biophys. 252*, 334−338]. To characterize this reaction further, we set out to determine the precise structures and mechanism of biosynthesis of the epoxy alcohol products. We compared the products formed from 15*R*- and 15*S*-HPETE by hematin (a nonenzymatic reaction), by liver microsomes isolated from control and phenobarbital-treated rats, and by purified cytochrome P450 2B1. Eight epoxy alcohol isomers were identified by mass spectrometry and ^{1}H NMR. In the hematin reaction, the major products are four epoxy alcohols with the epoxide in the *trans* configuration, diastereomers are formed in similar amounts, and the 15-HPETE enantiomers give indistinguishable patterns of products. By contrast, the liver microsomes and P450 2B1 enzyme form predominantly single diastereomers, and the configuration of the epoxide is dependent on the stereochemistry of the substrate. The main product formed from 15*S*-HPETE is 11*S*-hydroxy-14*S*,15*S*-*trans*-epoxyeicosa-5*Z*,8*Z*,12*E*-trienoic acid, and the amounts increase upon phenobarbital induction. The main products from 15*R*-HPETE are 11-hydroxy-14*S*,15*R*-epoxyeicosa-5*Z*,8*Z*,12*E*-trienoic acid from microsomes from control rats and 13-hydroxy-14*S*,15*R*-*cis*-epoxyeicosa-5,8,11-trienoic acid in microsomes from phenobarbital-induced rats. The P450 2B1 enzyme gave products similar to those from the phenobarbital-induced microsomes. Analysis of an incubation using the ^{18}O-labeled 15*S*-HPETE substrate demonstrated 97.6% retention of both hydroperoxy oxygens in the major product with progressively lower ^{18}O retentions in the minor products (74−32%), possibly reflecting degrees of enzymatic control of these reactions. These results establish a precedent for the stereoselective synthesis of epoxy alcohols by mammalian cytochrome P450s.

Cytochrome P450s have been implicated in the metabolism of fatty acid hydroperoxides in both plant and animal systems. In plants, a novel type of cytochrome P450 converts 13-hydroperoxylinolenic acid to an allene epoxide, a key intermediate in the biosynthesis of the signaling molecule, jasmonic acid (Song & Brash, 1991a; Hamberg & Gardner, 1992; Song et al., 1993a). This enzyme forms only the allene oxide from its natural hydroperoxy substrate, but certain other fatty acid hydroperoxides are also converted to epoxy alcohol derivatives. There is a potential mechanistic relationship between allene oxide and epoxy alcohol synthesis (Scheme 1) (Song et al., 1993b).

Scheme 1



Allene oxide synthesis occurs in some marine invertebrates [reviewed in Song and Brash (1991b)], but it has not been detected in mammalian systems. By contrast, there are many reports of the formation of epoxy alcohols in mammalian tissues (Pace-Asciak & Asotra, 1989; Pace-Asciak et al., 1995). What is less clear, however, is the extent to which this epoxy alcohol synthesis is enzymatic. Epoxy alcohols are readily formed from fatty acid hydroperoxides via nonenzymatic catalysis by a number of agents, including strong acid, ferrous iron, or hematin [reviewed in Gardner (1989)]. Clearly, there is the possibility for nonenzymatic catalysis by adventitious iron or free heme in biological

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systems. In general, the enzymatic and nonenzymatic reactions can be distinguished both by their different sensitivities to heat inactivation and by the different profiles of products formed. Nonenzymatic catalysis by iron or free heme gives similar amounts of epoxy alcohol diastereomers (e.g., Gardner & Kleiman, 1981; Gardner & Crawford, 1981; Van Os et al., 1982; Dix & Marnett, 1985), whereas the more stereocontrolled enzymatic syntheses give a marked preponderance of a single diastereomer (Galliard et al., 1975; Garssen et al., 1976; Hamberg et al., 1986a). This distinction is evident, for example, in the epoxy alcohol synthesis by the allene oxide synthase (Song et al., 1993b).

A report by Weiss et al. (1987) described the conversion of the 15*S*-hydroperoxide of arachidonic acid to epoxy alcohols and their trihydroxy hydrolysis products in rat liver microsomes. Metabolism was largely abolished by boiling the microsomes. These findings set a clear precedent for the enzymatic transformation of hydroperoxides to epoxy alcohols in mammalian systems. More recently, Reynaud et al. (1994) reported heat-labile activity in rat pineal gland that catalyzed the enantioselective metabolism of 12*S*-hydroperoxide to allylic epoxy alcohols. We have further investigated the rat liver microsome system with a view to examining the stereochemistry of the transformations and to providing additional evidence for the involvement of cytochrome P450s.

MATERIALS AND METHODS

Materials. Arachidonic acid and its methyl ester were obtained from Nuchek Prep Inc. (Elysian, MI), and [1-^{14}C]-arachidonic acid was purchased from NEN-Dupont. 15*S*-HPETE[1] was prepared from arachidonic acid using soybean lipoxygenase (Sigma type V), and 15*S*-HETE was formed by the reduction of 15*S*-HPETE with a molar excess of triphenylphosphine. 15*S*-[^{18}O]HPETE was prepared under an atmosphere of $^{18}O_2$ (Isotec Inc., Miamisburg, OH). The HPETE and HETE products were quantified by UV spectroscopy ($\epsilon = 23\,000$). Rat liver P450 2B1 was prepared as reported (Guengerich & Martin, 1980; Guengerich et al., 1982). (−)-Menthyl chloroformate and *R*- and *S*-malate were purchased from Aldrich. SPB-2250 and SPB-1 capillary columns used in GC−MS were purchased from Supelco (Bellefonte, PA)

