Comparisons of Hydroperoxide Isomerase and Monooxygenase Activities of Cytochrome P450 for Conversions of Allylic Hydroperoxides and Alcohols to Epoxyalcohols and Diols: Probing Substrate Reorientation in the Active Site†

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Received May 4, 2001; Revised Manuscript Received July 25, 2001

ABSTRACT: Ferric cytochrome P450 (P450) catalyzes intramolecular rearrangements of hydroperoxides to diols by heterolytic scission of the O−O bond and insertion of the terminal oxygen atom into the resulting alcohol. The goals of this work were to further characterize the regio- and stereochemistry of P450 isomerase activity using allylic hydroperoxides and to compare these reactions with NADPH-supported monooxygenations of the corresponding alcohols. Microsomes from phenobarbital-treated rats or purified P450 2B1 catalyzed the conversions of several peroxyquinols, including 2-*tert*-butyl-4-hydroperoxy-4-methylcyclohexa-2,5-dien-1-one (BMPOOH) and its 2,6-dimethyl derivative (TMPOOH), to diols and to α,*β*-epoxyquinols by predominant (>93%) *cis* addition of oxygen to the least hindered double bond. Monooxygenation of the 4-hydroxy analogues (quinols) yielded identical *cis*-epoxyquinols, and both isomerization and monooxygenation reactions exhibited similar enantioselectivities (32−74% enantiomer excess). Regioselectivities were similar for BMPOOH and BMPOH (epoxyquinol:diol ratios of 5.8−7.6), but quite different for TMPOOH and TMPOH (ratios of 0.6 and 6.4, respectively). Bulky peroxyquinols and quinols derived from the A-rings of 17*β*-estradiol and estrone were utilized to further compare these reactions. Both estradiol derivatives underwent approximately equal amounts of 6*β*-hydroxylation and 1,2-epoxidation. The estrone derivatives also underwent 6*β*-hydroxylation, but only estrone quinol yielded a second product consistent with hydroxylation at position 16. The results support several conclusions. (i) Allylic hydroperoxides may be isomerized to α,*β*-epoxyalcohols by a heterolytic O−O cleavage mechanism with high stereoselectivity. (ii) Hydroperoxide isomerization is an efficient process relative to monooxygenation. (iii) Isomerase substrates remain in proximity to the P450 oxoferryl intermediate and are rapidly captured by the oxidant. Monooxygenase substrates, on the other hand, may bind to ferric P450 in multiple orientations and undergo more extensive substrate reorientation prior to oxidative attack.

Cytochrome P450 (P450)[1] catalyzes monooxygenations of exogenous and endogenous substrates by inserting one atom of dioxygen as outlined in Figure 1 (*a* and *b*) (*1*). The widely accepted mechanism for this process involves substrate binding in the distal heme cavity, enzymatic one-electron reduction of iron, and formation of a ferrous−dioxygen complex. The latter species undergoes another one-electron

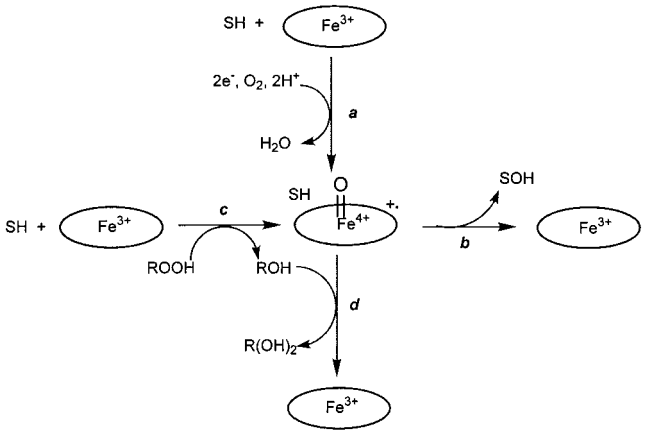

FIGURE 1: Scheme for monooxygenase (*a* + *b*), peroxygenase (*c* + *b*), and isomerase (*c* + *d*) activities of cytochrome P450 with substrate SH and hydroperoxide ROOH. The heme is represented by Fe centered in an ellipse.

† This work was supported by Grant CA41248 from the National Institutes of Health.

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[1] Abbreviations: P450, cytochrome P450; $(FeO)^{3+}$, putative oxoferryl−cation radical intermediate of P450; BHTOOH, 2,6-di-*tert*-butyl-4-hydroperoxy-4-methylcyclohexa-2,5-dien-1-one; BMPOOH, 2-*tert*-butyl-4-hydroperoxy-4-methylcyclohexa-2,5-dien-1-one; TMPOOH, 4-hydroperoxy-2,4,6-trimethylcyclohexa-2,5-dien-1-one; DMPOOH, 4-hydroperoxy-2,4-dimethylcyclohexa-2,5-dien-1-one; MPOOH, 4-hydroperoxy-4-methylcyclohexa-2,5-dien-1-one; BHTOH, BMPOH, TMPOH, DMPOH, and MPOH, 4-hydroxy analogues of the hydroperoxides; E1OOH, 10*β*-hydroperoxy derivative of estrone; E2OOH, 10*β*-hydroperoxy derivative of 17*β*-estradiol; E1OH and E2OH, 10*β*-hydroxy analogues of E1OOH and E2OOH, respectively; TMS, trimethylsilyl; PB microsomes, liver microsomes from phenobarbital-treated rats.

10.1021/bi010914d CCC: $20.00
Published on Web 08/30/2001

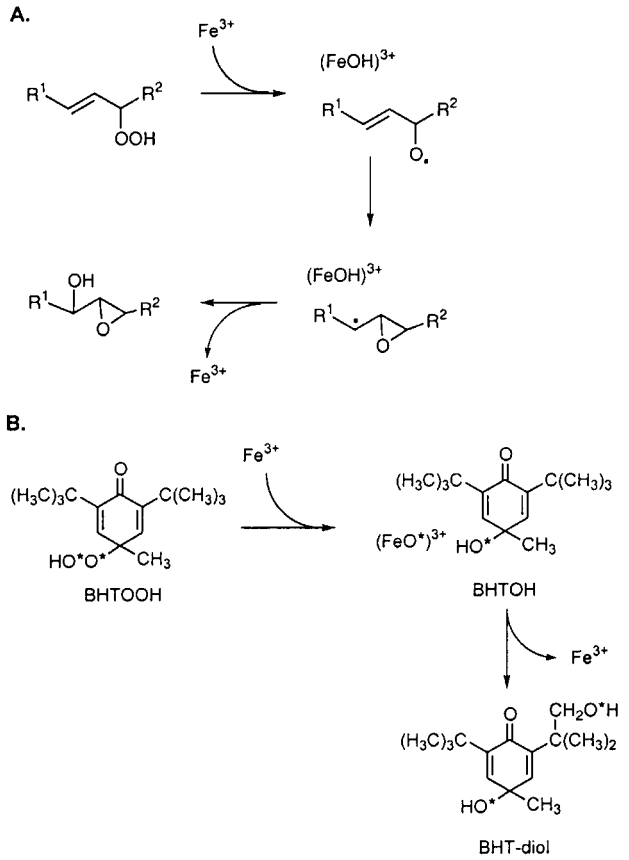


FIGURE 2: (A) Homolytic pathway for the isomerization of an allylic hydroperoxide to an α,*β*-epoxyalcohol and (B) heterolytic pathway for the isomerization of peroxyquinol BHTOOH to a diol where Fe^{3+} represents ferric P450. Asterisks denote ^{18}O atoms, and incubations were conducted with a mixture of unlabeled and ^{18}O-labeled peroxyquinol.

reduction followed by protonation and heterolysis of the O−O bond to form water and the P450 oxoferryl intermediate $(FeO)^{3+}$. The interaction of a hydroperoxide with ferric P450 also generates a heme complex (Figure 1, *c*) presumed to be identical to the intermediate formed during monooxygenation (*2*). Hydroperoxide-supported (i.e., peroxygenase) reactions yield products similar to those formed by reductase-supported monooxygenations, although differences in regio- and stereochemistry are frequently observed (*3*−*5*). Despite this drawback and the fact that results may be complicated by homolytic O−O bond cleavage followed by alkoxyl radical−substrate interactions and enzyme degradation (*6*−*8*), peroxygenase reactions have been utilized extensively to probe metabolic pathways and mechanisms of P450 monooxygenases.

Ferric P450 also catalyzes the rearrangement of hydroperoxides shown in Figure 1 (*c* and *d*). ^{18}O labeling demonstrates that this process is intramolecular with the terminal oxygen of the hydroperoxide inserted into the resulting alcohol before the latter migrates from the P450 active site. Examples include the conversions of cumyl hydroperoxide (*6*), 20-hydroperoxycholesterol (*9*), and 1-hydroperoxyhexane to vicinal diols (*10*). Hydroperoxide isomerization also may take place by homolytic O−O bond scission. For example, P450 2B1 catalyzes conversion of the lipoxygenase product of arachidonic acid, 15-hydroperoxyeicosa-5,8,11,13-tetraenoic acid (15-HPETE), to 13-hydroxy-14,15-epoxyeicosa-5,8,11-trienoic acid as outlined in Figure 2A (*11*). Isomerization of the endoperoxide prostaglandin H_2 by prostacyclin synthase and thromboxane synthase, both P450 enzymes, also involves homolysis of the O−O bond (*12*). The 1,4-peroxyquinol BHTOOH, a hydroperoxy derivative of the phenolic antioxidant BHT, is degraded by P450 2B1 along both heterolytic and homolytic pathways. The former results in diol formation by intramolecular rearrangement as demonstrated by the conversion of a mixture of BHTOOH and [$^{18}O_2$]BHTOOH to diols containing either two atoms of ^{16}O or two atoms of ^{18}O as outlined in Figure 2B (*13*); while homolysis generates an alkoxy radical leading to ring-enlarged and ring-contracted products (*14*), BHTOOH has served as a useful probe of factors that influence O−O bond cleavage by various heme proteins (*15*, *16*).

