Stereoselective oxidation of regioisomeric octadecenoic acids by fatty acid dioxygenases

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Abstract Seven Z-octadecenoic acids having the double bond located in positions 6Z to 13Z were photooxidized. The resulting hydroperoxy-E-octadecenoic acids [HpOME(E)] were resolved by chiral phase-HPLC-MS, and the absolute configurations of the enantiomers were determined by gas chromatographic analysis of diastereoisomeric derivatives. The MS/MS/MS spectra showed characteristic fragments, which were influenced by the distance between the hydroperoxide and carboxyl groups. These fatty acids were then investigated as substrates of cyclooxygenase-1 (COX-1), manganese lipoxygenase (MnLOX), and the (8R)-dioxygenase (8R-DOX) activities of two linoleate diol synthases (LDS) and 10R-DOX. COX-1 and MnLOX abstracted hydrogen at C-11 of (12Z)-18:1 and C-12 of (13Z)-18:1. (11Z)-18:1 was subject to hydrogen abstraction at C-10 by MnLOX and at both allylic positions by COX-1. Both allylic hydrogens of (8Z)-18:1 were also abstracted by 8R-DOX activities of LDS and 10R-DOX, but only the allylic hydrogens close to the carboxyl groups of (11Z)-18:1 and (12Z)-18:1. 8R-DOX also oxidized monoenoic C14-C20 fatty acids with double bonds at the (9Z) position, suggesting that the length of the omega end has little influence on positioning for oxygenation.

We conclude that COX-1 and MnLOX can readily abstract allylic hydrogens of octadecenoic fatty acids from C-10 to C-12 and 8R-DOX from C-7 and C-12. — Oliw, E. H., A. Wennman, I. Hoffmann, U. Garscha, M. Hamberg, and F. Jernerén. Stereoselective oxidation of regioisomeric octadecenoic acids by fatty acid dioxygenases. *J. Lipid Res.* 2011: 52: 1995–2004.

Supplementary key words chiral phase HPLC • cyclooxygenase • enzy-mology/enzyme mechanisms • fatty acid/oxygenation • linoleate diol synthase • manganese lipoxygenase • mass spectrometry

Monounsaturated fatty acids and their derivatives are of biological interest as nutrients, signal molecules, and precursors of biological mediators. Palmitoleic acid can regulate systemic metabolic homeostasis, and it potentiates the effects of insulin (1, 2). Oleic acid modulates inflammation, and both oleic acid and oleylethanolamine suppress food intake (3–6). Mono- and polysaturated fatty acids can be oxygenated by P450, heme-dependent fatty acid dioxygenases, and lipoxygenases to a long list of biological mediators, eicosanoids, and oxylipins. Oxidation of arachidonate in humans and linoleic and α-linolenic acids in plants has been studied extensively (7–10). In contrast to plants and animals, some microorganisms can also oxygenate monounsaturated fatty acids efficiently to oxylipins of physiological interest (11, 12).

Enzymatic oxidation of octadecenoic acids to hydroperoxides has been reported in bacteria (e.g., *Pseudomonas aeruginosa*) and fungi (e.g., *Gaummannomyces graminis*, aspergilli). *P. aeruginosa* expresses a prominent enzyme in the periplasm that oxidizes oleic acid sequentially to (10S)-hydroperoxy-(8E)-octadecenoic acid (10S-HpOME(E)) and (7S,10S)-dihydroxy-(8E)-octadecenoic acid (12). Oleic acid is oxidized by *G. graminis* and several aspergilli sequentially to (8R)-hydroperoxyoleic acid (8R-HpOME(E)) and to diols, e.g., (7S,8S)-dihydroxyoleic acid (7S,8S-DHOME), 5S,8S-DiHOME, and 8R,11S-DiHOME (13–15). These oxylipins affect sexual and asexual sporulation in *Aspergillus nidulans* (14), and the key intermediate, 8R-HpOME(E), is formed by two closely related enzymes, linoleate diol synthases (LDS) and linoleate 10R-dioxygenases (10R-DOX). These enzymes belong to the group of animal heme peroxidases/myeloperoxidases that also include cyclooxygenases (COX) of animals and α-DOX of plants (15, 16).

Abbreviations: CP, chiral phase; COX, cyclooxygenase; DOX, dioxygenase; HETE, hydroxyeicosatetraenoic acid; HpOEME(mZ), hydroperoxy-(mZ)-octadecenoic acid; HOME(mZ), hydroxy-(mZ)-octadecenoic acid; HpETE, hydroperoxyeicosatetraenoic acid; HpODE, hydroperoxyoctadecadienoic acid; KOME, ketoctadecenoic acid; LDS, linoleate diol synthase; LOX, lipoxygenase; MnLOX, manganese lipoxygenase; NL, normalized; NP, normal phase; PGH, prostaglandin H; RP, reverse phase; TIC, total ion current.