Racemic 15-HPETE was prepared from arachidonate methyl ester by the following route: (i) autoxidation of arachidonate methyl ester in the presence of α-tocopherol (Peers & Coxon, 1983); (ii) isolation of 15*RS*-HPETE methyl ester by SP-HPLC using an Alltech silica column (25 × 1 cm) and a solvent system of hexane/ethyl ether (85:15, v/v, 5 mL/min, UV detection at 235 nm); (iii) preparation of the free acid by treatment for 15 min with 0.5 M KOH in methanol/water (1:1) at room temperature, followed by acidification to pH 3.5 and extraction into dichloromethane; and (iv) purification of 15*RS*-HPETE by SP-HPLC (Alltech silica column, solvent system of 100:2:0.1 hexane/isopropyl alcohol/glacial acetic acid (v/v/v), flow rate of 1 mL/min, and UV detection at 235 nm). A second route to the preparation of racemic 15-HPETE methyl ester involved the conversion of 15*S*-HETE methyl ester to the mesylate derivative, followed by treatment with hydrogen peroxide in ether at −110 °C as described by Corey et al. (1980). The product was isolated by SP-HPLC as described earlier.

15R-HPETE was prepared by resolution of the 15*RS*-HPETE methyl ester using a Chiralcel OD column (Baker), a solvent of 100:2 hexane/isopropyl alcohol (v/v), and a flow rate of 1 mL/min. Aliquots of 200 μg of 15*RS*-HPETE methyl ester were injected onto the column; the *R* enantiomer eluted at approximately 11.5 min and the *S* enantiomer at 15.3 min. The free acid was prepared by alkaline hydrolysis as described earlier, repurified finally by SP-HPLC, and quantified by absorbance at 235 nm ($\epsilon = 23\,000$).

Epoxy alcohol standards were prepared by reacting 15*S*-HPETE (500 μg) with hematin (125 μg) in 1 mL of K_2HPO_4 (0.1 M). Reaction was allowed to proceed for 15 min at room temperature and was then terminated by acidification (pH 4) with 1 N HCl, followed by extraction with 2 vol of dichloromethane. The organic phase was washed with water until it reached neutral pH. Isolation and purification of the epoxy alcohol were carried out by RP-HPLC and SP-HPLC as described on the following page.

Preparation of Liver Microsomes. Sprague−Dawley rats of either sex were anesthetized with an intraperitoneal injection of pentabarbital, the peritoneal cavity was opened, and the portal vein was cannulated and perfused with 0.15 M KCl delivered manually from a 50 mL syringe until the liver was pale in color. The liver was removed, cleaned of all visible connective tissues and chopped into small pieces. The homogenization and centrifugation procedures were carried out as described by Capdevila et al. (1990). The microsomal pellets (100000*g* pellets) were stored at −80 °C. Microsomes were resuspended in the same buffer used for the incubations [50 mM Tris (pH 7.5) containing 10 mM $MgCl_2$ and 150 mM KCl] at a protein concentration of approximately 10 mg/mL. Protein concentration of the microsome suspension was determined by the Bio-Rad Bradford method using bovine serum albumin as standard. Liver microsomes were also prepared from rats that were allowed free access to water containing phenobarbital (1 mg/mL) for 1 week. The P450 contents of the phenobarbital-induced and the noninduced liver microsomes, determined from the 450 nm carbon monoxide difference spectrum, typically were 3-4 and 1 nmol/mg protein, respectively.

Incubation and Extraction Conditions. Microsomes were diluted to 2 mg of protein/mL in the 50 mM Tris, 10 mM $MgCl_2$, and 150 mM KCl buffer (pH 7.5) at 25 °C. Typical incubations were carried out in a volume of 2 mL for 2 min at 25 °C. Reaction was initiated by the addition of 100 μM 15-HPETE dissolved in 10 μL of methanol (with the 15*S* enantiomer, including 100 000 cpm of 15*S*-[1-^{14}C]HPETE). The incubation was terminated by adding 2.5 vol of methanol and placing the sample on ice. Products were extracted with a slight modification of the Bligh and Dyer (1959) procedure, in which dichloromethane was substituted for chloroform and an additional volume of water was added to help reduce the volume of emulsion at the interface. The final mixture, comprising of 5 mL of methanol, 5 mL of dichloromethane, and 6.5 mL of water was centrifuged, and the organic phase was removed and dried under nitrogen. Products were dissolved in methanol and stored a −80 °C under argon until further analysis by HPLC.

[1] Abbreviations: EET, epoxyeicosatrienoic acid; HETE, hydroxyeicosatetraenoic acid; HPETE, hydroperoxyeicosatetraenoic acid; HPLC, high-pressure liquid chromatography; P450, cytochrome P450; PFB, pentafluorobenzyl; RP-HPLC, reversed-phase HPLC; SP-HPLC, straight-phase HPLC; TMS, trimethylsilyl.

Incubations with P450 2B1 enzyme were carried out in a similar manner on a 0.5 or 1 mL scale, using an enzyme concentration of 1 nmol/mL with 100 μM 15-HPETE substrate. Incubation and extraction were carried out as described earlier.

HPLC Analysis. Products of incubations and the hematin reaction were analyzed initially by RP-HPLC with UV monitoring using a Hewlett-Packard 1040 diode array detector and an on-line Packard Flo-One radioactivity detector. A Beckman 5 μm ODS Ultrasphere column (25 $\times$ 0.46 cm) was used with a solvent system of methanol/water/acetic acid (75:25:0.01, by volume). The products were collected manually while running the same system at a flow rate of 0.5 mL/min, with UV detection using an LDC/Milton Roy variable wavelength instrument set at 205 nm. The main products were recovered from the reversed-phase solvent by extraction with dichloromethane. Further purification was carried out by straight-phase HPLC using an Alltech 5 μm silica column (25 $\times$ 0.46 cm), a solvent system of hexane/isopropyl alcohol/acetic acid (100:2:0.1, by volume), and a flow rate of 1 mL/min. UV detection was again carried out either on the diode array detector or on the variable wavelength detector set at 205 nm.

