Reaction of Hematin with Allylic Fatty Acid Hydroperoxides: Identification of Products and Implications for Pathways of Hydroperoxide-Dependent Epoxidation of 7,8-Dihydroxy-7,8-dihydrobenzo[*a*]pyrene†

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ABSTRACT: Reaction of 10-hydroperoxyoctadec-8-enoic acid (10-OOH-18:1) (50 μM) with hematin (0.5 μM) in sodium phosphate buffer containing Tween 20 (200 μM) generates 10-oxooctadec-8-enoic acid, 10-oxodec-8-enoic acid (10-oxo-10:1), and 10-hydroxyoctadec-8-enoic acid in relative yields of 79, 4, and 17%, respectively. The product profile and relative distribution are unaffected by 1 mM butylated hydroxyanisole. Approximately 5% of the hydroperoxide isomerizes from the 10- to the 8-position. 10-Oxo-10:1 most likely arises via β-scission of an intermediate alkoxyl radical to the aldehyde and the *n*-octyl radical. To test this, 10-hydroperoxyoctadeca-8,12-dienoic acid was reacted with hematin under identical conditions. 10-Oxooctadeca-8,12-dienoic acid, 10-oxodec-8-enoic acid, and 10-hydroxyoctadeca-8,12-dienoic acid are formed in relative yields of 50, 45, and 5%, respectively. The product ratios are constant with time and hydroperoxide to catalyst ratio and unaffected by inclusion of phenolic antioxidants. The higher yield of 10-oxo-10:1 from 10-OOH-18:2 compared to 10-OOH-18:1 is due to the higher rate of β-scission of the intermediate alkoxyl radical from the former to the resonance-stabilized octenyl radical. Two products of reaction of the 2-octenyl radical with O_2, octenal and octenol, were detected in 10% yield relative to 10-oxo-10:1. Inclusion of 7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene (BP-7,8-diol) led to epoxidation by both 10-OOH-18:1 and 10-OOH-18:2. Studies with isotopically labeled hydroperoxide or O_2 indicated approximately 65% of the epoxide oxygen was derived from O_2 and 35% from hydroperoxide oxygen, consistent with the involvement of peroxyl free radicals as the oxidizing agents. The available evidence indicates that hematin reduces the fatty acid hydroperoxides homolytically to alkoxyl radicals that are oxidized to ketones, reduced to alcohols, or undergo β-scission to aldehydes. Carbon radicals generated during these reactions couple to O_2, generating peroxyl free radicals that epoxidize BP-7,8-diol. The smaller percentage of epoxidation that results from hydroperoxide oxygen may arise from oxidation of the hydroperoxide group to peroxyl radicals or from heterolytic cleavage of the hydroperoxide to alcohol and an iron–oxo complex.

Fatty acid hydroperoxides arise in nature by lipid peroxidation (Tappel, 1973; Bus & Gibson, 1979; Svingen et al., 1979) or during prostaglandin and leukotriene biosynthesis (Hamberg & Samuelsson, 1974; Samuelsson et al., 1978; Gardner, 1980; Samuelsson, 1983). Alkyl hydroperoxides such as peroxy-Y-base, a component of $tRNA^{Phe}$ (Feinberg et al., 1974), and 4a-hydroperoxyflavin, an intermediate in flavin oxidase (Kemal & Bruice, 1976), have also been isolated in mammalian tissue. Alkyl hydroperoxides are mild oxidizing agents that are converted by transition metals and metalloproteins to a variety of powerful but transient oxidants (Dunford & Stillman, 1976; White et al., 1980; Lee & Bruice, 1985; McMurry & Groves, 1986; Marnett et al., 1986; Vaz & Coon, 1987). Such oxidants may contribute to xenobiotic metabolism, tissue degradation, or tumor promotion inter alia (Marnett, 1987; Sevanian & Hochstein, 1985; Kensler & Taffe, 1986).

Metal-catalyzed homolytic cleavage of hydroperoxides produces alkoxyl radicals (eq 1), whereas heterolytic cleavage generates alkoxide ions (eq 2) (Lee & Bruice, 1985). These

$$M^{n+} + ROOH \rightarrow M^{(n+1)+}{=}O + RO^{\bullet} \quad (1)$$

$$M^{n+} + ROOH \rightarrow M^{(n+2)+}{=}O + RO^{-} \quad (2)$$

reductions produce metal–oxo complexes as byproducts. Alkoxyl radicals and metal–oxo complexes are potent oxidants that approach the hydroxyl radical in reactivity (Pryor, 1986; Guengerich & Macdonald, 1984). Metal complexes can also oxidize hydroperoxides to produce peroxyl radicals and reduced metal (eq 3) (Kochi, 1978; Sheldon & Kochi, 1981; Howard,

$$M^{n+} + ROOH \rightarrow M^{(n-1)+} + ROO^{\bullet} \quad (3)$$

1972). Peroxyl radicals are more stable and selective in their reactivity than alkoxyl radicals and possess half-lives that should enable them to diffuse relatively far from the site of their production in cells (Pyror, 1986).

Our laboratory has been interested in the reaction of polyunsaturated fatty acid hydroperoxides with metals and metalloproteins. One of the reasons for our interest is the ability of such reactions to epoxidize dihydroaromatic compounds to mutagenic and carcinogenic epoxides (Dix et al., 1985; Panthananickal et al., 1983). For example, 7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene (BP-7,8-diol)[1] is a proximate carcinogenic metabolite of the ubiquitous polycyclic hydrocarbon benzo[*a*]pyrene. Oxidation of BP-7,8-diol produces dihydrodiol epoxides that represent the ultimate car-

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[1] Abbreviations: BP-7,8-diol, 7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene; 10-OOH-18:1, 10-hydroperoxyoctadec-8-enoic acid; 10-OOH-18:2, 10-hydroperoxyoctadeca-8,12-dienoic acid; 10-oxo-18:1, 10-oxooctadec-8-enoic acid; 10-OH-18:1, 10-hydroxyoctadec-8-enoic acid; 10-oxo-10:1, 10-oxodec-8-enoic acid; 10-oxo-18:2, 10-oxooctadeca-8,12-dienoic acid; 10-OH-18:2, 10-hydroxyoctadeca-8,12-dienoic acid; 13-OOH-18:2, 13-hydroperoxyoctadeca-9,11-dienoic acid; HQ, hydroquinone; BHA, butylated hydroxyanisole; BSTFA, bis(trimethylsilyl)-(trifluoromethyl)acetamide, DBPO, di-*tert*-butylperoxyoxalate; GC–MS, gas chromatography–mass spectrometry.

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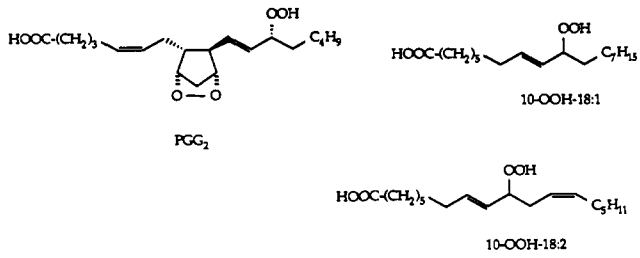
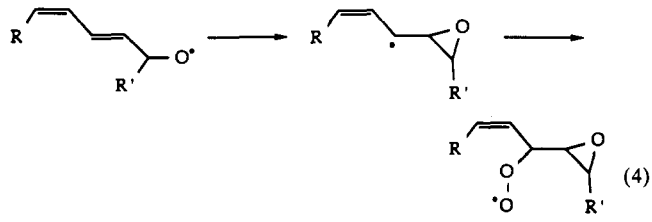


FIGURE 1: Structure of PGG_2, 10-OOH-18:1, and 10-OOH-18:2.

cinogenic forms of the parent hydrocarbon (Borgen et al., 1973; Sims et al., 1974). Dihydrodiol epoxide formation is catalyzed by NADPH-dependent mixed-function oxidases (Thakker et al., 1976; Yang et al., 1976), by prostaglandin H synthase (Marnett et al., 1979; Sivarajah et al., 1979; Marnett & Bienkowski, 1980; Marnett, 1981), and by metal–hydroperoxide reactions (Dix & Marnett, 1983a). The latter reactions provide pathways linking polyunsaturated fatty acid metabolism to metabolic activation of chemical carcinogens.

Mechanistic studies of BP-7,8-diol oxidation by 13-hydroperoxyoctadeca-9,11-dienoic acid (13-OOH-18:2) and hematin implicate peroxyl radicals as the epoxidizing agent (Dix & Marnett, 1981). Peroxyl radicals are well-precedented to epoxidize nonaromatic double bonds (Aringer & Eneroth, 1974; Dix & Marnett, 1981, 1983c; Kimura & Muto, 1981; Watabe et al., 1981, 1982; Reed et al., 1984; Ortiz de Montellano & Catalano, 1985; Ortiz de Montellano & Grab, 1987). A key step in the generation of peroxyl radicals from 13-OOH-18:2 is cyclization of an alkoxyl radical produced by one-electron reduction of the hydroperoxide by hematin (eq 4) (Dix & Marnett, 1983a, 1985). This allylic radical reacts



(4)

with either O_2 to form a peroxyl radical or a heme–oxo complex to form an alcohol (eq 4). Presumably, an important determinant of the ability of the alkoxyl radical to cyclize is the fact that it produces a resonance-stabilized allylic carbon radical. In the absence of allylic stabilization it is not known if alkoxyl radical cyclization occurs.