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COX transforms certain polyunsaturated C20 fatty acids sequentially to PGG and PGH compounds, and arachidonic acid is the most important physiological substrate (16). This enzyme also oxygenates (8Z,11Z,14Z)-20:3 to prostaglandins and Mead acid [\((5Z,8Z,11Z)-20:3\)], linoleic acid, and oleic acid to hydroperoxides (17-19). The 3D structure of COX-1 has been determined with polyunsaturated C20 and C18 fatty acids in the active site (20). Arachidonic and linoleic acids bind with the bisallylic methylene, from which hydrogen abstraction takes place, close to the catalytic Tyr-385, and the omega ends align with three phenylalanine residues.

In contrast to heme-dependent dioxygenases, lipooxygenases oxidize monoenoic fatty acids only at insignificant rates (16, 21). Lipooxygenases contain nonheme iron as catalytic metal with at least one exception, manganese lipoxygenase (MnLOX) (22). This metal may affect oxygenation of 18:1 as MnLOX can oxygenate octadecenoic acids to HpOME, and soybean LOX-1 mainly forms enones (19, 23).

Regioisomeric octadecenoic acids can be used to systematically determine the importance of the position of the double bond for hydrogen abstraction by fatty acid dioxygenases. These studies would be facilitated by methods for steric analysis of the products, either the hydroperoxides or the corresponding alcohols. Unfortunately, there is little information on chromatographic separation of enantiomers of HOME and HpOME.

Chiral phase-HPLC (CP-HPLC) is now routinely used for analysis of polyunsaturated hydroxy and hydroperoxy fatty acids, e.g., HETE and HpETE (24). Versatile matrices contain chiral selectors linked to cellulose or amylose, which are coated on silica, e.g., 3,5-dimethylphenyl carbamate (Chiralcel OD, Chiralpak AD, Reprosil Chiral-AM) and benzoate (Chiralcel OB). With different alcoholic modifiers, these matrices can separate a large number of hydroxy and hydroperoxy metabolites (24), but they cannot be eluted with high pressure and do not tolerate certain solvents. A more stable alternative is a chiral selector of “Pirkle type” on silica (Reprosil Chiral NR3), which can separate enantiomers of hydroperoxyecosatetraenoic and hydroperoxyoctadecadienoic acids, as well as enantiomers of many drugs (25, 26).

The first goal of the present study was to develop a method for CP-HPLC separation of enantiomers of HpOME and to study their fragmentation during LC-MS analysis. Racemic HpOME were obtained by photooxidation and by autoxidation. Seven octadecenoic acids were investigated [double bonds at positions (6Z)-(13Z), except at (10Z)], and the hydroperoxides were analyzed by CP-HPLC on Reprosil Chiral NR. Our second goal was to determine the position specificity of fatty acid dioxygenases for hydrogen abstraction of regioisomeric octadecenoic acids. We chose the 8R-DOX activities of LDS (7,8-LDS of G. graminis and 5,8-LDS of A. fumigatus) and 10R-DOX of A. fumigatus, all with olate 8R-DOX activities, and oive COX-1 and MnLOX. To facilitate the analysis, we silenced the hydroperoxide isomerase (P450) activities of LDS by point mutation (5,8-LDS Cys1006Ser) or truncation of the carboxyl end (7,8-LDS-1,673) (27). The heme-dependent dioxygenases catalyze antarafacial hydrogen abstraction and oxygenation, whereas MnLOX catalyzes suprafacial abstraction and oxygenation (28). Our third goal was to determine how the chain length affects the oxidation of monoenoic fatty acids by the 8R-DOX activity of LDS.

### Experimental Procedures

**Materials**

HPLC solvents (Lichrosolv) and routine chemicals were from Merck. (9Z)-18:1 (99%) and (11Z)-18:1 (99%) were from Larodan. (7Z)-18:1 (99%), (8Z)-18:1 (99%), and (12Z)-18:1 (99%) were from Lipidex. (6Z)-18:1 (99%), (13Z)-18:1 (99%), N-hydroxyphthalimide, ceric ammonium nitrate, L-terephthalic acid, and Dess-Martin periodinane were from Sigma-Aldrich. Fatty acids were dissolved in ethanol and stored in stock solutions (30-100 mM) at −20°C. Photooxidation of octadecenoic acids (0.2-3 mg) was performed with methylene blue in methanol (25).