Derivatization. Methyl esters were prepared by using ethereal diazomethane/methanol ($\approx$5:1). Catalytic hydrogenations were performed in 100 μL of ethanol using 1 mg of palladium on alumina and bubbling with hydrogen for 5 min at room temperature. Reaction was terminated by the addition of water and extraction with ethyl acetate. Trimethylsilyl (TMS) ester and TMS ether derivatives were prepared by treatment with bis(trimethylsilyl)trifluoracetamide (10 μL) and pyridine (5 μL) for 15 min at room temperature. Subsequently, the reagents were evaporated under a stream of nitrogen and the samples were dissolved in hexane for GC−MS analysis. Pentafluorobenzyl ester derivatives were prepared by treatment with 35 μL of 10% pentafluorobenzyl bromide in acetonitrile and 10 μL of 10% diisopropylethylamine in acetonitrile. Reaction was allowed to proceed for 10 min at room temperature under nitrogen; the reagents were then evaporated to dryness and the samples were dissolved in hexane for GC−MS analysis.

Oxidative Ozonolysis. The two 11-hydroxy-14,15-*trans*-epoxyeicosa-5,8,12-trienoic acids were isolated by RP-HPLC from reactions of 15*S*-HPETE with hematin, derivatized to the methyl esters by using ethereal diazomethane, and repurified by SP-HPLC. The following procedure, based on the oxidative ozonolysis approach originally described by Hamberg (1971), was developed with the aid of ^{14}C-labeled epoxy alcohol and then applied to samples of approximately 100 μg of each diastereomer. The methyl ester was taken to dryness in a 1 mL Reactivial and dissolved in 50 μL of toluene and 3 μL of pyridine. (−)-Menthyl chloroformate (1 μL) was then added with rapid mixing. After 30 min at room temperature, 0.8 mL of hexane was injected into the reaction mixture, and the cloudy suspension was immediately applied to a 500 mg Bond-Elut silica cartridge (Varian). The silica cartridge was preequilibrated in a solvent of hexane/ethyl acetate (80:20, v/v). The derivatized epoxy alcohol was not retained on this silica column and was collected in the first 3.5−4 mL of eluate, comprising the applied sample together with the first 3 mL of wash with column solvent. The methyl ester menthyl carbonate derivatives were purified by reversed-phase HPLC on a Beckman 5 μm ODS Ultrasphere column (25 $\times$ 0.46 cm) using 100% methanol as the mobile phase at a flow rate of 1 mL/min. In this system, 15-HETE methyl ester menthyl carbonate has a retention time of 9−10 min. The epoxy alcohol derivatives elute at approximately 7−8 mL, and quantities of 1 μg or more are readily detected by UV monitoring at 205 nm. By using these procedures, each 11-hydroxy diastereomer was recovered in approximately 50% yield as the methyl ester menthyl carbonate derivative.

Ozonolysis was performed by bubbling ozone through the sample (25 μg) in 0.5 mL of chloroform at −20 °C for 5 min. Products were dried under nitrogen and left overnight at 50 °C in glacial acetic acid and 30% H_2O_2 (100:20, by volume). Samples were dried under nitrogen and stored in ethyl acetate at −60 °C until further analysis. Methyl ester menthyl carbonate derivatives of racemic, *R*-, and *S*-malic acid were prepared as outlined earlier and partially purified by application to a Bond-Elut silica column and elution with 100% ethyl acetate.

GC−MS Analysis. Analysis of the methyl ester menthyl carbonate derivatives of malic acid was performed by using a Finnigan Incos 50 mass spectrometer equipped with a Hewlett-Packard 5890 gas chromatograph and an SPB-2250 capillary column (30 m $\times$ 0.25 mm id). The oven temperature was programmed from 150 to 250 °C at 10 °C/min, with a 20 min hold at the final temperature. The mass spectrometer was operated in the positive ion electron impact mode, and the methyl ester menthyl carbonate derivatives of *R*- and *S*-malic acid were detected by selected ion monitoring at *m/z* 138.

Epoxy alcohol derivatives were analyzed by using the same instrument and an SPB-1 fused capillary column (15 m $\times$ 0.25 mm id). The oven temperature was programmed from 150 to 300 °C at 15 °C/min.

The ^{18}O content of epoxy alcohol products was analyzed by GC−MS on the pentafluorobenzyl ester TMS ether derivatives using a Nermag R10-10C instrument operated in the negative ion/chemical ionization mode. Data were acquired by fast repetitive scans over the mass range *m/z* 404−414, comprising the prominent M−PFB ion of the unlabeled epoxy alcohol (*m/z* 407) and the ^{18}O-labeled species (*m/z* 409 and 411). Spectra collected during elution of the GC peak (typically about 30 scans) were averaged prior to the calculation of isotopic composition.

NMR. A Bruker AM-400 or an IBM/Bruker NR-300 instrument at 400 or 300 MHz, respectively, was used. Initially, $CDCl_3$ was used as the solvent. However, the acidity of $CDCl_3$ caused degradation of the samples, and substitution with C_6D_6 was required. The instrument parameters were similar to those described previously (Song et al., 1993b).