BP-7,8-diol is epoxidized moderately well when it is reacted with hematin and 10-hydroperoxyoctadec-8-enoic acid (10-OOH-18:1) (Dix et al., 1985), but the mechanism of the oxidation has not been elucidated. 10-OOH-18:1 is a structural analogue of PGG_2, a major naturally occurring hydroperoxide and the principal trigger for BP-7,8-diol epoxidation by PGH synthase (Figure 1) (Marnett, 1981; Marnett & Eling, 1983). If the mechanism of oxidant generation is analogous to that observed with 13-OOH-18:2, one anticipates cyclization of the alkoxyl radical to an oxacyclopropylcarbinyl radical. The limited number of studies performed on such radicals indicate they prefer to ring open, so it is not clear that sufficient driving force exists to support this pathway of oxidant generation (Barton et al., 1981; Kuivila, 1968).

We report here a series of investigations of the reaction of hematin with 10-OOH-18:1 and a structural analogue 10-hydroperoxyoctadeca-8,12-dienoic acid (10-OOH-18:2). The results demonstrate the multiplicity of chemical options available to fatty acid alkoxyl radicals and assist in the development of mechanistic probes for the reaction of hydroperoxides with metals and metalloproteins.

Experimental Procedures

Materials. Unlabeled oleic acid and linoleic acid were from Nu Chek Prep (Elysian, MN). Hematin, Tween 20, and L-cysteine (free base) were obtained from Sigma (St. Louis, MO). [1-^{14}C]Oleic acid (59 mCi/mmol) and [1-^{14}C]linoleic acid (59 mCi/mmol) were obtained from ICN (Irvine, CA), and $^{18}O_2$ (98%) was from MSD isotopes (St. Louis, MO). BSTFA was from Anspec (Ann Arbor, MI). Diazomethane was prepared by the KOH (40% in water) catalyzed decomposition of *N*-methylnitrosourea (ICN Pharmaceuticals) into diethyl ether. Silicic acid was from Merck (Rahway, NJ). Solvents used for chromatography were HPLC grade from Fisher (Detroit, MI). Other chemicals were reagent grade. Unlabeled and [7,10-^{14}C]BP-7,8-diol was obtained from Chemsyn Laboratories (Lenexa, KS) through the Cancer Research Program of the National Cancer Institute, Division of Cancer Cause and Prevention, Bethesda, MD.

Instrumentation. HPLC was performed on a Varian Model 5000 instrument with a Varian 5020 variable-wavelength absorbance monitor. Preparative work were performed with an LDC pump with an LDC Refractomonitor. Columns used included an Alltech Partisil 10 (10 μm silica, 4.6 × 250 mm), a Whatman Magnum S9 (10 μm silica), and an Altex Ultrasphere ODS (5 μm, 4.6 × 250 mm).

Radioactivity eluting from the HPLC columns was quantitated in a continuous fashion with a Radiomatic Flo-One HP radioactivity flow detector and Scinti Verse LC, premixed scintillant, from Fisher.

Gas chromatography was performed on a Varian Model 3700. Samples were eluted through a 2-ft 3% OV-17 packed column or a WCOT wide-bore SE-30 column (25 m × 0.33 mm) from Anspec. GC–MS was performed on a Kratos MS 80 at 70 eV.

Preparation of 10-OOH-18:1 and 10-OOH-18:2. An equal mixture of 9-hydroperoxyoctadec-11-enoic acid (9-OOH-18:1) and 10-hydroperoxyoctadec-8-enoic acid (10-OOH-18:1) was prepared by singlet oxygen oxidation of oleic acid. Oleic acid (500 mg, 16 μCi/mmol) and methylene blue (2 mg) were dissolved in 20–25 mL of methanol. The solution was cooled to 0–5 °C with oxygen bubbling and exposed to a 1000-W high-pressure sodium lamp, filtered to remove UV light. The reaction was monitored by TLC using silica gel plates developed in hexane/2-propanol/acetic acid (110/10/1). Detection was by phosphomolybdic acid (5 g/100 mL of 2-propanol) sprayed on the plates followed by heating for color development. A ferrous ammonium thiocyanate spray (Abraham et al., 1957) was used to detect hydroperoxides. The reaction was terminated after 12 h. Separation of hydroperoxides from unreacted fatty acid was achieved by passage through a 10-g silicic acid column. Unreacted starting material was eluted with 95/5 hexane/ethyl acetate followed by elution of hydroperoxides in 85/15 hexane/ethyl acetate. 10-OOH-18:1 (k' = 9.6) and 9-OOH-18:1 (k' = 10.7) were separated by normal-phase HPLC (hexane/2-propanol)acetic acid, 984/15/1). The purified hydroperoxides were reduced, methylated, silylated, and subjected to GC–MS analysis for identification.

In a similar manner, 10-hydroperoxy-8,12-octadecadienoic acid (10-OOH-18:2) was synthesized from linoleic acid. Linoleic acid (500 mg, 16 μCi/mmol) was photooxidized for 4 h. A mixture of four isomers, 13-OOH-18:2 (k' = 9.6), 12-OOH-18:2 (k' = 10.6), 10-OOH-18:2 (k' = 12.1), and 9-OOH-18:2 (k' = 13.4), that could be separated by normal-phase HPLC (hexane/2-propanol/acetic acid, 987/12/1)

was obtained. 10-OOH-18:2 was obtained in 7% yield.

Conversion of 10-OOH-18:1 and 10-OOH-18:2 to Products by Hematin. Hydroperoxide (50 μM) was placed in 0.1 M sodium phosphate, pH 7.8, containing 200 μM Tween 20 (buffer A). Reactions were initiated by addition of 0.5 μM hematin (final concentration) and allowed to continue at room temperature for 5 min with stirring. Termination was effected by addition of 10 μM BHA. The mixture was acidified to pH 3.5 with HCl followed by extraction with 4 × 1 volume of ethyl acetate.

Preparation of 10-[$^{18}O_2$]OOH-18:1. Oleic acid (200 mg, 7 μCi) and methylene blue (10–15 mg) were dissolved in 35 mL of CCl_4/MeOH (95/5). Air was removed from the solvent by 5 freeze–pump–thaw cycles, and $^{18}O_2$ was allowed to enter the system (Bienkowski, 1979). The solution was cooled to 0–5 °C and irradiated as described above for 6 h. The products were isolated and purified as described.

Reaction of 10-OOH-18:1 and 10-OOH-18:2 with Fe^{2+}/Cysteine. Alkoxyl radicals were generated according to the method described by Gardner et al. (1974, 1981). Hydroperoxide (3.2 mM) was placed in an oxygenated mixture of methanol/water (4/1) containing 12.8 mM cysteine. The reaction was initiated by addition of 0.05 mM $FeCl_3$ and allowed to continue for 25 min at room temperature. The products were extracted into methylene chloride, the organic layer was dried over $MgSO_4$, and the solvent was removed under vacuum.

Reaction of 10-OOH-18:1 with Di-tert-butylperoxyoxalate (DBPO). Peroxyl radicals were generated from 9-OOH-18:1 and 10-OOH-18:1 by reaction with DBPO based on the method described by Porter et al. (1976). Me-10-OOH-18:1 (20 mM) was dissolved in benzene and the solution saturated with oxygen for 15 min at room temperature. After addition of 5 mM of DBPO, prepared according to Bartlett et al. (1960), the reaction mixture was incubated for 3 h at room temperature. The products were obtained by removing the benzene under vacuum and purified by straight-phase HPLC (hexane/tetrahydrofuran, 9/1).

Oxidation of BP-7,8-diol under $^{18}O_2$. BP-7,8-diol (1 mg, 18 μM final concentration) and 10-OOH-18:1 (50 μM) were dissolved in 220 mL of buffer A. The mixture was placed in a 500-mL round-bottomed flask that was connected to a manifold containing two joints mounted on opposite sides with respect to each other. One of the side arms contained 50 mL of $^{18}O_2$ (98.4% atom excess) and the other contained hematin (110 nmol in 2 mL of buffer A). Buffer and the side arm containing hematin were degassed to 10 mTorr by 5 consecutive freeze–pump–thaw cycles. The system was then isolated from the vacuum pump and the $^{18}O_2$ allowed to enter the reaction flask to equilibrate with the solvent. In order to condense the oxygen, the reaction flask was cooled with liquid nitrogen. After the mixture was warmed, reaction was initiated by addition of the hematin solution (0.5 μM final concentration) into the bulk of the solvent and allowed to continue with stirring for 10 min at 25 °C. The reaction mixture was then quickly poured into a flask containing 10 μM BHA in ethyl acetate (200 mL). Products were further extracted into 3 × 1 volume of ethyl acetate. After removal of solvent, the reaction mixture was purified by HPLC (Dix & Marnett, 1983b). The BP tetrols were isolated, acetylated, and analyzed by direct probe mass spectrometry.