Reprosil Chiral NR and NR-R columns (8 µm; 250 × 2 mm and 250 × 4 mm respectively; Dr. Maisch GmbH, Ammerbuch, Germany) were purchased locally (Daiichichrom Tech). Recombinant MnLOX was expressed in *Pichia pastoris* (strain X-33) as a secreted protein containing 602 or 580 amino acids and purified as described (29, 30). Microsomes of ram seminal vesicles were prepared as described (31), and purified ovine COX-1 was from Cayman. Recombinant 5,8-LDS Cys10006Ser of *A. fumigatus* and 7,8-LDS-1,673 of *G. graminis* were expressed in *E. coli*, and 10R-DOX of *A. fumigatus* was expressed in insect cells (27, 32).

**HPLC and MS analysis**

NP-HPLC with MS/MS analysis was performed on a silica column (Kromasil-100SI; 250 × 2 mm, 5 µm, 100 Å), which was usually eluted at 0.3 ml/min with hexane/isopropanol alcohol/acetic acid, 96.9/3/0.1.

CP-HPLC/MS analysis of HpOME was performed with the Reprosl Chiral NR column (8 µm; 250 × 2 mm), which was usually eluted at 0.5 ml/min with hexane/isopropanol alcohol/acetic acid, 98.8/1.2/0.01. Preparative CP-HPLC was performed with the Reprosl Chiral NR-R column (8 µm; 250 × 4 mm; eluted at 2 ml/min), and HpOME were analyzed with online UV detection (210 nm; PDA plus detector, ThermoFisher). Fractions were collected and an aliquot (0.1%) was analyzed by direct injection to the mass spectrometer.

CP-HPLC/MS analysis of HOME required different columns and alcoholic modifiers: Reprosl Chiral AM [amylose tris(3,5-dimethylphenyl carbamate)-modified silica; 2 × 250 mm] eluted at 0.15-0.2 ml/min with hexane/ethanol/acetic acid, 95/5/0.01 or with hexane/methanol/acetic acid, 95/5/0.01, and Chiralcel OB-H [amylose trishexenate-modified silica; 250 × 4.6 mm; Daicel] eluted at 0.5 ml/min with hexane/isopropanol alcohol/acetic acid, 95/5/0.01.

The eluents from the columns were combined with isopropyl alcohol/water (5/2) from a second HPLC pump (33), and then introduced by electrospray into a linear ion trap mass spectrometer (LTQ, ThermoFisher). We mixed the column eluates with iso-propanol/water (60/40) in a ratio of ~2:1. This choice was based on the observed signal intensities of HpOME with mixing ratios of 11:1, 5:1, 2:1, 1:5:1, and 1:1. The last three ratios yielded

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5References to the separation of drugs on the Reprosil Chiral NR column and its chemical properties can be found at the home page of Dr. Maisch (http://www.dr-maisch.com/xedim.php?point=chiral_nr.html).
signals of almost equal intensities, whereas the signals intensities of the ratios 5:1 and 11:1 were reduced by 30 and 80%.

The transfer capillary was heated to 315°C, the ion isolation width was set at 1.5 for anions of HOME (m/z 297 → full scan) and 5 for anions of HpOME (m/z 313 → full scan) and 1.5 at the final selection of MS3 analysis of HpOME (m/z 313 → 295 → full scan). The collision energy was set at 1.7 V, and the ion tube lens at −110 to −130 V. We recorded five microscans and used the Gaussian algorithm for peak smoothing (Xcalibur Software). Prostaglandin F2α was infused for tuning.

Chemical oxidation and steric analysis

Photooxidation of 18:1 was performed with methylene blue in methanol as described (25). Autoxidation of (9Z)-18:1 was modified from the method of Punta et al (34). 0.2 M oleic acid [with methanol as described (25)]. Autoxidation of (9Z)-18:1 was shown in Figs. 1A and 2, respectively. For comparison, the separation of products formed from (12Z)- and (13Z)-18:1 by MnLOX is shown in Fig. 1B, C. This column also seemed to separate enantiomers of HpOME with cis double bond configuration (Fig. 2 and Table 1), and the elution order of 8S and 8R-HpOME (9Z) was deduced with aid of the 8RDOX activity of LDOC (Fig. 2, bottom chromatogram).

The chiral selection of HpOME was likely based on the absolute configuration of the 1-hydroperoxy-2-propene elements (illustrated in Fig. 1D) and not on the positions of these elements between C-6 and C-14 (Table 1).