RESULTS

Metabolism of 15S-HPETE. 15*S*-HPETE (100 μM) was incubated with rat liver microsomes (2 mg/mL) as described by Weiss et al. (1987) using an extraction procedure modified to eliminate acid-catalyzed hydrolysis of the epoxy alcohol products. As indicated by RP-HPLC analysis, in boiled microsomes there is little conversion of the 15-hydroperoxy substrate (Figure 1A), while complete conversion occurs in microsomes from phenobarbital-treated or control animals (Figure 1B,C). The radiolabeled peaks eluting near the end

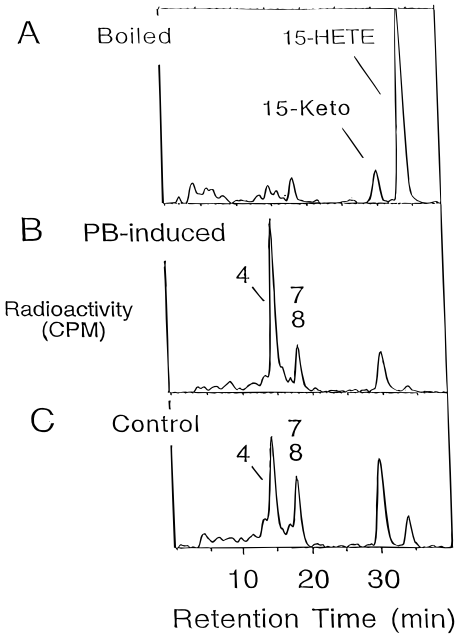


FIGURE 1: RP-HPLC analysis of the products of 15*S*-[^{14}C]HPETE metabolism in rat liver microsomes. The profile of ^{14}C-labeled products is shown for the incubation of 15*S*-[^{14}C]HPETE with boiled liver microsomes (A), microsomes from phenobarbital-treated rats (B), and control liver microsomes (C). The chromatograms were obtained by using a 5μm Ultrasphere ODS (25 × 0.46 cm) column with a 5 μm Bio-Rad ODS guard column, a mobile phase of methanol/water/glacial acetic acid (75:25:0.01, by volume), and a flow rate of 1 mL/min.

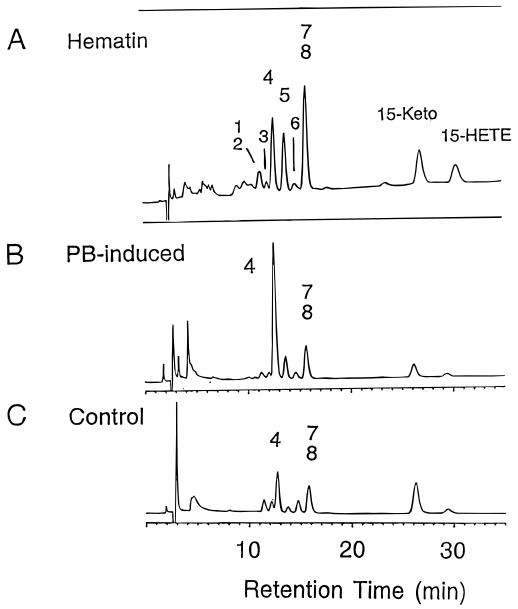


FIGURE 2: RP-HPLC profiles of products (UV detection at 205 nm) from reaction of 15*S*-HPETE with hematin and rat liver microsomes. The product profiles of 15*S*-HPETE incubations with hematin (A), liver microsomes from phenobarbital-treated rats (B), and control liver microsomes (C) are shown. The chromatographic conditions are same as for Figure 1. The epoxy alcohol products are numbered **1**−**8**. Eluting at approximately 27.5 min is 15-ketoeicosatetraenoic acid (labeled 15-keto), followed by 15-HETE at 29.5 min.

of the RP-HPLC chromatogram (at 30 and 34 min) are 15-ketoeicosatetraenoic acid and 15-hydroxyeicosatetraenoic acid (15*S*-HETE), respectively. The epoxy alcohol products chromatograph as a group in the middle of the chromatogram (12−18 min).

Identification of the individual products was made by HPLC and GC−MS comparison to a set of standards prepared by reaction of 15*S*-HPETE with hematin. Initial comparison of the hematin and microsomal products was made by RP-HPLC analysis with UV detection at 205 nm. The UV 205 nm profile gives a fairly accurate impression of the relative abundance of the different products (with the exception of the area of the chromatogram distorted by artifacts near the solvent front) and has the advantage that it is a higher resolution recording than the radioactivity profile. The products from a hematin reaction are shown in Figure 2A together with the profile from phenobarbital-treated and control rat liver microsomes (Figure 2B,C). Clearly, the microsomal incubations give UV 205 nm profiles that markedly differ from the nonenzymatic reaction and from each other.

Identification of the Hematin Products. In the hematin reaction, 15*R*-HPETE and 15*S*-HPETE give enantiomeric products with HPLC profiles that are indistinguishable (not shown). The products from 15*S*-HPETE were separated by RP-HPLC and straight-phase HPLC (Table 1) and characterized by oxidative ozonolysis, GC−MS, and ^{1}H NMR (supporting information). The structures of the products, and the stereochemistry as far as it is established, are presented in Figure 3.

In summary, the major hematin products comprise two 11-hydroxy-14,15-*trans*-epoxyeicosatrienoic acids (products **4** and **5**, Figure 1) and two 13-hydroxy-14,15-*trans*-epoxyeicosatrienoic acids (products **7** and **8**). Products **7** and **8** cochromatograph upon RP-HPLC, but are easily resolved by SP-HPLC (Table 1). The configuration of the hydroxyl group in products **4** and **5** was determined as 11*S* and 11*R*, respectively, by oxidative ozonolysis, and products **7** and **8** were designated as 13*S* (*erythro*) and 13*R* (*threo*), respectively, on the basis of the $J_{13,14}$ coupling constants by ^{1}H NMR (supporting information). The remaining four products are diastereomeric pairs of the corresponding *cis*-epoxy alcohols; the configuration of their hydroxyl groups was not defined (Figure 3).