Preparation of Derivatives for GC–MS. Hydroperoxides (100 μg) were reduced by treatment with $NaBH_4$ in methanol (1 mL) for 20 min at 0 °C, followed by 20 min at 25 °C. At the end of the reaction water was added, the mixture was acidified to pH 3.5, and the products were extracted into 4 × 1 volume of ethyl ether.

Fatty acids were methylated by treatment with ethereal diazomethane for 5 min at room temperature, followed by removal of the solvent with a stream of argon.

Alcohols were silylated by treatment with bis(trimethylsilyl)(trifluoromethyl)acetamide (BSTFA) for 30 min.

Catalytic hydrogenation was accomplished by dissolving the products in methanol and adding 100 μg of 10% Pd/C. The solution was hydrogenated for 15 min at room temperature.

Carbonyl-containing compounds were reacted with methoxyamine hydrochloride in pyridine (30 mg/mL) for 12 h at room temperature to form methoxime derivatives.

BP tetrols were converted to poly(acetates) as described by Marnett & Bienkowski (1980). The samples were dissolved in 500 μL of pyridine/acetic anhydride (1/1) and heated to 60 °C for 2 h.

Quantitation of 2-Octenol and 2-Octenal. [1-^{14}C]-10-OOH-18:2 (final concentration 50 μM) was placed in 260 mL of buffer A. The reaction was initiated by addition of 0.5 μM hematin and allowed to continue for 5 min. The pH was adjusted to 9, and the products were extracted into 4 × 1 volume of hexane. The organic layer was dried over $MgSO_4$. The residue obtained after solvent removal was reconstituted in 50 μL of hexane and injected on a WCOT wide-bore SE-30 column (25 m × 0.33 mm) and analyzed by GC–MS.

The efficiency of extraction of 2-octenol and 2-octenal was measured as follows: 1 mg of 2-octenol and 1 mg of 2-octenal (respectively 2 mg of 2-octenol and 2 mg of 2-octenal) were dissolved in 200 mL of buffer A. After 5 min, the mixture was extracted with hexane (4 × 1 volume). The residue was reconstituted in 50 μL of hexane, and 250 μg (respectively 500 μg) of nonen-1-ol (internal standard) was added. The mixtures were chromatographed and the area ratios measured. From calibration curves constructed with known amounts of 2-octenol and 2-octenal, their efficiency of extraction was calculated.

Results

Identification of the Major Products of the Reaction of 10-OOH-18:1 with Hematin. When 10-OOH-18:1 was incubated with hematin in phosphate buffer containing Tween 20, three products, represented by peaks 1, 3, and 4 in Figure 2, were detected in 44, 2, and 10% yields following separation by HPLC. Peak 1 was identified as 10-oxooctadec-8-enoic acid (10-oxo-18:1) by NMR, IR, UV, and MS of the methylated derivative. Resonances were observed in the ^{1}H NMR ($CDCl_3$, 300 MHz) at δ 6.83 (dt, J = 15.8, 7 Hz, 1 H), 6.1 (d, J = 15.8 Hz, 1 H), 3.69 (s, 3 H), 2.54 (t, J = 7.4 Hz, 2 H), 2.33 (t, J = 7.4 Hz, 2 H), 2.3 (dq, J = 7.2, 1.1 Hz, 2 H), and 1–2.2 (m, 23 H) and in the ^{13}C NMR ($CDCl_3$, 75.4 MHz) frequencies at δ 200.8 (C10), 174.5 (C1), 147 (C8), 130.5 (C9), and 51.8 (OCH_3); IR absorbances were detected at 1737, 1700 (ν_{CO}), and 1628 cm^{-1} (ν_{CC}). The compound exhibited a UV maximum at 223 nm (MeOH, ε = 12000 mol^{-1} L cm^{-1}). Electron impact mass spectra were obtained by direct probe or GC–MS of methyl ester and methyl ester methoxime derivatives of 1 (the GC profile showed two peaks of identical mass spectra corresponding to syn and anti isomers). Major ions were observed at m/z 311 (M + 1), 279 (M − OCH_3), 251 (M − $COOCH_3$), 212 [CH_2=C(OH)CH=CH$(CH)_6$C-$OOCH_3$], 197 [$^+$O=CCH=CH$(CH_2)_6COOCH_3$], and 141 ($^+$O=CC_8H_{17}) for Me-10-oxo-18:1 and 339 (M^+) and 308 (M − OCH_3) for its methoxime derivative.

Peak 2 cochromatographed with starting material. It exhibited an identical ^{1}H NMR and was reduced to peak 4

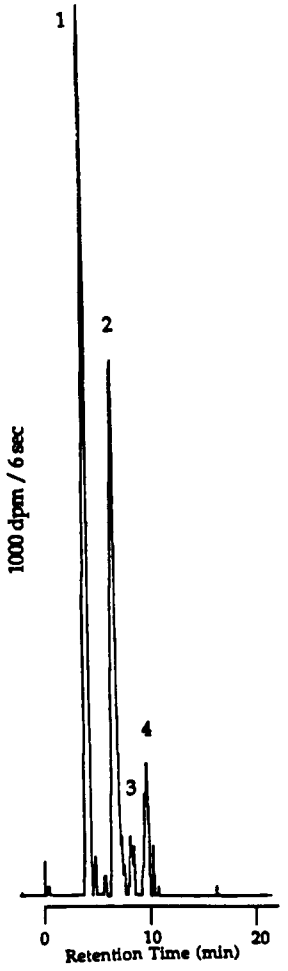


FIGURE 2: Radioactivity profile of products of hematin with 10-OOH-18:1. Elution was performed with hexane/tetrahydrofuran/acetic acid (885/110/5) at a flow rate of 1.5 mL/min on an Alltech Partisil (10 μm silica, 4.6 × 250 mm) column.

following $NaBH_4$ reduction. The amounts of product 3 were too low to permit complete spectroscopic characterization. We hypothesized that 3 was derived from an alkoxyl radical, so we attempted to increase its production by reacting 10-OOH-18:1 with Fe^{2+}–cysteine, a metal complex that Gardner and associates have found to smoothly convert fatty acid hydroperoxides to alkoxyl radicals (Gardner et al., 1974, 1981). Indeed, reaction of 10-OOH-18:2 with Fe^{2+}–cysteine generated a product in 20% yield that cochromatographed with 3. This product was subjected to spectroscopic analysis. Resonances in the ^1H NMR ($CDCl_3$, 300 MHz) were detected at δ 9.48 (d, J = 8.04 Hz, 1 H), 6.82 (dt, J = 15.6, 6.8 Hz, 1 H), and 6.1 (ddt, J = 15.5, 8.05, 1.3 Hz, 1 H) (Figure 3). Absorbances in the IR were observed at 2860, 2740 (ν_{CH}), 1692 (ν_{CO}), and 1634 cm^{-1} (ν_{CC}). The chemical ionization mass spectrum of product 3 (Figure 4) was obtained by direct-probe mass spectrometry. Major ions were observed at m/z 185 (M + 1), 167 (M – •OH), 139, and (M – HO – •C=O). The GC–MS of the methyl ester of 3 and its methoxime derivative showed, respectively, molecular ions at m/z 198 and 227. On the basis of its spectroscopic properties, this compound was identified as 10-oxodecenoic acid (10-oxo-10:1). The GC–MS of the methyl ester of peak 3 (Figure 2) generated in the hematin reaction was identical with the GC–MS of 10-oxo-10:1 generated by Fe^{2+}–Cysteine.