MS fragmentation of HpOME and KOME

The MS/MS spectra (m/z 313 → full scan) confirmed that HpOME were dehydrated to KOME as judged from the main ion at m/z 295 analogous to dehydration of other hydroperoxy fatty acids (25, 37). Important ions in the MS3 spectra (m/z 313 → 295 → full scan) of HpOME are summarized in Tables 2 and 3.

The MS3 spectra of HpOME with the hydroperoxide group located at the omega end of the trans or cis double bonds showed a fragmentation pattern, which was consistently changed from 7-HpOME to 14-HpOME (Table 2).

| Oxidized precursor | HpOME(£)a | HpOME(Z)b |
|--------------------|-----------|-----------|
| (m/z)-18:1 | Position and configuration of the hydroperoxide group |
| (6Z)-18:1 | 7S | 7R | 6S | 6R | – | – |
| 0.66 | 0.75 | 0.90 | 1 |
| (7Z)-18:1 | 8S | 8R | 7S | 7R | – | – |
| 0.71 | 0.84 | 0.89 | 1 |
| (8Z)-18:1 | 9S | 9R | 8S | 8R | 7S | 7R |
| 0.52 | 0.59 | 0.90 | 1 | 0.75 | 0.83 |
| (9Z)-18:1 | 10S | 10R | 9S | 9R | 8S | 8R |
| 0.76 | 0.90 | 0.88 | 1 | 0.84 | 0.91 |
| (11Z)-18:1 | 12S | 12R | 11S | 11R | 10S | 10R |
| 0.71 | 0.82 | 0.86 | 1 | 0.76 | 0.85 |
| (12Z)-18:1 | 13S | 13R | 12S | 12R | – | 11R |
| 0.82 | 0.95 | 0.87 | 1 | – | 11R |
| (13Z)-18:1 | 14S | 14R | 13S | 13R | – | 12R |
| 0.74 | 0.87 | 0.86 | 1 | – | 0.86 |

aHpOME(£) were obtained by photooxidation. The retention volumes of the last eluting isomers were 8–9.5 ml (with retention times of 16–19 min) on the analytical Reprosil Chiral NR column. The absolute configurations of HpOME(£) were determined by chemical methods after separation of enantiomers on a preparative Reprosil Chiral NR-R column.

bHpOME(Z) were obtained by oxidation with the 8RDOX of LDOC and MnLOX.
The MS\textsuperscript{3} spectrum of 13-HpOME(11E) is illustrated in Fig. 3A. A plausible fragmentation mechanism, based on keto-enol tautomerism and \(\alpha\)-fragmentation, is summarized in Fig. 3B.

The MS\textsuperscript{3} spectra of HpOME with their hydroperoxide groups located at the carboxylic side of the trans or cis double bond, e.g., from 6-HpOME(7E) to 13-HpOME(14E), could not be explained by a common fragmentation mechanism. Inspection of Table 3 suggests that hydroperoxides positioned from C-11 to C-13 yielded a similar fragmentation. The MS\textsuperscript{3} spectrum of 12-HpOME(13E) is illustrative (Fig. 4A), and a plausible mechanism for its fragmentation is suggested (Fig. 4B). Hydperoxides positioned at C-6 to C-9 yielded a series of unique and rather intense signal pairs (e.g., \(m/z\) 211 and 193, 197 and 179, 183 and 165, and 169 and 151, respectively; Table 3).

We confirmed that the MS\textsuperscript{2} spectrum of 7-KOME(8E) was identical to the MS\textsuperscript{3} spectrum of 7-HpOME(8E). The MS\textsuperscript{2} spectrum (\(m/z\) 295 \(\rightarrow\) full scan) of 7-KODE(8E) is illustrated in Fig. 5A. MS\textsuperscript{2} analysis of \(m/z\) 197 (\(m/z\) 295 \(\rightarrow\) full scan) and \(m/z\) 179 (\(m/z\) 295 \(\rightarrow\) full scan; Fig. 5B) showed that both ions could lose water. It seems likely that the characteristic ions at \(m/z\) 211 (295-84) of 6-HpOME, 197 (295-99) of 7-HpOME, 183 (295-113) of 8-HpOME and 169 (295-127) of 9-HpOME could be due to rearrangement with loss of an uncharged species containing part of the carboxyl end (e.g., \(\text{O} = \text{CH-(CH}_2\text{)}_n\text{CH=CH}_2\text{n = 2-5}\)) with transfer of O\textsuperscript{\textendash}to the \(\omega\) end. This hypothesis is illustrated in Fig. 5C.