Table 1: SP-HPLC Retention Times of Epoxy Alcohols Derived from 15*S*-HPETE[a]

retention time (min)	product designation no.	epoxy alcohol structure
3.2	15-keto-20.4[b]	
3.8	15-HETE	
5.4	7	13S-hydroxyy (*erythro*) 14,15-*trans*-epoxy
6.6	8	13R-hydroxy (*threo*) 14,15-*trans*-epoxy
8.5	2	13-hydroxy 14,15-*cis*-epoxy
9.1	6	13-hydroxy 14,15-*cis*-epoxy
10.2	4	11*S*-hydroxy 14,15-*trans*-epoxy
10.8	5	11*R*-hydroxy 14,15-*trans*-epoxy
11.7	1	11-hydroxy 14,15-*cis*-epoxy
12.2	3	11-hydroxy 14,15-*cis*-epoxy

[a] Column: Alltech 5 μm silica (25 × 0.46 cm). Solvent: hexane/isopropyl alcohol/glacial acetic acid (100:2:0.1, by volume). Flow rate: 2 mL/min. Detector: UV 205 nm. The retention time of authentic α-ketol (14-keto-15-hydroxyeicosa-5,8,11-trienoic acid, the hydrolysis product of the allene oxide derived from 15-HPETE) is 4.9 min in this SP-HPLC system. [b] 15-keto-20.4 is 15-ketoeicosa-5,8,11,13-tetraenoic acid.

Identification of Epoxy Alcohols from Incubations of 15S-HPETE of Microsomes. The most prominent products from 15*S*-HPETE incubations with microsomes from control rats

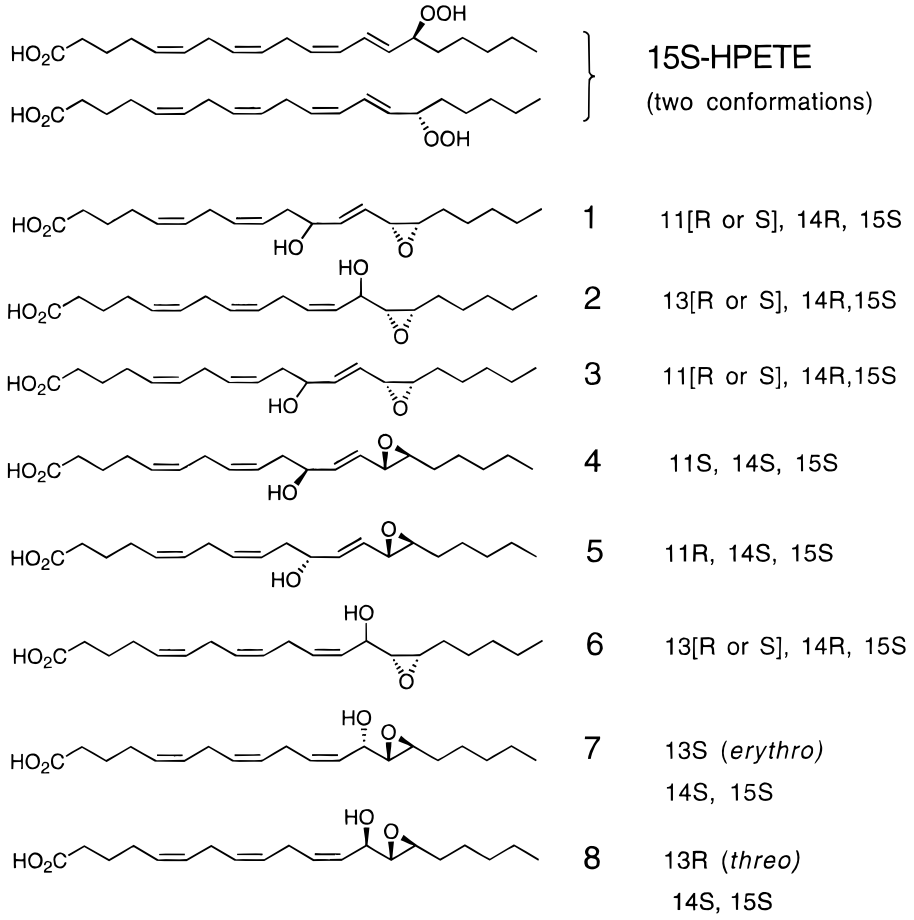


FIGURE 3: Structures of epoxy alcohols **1**−**8** formed from 15*S*-HPETE,

cochromatograph on RP-HPLC with epoxy alcohol **4** and the peak of **7** + **8**. Phenobarbital treatment markedly induces the synthesis of the first of these products (Figures 1 and 2). This compound also cochromatographed with hematin product **4** on SP-HPLC. The methyl ester TMS ether derivative had the same GC retention time and an EI mass spectrum indistinguishable from that of hematin product **4**. On this basis, the major product from 15*S*-HPETE in liver microsomes from the phenobarbital-treated animals was identified as 11*S*-hydroxy-14*S*,15*S*-*trans*-epoxyeicosa-5*Z*,8*Z*,12*E*-trienoic acid.

The products from the microsome incubations that cochromatographed with hematin standards **7** + **8** upon RP-HPLC resolved into two peaks upon SP-HPLC in proportions of approximately 1:3 in the order of elution. On the basis of HPLC and GC−MS comparison to the hematin standards, these products were identified as the *erythro* (13*S*) and *threo* (13*R*) diastereomers, respectively, of 13-hydroxy-14*S*,15*S*-*trans*-epoxyeicosa-5*Z*,8*Z*,11*Z*-trienoic acids.