Peroxygenase and isomerase reactions of P450 have been demonstrated in rat hepatocytes (*17*, *18*), and in many cases, the latter are fast compared to monooxygenation (*9*, *13*, *19*). Despite the potential significance of isomerization in vivo and the value of this reaction as a mechanistic probe in vitro (*13*, *14*), little is known about the formation of products other than diols. In addition, products of hydroperoxide isomerization have not been extensively compared with those from monooxygenation of the corresponding alcohols, although the resulting information could provide additional insight into substrate movement in the presence of the oxoferryl intermediate. There is evidence (to be discussed later) that during monooxygenation, substrate reorientation and/or dissociation from the active site can occur after formation of the $(FeO)^{3+}$ (*20*−*22*), but during isomerization, substrates are captured by the oxo species before significant repositioning occurs (*9*, *10*, *13*).

The goals of this work were to further characterize P450 isomerase activity utilizing peroxyquinols with smaller alkyl substituents relative to BHTOOH. These bis-allylic hydroperoxides are reduced more efficiently by ferric P450 2B1 and exhibit higher ratios of heterolytic:homolytic O−O bond cleavage. In addition, the double bonds are susceptible to epoxidation so both α,*β*-epoxyalcohols and diols are formed, enabling detailed comparisons of regio- and stereochemistry for peroxyquinol isomerization with monooxygenation of the corresponding quinols (i.e., the 4-hydroxy analogues of peroxyquinols). Finally, peroxyquinols and quinols derived from the phenolic A-rings of estrone and 17*β*-estradiol (*23*, *24*) were investigated in an effort to compare the courses of isomerase and monooxygenase reactions for larger substrates having several possible sites for P450 attack.

MATERIALS AND METHODS

Chemicals and Analytical Procedures. Unless stated otherwise, chemicals were purchased from Aldrich (Milwaukee, WI) or Sigma (St. Louis, MO), and HPLC grade solvents were obtained from Fisher (Pittsburgh, PA). The HPLC separations were conducted on a Hewlett-Packard (Palo Alto, CA) 1090 system equipped with a photodiode array detector and utilizing a 4.6 mm × 250 mm Beckman Ultrasphere ODS column (Fullerton, CA) at a flow rate of 1.0 mL/min for analytical work, or a 10 mm × 250 mm Ultrasphere ODS column with a flow rate of 2.5 mL/min for preparative work. For the first 15 min, the mobile phase was held at a 35:65 or 18:82 acetonitrile:water ratio for BMPOOH- or TMPOOH-derived products, respectively,

followed by a linear gradient to 80% acetonitrile from 15 to 40 min. Products of DMPOOH and MPOOH were analyzed under similar conditions except the initial mobile phase was 10% acetonitrile in water. Steroid substrates and products were analyzed with a gradient of 10 to 30% acetonitrile in water over the course of 20 min and 30 to 90% acetonitrile from 20 to 30 min and held at 90% for an additional 5 min. The UV spectra were obtained during HPLC analysis. Proton NMR spectra were recorded in [^{2}H]chloroform (unless indicated otherwise) with a Varian 500 MHz INOVA spectrometer. The GC−MS analyses were conducted on a Hewlett-Packard 5988 mass spectrometer equipped with an Alltech (Deerfield, IL) 30 m × 0.25 mm SE-54 column held at 80 °C for 2 min and then heated to 200 °C at a rate of 6 °C/min or to 290 °C at a rate of 8 °C/min for TMS ethers of the steroid derivatives. The latter were prepared by heating in Sylon BFT (Supelco, Bellefonte, PA) at 110 °C for 1 h. Unless indicated otherwise, spectra were obtained under EI or CI conditions with isobutane at 0.5 Torr and a source temperature of 250 °C.

Peroxyquinols. The preparations of BMPOOH, TMPOOH, and DMPOOH were described previously (*16*, *25*, *26*). MPOOH was synthesized by photooxygenation (*25*, *27*). Briefly, a stream of O_2 was directed through a solution of 4-methylphenol (33 mmol) in 120 mL of methanol containing 25 mg of the sensitizer rose bengal with irradiation by a 275 W sunlamp for 8 h. Solvent was evaporated and the residue purified by flash chromatography on a column of silica gel with an ethyl acetate/hexane mixture (1:3) to yield a product with the following spectra: UV λ_{max} 229 nm; EI MS *m*/*z* (relative abundance) 108 (62%, M − O_2), 107 (100%, M − OOH), 79 (10%), 77 (17%); ^{1}H NMR δ 1.43 (s, 3H), 6.30 (2H, d, J = 10 Hz), 6.91 (2H, d, J = 10 Hz). Further evidence of the hydroperoxide was provided by the reduction of MPOOH to MPOH upon treatment with excess triphenylphosphine in methylene chloride. E1OOH also was prepared by photooxygenation of estrone (*28*) and purified by flash chromatography on silica gel with an ethyl acetate/dichloromethane mixture (3:7) followed by semipreparative HPLC to yield the following spectral data: UV λ_{max} 243 nm; EI MS *m*/*z* 302 (M^+); ^{1}H NMR (CD_3OD) δ 6.12 (1H, d, J = 1.8 Hz, H-4), 6.31 (1H, dd, J = 10, 1.8 Hz, H-2), 7.12 (1H, d, J = 10 Hz, H-1). E2OOH was prepared from estradiol and purified as described for E1OOH to yield the following spectra: UV λ_{max} 244 nm; EI MS *m*/*z* 304 (M^+); ^{1}H NMR δ 6.09 (1H, bs, H-4), 6.36 (1H, dd, J = 10, 2.0 Hz, H-2), 7.31 (1H, d, J = 10.5 Hz, H-1).

Quinols. The synthesis of TMPOH was reported previously (*25*). BMPOH was prepared by adding [bis(trifluoroacetoxy)-iodo]benzene (2.2 mmol) to a solution of 2-*tert*-butyl-4-methylphenol (1.8 mmol) in 8 mL of a tetrahydrofuran/water mixture (4:1) at 0 °C. The resulting solution was stirred for 1 h and then partitioned between water and diethyl ether. The organic layer was separated, the solvent evaporated, and the crude product purified by flash chromatography on silica gel with a methylene chloride/ethyl acetate mixture (95:5) to yield a white solid: UV λ_{max} 232 nm; EI MS *m*/*z* (relative abundance) 180 (27%, M^+), 165 (45%, M − 15), 147 (22%), 137 (100%), 123 (50%), 109 (18%), 107 (15%), 95 (16%); ^{1}H NMR δ 1.20 (s, 9H), 1.43 (s, 3H), 6.02 (1H, d, J = 9.8 Hz), 6.61 (1H, d, J = 3.2 Hz), 6.75 (1H, dd, J = 9.8, 3.2 Hz). DMPOH was prepared by treating a solution of 2,4-dimethylphenol (0.97 mmol) in 4 mL of a tetrahydrofuran/water mixture (4:1) with [bis(trifluoroacetoxy)iodo]benzene (1.1 mmol) as described for the synthesis of BMPOH. Purification by flash chromatography on silica gel with a dichloromethane/ethyl acetate mixture (82:18) yielded a white solid: UV λ_{max} 232 nm; EI MS *m*/*z* (relative abundance) 138 (28%, M^+), 123 (100%, M − 15), 110 (40%), 95 (58%); ^{1}H NMR δ 1.47 (3H, s), 1.88 (3H, s), 6.12 (1H, d, J = 9.5 Hz), 6.6 (1H, m), 6.85 (1H, dd, J = 10, 2.8 Hz). MPOH was prepared from 4-methylphenol by the procedure outlined for BMPOH except the reaction was carried out in a 5:2 tetrahydrofuran/water mixture and the product was purified by flash chromatography with an ethyl acetate/hexane mixture (1:2) and recrystalized from the same solvent to yield the product: UV λ_{max} 229 nm; EI MS *m*/*z* (relative abundance), 124 (27%, M^+), 109 (100%, M − 15), 96 (36%), 95 (15%), 81 (39%), 53 (11%); ^{1}H NMR δ 1.49 (3H, s), 6.14 (2H, d, J = 9.5 Hz), 6.89 (2H, d, J = 10 Hz). E1OH was prepared by treating a solution of estrone in 1 M sodium carbonate with a 3-fold excess of [bis(trifluoroacetoxy)iodo]benzene. The product was purified as described for E1OOH above to yield the following spectral data: UV λ_{max} 242 nm; EI MS *m*/*z* 286 (M^+); ^{1}H NMR δ 6.00 (1H, d, J = 1.8 Hz, H-4), 6.17 (1H, dd, J = 10.5, 1.8 Hz, H-2), 7.09 (1H, d, J = 10.5 Hz, H-1). E2OH was prepared and purified by the same procedures to yield the product: UV λ_{max} 243 nm; EI MS *m*/*z* 288 (M^+); ^{1}H NMR δ 5.98 (1H, bs, H-4), 6.18 (1H, dd, J = 10, 2.1 Hz, H-2), 7.04 (1H, d, J = 10 Hz, H-1).