HpOME with retention of the cis double bond of the substrates were obtained as major products by oxidation of (8Z)-, (9Z)-, (11Z)-, (12Z)-, and (13Z)-18:1 with 8R-DOX and MnLOX. The MS\textsuperscript{3} spectra of cis and trans HpOME seemed to be identical.

**Oxidation of octadecenoic acids by LDS, 10R-DOX, and ovine COX-1**

The preferred octadecenoic acids of 8R-DOX and COX-1 were, as expected, (9Z)-18:1 and (12Z)-18:1, respectively, which were oxidized by hydrogen abstraction and insertion of molecular oxygen at C-8 and C-11 (and in a few percentages at C-10 and C-13, respectively). We first evaluated the effect of shifting the double bond toward the carboxyl group. 8R-DOX and COX-1 oxidized (8Z)- and (11Z)-18:1 by hydrogen abstraction and oxygenation at C-7 and C-10, respectively, but significant oxygenation also occurred at C-8 of (8Z)-18:1 and C-11 of (11Z)-18:1. This was deduced from analysis of the corresponding HOME, as HpOME were largely reduced to alcohols by the peroxidase activity of microsomal and purified COX-1. This suggests that these enzymes abstracted both allylic hydrogens. The oxidation of (8Z)-18:1 by 8R-DOX is shown...
in Fig. 6A, and the oxidation of (11Z)-18:1 by COX-1 to almost equal amounts of 10S-HOME and 11R-HOME (12E) is shown in Fig. 6B. COX-1 also formed significant amounts of 12S-HOME (10E) (19). 8R-DOX of 5,8:10S-C1006S oxidized (7Z)-18:1 slowly and with little stereospecificity. (6Z)-18:1 was not oxidized. 7,8-LDS-1-673 of G. graminis yielded the same products as 5,8-LDS-C1006S of A. fumigatus.

We next examined the effect of positioning the double bond from the optimal positions [(9Z) and (12Z), respectively] toward the omega end. 8R-DOX mainly oxidized C-10 of (11Z)-18:1 (Fig. 6C) and C-11 of (12Z)-18:1, but the latter was a poor substrate; the rate of oxygenation by 8R-DOX seemed to be (8Z)-18:1 = (9Z)-18:1 ≈ (11Z)-18:1 > (12Z)-18:1. (13Z)-18:1 was not oxidized. COX-1 oxidized (13Z)-18:1 by hydroxy abstraction at C-12 to 12SHpOME (13Z) and 14SHpOME (12E), and this occurred more efficiently than the oxidation of (11Z)-18:1; the rate of oxygenation was (12Z)-18:1 > (13Z)-18:1 >(11Z)-18:1 by COX-1 of microsomes of ram seminal vesicles.

We conclude that 8R-DOX efficiently catalyzes hydrogen abstraction from C-7 of (8Z)-18:1 to C-10 of (11Z)-18:1, and COX-1 from C-10 of (11Z)-18:1 to C-12 of (13Z)-18:1.

**Oxidation of octadecenoic acids by MnLOX**

MnLOX oxidized (11Z)-, (12Z)-, and (13Z)-18:1 to 10R-HpOME(Z), 11R-HpOME(Z), and 12R-HpOME(Z), respectively, as major products, along with significant amounts of hydroperoxides with 3R-hydroperoxy-1-propene structure elements counted from the carboxyl group, e.g., 12HpOME(10E), 13HpOME(11E), and 14HpOME(12E). Some of these cis and trans HpOME were poorly resolved on the Reprosil Chiral NR column (Fig. 1 and Table 1), but could be identified by aid of characteristic signals (e.g., m/z 167, 179, and 193, respectively; see under X-18 in Table 2).

**Oxidation of monoenoic C_{14}, C_{16}, and C_{20} fatty acids by 8R-DOX**

The 8R-DOX of 7,8-LDS-1-764 rapidly oxidized (9Z)-14:1 to its 8-hydroperoxy metabolite. Previous work has demonstrated that (9Z)-16:1, (9Z)-20:1, and (11Z)-20:1 were also oxygenated (27). These results, and the effect of the position of the double bond on hydrogen abstraction, are summarized in Fig. 7.

**Separation of stereoisomers of HOME**

An advantage of CP-HPLC analysis of hydroperoxides on Reprosil Chiral NR was resolution of all studied HpOME enantiomers. In contrast, enantiomers of HOME were not generally resolved on this column.