Peak 4 cochromatographed with 10-hydroxyoctadec-8-enoic acid (10-OH-18:1). The structure of the methyl ester of this metabolite was confirmed by ^1H NMR ($CDCl_3$, 300 MHz)

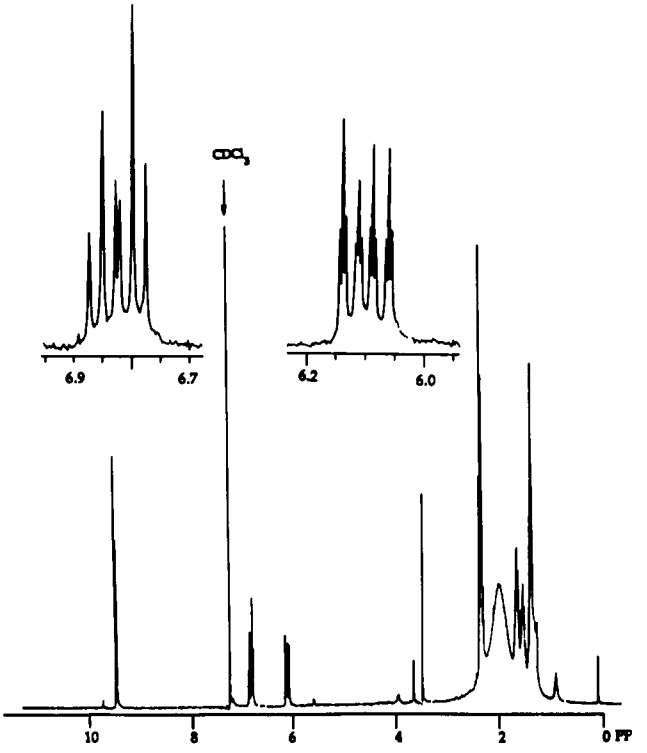


FIGURE 3: ^1H NMR spectrum of 10-oxo-10:1.

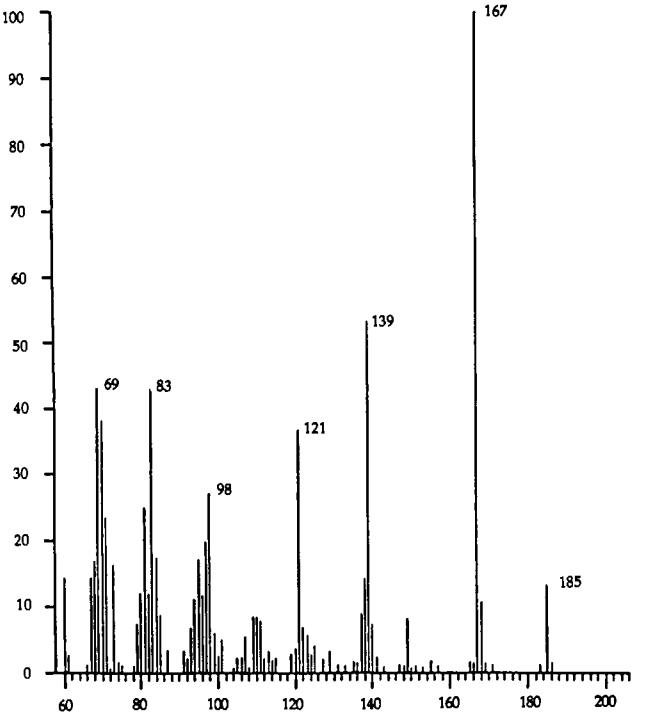


FIGURE 4: Chemical ionization spectrum of 10-oxo-10:1.

with δ 5.65 (dt, J = 15, 7 Hz, 1 H), 5.48 (dd, J = 15, 7 Hz, 1 H), 4.05 (q, J = 7 Hz, 1 H), and 3.69 (s, 3 H) and mass spectrometry of the methyl ester trimethylsilyl ether derivative with m/z 384 (M^+), 369 (M – CH_3), 354 (M – •OCH_3), and 271 [$H_3COOC(CH_2)_6CH$=$CHHC$=O^+TMS]. The structures of the compounds designated as peaks 1–4 are displayed in Figure 5.

Previous studies indicate that hematin catalyzes positional isomerization of 13-hydroperoxyoctadeca-9,11-dienoic acid (13-OOH-18:2) (Dix & Marnett, 1985). Therefore, the HPLC peak containing unreacted 10-OOH-18:1 (Figure 2,

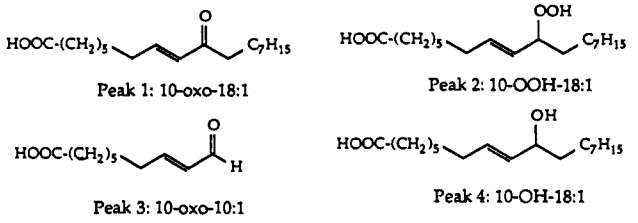


FIGURE 5: Structures of the major products from the reaction of 10-OOH-18:1 and hematin.

Table I: Quantitation of the Minor Products from the Reaction of 10-OOH-18:1 with Hematin

	ions	% abund	isomers
10-OOH-18:1	273 + 215	99.2	Me-10-OTMS-18:0
(starting material)	245 + 243	0.8	Me-8-OTMS-18:0
10-OOH-18:1	273 + 215	94	Me-10-OTMS-18:0
(recovered from hematin reaction)	245 + 215	6	Me-8-OTMS-18:0
10-oxo-18:1	273 + 215	95	Me-10-OTMS-18:0
(from hematin reaction)	245 + 243	5	Me-8-OTMS-18:0

peak 2) was analyzed by GC–MS. Since unsaturated trimethylsilyl ether derivatives have been reported to isomerize in the mass spectrometer, catalytic hydrogenation of peak 2 was carried out before silylation. The resulting saturated alcohol was methylated, silylated, and subjected to GC–MS. The isomeric composition was determined by monitoring the following pair of ions via GC–MS: m/z 273 [$H_3COOC(CH_2)_8CH{=}O^+TMS$] and 215 [$TMSO^+{=}CH(CH_2)_7CH_3$] for Me-10-OTMS-18:1 compared to m/z 245 [$H_3COOC(CH_2)_6CH{=}O^+TMS$] and 243 [$TMSO^+{=}CH(CH_2)_9CH_3$] for Me-8-OTMS-18:1.

Hydroperoxide isomerization occurs via peroxyl radical intermediates. Peroxyl radicals can be generated independently by reacting the hydroperoxide with di-*tert*-butylperoxyoxalate (DBPO) (Porter et al., 1976). The principal products are the corresponding ketones and alcohols. When Me-10-OOH-18:1 was reacted with DBPO in benzene, five major products were detected. They were identified by ^1H NMR and mass spectrometry as Me-10-oxo-18:1 (12%), Me-8-oxo-18:1 (8%), Me-8-OOH-18:1 (20%), and Me-8-OH-18:1 and Me-10-OH-18:1 (7% total yield). The presence of Me-8-oxo-18:1 in the DBPO reaction prompted us to examine the HPLC peak containing 10-oxo-18:1 from the hematin reaction (Figure 2, peak 1) in addition to the peak containing recovered hydroperoxide. The ketone peak was hydrogenated, reduced with sodium borohydride, methylated, converted to a trimethylsilyl ether, and analyzed by GC–MS. The pairs of ions (m/z 273, 215 vs 245, 243) were compared, and the results are listed in Table I. When 10-OOH-18:1 was reacted with hematin in phosphate buffer, 8-OOH-18:1 and 8-oxo-18:1 accounted for 5 and 4% of the total products. When peroxyl radicals were generated by reaction of 10-OOH-18:1 with DBPO, we observed 40% isomerization [8%/(8% + 12%)]. Thus, the 5% isomerized hydroperoxide in the hematin reaction resulted from approximately twice that number of peroxyl radicals. This corresponds to a total peroxyl radical yield of ~10%.

No other products were detected in the reaction of 10-OOH-18:1 with hematin; 85–92% of the radioactivity was recovered after extraction. The product profile and relative distribution of products were essentially the same in the presence of 1 mM butylated hydroxyanisole (BHA). To the extent that alkoxyl radicals are generated by reaction with hematin, they do not appear to cyclize and couple to molecular oxygen to form epoxyols. Even when the buffer solution was

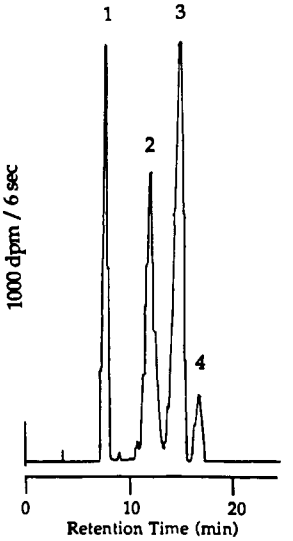


FIGURE 6: Radioactivity profile of products of reaction of hematin with 10-OOH-18:2. Elution was performed with hexane/tetrahydrofuran/acetic acid (885/110/5) at a flow rate of 1.2 mL/min on an Alltech Partisil (10 μm silica, 4.6 × 250 mm) column.

saturated with O_2 prior to addition of hematin, in an attempt to trap transient carbon-centered radicals formed by cyclization, no epoxyols were detected.

Identification of the Major Products from the Reaction of 10-OOH-18:2 with Hematin. 10-Oxo-10:1 almost certainly arises via β-scission of an alkoxyl radical. One of the major determinants of the extent of β-scission of alkoxyl radicals is the stability of the radical produced by fragmentation. Introduction of a double bond at the 12,13-position should yield an alkoxyl radical that can undergo β-scission to a highly resonance-stabilized allylic radical. We, therefore, synthesized 10-hydroperoxy-8,12-octadecadienoic acid (10-OOH-18:2) and subjected it to reaction with hematin under standard conditions. The straight-phase HPLC profile of the reaction mixture revealed four products (Figure 6).