Epoxyquinols. *cis*-BMP-epoxyquinol was prepared by dissolving 2-*tert*-butyl-4-methylphenol (1.60 mmol) and potassium *tert*-butoxide (1.60 g) in 25 mL of hexamethylphosphoramide and bubbling oxygen gas through the stirred solution at 25 °C for 6 h (*29*). The reaction was quenched with an ice/water mixture and the product purified by flash chromatography on silica gel using a dichloromethane/ethyl acetate mixture (98:2) to yield a white solid yielding a single peak by HPLC and GC−MS and the following spectra: UV λ_{max} 242 nm; CI MS *m*/*z* (relative abundance) 197 (100%, MH^+), 182 (10%, M − CH_3), 179 (12%, MH − H_2O), 164 (15%, MH − CH_3 − H_2O); ^{1}H NMR δ 1.17 (9H, s), 1.43 (3H, s), 2.33 (s, OH), 3.48 (1H, d, J = 4.0 Hz), 3.60 (1H, dd, J = 4.0, 2.7 Hz), 6.21 (1H, d, J = 3 Hz). This compound also was prepared by dropwise addition of H_2O_2 (40 mL of a 50% aqueous solution) to BMPOH (10 mg) and anhydrous sodium carbonate (8 mg) in 2 mL of ethanol as described previously (*30*). The resulting mixture was stirred at 40 °C for 5 h, diluted with water, and extracted into ethyl ether. The solvent was evaporated and the product purified as above to yield 4.7 mg of *cis*-BMP-epoxyquinol. *cis*/*trans*-BMP-epoxyquinols were prepared from the 4-*O*-methyl derivative of BMPOH obtained by treating a solution of the quinol (0.67 mmol) containing methyl iodide (6.7 mmol) in 10 mL of DMSO with anhydrous KOH (1.0 mmol) and stirring at 25 °C for 1 h. The solution was then diluted with saturated aqueous sodium chloride and the product extracted into diethyl ether. The solvent was evaporated and the product eluted through a column of silica gel with methylene chloride to yield $BMPOCH_3$ as a white solid: UV λ_{max} 230 nm; EI MS *m*/*z* (relative abundance) 194 (23%, M^+), 179 (100%, M − CH_3), 151 (86%), 121 (80%). The epoxidation of $BMPOCH_3$ was carried out with H_2O_2 as described for

BMPOH and the crude product purified by semipreparative HPLC using 60% acetonitrile in water to yield a *cis*/*trans* epoxide mixture. This material eluted as two peaks by GC−MS analysis in a 1:1 ratio and produced spectra consistent with epimeric epoxides of $BMPOCH_3$ each yielding MS ions at *m*/*z* 210 (M^+), 195 ($M - CH_3$), 181, 177, 139, and 109. The mixture of $BMPOCH_3$ epoxides (2.50 μmol in 25 μL of DMSO) was O-demethylated enzymatically by incubating with PB-microsomes (20 nmol of P450 in a total volume of 10 mL) as described below. Products were extracted from the reaction mixture with ethyl acetate and analyzed by HPLC and GC−MS, giving two (essentially) equal size peaks. The first peak in both cases exhibited the same chromatographic and spectral properties as *cis*-BMP-epoxyquinol. The second peak produced the same UV spectrum and EI MS ions at *m*/*z* (relative abundance) 163 (16%, $M - CH_3 - H_2O$), 153 (62%), 125 (39%), 111 (100%), and 109 (83%), consistent with *trans*-BMP-epoxyquinol.

Syntheses of *cis*- and *trans*-TMP-epoxyquinols from TMPOH were carried out using dimethyldioxirane prepared in situ (*31*, *32*). A typical reaction involved preparation of a mixture of the quinol (1 mmol) in 7 mL of an acetone/water mixture (3:4) containing 1 g of $NaHCO_3$. This mixture was cooled in an ice bath and 5.8 mmol of potassium peroxymonosulfate added slowly with vigorous stirring. The progress of the reaction was monitored by HPLC with the epimeric products eluting at 8.8 and 11.7 min and generating identical UV spectra ($\lambda_{max} = 244$ nm). GC−MS analysis of the reaction mixture yielded chromatographic peaks at 9.7 and 10.9 min for the these epoxyquinols. The epimeric mixture was isolated by extraction with diethyl ether; isomers were separated by flash chromatography on silica gel with a dichloromethane/ethyl ether mixture (9:1) and recrystallized from hexane. Spectral data for *cis*-TMP-epoxyquinol are as follows: EI MS *m*/*z* (relative abundance) 168 (1%, M^+), 153 (10%, $M - 15$), 139 (64%), 125 (100%), 123 (15%), 111 (14%), 97 (28%), 85 (31%); ^{1}H NMR δ 1.44 (3H, s), 1.52 (3H, s), 1.80 (3H, d, $J = 1.2$ Hz), 3.46 (1H, d, $J = 3.0$ Hz), 6.20 (1H, dd, $J = 3.0$, 1.2 Hz). Spectral data for *trans*-TMP-epoxyquinol are as follows: EI MS *m*/*z* (relative abundance) 168 (2%, M^+), 153 (13%), 139 (54%), 125 (100%), 123 (19%), 111 (23%), 97 (31%), 85 (29%); ^{1}H NMR δ 1.51 (3H, s), 1.52 (3H, s), 1.84 (3H, s), 3.45 (1H, d, $J = 3.0$ Hz), 6.20 (1H, d, $J = 3.0$ Hz).

The syntheses of *cis*- and *trans*-6-methyl-DMP-5,6-epoxyquinols and *cis*-2-methyl-DMP-5,6-epoxyquinol were conducted by treating DMPOH as described for the preparation of TMP-epoxyquinol. The epimers of 6-methyl-DMP-5,6-epoxyquinol (UV $\lambda_{max} = 229$ nm) eluted via GC−MS at 8.2 and 10.7 min for *cis* and *trans* isomers, respectively, and *cis*-2-methyl-DMP-5,6-epoxyquinol eluted at 9.6 min. These isomers were separated and purified by silica gel flash chromatography using a methylene chloride/ethyl acetate mixture (3:1). Spectral data for *cis*-6-methyl-DMP-5,6-epoxyquinol are as follows: EI MS *m*/*z* (relative abundance) 154 (3%, M^+), 139 (4%), 125 (40%), 111 (100%), 83 (23%); ^{1}H NMR δ 1.47 (3H, s), 1.51 (3H, s), 3.47 (1H, d, $J = 2.9$ Hz), 5.83 (1H, d, $J = 10$ Hz), 6.42 (1H, dd, $J = 10.7$, 2.9 Hz). Spectra for *trans*-6-methyl-DMP-5,6-epoxyquinol are as follows: EI MS *m*/*z* (relative abundance) 139 (68%, $M - CH_3$), 125 (67%), 111 (100%), 69 (40%); ^{1}H NMR δ 1.51 (3H, s), 1.56 (3H, s), 3.46 (1H, d, $J = 3.0$ Hz), 5.92 (1H, d, $J = 11$ Hz), 6.42 (1H, dd, $J = 11$, 3.0 Hz). The spectral data for *cis*-2-methyl-DMP-5,6-epoxyquinol (no *trans* isomer was detected) are as follows: UV λ_{max} 244 nm; EI MS *m*/*z* (relative abundance) 154 (8%, M^+), 139 (38%), 125 (47%), 111 (100%), 83 (27%); ^{1}H NMR δ 1.55 (3H, s), 1.82 (3H, s), 3.50 (1H, m), 3.60 (1H, m), 6.24 (1H, m).

The preparation of *cis*/*trans*-MP-epoxyquinols was carried out by treating MPOH with oxone as described for the synthesis of TMP-epoxyquinols. The products were separated by HPLC with retention times of 5.2 (*cis*) and 6.6 min (*trans*), and the GC−MS retention times were 8.0 (*cis*) and 9.4 min (*trans*). The epimers were purified by flash chromatography using a dichloromethane/ethyl acetate mixture (3:1) to give *cis*-MP-epoxyquinol: UV λ_{max} 230 nm; EI MS *m*/*z* (relative abundance) 140 (11%, M^+), 125 (19%, M − methyl), 111 (54%), 97 (100%), 71 (17%); ^{1}H NMR δ 1.49 (3H, s), 2.43 (s, OH), 3.51 (1H, ddd, $J = 3.9$, 2.0, 1.0 Hz), 3.64 (1H, t, $J = 2.9$ Hz), 5.82 (1H, dd, $J = 9.8$, 2.0 Hz), 6.48 (1H, dd, $J = 10.7$, 2.9 Hz). Data for *trans*-MP-epoxyquinol are as follows: UV λ_{max} 229 nm; EI MS *m*/*z* (relative abundance) 140 (6%), 125 (18%), 111 (29%), 97 (100%), 71 (11%); ^{1}H NMR δ 1.60 (3H, s), 1.86 (s, OH), 3.49 (1H, m), 3.62 (1H, m), 5.92 (1H, dd, $J = 10$, 1.5 Hz), 6.48 (1H, dd, $J = 10$, 2.9 Hz).