The resolution of enantiomers of HOME required assessment of different CP-HPLC columns and alcoholic modifiers. We evaluated Reprosil Chiral AM, eluted with 5% ethanol or 5% methanol in hexane, and Chiralcel OB-H, eluted with 5% isopropanol in hexane (38). A systematic investigation on resolution of HOME by CP-HPLC was beyond the scope of this investigation, but the observations described below are illustrative.

The Reprosil Chiral AM column with methanol as modifier readily baseline separated R and S stereoisomers of 10-HOME (11Z) (see Fig. 6B), 11-HOME (12Z), and 12-HOME (13Z). The elution order was as expected, R before S (24). This was deduced from reference compounds based on the suprafacial oxygenation by MnLOX (to R enantiomers) and the antarafacial oxygenation by COX-1 (to S enantiomers). Under these conditions, 11R-HOME (12Z) eluted before the stereoisomers of 12-HOME (13E), which were well separated, whereas 11S-HOME (12Z) coeluted with the first eluting isomer of 12-HOME (13E). The stereoisomers of 13-HOME (11E) were not resolved. Ethanol as alcoholic modifier did not improve the situation, as enantiomers of HOME(E), which were obtained by photooxidation of (11Z)-, (12Z)-, and (13Z)-18:1, were not resolved.

Chiralcel OB-H resolved the isomers of 11-HOME (12E) and 12-HOME (13E). Separation of HOME with 1-hydroxy-2-propene elements seemed less demanding than separation of HOME with 3-hydroxy-1-propene elements. The Chiralcel OB-H column thus resolved the isomers of 13-HOME (11E) but not the isomers of 12-HOME (10E).
TABLE 3. Structurally important ions in the MS3 spectra of hydroperoxides of regioisomeric octadecenoic acids with 1-hydroperoxy-2-propene elements

| HpOMEa | X-18 | X3 | Y-18 | Y3 |
|--------|------|----|------|----|
| 6-HpOME(7E) | – | 109 (ND) | 127 (5) | 99 (1) | 143 (<1) |
| 7-HpOME(8E) | 97 (10) | 123 (ND) | 141 (1) | 113 (2) | 157 (1) |
| 8-HpOME(9E) | 111 (35) | 137 (ND) | 155 (4) | 127 (30) | 171 (25) |
| 9-HpOME(10E) | 125 (55) | 151 (90)c | 169 (10)d | 141 (40) | 185 (45) |
| 10-HpOME(11Z) | 139 (90) | 165 (90)d | 183 (<1) | 155 (55)d | 199 (70) |
| 11-HpOME(12E) | 153 (30) | 179 (100) | 193 (<1) | 169 (29) | 213 (30) |
| 12-HpOME(13E) | 167 (40) | 193 (100) | 211 (<1) | 183 (10) | 227 (12) |
| 13-HpOME(14E) | 181 (40) | 207 (100) | 225 (<1) | 197 (10) | 241 (15) |

| HpOME | Z-18 | Z3 |
|--------|------|----|
| 6-HpOME(7E) | 193 (48) | 211 (13) |
| 7-HpOME(8E) | 179 (100) | 197 (6) |
| 8-HpOME(9E) | 165 (100) | 183 (15) |
| 9-HpOME(10E) | 151 (90)c | 169 (10)d |  |
| 10-HpOME(11Z) | 165 (90)d | 155 (55)d |  |
| 11-HpOME(12E) | 123 (6) | 151 (1) |  |
| 12-HpOME(13E) | 109 (1) | 137 (ND) |  |
| 13-HpOME(14E) | 95 (ND) | 123 (ND) |  |

ND, not detected.

aThe mass spectra of the corresponding hydroperoxides with a cis double bond were virtually identical (19).
bThese ions (X series) were postulated as intermediates, which formed strong fragment ions after loss of water or CO2 in some of the spectra.
cThese ions (Y series) were formed by loss of the omega end.
dThis ion occurs in two columns and could be formed by two mechanisms. The base peaks of the MS3 spectra were usually as listed in the table, except m/z 251 (313-18-44) for 9-HpOME and m/z 277 (313-44) for 6-HpOME.
eThese ions (Z series) contained the omega end and gave rise to intense signals after loss of water in some spectra.