Metabolism of 15R-HPETE. As noted earlier, the nonenzymatic hematin reaction does not discriminate between 15*R*- and 15*S*-HPETE, whereas a characteristic of enzymatic transformations is their enantioselectivity (e.g., Baertschi et al., 1988). To examine this feature of HPETE metabolism in rat liver microsomes, we examined the fate of 15*R*-HPETE. This substrate was metabolized at a rate similar to the 15*S* enantiomer, but a distinctly different profile of products was formed in both microsomes from control and phenobarbital-treated rats (Figure 4). The most abundant products differ from the main products with 15*S*-HPETE and also they correspond to minor products of the hematin reaction.

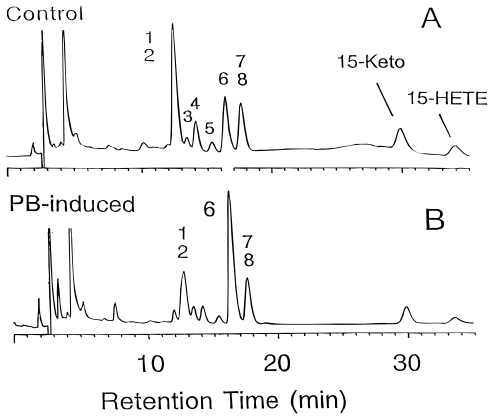


FIGURE 4: RP-HPLC analysis (UV detection at 205 nm) of products from reaction of 15*R*-HPETE with rat liver microsomes. The product profiles of 15*R*-HPETE from phenobarbital-treated rats (A) and control liver microsomes (B) are shown. The chromatographic conditions are given in the legend to Figure 1. The composition of the combined peak of **1** and **2** differs in panels A and B. In panel A, SP-HPLC analysis indicated that the first epoxy alcohol peak is mainly product **1**; product **2** is a minor component. In panel B, product **2** predominates.

In the control microsomal incubations, the main 15*R* metabolite cochromatographs on RP-HPLC with a hematin peak that contains two poorly resolved epoxy alcohols (hematin products **1** and **2**, Figure 1A). These two components are easily resolved on SP-HPLC (Table 1), and the main 15*R* metabolite from control microsomes cochromatographs with hematin product **1**. On the basis of the GC−

Table 2: Retention of Oxygen-18 in Products Formed from 15*S*-[$^{18}O_2$]Hydroperoxy Substrate in Liver Microsomes from Phenobarbital-Treated Rats

	15*S*-HPETE substrate	**4**	**5**	**7**	**8**
% content of 2-^{18}O	84	82	26	32	62
% retention of 2-^{18}O	100	97.6	31	38	74

MS analyses and the ^{1}H NMR analysis of hematin product **1**, the main 15*R*-HPETE metabolite in control microsomes was identified as 11-hydroxy-14*S*,15*R*-*cis*-epoxyeicosa-5*Z*,8*Z*,12*E*-trienoic acid.

In the incubation of phenobarbital-induced microsomes, the main 15*R*-HPETE product (product **6**) corresponds to a minor peak in the hematin reactions (cf. Figures 1 and 4). The mass spectrum of the methyl ester TMS ether derivative of product **6** was consistent with that of a 13-hydroxy-14,15-epoxyeicosatrienoic acid (not shown). As product **6** could not be produced in large amounts in the hematin reaction, we prepared sufficient product for NMR analysis in a 30 mL microsomal incubation with 1 mg of 15*R*-HPETE. On the basis of the GC−MS and ^{1}H NMR data, product 6 was identified as 13-hydroxy-14*S*,15*R*-*cis*-epoxyeicosa-5*Z*,8*Z*,11*Z*-trienoic acid (supporting information).

Metabolism of [^{18}O]Hydroperoxy Substrate. In the formation of epoxy alcohols from hydroperoxy substrates, the oxygen of the hydroxy group can originate from the hydroperoxy group, from molecular oxygen, or from the solvent (Gardner, 1989). This information affords insight into the mechanism of the reaction (free radical or ionic) and can also reflect the degree of enzymatic control (Gardner, 1989; Dix & Marnett, 1985; Song et al., 1993b). To determine the source of the oxygen in the different epoxy alcohol products from 15*S*-HPETE, incubations of 15*S*-[^{18}O]-HPETE were carried out with liver microsomes from phenobarbital-treated rats. The products showed different percent retentions of the two original peroxyl oxygens of the 15*S*-[^{18}O]HPETE (Table 2). In general, the more abundant the product, the higher the retention of the two peroxyl oxygens. The major product (product **4**) from the phenobarbital-induced microsomes contained 82% 2-^{18}O, which, by comparison to the substrate (84% 2-^{18}O), corresponds to a percent retention of both ^{18}O labels of 82/84 = 97.6%. In contrast, its diastereomer, product **5**, showed only 31% 2-^{18}O. Of the two 13-hydroxy 14,15-*trans*-epoxides, the more prominent *threo* isomer retained 74% 2-^{18}O, while the minor *erythro* isomer retained only 38%.

Metabolism of 15R- and 15S-HPETE by Cytochrome P450 2B1. Figure 5 shows the reversed-phase HPLC profiles of products obtained by using 1 μM purified P450 2B1 incubated for 2 min with 100 μM 15*R*- and 15*S*-HPETE. The identification of products was confirmed by GC−MS analyses (not shown). With 15*S*-HPETE, the 2B1 enzyme gives a product profile indistinguishable from that of phenobarbital-induced microsomes, with the formation of product **4** (major product) and products **7** and **8** (minor products). With 15*R*-HPETE, the enzyme forms a pattern of products that is intermediate between those of the control and the phenobarbital-induced microsomes. Products **1** and **6** are formed in approximately equal abundance with the purified 2B1 enzyme.