Microsomes and Incubations. Male Spraque-Dawley rats (180−220 g) purchased from Sasco (Omaha, NE) were treated with phenobarbital as described previously (*14*) or pyrazole (200 mg/kg in saline, ip) for 3 days and killed on day 4. Livers were removed, microsomes prepared, and the concentrations of protein and P450 determined as described previously (*16*). Induction of P450 2B1 or 2E1 in PB microsomes or microsomes from pyrazole-treated rats, respectively, was verified with the substrates 7-pentoxyresorufin (*33*) or *p*-nitrophenol (*34*). Incubations were conducted by adding 10 μL of a 100 mM solution of the substrate dissolved in DMSO to 4 nmol of P450 in pH 7.4 phosphate buffer (50 mM) in a total volume of 2 mL with shaking at 37 °C for 10 min. Incubations with purified P450 2B1 obtained from M. A. Correia (University of California, San Francisco, CA) contained 0.2 nmol of enzyme in a volume of 1 mL and did not contain P450 reductase. With quinols as substrates, incubations contained microsomes (as above) and an NADPH-generating system (*16*) and were carried out for 30 min. Reactions were terminated by the addition of 2 volumes of ice-cold diethyl ether containing 10 μL of a 100 mM solution of either 2,4-di-*tert*-buylphenol or 2,4,6-trimethylphenol as an internal standard. The organic layer was separated and the aqueous layer extracted two additional times with diethyl ether; the extracts were combined, and the solvent was evaporated at 30 °C under a stream of nitrogen. The resulting residue was dissolved in 100 μL of acetonitrile for HPLC analysis.

Product Identification. Extracts were analyzed by HPLC as described above and compound identities based on comparisons of retention data, UV spectra, and mass spectra with known standards. Quantitation was performed by measuring product:internal standard HPLC peak area ratios and converting them to nanomolar amounts using a calibration curve or response factor derived for each compound (*16*). The stereochemical composition of epoxyquinols was determined by GC−MS, with isomers separated on a 30 m ×

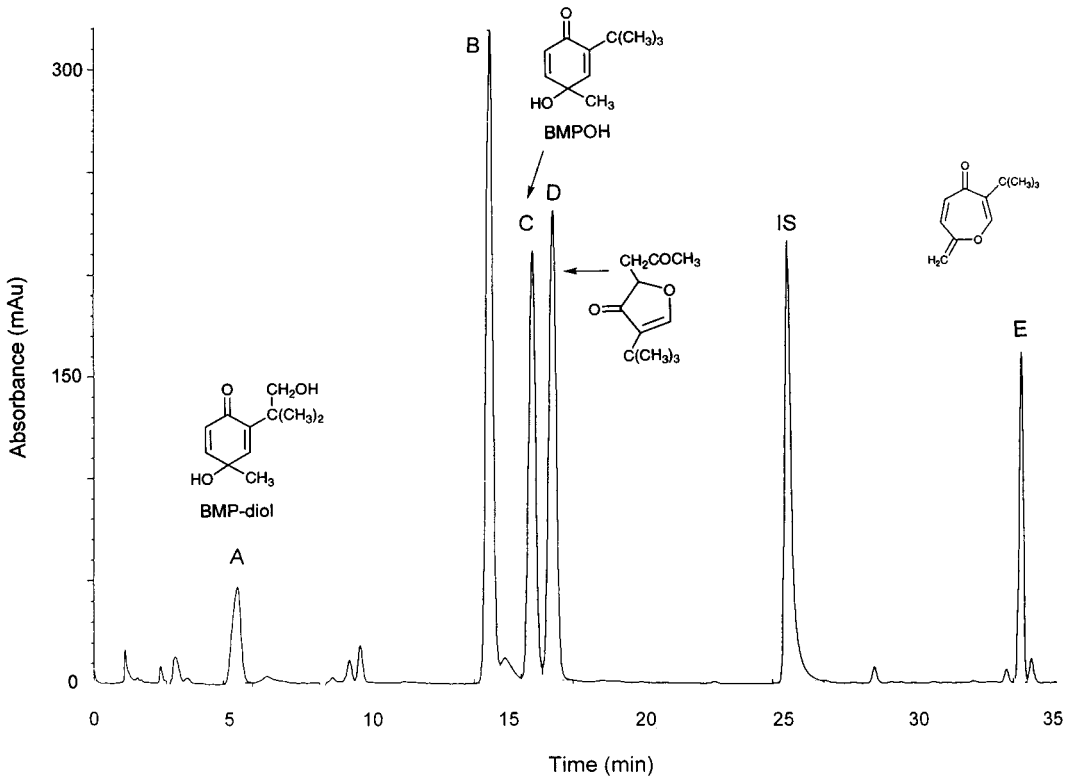


FIGURE 3: HPLC analysis over the range of 230−290 nm of BMPOOH-derived products formed by incubation with PB microsomes at 37 °C as described in Materials and Methods. The substrate (retention time of 19.4 min) was completely consumed. Incubations conducted at 25 °C for 10 min with 0.4 nmol of P450 2B1 contained approxomately 80% unreacted substrate along with the same products formed with microsomes. Identification of BMPOH (peak C) was based on comparisons of ^{1}H NMR, MS, and UV spectra with those of a standard, and the structures of BMP-diol (peak A) and rearranged products D and E are based on comparisons of spectral data with those of the analogous products from BHTOOH described in detail in refs *13* and *14*. The internal standard (IS) is 2,4,6-trimethylphenol.

0.25 mm Advanced Separation Technologies (Whippany, NY) β-cyclodextrin type B-PH chiral phase column using an injector split ratio of 100:1 and the following temperature program: BMP-epoxyquinols heated from 90 °C at a rate of 0.2 °C/min, TMP-epoxyquinols heated from 80 °C at a rate of 2 °C/min, DMP-epoxyquinols heated from 60 °C at a rate of 1 °C/min, and MP-epoxyquinols heated from 80 °C at a rate of 2 °C/min. Compounds eluting from the column were monitored by MS in the selected ion mode and peak area ratios compared. The following ions were monitored: BMP-epoxyquinols, *m*/*z* 153 and 111; TMP-epoxyquinols, *m*/*z* 139 and 125; DMP-epoxyquinols, *m*/*z* 139, 125, and 111; and MP-epoxyquinols, *m*/*z* 111 and 97.

^{18}O Labeling Experiments. The preparation of [$^{18}O_2$]-BMPOOH containing ^{18}O in the peroxy group involved preparation of a mixture of 2-*tert*-butyl-4-methylphenol (3.4 mmol) in 20 mL of ethanol containing anhydrous potassium carbonate (50 mg) and removal of dissolved O_2 by subjecting the solution to five freeze−thaw cycles under high vacuum. While the solution was frozen, 100 mL of $^{18}O_2$ gas (97−98% isotopically pure, Cambridge Isotope Laboratories, Woburn, MA) was introduced and the sealed flask stored at 5 °C for 18 h. The reaction mixture was then diluted by addition of several volumes of water, and the products were isolated by extraction with ethyl ether and purified by preparative HPLC. The isotopic composition was determined by MS analysis on a Hewlett-Packard 5988 instrument equipped with a thermospray ion source operated at 276 °C in the filament-on mode and with an acetonitrile/water mixture (2:3) flowing at a rate of 1.0 mL/min. This material was mixed with sufficient unlabeled peroxyquinol to produce a [$^{18}O_2$]BMPOOH/[$^{18}O^{16}O$]BMPOOH/[$^{16}O_2$]BMPOOH mixture with a 44.7:6.1:49.2 composition determined by thermospray MS analysis. This substrate was incubated with PB microsomes; products were isolated as described above, and the isotope content of BMP-diol and *cis*-BMP-epoxyquinol was determined by CI GC−MS analysis.

RESULTS

Epoxyquinol Formation. BMPOOH was efficiently converted by PB microsomes to products expected from both heterolysis (Figure 3, peaks A and C) and homolysis of the O−O bond (peaks D and E) as described for BHTOOH (*13*, *14*). The structure of the major product (peak B), however, is different than any of the BHTOOH-derived products, displaying a UV λ_{max} at 242 nm (compared to 232 nm for the substrate) and a molecular ion at *m*/*z* 196 (equivalent to the substrate). Incubation of a mixture of BMPOOH and [$^{18}O_2$]BMPOOH followed by MS analysis of both BMP-diol and the unidentified compound demonstrated retention of both hydroperoxide oxygens (Table 1). This finding is consistent with intramolecular transfer of the terminal oxygen of the hydroperoxide to the resulting quinol before it migrates out of the active site.