DISCUSSION

We demonstrated that enantiomers of HpOME can be conveniently separated by CP-HPLC on the Reprosil Chiral NR matrix and that the elution order was consistently “S” before “R”. The chiral selector has not been disclosed, but it is described as an aromatic chiral phase with π-donor and π-acceptor groups of Pirkle type. This column can be operated in both reverse and normal phase modes, but separation of enantiomers of oxygenated fatty acids has so far been reported only in normal phase mode. Reprosil Chiral NR also resolves the enantiomers of

Fig. 3. MS3 spectrum of 13-HpOME(11E) and an interpretation of the fragmentation mechanism. A: MS3 spectrum (m/z 313 → 295 → full scan). B: A hypothetical fragmentation mechanism: 13-HpOME is dehydrated to 13-KOME, and the latter might fragment as indicated. The fragment ions are found in the X and Z series of ions in Table 2.
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10-HODE, 9-HpODE, 13-HpODE, HpETE, and many drugs (25, 26).

The absolute configuration of enantiomers of HpOME obtained following chromatography on Reprosil Chiral NR was determined by chemical methods and by comparison with enzymatic products described above. The chemical method was based on (−)-menthoxyacetyl derivatives of the corresponding HOME methyl esters, followed by ozonolysis of the double bond, methylation, and separation of the diastereoisomers by gas chromatography (36). This procedure showed that the S stereoisomers of 14 regioisomeric HpOME with the double bond in trans configuration eluted before the R stereoisomers on Reprosil Chiral NR. As far as it is known, this elution order also applies to HpOME(Z) (Table 1).

Decisive for chiral separation is interaction of the analyte to the chiral selector at a minimum of three positions with at least one position dependent on stereochemistry (39). The two oxygens of the hydroperoxide group and the double bond seem to interact with the chiral selector in this way. HpOME with a 1S-hydroperoxy-2-propene element eluted before the enantiomer having the 1R-hydroperoxy-2-propene element.

Fig. 4. MS3 spectrum of 12-HpOME(15E) and an interpretation of the fragmentation mechanism. A: MS3 spectrum (m/z 313 → 295 → full scan). B: A hypothetical fragmentation mechanisms by which 12-HpOME is dehydrated to 12-KOME and the latter fragments as indicated (see Fig. 3B). The fragment ions are found in the X and Y series of ions in Table 3.

Fig. 5. MS analysis of 7-KOME(8E). A: The MS3 spectrum (m/z 295 → full scan) shows two characteristic ions at m/z 197 and 179 (197-18). The MS3 spectrum of 7-HpOME(8E) was identical. B: MS3 analysis (m/z 295 → 179 → full scan). The signal at m/z 161 (179-18) suggests loss of water. C: A hypothetical fragmentation mechanism of 7-KOME(8E) based on rearrangement and cleavage between C6 and C7 (see Table 3 and its series of Z ions).
Fig. 6. Oxidation of (8Z)- and (11Z)-18:1 by LDS and COX-1. A: CP-HPLC separation (Reprosil Chiral-NR) of the two main HpOME formed by oxidation of (8Z)-18:1 by the 8R-DOX domain of 5,8-LDS. B: CP-HPLC separation (Reprosil Chiral AM) of 10R- and 10S-HOME(11Z) and simultaneous detection of 11-HOME(12E) formed by COX-1. The stereoisomers of 11-HOME(12E) were not resolved on Reprosil Chiral AM. C: CP-HPLC separation (Reprosil Chiral-NR) of the two main HpOME formed by oxidation of (11Z)-18:1 by the 8R-DOX domain of 7,8-LDS. The MS$^3$ spectra of the two stereoisomers were identical. NL, normalized absolute intensities. Panels A–C show selected ion chromatograms as indicated by insets of the selected ions.

Fig. 7. Summary of oxygenation of monounsaturated fatty acids by LDS. The carbons, which were subjected to hydrogen abstraction by 8R-DOX activity of LDS, are marked by *. Data on C$_{16}$ and C$_{20}$ fatty acid are from Ref 27.

element, and the position of this hydroperoxy-propene unit along the carbon chain from C-6 to C-14 did not change the elution order (Fig. 1D and Table 1). Interestingly, chiral separation was lost when the hydroperoxide group of HpOME was replaced by an alcohol. This seems to be a unique property of Reprosil Chiral NR compared with chiral selectors based on derivatized amylose (24). Enantiomers of 10-HOME(8Z) thus interacted with the chiral NR selector without separation, but the stereoisomers of 10-hydroxy-8E,12Z-octadecadienoic acid, with an addition double bond at 12Z, were resolved (35), presumably by three-point interaction of the hydroxyl group and the two double bonds with the chiral selector. It follows that enantiomers of polyunsaturated fatty acid hydroperoxides with 1-hydroperoxy-2E,4Z-pentene elements and additional double bonds (e.g., HpETE) may not necessarily elute in the same “S” and “R” order as HpOME, 9-HpODE, and 13-HpODE (see Ref. 25).