The rate of reaction of 15*S*-HPETE was determined by using a spectroscopic assay measuring the loss of the conjugated diene chromophore (decrease in absorbance at 235 nm). The initial rate of reaction of 50 μM 15*S*-HPETE with the P450 2B1 enzyme (1 μM) corresponded to a turnover number of approximately 50 nmol of product/nmol of enzyme/min.

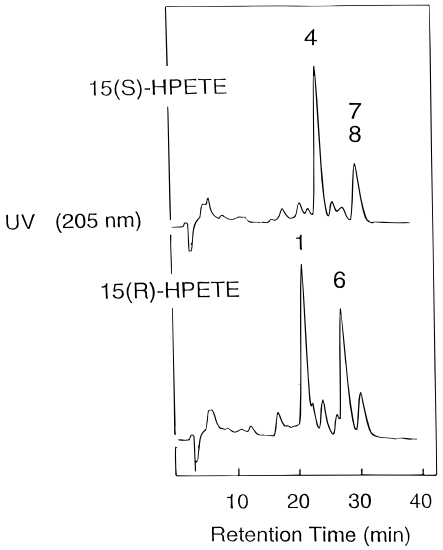


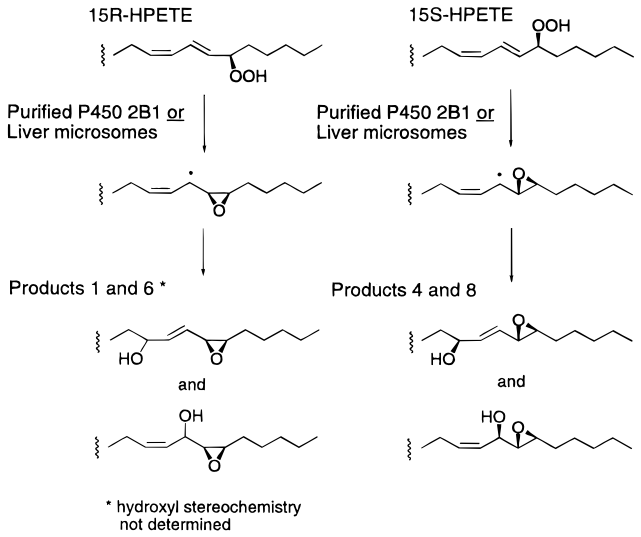
FIGURE 5: RP-HPLC analysis (UV detection at 205 nm) of products formed from 15*R*-HPETE and 15*S*-HPETE by purified cytochrome P450 2B1. The product profiles of 15*S*-HPETE (A) and 15*R*-HPETE (B) incubations are shown. The chromatographic conditions were similar to those of Figure 1, except that the mobile phase was changed to methanol/water/glacial acetic acid (70:30:0.01 by volume). In panel B, SP-HPLC analysis indicated that the first epoxy alcohol peak is mainly product **1**.

DISCUSSION

In mammalian systems, there are many reports of epoxy alcohol synthesis from fatty acid hydroperoxides, but little information on the potential enzymes involved. While epoxy alcohols are readily formed via nonenzymatic transformations, in general these reactions lack stereocontrol and they do not lead to the synthesis of the distinct diastereomers that are likely to be involved in cell signaling or other biological functions. The present study was prompted by our observation that a plant allene oxide synthase, a cytochrome P450, was capable of synthesizing specific epoxy alcohol diastereomers (Song et al., 1993a,b) and by the report of Weiss et al. (1987) that rat liver microsomes convert 15*S*-HPETE to epoxy alcohol derivatives. Our aims were to determine the possible stereoselectivity of the reactions in rat liver microsomes and to use a purified enzyme to examine the possible involvement of cytochrome P450. Besides the allene oxide synthase mentioned earlier, the only enzymes known to catalyze the synthesis of specific epoxy alcohols from fatty acid hydroperoxides are the L-1 lipoxygenase of soybeans (Garssen et al., 1976) and a hydroperoxide isomerase of the fungus *Saprolegnia parasitica* (Hamberg et al., 1986b).

Enzymatic Reaction in Rat Liver Microsomes. There were several indications that specific enzymatic processes are involved in epoxy alcohol synthesis in this system: (i) as noted by Weiss et al. (1987), metabolism is markedly inhibited by prior heat treatment of the microsomes (Figure 1); (ii) the product profiles distinctly differ from that of the nonenzymatic hematin reaction; (iii) different products were

Scheme 2



formed from 15*R*- and 15*S*-HPETE (whereas in the nonenzymatic hematin reaction the product profiles from 15*R*- and 15*S*-HPETE are identical); (iv) phenobarbital pretreatment of the animals markedly changes the profile of products; (v) in the experiment with 15*S*-[^{18}O]HPETE, the retention of the hydroperoxyl oxygens is very high for the main product, possibly reflecting a high degree of enzymatic control of the reaction (discussed later); and (vi) purified P450 enzyme (P450 2B1) produced a similar pattern of products.

Involvement of Multiple Catalysts. In the boiled microsomes, the profile in the epoxy alcohol region of the reversed-phase chromatogram looks like a miniature version of the hematin result, implying that some nonenzymatic formation normally contributes to the product profile, albeit most of the transformation is enzyme catalyzed. The contribution of different enzymes to the product profile is evident when comparing the product profiles formed by the liver microsomes and pure P450 2B1. Phenobarbital-induced liver microsomes and P450 2B1 give a similar product profile with 15*S*-HPETE as the substrate. However, with 15*R*-HPETE, the product profile of P450 2B1 is intermediate between the profiles with control liver microsomes and phenobarbital-induced microsomes (cf. Figures 4 and 5), indicating that P450 2B1 is not the the only P450 enzyme in the liver microsomes that catalyzes the transformation of 15-HPETE.