The combination of chromatographic, spectral, and isotope data suggests that BMPOOH was isomerized to an epoxyquinol by either of the pathways shown in Figure 4A. The ^{1}H NMR data are consistent with published spectra for the *cis*-5,6-epoxy-4-hydroxy structures shown in Table 2 (*29*, *35*), and confirmation was obtained through synthesis. This

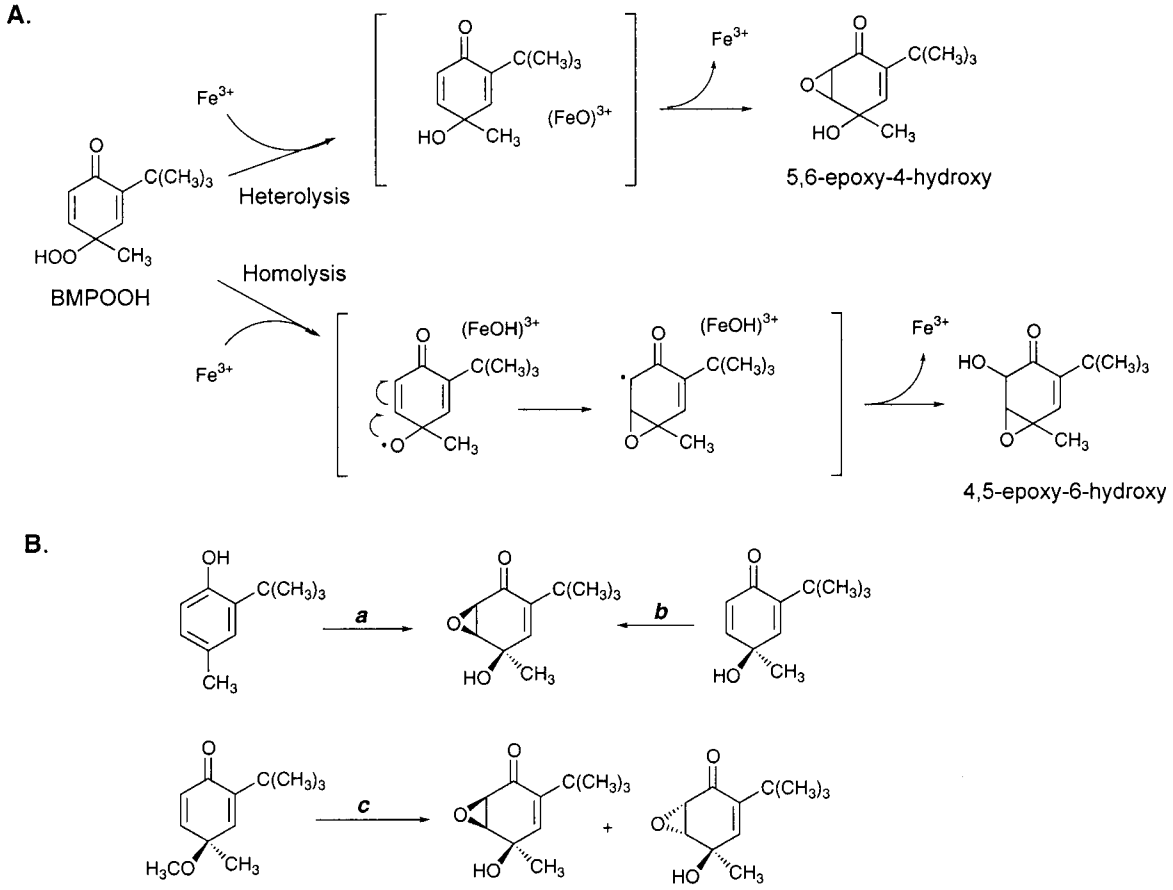


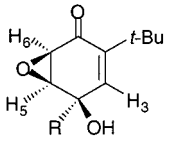
FIGURE 4: (A) Alternative pathways for the conversion of BMPOOH to an epoxyquinol by ferric P450 (Fe^{3+}). (B) Summary of synthetic reactions. (*a*) BMP treated with O_2 in the presence of potassium *tert*-butoxide generates the *cis*-epoxyquinol. (*b*) BMPOH is converted to the *cis*-epoxyquinol by H_2O_2 and sodium carbonate. (*c*) The 4-methoxy derivative of BMPOH treated as in reaction *b* followed by enzymatic O-demethylation led to a mixture of *cis* and *trans* products.

Table 1: Isotope Content of Products Formed from a Mixture of BMPOOH and[$^{18}O_2$]BMPOOH[a]

	% isotope composition		
compound	^{16}O, ^{16}O	^{16}O, ^{18}O	^{18}O, ^{18}O
BMPOOH	49.2	6.1	44.7
BMP-diol	50.5	6.8	42.7
unidentified (peak B)	51.5	5.2	43.3

[a] The substrate mixture was incubated with PB microsomes, and products were isolated and analyzed by MS as described in Materials and Methods.

Table 2: ^1H NMR Data for *cis*-Epoxyquinols



	δ (ppm)[a]			J (Hz)	
R	H_3	H_5	H_6	$H_{3,5}$	$H_{5,6}$
H[b]	6.23	3.81	3.48	–	–
$C(CH_3)_3$[c]	6.08	3.70	3.46	3	4
CH_3[d]	6.21	3.60	3.49	2.7	4.1

[a] Spectra recorded as described in Materials and Methods. [b] From ref *30*; coupling constants were not reported. [c] From ref *24*. [d] From the work presented here.

procedure (shown as conversion *a* of Figure 4B) had been developed previously for converting 2,6-di-*tert*-butylphenol to the corresponding epoxyquinol exhibiting a *cis* relationship of epoxy and hydroxy groups (*29*).

To substantiate the specificity of the enzymatic reaction for *cis*- versus *trans*-epoxyquinol, a mixture of the epimers was prepared. Oxidation of BMPOH with alkaline H_2O_2 (Figure 4B, conversion *b*) produced only the *cis* isomer, but carrying out this reaction on the 4-methoxy derivative followed by O-demethylation (Figure 4B, conversion *c*) yielded a mixture of *cis*- and *trans*-BMP-epoxyquinols. The *trans* isomer coeluted with BMPOH on HPLC, but both isomers were easily resolved from each other and from other components by GC−MS. In addition, the epimers were further separated into enantiomers with a chiral phase GC column as shown in Figure 5. Chiral analysis of the enzymatic products of BMPOOH confirmed very high selectivity for *cis*- versus *trans*-BMP-epoxyquinol and revealed enantioselectivity in the formation of both epimers.

With purified P450 2B1, the relative amounts of BMPOH, BMP-epoxyquinol, and BMP-diol formed were 22, 71, and 7%, respectively, in close agreement with the microsomal incubations summarized in Table 3. BMPOOH also was converted to the epoxyquinol by microsomes from pyrazole-treated rats, but no epoxyquinol was generated in incubations with boiled microsomes or with metmyoglobin as the heme source.

Influence of Alkyl Substituents on Peroxyquinol Reactivity. BMPOOH was reduced most rapidly, whereas peroxyquinols containing a hydrogen or methyl group at positions 2 and 6

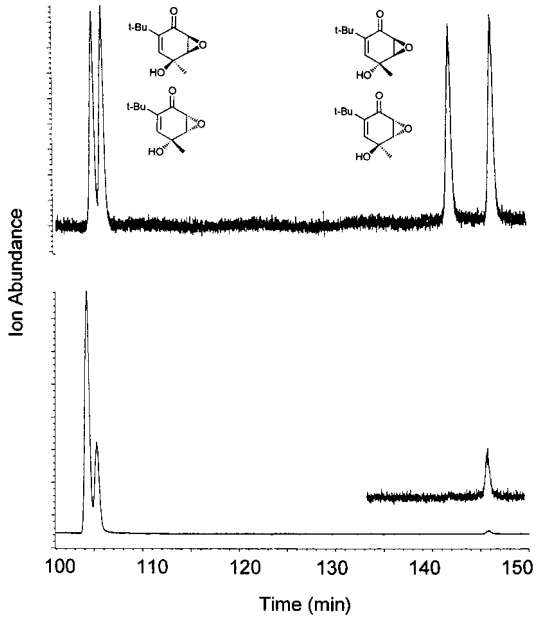


FIGURE 5: Analysis of BMP-epoxyquinol stereoisomers by chiral phase GC−MS. The synthesized epoxyquinol (top chromatogram) was resolved into its four stereoisomers with the *cis*-epoxyquinol enantiomers eluting first and the *trans*-epoxyquinol enantiomers eluting last. Chiral analysis of the product mixture from BMPOOH isomerization (bottom chromatogram) demonstrates nearly total specificity for the *cis* epimer and substantial enantioselectivity. A trace amount of the *trans* epimer also was formed as shown in the inset (abundance scale expanded 10-fold) with very high enantioselectivity.

Table 3: Product Distributions for Variously Substituted Peroxyquinols

	product distribution[a] (%)		
peroxyquinol	quinol	epoxyquinol	diol
BMPOOH	23	68	9
TMPOOH	12	34	54
DMPOOH	42	58[b]	nd[c]
MPOOH	77	23	−

[a] Incubations were conducted with PB microsomes, and products were analyzed as described in Materials and Methods. [b] The 2-methylepoxyquinol:6-methylepoxyquinol ratio was 6.2. [c] No diol detected (<0.5%).

of the cyclohexadienone ring (Figure 6) were consumed at rates that decreased in the order of decreasing alkyl substitution (Table 3). In contrast to BMPOOH which underwent significant homolysis (Figure 3), <10% of the products from TMPOOH were derived from homolytic O−O bond cleavage and no homolytic products were detected from DMPOOH and MPOOH. Isomerization efficiency, i.e., the percentage of quinol undergoing epoxidation or hydroxylation, was highest for BMPOOH and TMPOOH and lowest for MPOOH. No diol was detected with DMPOOH, but two regioisomeric epoxyquinols were formed through oxidation of the 2,3- or 5,6-double bond, with the latter predominating by a factor of ∼6.