Peak 1 was shown by GC–MS to be 10-oxo-8,12-octadecadienoic acid (10-oxo-18:2). Electron impact mass spectrometry of its methyl ester displayed a molecular ion at m/z 308. The spectrum was featureless, possibly due to enolization of the ketone, so the methoxime derivative was prepared. Ions were observed at m/z 337 (M^+), 306 ($M - OCH_3$), 266 [$H_3COOC(CH_2)_6CH{=}CHC(=NOCH_3)CH{=}CHCH_2^+$], 236 [$M - H_3COOC(CH_2)_3^\bullet$], 222 [$M - H_3COOC(CH_2)_4^\bullet$], and 194 [$CH_2{=}CHC(N{=}O^+CH_3){=}CHCH{=}CH(CH_2)_4CH_3$].

Peak 2 corresponded to unreacted starting material and peak 4 to its alcohol reduction product. The identity of peak 3 was confirmed by comparing its GC–MS properties with an authentic sample. This metabolite was shown to be identical with 10-oxo-8-decenoic acid.

The time course of reaction of 10-OOH-18:2 with hematin (Figure 7) was similar to that of 10-OOH-18:1. Hydroperoxide consumption slowed markedly by 30 s and ceased after 2 min due to consumption of catalyst. Addition of fresh hematin after 5 min led to further hydroperoxide disappearance. The ratio of yields of 10-oxo-18:1 to 10-oxo-10:1 remained constant over the period of time examined after the first addition of hematin.

The effect of varying the ratio of hydroperoxide to heme is reported in Table II. Little or no difference in the yield of 10-oxo-18:1 was observed when this ratio was decreased from 100:1 to 1:1. At equimolar hydroperoxide to heme, a reaction was conducted in the presence of a 2000-fold excess

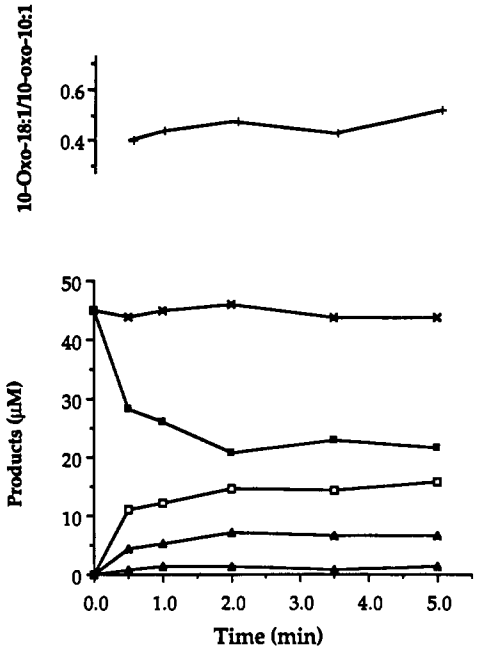


FIGURE 7: Time course for the reaction of hematin with 10-OOH-18:2. The concentrations were 0.5 μM hematin and 50 μM 10-OOH-18:2. 10-Oxo-10:1 (opened squares), 10-oxo-18:2 (opened triangles), 10-OH-18:2 (closed triangles), and 10-OOH-18:2 (closed squares). The recovery of products for each time point is indicated by ×. The ratio of 10-oxo-18:2 to 10-oxo-10:1 for each time point is represented by +. The values are the means of duplicates.

Table II: Effect of Varying Hydroperoxide:Heme Ratios on the Products of 10-OOH-18:2 Metabolism[a]

[hydroperoxide]:[heme]	10-oxo-18:2	10-oxo-10:1	10-OH-18:2
100:1	50 ± 5	47 ± 4	5 ± 2
50:1	52 ± 1	47 ± 2	1 ± 1
10:1	40 ± 4	54 ± 5	6 ± 3
5:1	42 ± 3	50 ± 4	9 ± 3
1:1	45 ± 2	42 ± 2	12 ± 1
1:1 + 1 mM HQ	54 ± 2	34 ± 2	12 ± 2
100:1 + 1.5 mM BHA	27 ± 3	73 ± 4	2 ± 1

[a] Reactions were performed at 25 °C with 0.5 μM heme and the indicated concentrations of [1-^{14}C]-10-OOH-18:2 in sodium phosphate buffer pH 7.8 containing 200 μM Tween 20. HQ, hydroquinone; BHA, butylated hydroxyanisole. The values reported are percentages of products based on recovered starting material. The percentages of recovered hydroperoxide for each experiment are as follows: 100:1, −53%; 50:1, 17%; 10:1, 4%; 5:1, 10%; 1:1, 3%;, 1:1 + HQ, 5%; 100:1 + BHA, 22%.

of hydroquinone. Hydroquinone was added to reduce any metal–oxo derivatives produced during the reaction. No alteration of the product profile was detected. Inclusion of a 3000-fold excess of butylated hydroxyanisole at a hydroperoxide to heme ratio of 100:1 actually increased the yield of 10-oxo-10:1 in addition to stimulating the overall conversion of hydroperoxide.

The higher yield (59%) of 10-oxo-10:1 in the reaction of 10-OOH-18:2 with hematin is consistent with the former arising by β-scission of an intermediate alkoxyl radical. The other putative cleavage product is the 2-octenyl radical, which should couple to O_2 and form inter alia 2-octen-1-ol and 2-octenal. Reaction mixtures were extracted with hexane prior to acidification and analyzed by GC on a capillary column. Peaks were detected at the retention times of 2-octen-1-ol and 2-octenal that gave mass spectra identical with that of authentic standards when analyzed by GC–MS (chemical ionization and electron impact). The yields of the alcohol and aldehyde were approximately 2% each. The yields of 2-octen-1-ol and 2-octenal, based on the amount of 10-oxo-10:1, are each 5% of theoretical for every β-scission event.

Table III: Incorporation of $^{18}O_2$ or R^{18}O^{18}OH into Products of BP-7,8-diol Oxidation by Hematin and 10-OOH-18:1 or 10-OOH-18:2

	atom % excess ^{18}O[a]			
	TA	TS	CA	CS
10-^{11}O^{16}OH-18:1, $^{18}O_2$	62	58	62	62
10-^{18}O^{18}OH-18:1, $^{16}O_2$	32	ND	42	55
10-^{16}O^{16}OH-18:2, $^{18}O_2$	66	ND	63	62

[a] TA, TS, CS, and CA represent, respectively, *trans-anti-*, *trans-syn-*, *cis-anti-*, and *cis-syn*-tetrols (7,8,9,10-tetrahydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene). ND, not detected.

BP-7,8-diol Oxidation. Reaction of unsaturated fatty acid hydroperoxides with hematin generates oxidants that epoxidize the nonaromatic double bond of BP-7,8-diol. A comparison was made of the ability of 13-OOH-18:2, 10-OOH-18:1, and 10-OOH-18:2 to epoxidize BP-7,8-diol. Hydroperoxide (50 μM) was added to a solution of hematin (0.5 μM) and [^{14}C]-(±)-BP-7,8-diol (7.6 nmol, 18 μM, 0.5 μCi) in 430 μL of buffer A. The incubation was allowed to continue for 10 min at 25 °C. The products were extracted into ethyl acetate and, after removal of the solvent, separated by HPLC. The anti and syn dihydrodiol epoxides each solvolyze to *cis-* and *trans*-tetrahydrotetrols that are stable and separable by HPLC. The radioactivity that eluted in zones corresponding to the unlabeled standards of the four possible tetrahydrotetrols was used to quantitate the extent of epoxidation. The extent of epoxidation was highest with 13-OOH-18:2 (1.5 ± 0.1 nmol/mL tetrols generated) among a series of unsaturated fatty acid hydroperoxides. The yields of tetrols produced from 10-OOH-18:2 and 10-OOH-18:1 were 1.1 ± 0.1 and 0.85 ± 0.1 nmol/mL, respectively.