Structural Features of the Epoxy Alcohol Products. The epoxy alcohols formed from 15*S*-HPETE are mainly *trans*-epoxides, while 15*R*-HPETE is converted mainly to *cis*-epoxides (Scheme 2). By contrast, in the hematin reaction 15*R*- and 15*S*-HPETE gave identical patterns of products.

Further evidence of enzymatic control is seen in the configuration of the epoxy alcohol hydroxyls. Our finding of the enzymatic synthesis of predominantly a single diastereomer of the allylic epoxy alcohols, product **4** from 15*S*-HPETE and product **1** from 15*R*-HPETE, is unprecedented. Rat pineal gland is known to form predominantly allylic epoxy alcohols, with similar amounts of each diastereomer (Reynaud et al., 1994).

Both the rat liver microsomes and the P450 2B1 enzyme formed the α-hydroxy epoxy alcohols with control of the hydroxyl stereochemistry. This type of stereoselective biosynthesis has been seen before with various plant enzymes (Song et al., 1993b; Galliard et al., 1975; Garssen et al., 1976; Hamberg et al., 1986b). The results described here constitute the first report in an animal system.

Origin of Multiple Products. The results of incubations with pure P450 2B1 prove that several products are formed from a single enzyme. Although this might be ascribed to a stereoselective enzymatic reaction overlaid by a nonenzymatic component, the results with 15*R*-HPETE argue against this possibility. The point here is that the main products from 15*R*-HPETE are *cis*-epoxides, which constitute extremely minor components of a nonenzymatic reaction. By using the 2B1 enzyme, 15*R*-HPETE is converted to two main products: single diastereomers of an 11-hydroxy *cis*-epoxide and a 13-hydroxy *cis*-epoxide. It appears that the enzyme catalyzes the synthesis of the 14,15-*cis*-epoxide with high specificity, but it hydroxylates the intermediate at both the C-11 and C-13 positions (Scheme 2).

Abundance of Products and Hydroperoxide Oxygen Retention. Dix and Marnett (1985) reported the ^{18}O retentions in hematin-catalyzed formation of epoxy alcohols from 13-[^{18}O]-hydroperoxylinoleic acid, and in a separate experiment they also measured the incorporation of oxygen from $^{18}O_2$ into the epoxy alcohols. They found that, in the epoxy alcohol derivatives equivalent to the 13-hydroxy derivatives from 15-HPETE, there was a very high retention of both hydroperoxy oxygens (93%) and, in reasonable agreement with this result, a correspondingly low incorporation of oxygen from $^{18}O_2$ (18%). Their figures for an allylic epoxy alcohol (measured in a hydrolysis product) were 66% retention of 2-^{18}O from the hydroperoxide and 32% incorporation of oxygen from $^{18}O_2$ (Dix & Marnett, 1985). These results clearly indicate that a high retention of hydroperoxy oxygen can occur in a nonenzymatic reaction.

We measured the retention of ^{18}O from 15*S*-[15-^{18}O]-HPETE after metabolism in microsomes from phenobarbital-treated rats. The major product, an allylic epoxy alcohol (product **4**), retained 98% of the original hydroperoxy oxygens, while the minor diastereomer (product **5**) retained only 31%. Similarly, for the two 13-hydroxy 14,15-*trans*-epoxides (products **7** and **8**), the more abundant *threo* product showed a retention of 74% 2-^{18}O, compared to only 38% for the *erythro* isomer. Thus, formation of the more abundant diastereomers is associated with a high retention of the original hydroperoxide oxygens. This may reflect efficient hydroxylation with the original hydroperoxide oxygen in the enzyme active site. It is worth emphasizing again, however, that high retention of the hydroperoxy oxygens, per se, is not indicative of enzymatic control. Other points such as those listed earlier in this discussion also need to be considered.

Epoxy Alcohols and Allene Oxides. The potential connection between epoxy alcohol and allene oxide biosynthesis (indicated earlier in Scheme 1) naturally prompted us to look for evidence of allene oxide formation from 15*R*- and 15*S*-HPETE. As 15*S*-HPETE is a good substrate for the plant allene oxide synthases, we could prepare an authentic standard of the α-ketol hydrolysis product of the extremely unstable epoxide. Upon RP-HPLC this α-ketol chromatographs very close to epoxy alcohol product **6**, and upon SP-HPLC it elutes before any of the epoxy alcohols (Table 1, footnote a). The HPLC chromatograms were examined carefully for the appearance of the α-ketol, but it was not

detected in extracts of the microsomal incubations, nor as a product of the purified P450 2B1.

It has been noted previously that the allene oxide synthase is a P450 specialized for the metabolism of fatty acid hydroperoxides (Song et al., 1993a), and this enzyme certainly sets a striking contrast in rates of reaction in comparison to a liver microsomal P450. The flax seed allene oxide synthase can approach 1000 turnovers/s with 15*S*-HPETE as substrate, whereas the 2B1 enzyme reacted at ≈1 turnover/s under the same conditions. Nonetheless, a rate of 1 turnover/ is comparable to the rates for NADPH-supported reactions by P450 2B1.

There appears to be a common intermediate in epoxy alcohol and allene oxide synthesis (Scheme 1), and although the desaturation pathway leading to the allene oxide was not detected in our experiments, the precedent does exist for the catalysis of desaturation reactions by mammalian P450s (Nagata et al., 1986; Rettie et al., 1987; Korzekwa et al., 1990; Guengerich & Kim, 1991; Wang et al., 1991; Kassahun & Baillie, 1993). Future investigations can be directed toward the study of these P450s in the metabolism of hydroperoxy fatty acids.

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SUPPORTING INFORMATION AVAILABLE

Detailed descriptions of the GC−MS and ^{1}H NMR data used in the identification of hematin products and metabolites of 15*R*-HPETE microsome incubations (16 pages). Ordering information is given on any current masthead page.

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