Comparison of the Isomerase and Monooxygenase Reactions of P450. The diols and epoxyquinols also were formed under monooxygenase conditions with quinols as substrates, although these reactions were considerably slower than peroxyquinol isomerization. For example, the conversions of BMPOOH and TMPOOH to epoxyquinols were complete

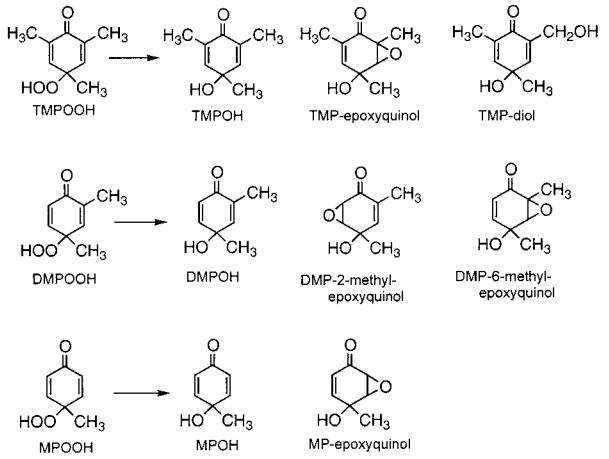


FIGURE 6: Structures of additional peroxyquinols that were investigated, the corresponding quinols, and the products of isomerization.

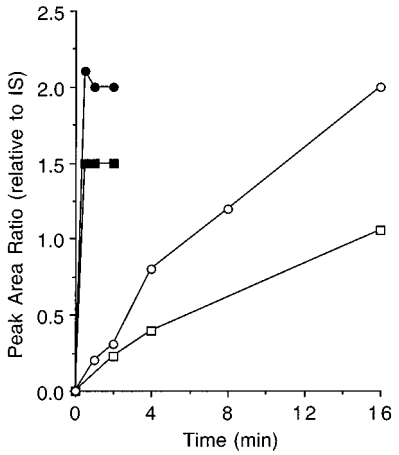


FIGURE 7: Time courses for epoxyquinol formation via isomerase and monooxygenase reactions. BMPOOH (●), BMPOH (○), TMPOOH (■), and TMPOH (□) were incubated (0.5 mM) with PB microsomes (2 nmol of P450/mL) at 37 °C containing NADPH for quinol incubations, and the extent of epoxyquinol formation was determined at the times shown. Values are HPLC peak area ratios for products relative to the internal standard 2,6-di-*tert*-butylphenol.

within 0.5 min at 37 °C, but at least 16 min was required to achieve comparable product levels in the reductase-supported reaction (Figure 7). The regioselectivity of BMPOH monooxygenation by PB microsomes was similar to that of BMPOOH isomerization, with epoxidation predominating over hydroxylation as shown in Table 4. On the other hand, TMPOOH isomerization produced substantially different regioselectivity compared to the NADPH-supported oxidation of TMPOH, with the former yielding mainly TMP-epoxyquinol and the latter TMP-diol. Both DMPOOH and DMPOH underwent epoxidation predominantly at the unsubstituted 5,6-double bond, and no diol was detected from either reaction. Analysis of all epoxyquinols by chiral phase GC−MS demonstrated that one enantiomer was formed preferentially in all cases, and the same enantiomer predominated in both isomerization and monooxygenation. Enantioselectivty was generally higher in the former reaction.

Reactions of Estrogen-Derived Peroxyquinols and Quinols. To investigate isomerization reactions with larger substrates having more potential sites for oxidative attack, the phenolic A-rings of 17β-estradiol and estrone were oxidized to

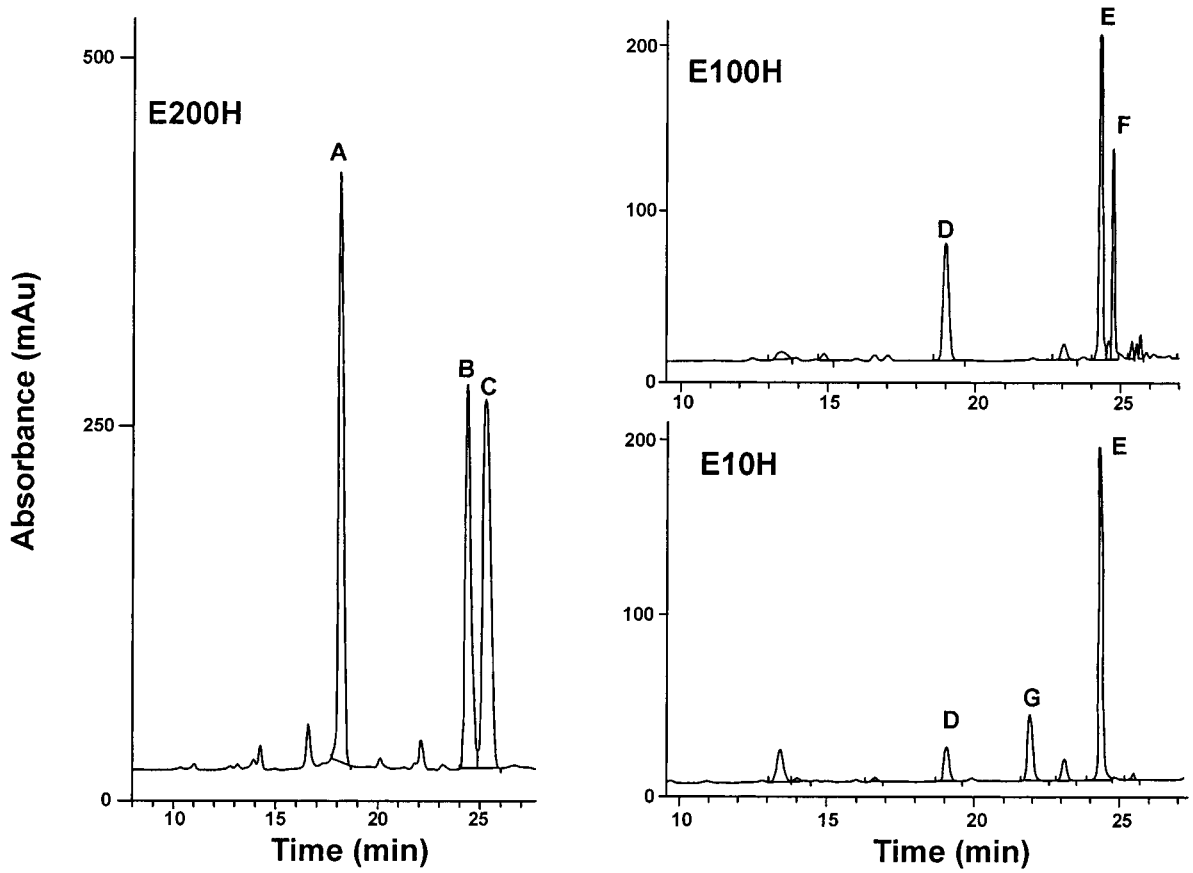


FIGURE 8: Chromatograms from HPLC analysis of products from estrogen derivatives (0.5 mM) formed with PB microsomes (2 nmol of P450/mL) at 37 °C. Products from E2OOH are (A) 6β-hydroxy-E2OH, (B) E2OH, and (C) E2-epoxyquinol. E1OOH products are (D) 6β-hydroxy-E1OH, (E) E1OH, and (F) unreacted E1OOH. Products formed from microsomal incubations of E1OH containing NADPH are (D) 6β-hydroxy-E1OH, (G) 16-hydroxy-E1OH, and (E) unreacted E1OH.

Table 4: Regio- and Stereoselectivity of Isomerase and Monooxygenase Reactions

substrate[a]	product ratio (epoxyquinol:diol)	epimer composition (% *cis*)	enantiomer excess[b] (%)
BMPOOH	7.6	99.0	48
BMPOH	5.8	97.5	34
TMPOOH	0.6	98.4	56
TMPOH	6.4	99.7	32
DMPOOH	6.2[c]	>99.8[d]	62[e]
DMPOH	15[c]	>99.8[d]	36[e]
MPOOH	–	92.9	74
MPOH	–	98.8	68

[a] Incubations were conducted with microsomes in the absence (peroxyquinols) or presence (quinols) of NADPH as described in Materials and Methods. [b] Enantiomer excess (*cis* epimer) calculated as the percentage excess of the major enantiomer over the racemate. The same enantiomer predominated in both isomerase and monooxygenase reactions. [c] No diol was detected for DMPOOH or DMPOH. Values are ratios of DMP-6-methyl-5,6-epoxyquinol to DMP-2-methyl-5,6-epoxyquinol. [d] No *trans* epimer detected. [e] Only the major regioisomer (DMP-2-methyl-5,6-epoxyquinol) was examined.

peroxyquinols with the hydroperoxy group in position 10β as shown in Figure 9 (*28*). E2OOH was extensively metabolized by PB microsomes with the formation of E2OH and two other major products as shown in Figure 8. The compound eluting at 18.2 min was determined to be a hydroxylated derivative of E2OH by MS analysis, with an MH^+ at *m*/*z* 305 (relative to *m*/*z* 289 for the quinol). The presence of three hydroxyl groups was demonstrated by MS analysis of the tri-TMS ether derivative. Data summarized in Table 5 support hydroxylation at position 6 of E2OH. The UV absorbance was shifted relative to E2OH, and the ^{1}H NMR spectrum differed from that of the quinol by the presence of a triplet at 4.60 ppm that is consistent with an allylic hydroxymethylene (CHOH) proton. Confirmation of the structure as 6β-hydroxy-E2OH (Figure 9) was accomplished by chemical oxidation of a standard sample of 6β-hydroxy-17β-estradiol to the corresponding quinol.