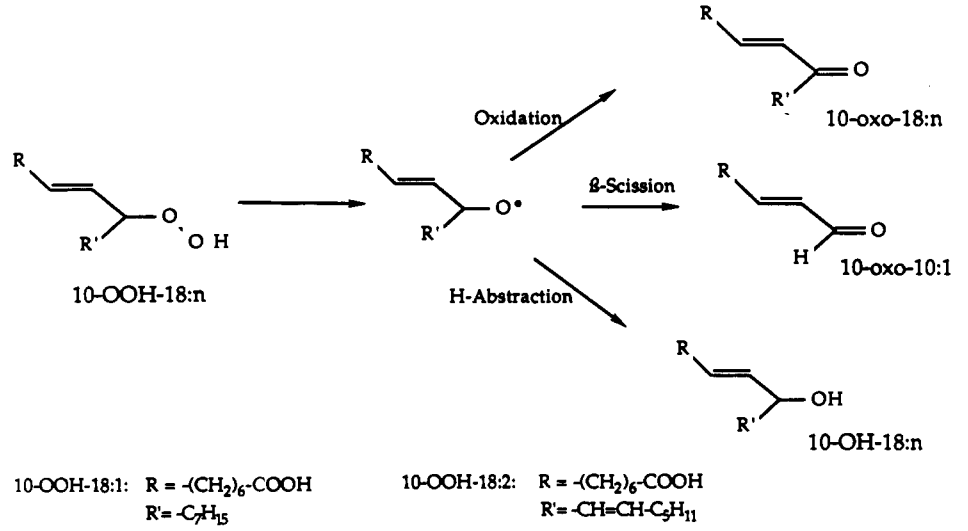
The principal source of the epoxide oxygen (~90%) introduced during the oxidation of BP-7,8-diol by 13-OOH-18:2 and hematin is O_2; approximately 10% originates from the hydroperoxide oxygens (Dix et al., 1985). A series of experiments was performed to determine the origin of the epoxide oxygen introduced in the hematin-catalyzed oxidations by 10-OOH-18:1 and 10-OOH-18:2. Separate incubations were carried out with ^{18}O-labeled hydroperoxides or $^{18}O_2$. The isotopic abundance of ^{18}O in 10-^{18}O^{18}OH-18:1 was 92 atom % excess. The solvolysis products were isolated by HPLC and the incorporation of ^{18}O determined by direct probe mass spectrometry of peracetyl derivatives. Approximately 30–35% of the epoxide oxygen was derived from hydroperoxide oxygen and 60–65% from molecular oxygen (Table III).

The major oxidation product in all cases is the *trans-anti*-tetrol, which makes quantitation of its isotopic abundance most reliable of all of the tetrols. The yields of *trans-syn-*, *cis-anti-*, and *cis-syn*-tetrols were in general low, which made the determination of the atom percent excess difficult due to high background in the mass spectra. This could explain the variations in some of the values in Table III.

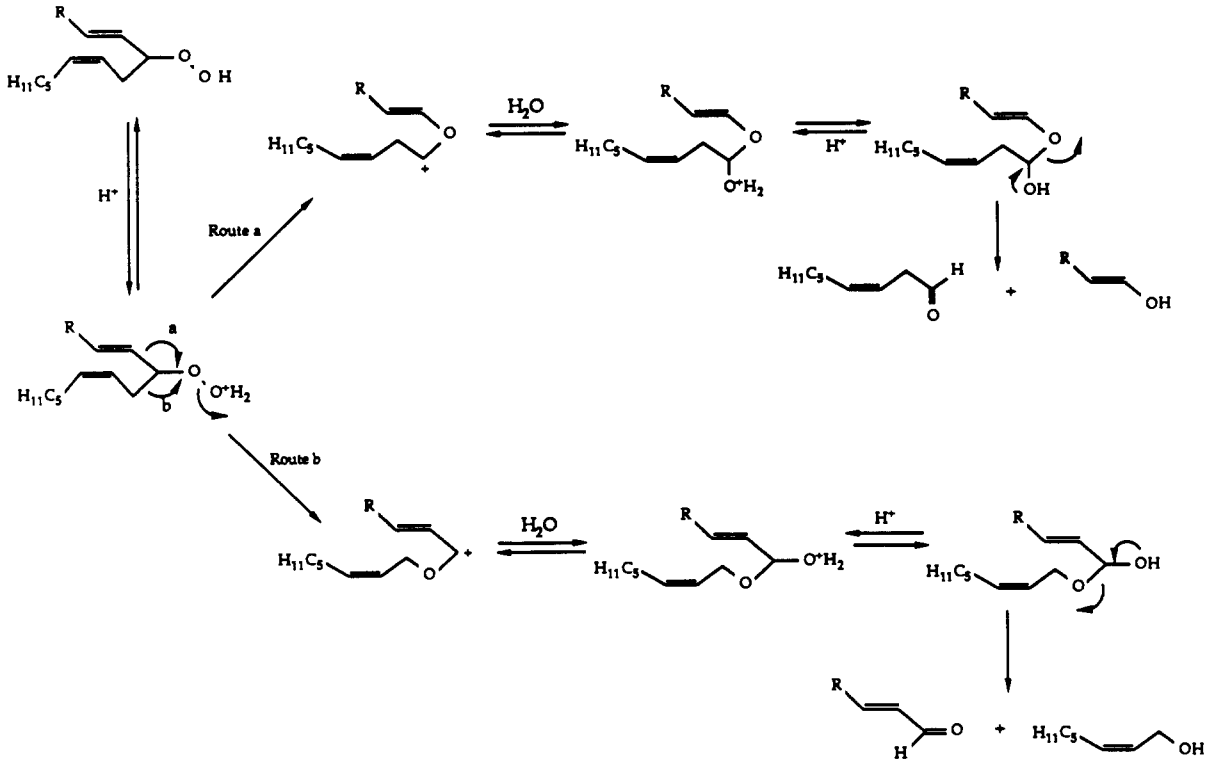
Discussion

Reaction of 13-OOH-18:2 with hematin in detergent-containing buffers produces epoxy alcohols in 65% yield (Dix & Marnett, 1985). The epoxy alcohols result from cyclization of alkoxyl pentadienyl radicals to epoxy allylic radicals that are trapped by O_2 or Fe^{4+}=O. The present study demonstrates the importance of allylic stabilization to cyclization of alkoxyl radicals. No epoxide-containing products were detected in the reaction of hematin with 10-OOH-18:1 or 10-OOH-18:2. The exclusive products are ketones and alcohols

Scheme I: Proposed Mechanism for the Hematin-Catalyzed Decomposition of 10-OOH-18:1 or 10-OOH-18:2



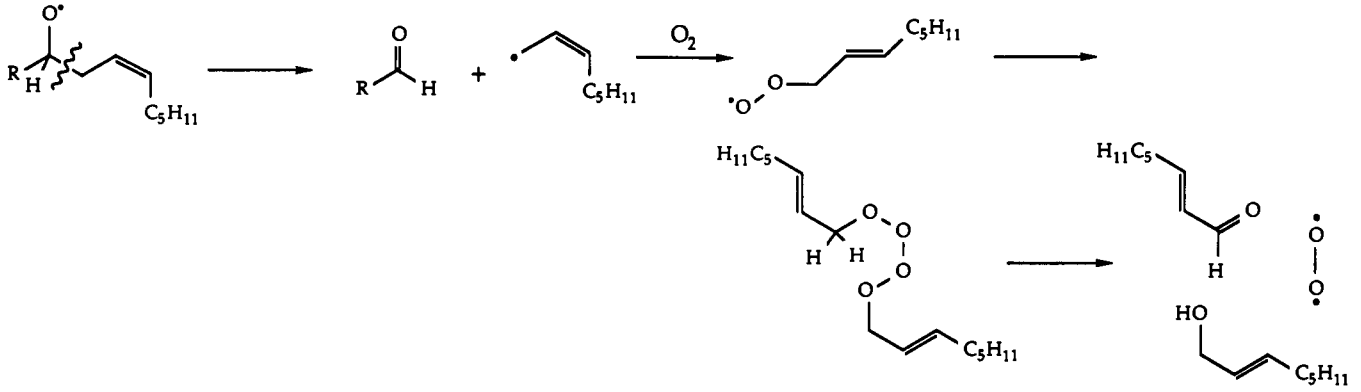
Scheme II: Possible Mechanisms for the Lewis Acid Catalyzed Decomposition of 10-OOH-18:2



that result from dehydration and reduction, respectively, and an aldehyde that results from β-scission of the intermediate alkoxyl radical (Scheme I). The amount of the β-scission product is determined by the stability of the carbon radical produced in the cleavage (Walling, 1967). When the radical is primary (from 10-OOH-18:1) low yields (4%) are obtained, but when it is allylic (from 10-OOH-18:2) high yields are observed (59%). The fact that the yields of products (ketone/alcohol/aldehyde) are reciprocally related implies that they are derived from a single reactive intermediate—an alkoxyl radical. Additional support for a single reaction intermediate is the finding that the product ratios are constant with time (Figure 7). If two separate reaction pathways operate, one anticipates the ratios would vary unless the rates of both reactions are identical. The probability of this seems low, but the rapidity with which the reactions proceed makes it difficult to determine the product profile at very early times when the extent of conversion seems low.