COX-1 and the oleate 8R-DOX activities of 7,8-LDS, 5,8-LDS, and 10R-DOX initiate oxidation of octadecenoic acids by hydrogen abstraction, which likely is catalyzed by a tyrosyl radical (16, 40, 41). The selective oxidation of three octadecenoic acids by ovine COX-1 proved that C-11 of (12Z)-18:1, C-12 of (13Z)-18:1, and both C-10 and C-13 of (11Z)-18:1 were positioned sufficiently close to Tyr-385 for hydrogen abstraction. This was not unexpected, as the 3D structure of COX-1 with linoleic acid in the active site shows that C-11 to C-13 are within relatively short distances from Tyr-385 (20). A double bond at the 11Z, 12Z, or 13Z position seems to be required for correct positioning, as (9Z)-18:1 was not oxidized by COX-1.

The 8R-DOX domain of 7,8-LDS, 5,8-LDS, and 10R-DOX readily oxidized 18:1 with double bonds at position from (8Z) to (11Z). Significant oxidation by LDS also occurred at the (12Z) position. We do not know whether the active site of 8R-DOX mimics the COX-1 “tail first” orientation of fatty acids.
in the active site. A critical factor for 8R-DOX catalysis is the presence of a saturated carbon chain of 6-10 carbons between the carboxyl group and the first double bond (13, 27, 42), but the total chain lengths of monounsaturated fatty acids from 14:1 and 20:1 or additional double bonds at the ω end do not affect catalysis.

It is interesting to compare 8R-DOX with lipoxygenases. Newcomer and coworkers propose from crystal studies of arachidonate 8R-LOX of Pleaxura homomalla that all lipoxygenases may bind their substrates in a U-shaped channel (43). Each lipoxygenase may have one end of the U-shaped channel closed with the carbonyl group of the substrate exposed to solvents at the open channel with the carbon chain in the U-shaped channel. MnLOX likely binds fatty acids with the carbonylates at the entrance, and the omega ends could be buried in a substrate channel (44). Analogous to COX-1, MnLOX oxidized (11Z)-18:1, (12Z)-18:1, and (15Z)-18:1 to hydroperoxides at the allylic carbon of the carboxyl ends, albeit with R configuration (19). 8R-DOX may also bind their substrates in this way, but we cannot exclude that the carbonylate could be buried in the interior analogous to α-DOX (45).

LC-MS is a convenient and powerful tool for identification of oxylipins. The fragmentation of a large number of hydroxy and dihydroxy fatty acids during MS/MS analysis has now been characterized (37). Informative fragments are often obtained by α-cleavage at the oxidized carbons. Hydroperoxy fatty acids are dehydrated to keto fatty acids during MS/MS analysis (37, 46). We recorded the MS3 spectra of HpOME and found that keto-enol tautomerism and α-fragmentation could explain many fragments, as illustrated by the MS3 spectra of 12-HpOME(13E) and 13-HpOME(11E) (Figs. 3 and 4).

The MS3 fragmentation of 6-HpOME, 7-HpOME, 8-HpOME, and possibly 9-HpOME differed from the other HpOME with 1-hydroperoxy-2-propene elements. In all four spectra, one of the most intense ions seemed to be formed by rearrangement with loss of a short chain of 5 to 8 carbons and oxygen transfer to the ω end (Table 3). We confirmed that 7-KOME(8E) and 7-HpOME(8E) yielded identical MS2 and MS3 spectra, respectively. The fragmentation of HpOME with 1-hydroperoxy-2-propene elements thus varies with the position. For comparison, 7,10-DiHOME(8E) fragments in a different way than 5,8-DiHOME, 7,8-DiHOME, and 8,11-DiHOME (47). If needed, GC-MS analysis can usually be used to identify regioisomeric HOME and other oxylipins without ambiguity (48). The MS spectra with electrospray ionization can be less informative than electron impact MS spectra. Comparison of the LC-MS spectra of two different compounds in Figs. 3A and 5A illustrates this point.

We conclude that 8R-DOX and COX-1 oxygenate octadecenoic acids to HpOME with stereoselectivity, provided the double bond is located at positions (8Z) to (12Z) for 8R-DOX and at positions (11Z) to (13Z) for COX-1. Octadecenoic acids can thus be used to determine the positional effect of a double bond on enzymatic catalysis. Racemic HpOME standards are readily available by photo- or autolysis. Enantiomers of HpOME can also be separated by CP-HPLC, and HpOME of biological origin can readily be analyzed for stereoisomers by this method.

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