From spectral data (summarized in Table 5), the other main product of E2OOH eluting at 25.3 min in Figure 8 was identified as E2-epoxyquinol resulting from epoxidation of the 1,2-double bond of E2OH. MS analysis indicated the presence of an additional oxygen relative to E2OH, but trimethylsilylation demonstrated only two free hydroxyl groups. The UV λ_{max} was shifted ~10 nm to a longer wavelength relative to that of E2OH, analogous to the epoxyquinols discussed above. Epoxidation of the 1,2-double bond of E2OH was confirmed by ^{1}H NMR as the H-1 and H-2 vinyl proton signals were replaced by a doublet at 3.89 ppm and a doublet of doublets centered at 3.47 ppm. A β-configuration for the A-ring epoxide was assumed on the basis of the selective formation of *cis*-epoxyquinols with other peroxyquinols. The NADPH-supported metabolism of E2OH with PB microsomes yielded identical products in approximately the same ratio obtained by isomerization.

Reduction of E1OOH by PB microsomes generated E1OH and only one other major product identified as 6β-hydroxy-

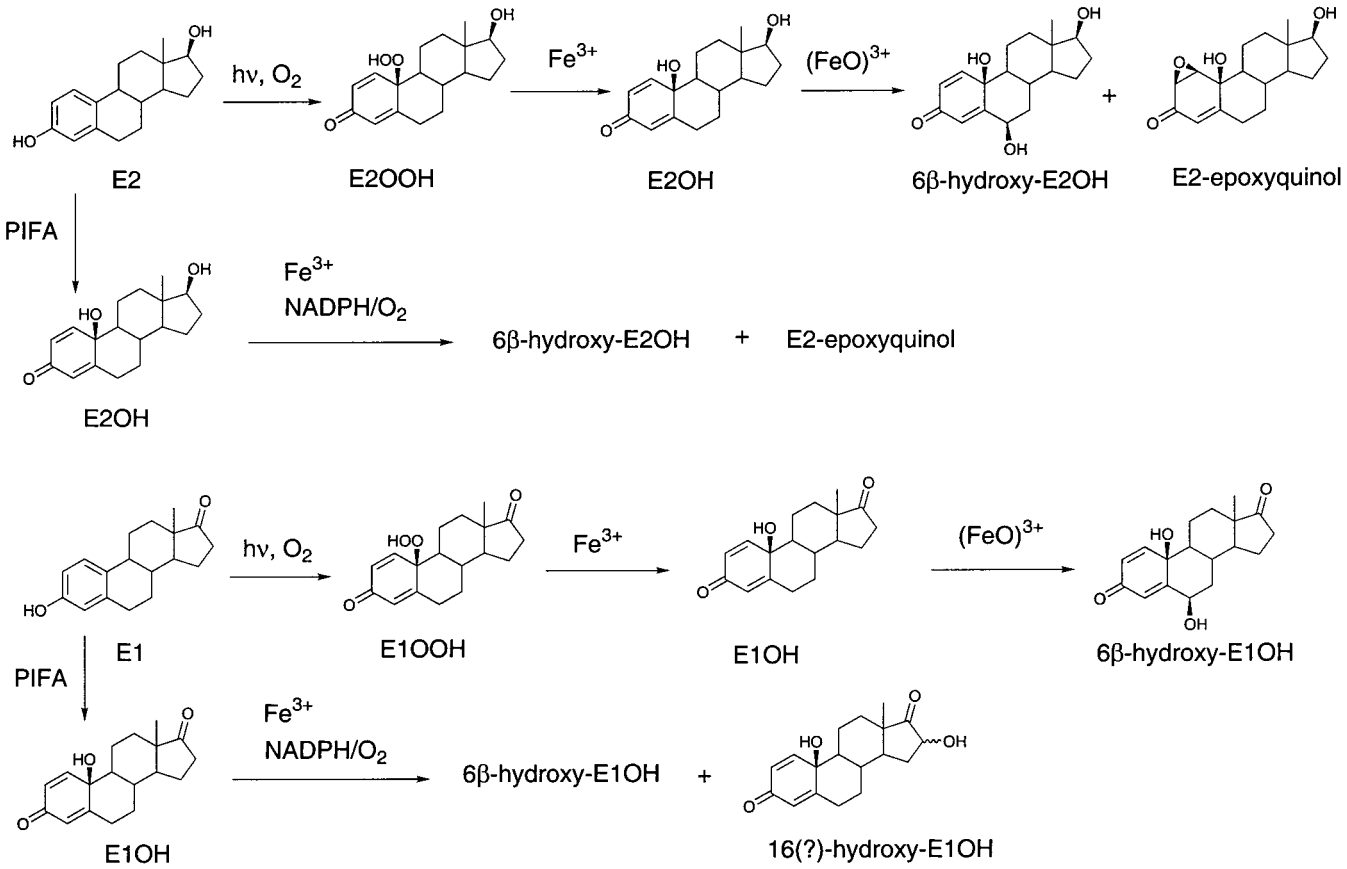


FIGURE 9: Synthesis and metabolism of peroxyquinols and quinols derived from 17β-estradiol and estrone. PIFA represents [bis-(trifluoroacetoxy)iodo]benzene, and Fe represents cytochrome P450.

Table 5: Analytical Data for Metabolites of the Estrogen Derivatives

metabolite	UV[a] λ_{max} (nm)	MS[b] (*m*/*z*)	NMR[c] δ (ppm)
6β-hydroxy-E1OH	232	303	4.58 (1H, t, J = 3.0 Hz, H-6), 6.07 (1H, d, J = 1.8 Hz, H-4), 6.15 (1H, dd, J = 10.5, 1.8 Hz, H-2), 7.14 (1H, d, J = 10.5 Hz, H-1)
	256 (sh)	447 (di-TMS)	
6β-hydroxy-E2OH	230	305	4.60 (1H, t, J = 3.3 Hz, H-6), 6.05 (1H, d, J = 1.8 Hz, H-4), 6.18 (1H, dd, J = 10.2, 1.8 Hz, H-2), 7.03 (1H, d, J = 10.2 Hz, H-1)
	255 (sh)	521 (tri-TMS)	
E2-epoxyquinol	250	305	3.47 (1H, dd, J = 3.9, 2.1 Hz, H-2), 3.89 (1H, d, J = 3.9 Hz, H-1), 5.68 (1H, bs, H-4)
		448 (di-TMS)	

[a] Recorded in an acetonitrile/water mixture during HPLC analysis. [b] Protonated molecular ion obtained either in the CI mode with a direct-insertion probe or by thermospray MS. Spectra of TMS ether derivatives recorded by GC−MS in the CI mode with a 30 m × 0.32 mm SE-54 column and temperature programming from 80 to 285 °C at a rate of 5 °C/min. [c] Only partial data are reported. Spectra recorded as described in Materials and Methods.

E1OH (Table 5 and Figure 9). Monooxygenation of E1OH, on the other hand, produced 6β-hydroxy-E1OH together with at least one additional product constituting peak G of the chromatogram in Figure 8. The UV spectrum was essentially the same as that of the substrate, and MS analysis demonstrated the introduction of a second hydroxyl group resulting in formation of a di-TMS ether derivative. These results indicate that hydroxylation of E1OH occurred at a position that does not influence the A-ring chromophore. The ^{1}H NMR spectrum varied from that of E1OH by the presence of a multiplet at 4.58 ppm, consistent with hydroxylation adjacent to the 17-keto group. Additional confirmatory data could not be obtained due to the small quantities of pure product available and the lack of an authentic standard. The structure of this metabolite as a 16-hydroxy-E1OH derivative was tentatively assigned with these data and by analogy with a major P450 2B1 metabolite of the structurally similar steroid androstenedione (*36*).

DISCUSSION

The results demonstrate that bis-allylic hydroperoxides derived from alkylphenols are isomerized to α,β-epoxyalcohols by P450 2B1 and PB microsomes. These rearrangements are intramolecular, occurring by heterolytic O−O bond scission and *cis* addition of the terminal peroxy oxygen to the least hindered double bond. In contrast, P450 2B1-catalyzed isomerizations of 15*S*- and 15*R*-HPETE to α,β-epoxyalcohols occurred by homolytic cleavage with allylic rearrangement of the alkoxy radical (Figure 2A) (*11*, *19*). The latter process is similar to nonenzymatic reactions of allylic hydroperoxides with various heme-containing compounds (*37*, *38*), although the enzymatic reaction exhibits

high stereoselectivity. The results presented here extend previous reports of hydroperoxide isomerization by ferric P450 in demonstrating conversions of allylic hydroperoxides to α,β-epoxyalcohols according to the same general mechanism described for diol formation (*9*, *10*, *13*). These P450 reactions are analogous to rearrangements catalyzed by heme protein isomerases of fungal and plant origin that catalyze conversions of fatty acid-derived allylic hydroperoxides to epoxyalcohols and diols (*39*−*41*).

The two largest, most lipophilic substrates (BMPOOH and TMPOOH) were completely consumed within a few seconds at 37 °C, and approximately 80−90% of the quinol formed in each case underwent further oxidation (Table 3 and Figure 7). These and previous results (*6*, *9*) demonstrate that isomerization may be highly efficient relative to monooxygenation. Little or no homolysis was detected during interactions of P450 with compounds smaller than BMPOOH or with estrogen derivatives. This result is consistent with previous findings which showed that peroxyquinols containing a methyl or hydrogen at position 2 and/or 6 exhibit high ratios of heterolytic:homolytic O−O bond scission by P450 2B1 (*16*). A possible explanation is that additional water coexists with substrate in the active site to facilitate protonation of the developing negative charge on the internal oxygen.