An aldehyde could conceivably be formed from 10-OOH-18:2 by a process known as the Hock cleavage in which heme acts as a Lewis acid rather than a redox catalyst (Hock & Scharder, 1936; Hock & Kropf, 1957). This reaction proceeds via the Criegee rearrangement (Ohloff et al., 1970; Turner & Herz, 1977; Frimer et al., 1979; Bartlett & Frimer, 1978) in which the vinyl group migrates to electropositive oxygen. In the case of 10-OOH-18:2, this would produce 3-nonenal and 9-oxo-9:0 (route a, Scheme II) (Wurzenberger & Grosch, 1986). Neither product was detected in the reaction with hematin. 10-Oxo-10:1 could be produced if alkyl rather than vinyl migration occurs (route b, Scheme II), but the only side product would be 2-octenol. Besides the fact that alkyl migration is unprecedented, the formation of 2-octenal in amounts equal to 2-octenol argues against the operation of such a mechanism. The most likely explanation for the formation

Scheme III: Proposed Mechanism for the Formation of 2-Octenol and 2-Octenal



of equal amounts of octenol and octenal is that the allylic radical formed by β-scission of the alkoxyl radical couples to O_2 to form a peroxyl radical. Two peroxyl radicals then undergo a Russell reaction to form equal amounts of alcohol and aldehyde (Scheme III) (Russell, 1957). It is also important to note that the yields of 2-octenal and 2-octenol are not equivalent to those of 10-oxo-10:1. This is probably because generation of carbon radicals in the presence of several potent oxidizing agents leads to a multiplicity of products. Thus, the available evidence suggests that the reaction of hematin with 10-OOH-18:1 and 10-OOH-18:2 proceeds via the mechanism illustrated in Scheme I in which the metal complex functions as a redox catalyst.

Although 10-oxo-18:2 is nominally a dehydration product of the hydroperoxide, we propose it arises by oxidation of the alkoxyl radical by the Fe^{4+}=O, which is the oxidized heme formed during hydroperoxide reduction. Oxidation of the alkoxyl radical would regenerate hematin for another cycle of catalysis. The importance of the metal complex in ketone formation is indicated by the finding that greatly reduced amounts of 10-oxo-18:2 are formed in the reaction of the 10-OOH-18:2 with Fe^{2+}–cysteine (8 vs 55% hematin). The alcohols detected in the reaction of hematin with both hydroperoxides may result from H abstraction by the alkoxyl radical, heterolytic cleavage of the hydroperoxides, or homolytic cleavage to alcohol and hydroxyl radical. Evidence supporting H abstraction by the alkoxyl radical is the dependence of the yield of alcohol on the fate of the alkoxyl radical and the detection of hydroperoxide *oxidation* products of uncertain origin. Evidence against H abstraction by the alkoxyl radical is that carrying out the reactions in the presence of excess H donors (hydroquinone or butylated hydroxyanisole) does not increase the yield of alcohol. Considering the low yields of alcohols derived from each hydroperoxide, it is difficult to definitively assign their pathway of formation.

The finding that the major products of reaction of hematin with fatty acid hydroperoxides are derived from alkoxyl radicals implies heme cleaves the hydroperoxide bond homolytically (eq 1). It is conceivable that alkoxyl radicals are generated following heterolytic cleavage as a result of oxidation of the alcohol by the heme–oxo complex (eq 5). A related

$$\text{ROOH} + \text{Fe}^{3+}\text{-PPIX} \longrightarrow \text{ROH} + \text{O=Fe}^{4+}\text{-PPIX}^{\bullet+} \longrightarrow \text{RO}^{\bullet} + \text{O=Fe}^{4+}\text{-PPIX} \quad (5)$$

reaction occurs in the metabolism of organic hydroperoxides to aldehydes by catalase (Schonbaum & Chance, 1976). In the catalase reaction, oxidation of primary alcohols proceeds directly to aldehydes without the apparent intermediacy of alkoxyl radicals. The analogous reaction with secondary hydroperoxides (such as 10-OOH-18:1 and 10-OOH-18:2) would produce the ketones 10-oxo-18:1 and 10-oxo-18:2. We probed for alcohol oxidation by adding unlabeled 10-OOH-18:2 to hematin in the presence of [1-^{14}C]-10-OH-18:2. The labeled alcohol was recovered unchanged from these reaction mixtures, and no oxidation products were detected.[2] Thus, we can uncover no direct evidence for heterolytic cleavage of the hydroperoxide bond by hematin (vide infra).

A number of literature reports describe reactions of iodosylbenzene and peracids with transition-metal complexes and metalloproteins that are best interpreted by invoking heterolytic cleavage (Groves, 1980; McMurry & Groves, 1986; Traylor et al., 1984). However, the mechanism of alkyl hydroperoxide reduction by metals appears to depend on the structure of the hydroperoxide and the coordination sphere of the metal (White & Coon, 1980; Lee & Bruice, 1985; Mansuy et al., 1982, 1984). Recently, Traylor and Xu proposed that alkyl hydroperoxides are reduced heterolytically by iron porphyrins, but the metal–oxo complex generated (analogous to horseradish peroxidase compound I) reacts with a second molecule of hydroperoxide to produce peroxyl radicals and a one-electron-reduced iron–oxo complex (analogous to horseradish peroxidase compound II) (Traylor & Xu, 1987). Thus, some of the chemistry we observe may result from such secondary reactions of iron–oxo complexes and hydroperoxide. If such reactions occurred in the present case, one would anticipate that they would be most important at high ratios of hydroperoxide to heme. The data in Table II demonstrate that the product profile is invariant from ratios of 100:1 to 1:1. At hydroperoxide to heme ratios of 100:1 and 1:1, inclusion of substantial excesses of phenolic antioxidants, which are superior to hydroperoxides as H atom donors, does not increase the production of the heterolytic reduction product 10-OH-18:2. At equimolar concentrations of 10:OOH-18:2 and hematin in the presence of 1 mM hydroquinone, the product detected should most accurately reflect the chemistry of the primary reaction of the hydroperoxide with the catalyst. Under these conditions, the products are predominantly those of homolytic hydroperoxide cleavage.

Metabolism of polyunsaturated fatty acids by plant and animal tissue to shorter chain aldehydes has been reported. Grosch and co-workers have shown that a protein fraction from mushrooms (*Psalliota bispora*) cleaves linoleic and linolenic acids into 10-oxo-10:1 (Wurzenberger & Grosch, 1982, 1984, 1986). 10-OOH-18:2 and 10-hydroperoxy-8,12,15-octadecatrienoic acid (10-OOH-18:3) are also substrates for the enzyme and are likely intermediates in the transformation of the fatty acids to aldehydes.[3] Glasgow et al. (1986) and Fruteau de

[2] R. Labeque, unpublished result.

Laclos et al. (1987) have recently reported that arachidonic acid stimulated porcine leukocytes or human platelets generate 12-oxododeca-5,8,10-trienoic acid (12-oxo-12:3), a shorter chain aldehyde structurally analogous to 10-oxo-10:1. Besides the formation of 12-oxo-12:3, the reaction of arachidonic acid with human platelets leads to the production of 12-hydroxyeicosatetraenoic acid (12-OH-20:4), 12-oxoeicosatetraenoic acid (12-oxo-20:4), 12-hydroxyheptadecatrienoic acid, 10-hydroxy-11,12-epoxyeicosatrienoic acid, and thromboxane B_2. 12-Oxo-12:3, 12-OH-20:4, 12-oxo-20:4, and 10-hydroxy-11,12-eicosatetraenoic acid are believed to be formed from 12-hydroperoxyeicosatetraenoic acid (12-OOH-20:4), a lipoxygenase oxidation product of arachidonic acid. 12-Oxo-20:4 and 12-oxo-12:3 are also obtained from reaction of 12-OOH-20:4 with cytochrome *c*, hemoglobin, horseradish peroxidase, or heat-denatured platelets. These findings strongly suggest that the carbonyl-containing compounds are products of metal-catalyzed reactions and that the metal is probably heme iron.

The transformations of 10-OOH-18:1 and 10-OOH-18:2 provide information about the identity of the oxidants generated in the reactions with hematin. Epoxidation of BP-7,8-diol can be effected by peroxyl radicals or by metal–oxo complexes (Marnett, 1987; Catalano & Ortiz de Montellano, 1987). Isotopic labeling experiments indicate that most of the epoxide oxygen (~60–65%) derives from O_2, which is consistent with peroxyl radical intermediates. There are two possible mechanisms of peroxyl radical generation. β-Scission of the alkoxyl radicals from 10-OOH-18:1 and 10-OOH-18:2 produces carbon radicals that couple to O_2 forming peroxyl radicals. Because of the much greater extent of β-scission detected with 10-OOH-18:2, one anticipates that this hydroperoxide should trigger significantly greater epoxidation than 10-OOH-18:1. The 37% greater epoxidation observed does not correlate to the 15-fold higher β-scission. However, the carbon radicals that form the peroxyl radicals are different for the two hydroperoxides. A primary radical results from 10-OOH-18:1, whereas an allylic radical results from 10-OOH-18:2. These radicals, which differ greatly in stability, may suffer different fates in the solvent cage thereby affecting the yield of peroxyl radicals. Alternatively, the peroxyl radicals may undergo termination at differential rates. Epoxidation of BP-7,8-diol may be competitive with bimolecular consumption of peroxyl radicals via the Russell reaction. The rate of the Russell reaction increases as the square of peroxyl radical concentration, whereas the rate of epoxidation is linearly dependent on peroxyl radical concentration. Therefore, even though the extent of β-scission is much higher for alkoxyl radicals derived from 10-OOH-18:2 than 10-OOH-18:1, the peroxyl radicals formed may be consumed in termination reactions. Although we can only account for 10% of the β-scission events of the former as peroxyl radicals that produce 2-octenal and 2-octenol via the Russell reaction, similar considerations would apply to isomeric peroxyl radicals generated by O_2 coupling at the other allylic terminus.

The second mechanism for peroxyl radical generation is oxidation of the hydroperoxide. Hydroperoxide oxidation was detected in low yield (~10%) in our previous studies of the reaction of hematin with 13-OOH-18:2 (Dix & Marnett, 1985). In that case, the epoxide oxygen was not quantitatively derived from the hydroperoxide oxygen because the oxygen of the pentadienyl peroxyl radical equilibrates with O_2 via a β-scission-readdition process first described by Chan (Chan et al., 1979). The percentage incorporation of hydroperoxide oxygen is higher in the present experiments (~35%). Porter and associates recently reported that allylic peroxyl radicals do not equilibrate the peroxyl oxygen atoms with O_2, so one expects a higher incorporation of epoxide oxygen when the oxidants are peroxyl radicals generated by oxidation of the hydroperoxide group (Porter & Wujek, 1987). The identity of the oxidizing agent that removes the H atom is unknown. Metal–oxo complexes or alkoxyl radicals are likely candidates. Most of the alkoxyl radicals appear to undergo β-scission, so metal–oxo complexes (compound I- or II-like) may be quantitatively more important.

We cannot rule out the possibility that the epoxide oxygen derived from the hydroperoxide group represents epoxidation by a ferryl–oxo complex (compound I-like). Production of this oxidant requires heterolytic cleavage of the hydroperoxide, but the available evidence implies that peroxide heterolysis is not a major pathway for hematin-catalyzed hydroperoxide consumption. The amount of BP-7,8-diol oxidized is low compared to the amount of hydroperoxide turned over, so it is conceivable that minor pathways of hydroperoxide metabolism may contribute to oxidant generation. One possibility for differentiating peroxyl radical and ferryl–oxo oxidants is to carry out the epoxidation of *cis*-stilbene with isotopically labeled hydroperoxide. The ferryl–oxo complex of hemoglobin oxidizes *cis*-stilbene to *cis*-stilbene oxide with incorporation of oxygen from hydroperoxide, whereas peroxyl radicals generated by hydroperoxide oxidation oxidize it to *trans*-stilbene oxide with incorporation of hydroperoxide oxygen (Catalano & Ortiz de Montellano, 1987). Unfortunately, *cis*-stilbene is not oxidized by hematin in the presence of any of the fatty acid hydroperoxides that we have used.[4]

SUMMARY

13-OOH-18:2, 10-OOH-18:2, and 10-OOH-18:1 epoxidize BP-7,8-diol to comparable extents in the presence of hematin. The common feature of the oxidations is that hematin reduces the hydroperoxide bond homolytically to alkoxyl radicals, which are converted to peroxyl radicals. The latter serve as the ultimate oxidizing agents. Rather dramatic differences exist in the pathways that the alkoxyl radicals follow to peroxyl radicals. Exploration of these pathways explains the biosynthesis of several novel metabolites of polyunsaturated fatty acids and provides a potentially useful diagnostic probe of metal–hydroperoxide interactions (Labeque & Marnett, 1987).

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[3] The formation of 10-hydroperoxy derivatives of linoleic and linolenic acids is interesting because these compounds result chemically from singlet oxygen oxidation but not free-radical autoxidation. All of the well-characterized lipoxygenases produce free-radical oxygenation products so the mushroom enzyme may oxygenate unsaturated fatty acids by a novel mechanism.

[4] R. Labeque, unpublished result.

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Isolation and Identification of Seven Metabolites of 25-Hydroxydihydrotachysterol$_3$ Formed in the Isolated Perfused Rat Kidney: A Model for the Study of Side-Chain Metabolism of Vitamin D†

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ABSTRACT: The in vivo metabolism of dihydrotachysterol$_3$, an analogue of vitamin D$_3$ and a potent calcemic factor, has been studied in the rat. This in vivo metabolism is compared to the in vitro metabolism of 25-hydroxydihydrotachysterol$_3$ in the perfused rat kidney. Using mass spectrometry and ultraviolet spectroscopy, we have identified seven novel metabolites derived from 25-hydroxydihydrotachysterol$_3$. The seven compounds represent intermediates on two renal pathways (24-oxidation and 26,23-lactone formation) also observed for 25-hydroxyvitamin D$_3$. No evidence was found for the renal synthesis of a 1-hydroxylated metabolite of 25-hydroxydihydrotachysterol$_3$ analogous to the hormone 1,25-dihydroxyvitamin D$_3$. Two of the compounds formed in vitro, 24,25-dihydroxydihydrotachysterol$_3$ and 25-hydroxydihydrotachysterol 26,23-lactone, were also formed in vivo. In vivo studies also revealed the formation of two other unidentified metabolites which are presumed to be formed nonrenally and may be calcemic factors. This work shows that dihydrotachysterol$_3$ metabolism is complex and probably utilizes the same side-chain enzymes as vitamin D$_3$. In addition, our work also confirms that intermediates postulated to lie on pathways to 26,23-lactone in the vitamin D$_3$ series are also formed for the side chain in dihydrotachysterol$_3$.

Although dihydrotachysterol$_2$ (DHT$_2$)[1] has been used for the treatment of hypocalcaemia associated with chronic renal disease for many decades (Cordy & Hodsman, 1984) since it was first introduced by E. Merck in 1934 (Fieser & Fieser, 1959), little is known about its metabolism or mechanism of action. Early in vivo metabolic studies were carried out with dihydrotachysterol$_3$ (DHT$_3$), which is more stable and easier to synthesize than DHT$_2$ (Hallick & DeLuca, 1971; Lawson & Bell, 1974). Both these groups reported the presence of metabolites more polar than 25-hydroxydihydrotachysterol$_3$ (25-OH-DHT$_3$), which had been shown to be formed from DHT$_3$ by rat liver homogenates (Bhattacharyya & DeLuca, 1973). Suda et al. (1970) had synthesized 25-OH-DHT$_3$ and showed that it stimulated intestinal calcium transport and bone calcium mobilization in rats. It was therefore suggested (Wing et al., 1974) that the active calcaemic metabolite of DHT was the 25-hydroxylated compound produced in the liver, which

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[1] Abbreviations: DHT, dihydrotachysterol$_2$ or dihydrotachysterol$_3$; DHT$_2$, dihydrotachysterol$_2$; DHT$_3$, dihydrotachysterol$_3$; 25-OH-DHT$_3$, 25-hydroxydihydrotachysterol$_3$; 1,25-(OH)$_2$DHT$_3$, 1,25-dihydroxydihydrotachysterol$_3$; 23,25-(OH)$_2$DHT$_3$, 23,25-dihydroxydihydrotachysterol$_3$; 24,25-(OH)$_2$DHT$_3$, 24,25-dihydroxydihydrotachysterol$_3$; 23,25,26-(OH)$_3$DHT$_3$, 23,25,26-trihydroxydihydrotachysterol$_3$; 24-oxo-25-OH-DHT$_3$, 24-oxo-25-hydroxydihydrotachysterol$_3$; 24-oxo-23,25-(OH)$_2$DHT$_3$, 24-oxo-23,25-dihydroxydihydrotachysterol$_3$; 25-OH-DHT$_3$-26,23-lactone, 25-hydroxydihydrotachysterol$_3$ 26,23-lactone; 25-OH-DHT$_3$-26,23-lactol, 25-hydroxydihydrotachysterol$_3$ 26,23-lactol; 25-OH-D$_3$, 25-hydroxyvitamin D$_3$; 1,25-(OH)$_2$D$_3$, 1,25-dihydroxyvitamin D$_3$; 23,25-(OH)$_2$D$_3$, 23,25-dihydroxyvitamin D$_3$; 24,25-(OH)$_2$D$_3$, 24,25-dihydroxyvitamin D$_3$; 25,26-(OH)$_2$D$_3$, 25,26-dihydroxyvitamin D$_3$; 24-oxo-23,25-(OH)$_2$D$_3$, 24-oxo-23,25-dihydroxyvitamin D$_3$; 25-OH-D$_3$-26,23-lactone, 25-hydroxyvitamin D$_3$ 26,23-lactone; 25-OH-D$_3$-26,23-lactol, 25-hydroxyvitamin D$_3$ 26,23-lactol; BSTFA, *N,O*-bis(trimethylsilyl)trifluoroacetamide; TSIM, (trimethylsilyl)imidazole; TMCS, trimethylchlorosilane.

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