The ability of hydroperoxide-supported (i.e., peroxygenase) reactions of P450 to mimic NADPH-supported monooxygenations has been investigated for a wide variety of substrates, and as mentioned earlier, product distributions often differ (*1*−*5*). If the structure of the oxoferryl intermediate and any changes in active site architecture that accompany its formation are identical in both reactions, influences on the formation of productive substrate complexes by water (monooxygenation) or by the alcohol (isomerization) may be factors. For hydroperoxide isomerization, it is not clear whether products should mimic monooxygenase products more or less closely than the products of peroxygenation. On one hand, substrate is present in the active site at the instant of oxoferryl formation in both isomerase and monooxygenase reactions, suggesting a high degree of product similarity, but on the other hand, the initial substrate positions relative to the heme differ in these reactions. For isomerization to proceed, the hydroperoxide must interact with iron, thereby defining the general orientation of the resulting alcohol in the proximity of the oxidizing species. Evidence indicates that oxo attack is rapid, ^{18}O-labeled substrates underwent little or no dissociation prior to product formation (*9*, *10*, *13*), and 1-hydroperoxyhexane isomerization by P450 2B4 yielded 1,2-dihydroxyhexane whereas 1-hexanol monooxygenation produced 1,3-dihydroxyhexane (*10*). These data, together with several other reports of hydroperoxide to vicinal diol conversions (*6*, *9*, *13*), suggest that $(FeO)^{3+}$ attacks the closest oxidizable position before significant substrate movement takes place.

In contrast, monooxygenase substrates may bind initially to ferric P450 in several orientations (*42*) and possibly at a significant distance from the heme such that movement is relatively facile (*43*). Intramolecular isotope effects have demonstrated that substrate reorientation may be faster than oxo attack (*20*−*22*). When hydrogens at one or more hydroxylation sites are replaced with deuterium atoms, the decrease in metabolism at these sites is offset by more extensive oxidation of alternative positions. As the distance separating the sites increases, however, repositioning becomes rate-limiting and intramolecular isotope effects are suppressed (*44*). Comparisons of isomerization and monooxygenation reactions constitute a different approach to probing substrate reorientation as no inherent kinetic barrier to oxidation exists (i.e., deuterium for hydrogen substitution), only differences in substrate positioning within the distal cavity at the instant of $(FeO)^{3+}$ formation.

Both peroxyquinol isomerization and quinol monooxygenation generated *cis*-epimers of epoxyquinols. In the former case, this stereoselectivity is readily explained by transfer of the hydroperoxide oxygen to iron followed by rapid addition to the same face of the quinol. The high stereoselectivity of quinol monooxygenation was unexpected, however, and demonstrates that the binding complex leading specifically to epoxidation involves orientation of the 4-hydroxyl group toward the heme, possibly due to hydrogen bonding interactions with the iron or another polar group near the heme, as well as repulsion from predominantly nonpolar residues in the distal cavity of P450 2B1 (*45*). Enantioselectivities associated with *cis*-epoxyquinol formation (Table 4) also were similar for isomerase and monooxygenase reactions with both chiral (BMPOOH/OH and DMPOOH/OH) and prochiral substrates (TMPOOH/OH and MPOOH/OH). These results are consistent with the idea that enantioselectivity reflects the chiral architecture of the active site, so the structure of the ultimate binding complex leading to *cis* epoxidation should be very similar regardless of the position of the quinol at the instant of oxo formation.

Regioselectivities of isomerase and monooxygenase reactions were nearly identical for BMPOOH and its quinol, with epoxidation predominating over hydroxylation by factors of 5.8−7.6 (Table 4). These results are consistent with the energetics of oxygen addition to a double bond versus hydrogen abstraction from a methyl group. For DMPOOH and DMPOH, epoxidation of the unsubstituted 5,6-double bond also was the predominant reaction, but in this case, epoxidation of the substituted 2,3-double bond occurred to a small extent and no hydroxylation was detected. A small substituent at position 2 (i.e., methyl vs *tert*-butyl), therefore, hinders but does not prevent epoxidation in preference to hydroxylation of the allylic methyl. With methyl groups on both double bonds, however, TMPOOH underwent epoxidation and hydroxylation with a modest preference for the latter (epoxidation:hydroxylation ratio of 0.6). In contrast, monooxygenation of TMPOH strongly favored epoxidation (ratio of 6.4). This difference in regioselectivity might be explained if NADPH-supported epoxidation involves an iron−peroxo species capable of addition to a double bond (*46*). Alternatively, it is reasonable to assume that the energetics of oxygen addition to a sterically hindered double bond and hydrogen abstraction from an allylic methyl are comparable. Monooxygenation regioselectivity, therefore, may be highly susceptible to influences of protein structure leading to preferential epoxidation in the case of TMPOH. Isomerization, on the other hand, involves rapid attack on the quinol which occupies a defined location in the proximity of the oxo species, and regioselectivity is influenced mainly by the chemical reactivities of alternative oxidation sites. The results are consistent with the proposal that isomerization involves rapid capture of the substrate in the proximity of

the oxo species, whereas multiple (and perhaps "looser") complexes formed with ferric P450 during monooxygenation allow more extensive substrate movement after oxo formation and prior to oxidative attack.

The regioselectivities of isomerase and monooxygenase reactions were further compared using larger substrates, i.e., peroxyquinols and quinols derived from the phenolic A-rings of 17β-estradiol and estrone. The only prominent metabolites of E2OOH resulted from attack at reactive sites in the A- and B-rings, i.e., the unsubstituted 1,2-double bond and allylic 6-methylene group (Figure 9). Oxidations at these sites are predictable because the 10-hydroperoxy group must interact with the heme placing the A/B-rings into proximity with the oxo species. Essentially the same regioselectivity was observed with E2OH monooxygenation. Interestingly, only 6β-hydroxylation occurred during E1OOH isomerization. Molecular modeling[2] demonstrated no structural effects of 17β-hydroxyl versus 17-oxo substitution on the A-rings of E2OH and E1OH. The NADPH-supported oxidation of E1OH also failed to yield an epoxyquinol but did produce 6β-hydroxy-E1OH. In the absence of evidence for differing reactivities of the 1,2-double bonds in these quinols, it can be concluded that the 17-oxo group influences E1OH orientation such that the 1,2-olefinic bond is not accessible to the oxidant during either reaction.

Monooxygenation of E1OH also resulted in at least one significant product not formed during E1OOH isomerization and believed to be 16-hydroxy-E1OH. Hydroxylation in the D-ring of E1OH during monooxygenation, but not during E1OOH isomerization, can be rationalized by evoking different initial complexes in the former reaction with either the A- and B-rings or the D-ring near the heme. To generate a D-ring metabolite from E1OOH, it would be necessary for the resulting quinol to dissociate and reassociate in the alternative orientation, so these results support ^{18}O data from other hydroperoxides demonstrating no dissociation prior to oxo attack (*9*, *10*, *13*). Studies of testosterone metabolism by P450 2C11 led to the conclusion that hydroxylation at opposite ends of the steroid nucleus does not involve substrate reorientation within the active site or formation of different initial complexes, but occurs by dissociation/association after $(FeO)^{3+}$ formation (*47*). The monooxygenation results in this work also can be explained by the formation of an initial E1OH−P450 complex that dissociates after oxo formation and re-forms in the alternative orientation, whereas the location of the hydroperoxide-derived quinol near the oxidant results in rapid 6β-hydroxylation during isomerization.

In conclusion, the results demonstrate that ferric P450 may catalyze rapid and stereoselective intramolecular rearrangements of allylic hydroperoxides to α,β-epoxyalcohols and diols by a heterolytic mechanism similar to that described previously for fungal and plant isomerases. Product distributions from P450 isomerase and monooxygenase reactions were compared. Although the possibility of a peroxo-type intermediate in quinol monooxygenation cannot be discounted, the similarities in regio- and stereochemistry suggest that the same oxoferryl intermediate exists in both reactions and that protein−substrate complexes differ only in the position of the substrate alcohol relative to the oxo species. The product data from this study and a previous study (*10*) indicate that oxo attack during isomerization occurs prior to any substantial substrate reorientation. It is proposed, therefore, that hydroperoxide-derived substrates remain in the proximity of the oxo species and product distributions are governed mainly by the relative chemical reactivities of alternative sites. Monooxygenase substrates, on the other hand, may bind to ferric P450 in multiple orientations and possibly at a greater distance from the heme. When relative reactivities of substrate sites are very different, the pattern of products is similar to that of isomerization, but when reactivities are similar, monooxygenation regioselectivity is greatly influenced by the distribution of competing complexes near the oxo species. Differences in regioselectivity are especially likely for large substrates such as steroids where facile reorientation within the distal cavity does not occur. In such cases, completely different patterns may result when monooxygenase products are determined by the contributions of competing initial complexes or by dissociation/reassociation.

[2] Energy minimized using the Discover module of Insight II (Molecular Simulations, Inc.).

ACKNOWLEDGMENT

Purified P450 2B1 was kindly supplied by Dr. M. A. Correia. We thank Dr. P. R. Ortiz de Montallano for helpful discussions and Dr. T. DeBeer for molecular modeling assistance. Access to the UCHSC Magnetic Resonance Center, partially supported by NIH Grant CA46934, is acknowledged.

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BI010914D