A one-step method of 10,17-dihydro(pero)xydocosahexa-4Z,7Z,11E,13Z,15E,19Z-enoic acid synthesis by soybean lipoxygenase

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Abstract A product of lipoxygenase (LOX) oxidation of docosahexaenoic acid (DHA), 10,17-dihydro(pero)xydocosahexa-4Z,7Z,11E,13Z,15E,19Z-enoic acid [10,17-(S)-dihydro(pero)xydocosahexa-4Z,7Z,11E,13Z,15E,19Z-enoic acid (DHA)] was obtained through various reaction pathways that involved DHA, 17(S)-hydro(pero)xydocosahexa-4Z,7Z,11E,13Z,15E,19Z-enoic acid (sLOX), and potato tuber lipoxygenase (ptLOX) in various combinations. The structure of the product was confirmed by HPLC, ultraviolet (UV) light spectrometry, GC-MS, tandem MS, and NMR spectroscopy. It has been found that 10,17(S)-dihydro(pero)xydocosahexa-4Z,7Z,11E,13Z,15E,19Z-enoic acid (DHA) was apparently identical to the product of ptLOX oxidation of the latter. The sLOX- and ptLOX-derived samples of 10,17-diHDHAs coeluted under the conditions of normal, reverse, and chiral phase HPLC analyses, displayed identical UV absorption spectra with maxima at 260, 270, and 280 nm, and had similar one-dimensional and two-dimensional proton NMR spectra. Analysis of their NMR spectra led to the conclusion that 10,17-dihydro(pero)xydocosahexa-4Z,7Z,11E,13Z,15E,19Z-enoic acid was apparently identical to the previously published structure of its ptLOX-derived counterpart. Based on the cis,trans geometry of the reaction products, the conclusion is made that in the tested conditions sLOX catalyzed direct double dioxygenation of DHA. Compared with the previously described two-enzyme method that involved sLOX and ptLOX, the current simplified one-enzyme procedure uses only sLOX as the catalyst of both dioxygenation steps.

Supplementary key words docosahexaenoic acid • 10,17-dihydroxydocosahexaenoic acid • 10,17(S)-docosatriene • neuroprotectin D1 • nuclear magnetic resonance • mass spectrometry • protectin D1

Recently, a series of monohydroxylated, dihydroxylated, and trihydroxylated compounds formed from docosahexaenoic acid (DHA) by lipoxygenase (LOX) and/or aspirin-treated (acytlated) cyclooxygenase-2 has been described (1–8). Some of the derivatives were shown to have potent antiinflammatory and antiapoptotic activity in various cells and tissues and can be considered as a novel class of bio-regulatory compounds and potential drug candidates. Among those, 10,17(S)-dihydroxydocosahexaenoic acid with postulated 4Z,7Z,11E,13E,15Z,19Z geometry of the double bonds, which was termed neuroprotectin D1 and 10,17(S)-docosatriene in previous publications (2–5, 8), is of special interest in that it was shown that this compound appears to be produced by a variety of mammalian cells. Added exogenously, it had potent antiapoptotic activity, effectively protected the cells during oxidative stress (3), and increased the healing rate of damaged mouse cornea by a mechanism that differed from its antiinflammatory activity (9). The compound protected rat brain from oxidative stress during ischemia-reperfusion by upregulating antiapoptotic Bcl-2 and Bcl-xL proteins (3, 8).

At the same time, questions remain regarding the exact structure of 10,17(S)-docosatriene (especially the cis,trans geometry of its double bonds) and the mechanisms of its biosynthesis. A role for a mammalian LOX-type enzyme activity in these transformations was implied (2–9), and an epoxidation-isomerization mechanism similar to the one leading to leukotriene B4 formation was proposed to explain the biosynthesis of 10,17(S)-docosatriene (2–8). This mechanism supposedly involves an enzyme-catalyzed epoxidation of 17(S)-hydroperoxydocosahexa-4Z,7Z,11E,13Z,15Z,19Z-enoic acid [17(S)-HPDHA] in a 16,17-epoxy-DHA intermediate, with subsequent hydrolysis of the

Abbreviations: BSTFA, bis(trimethylsilyl)trifluoroacetamide; C13E10, monododecyl ether of decaoxyethylene glycol; 1D, one-dimensional; 2D, two-dimensional; DHA, docosahexaenoic acid (ω-5 C22:6); 10,17-dihydro(pero)xydocosahexa-4Z,7Z,11E,13Z,15Z,19Z-enoic acid; EI, electron-impact; ESI, electrospray ionization; 1H, 1H-DQCOSY, double quantum correlation spectroscopy; LOX, lipoxygenase; neuroprotectin D1 and 10,17(S)-docosatriene, 10,17(S)-dihydroxydocosahexaenoic acid with postulated 4Z,7Z,11E,13E,15Z,19Z geometry of the double bonds; NP HPLC, normal phase high performance liquid chromatography; ptLOX, potato tuber lipoxygenase; 17(S)-HPDHA, 17(S)-hydro(pero)xydocosahexa-4Z,7Z,11E,13Z,15Z,19Z-enoic acid; sLOX, soybean lipoxygenase; UV, ultraviolet.

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epoxide and its rearrangement in a conjugated triene with 11E,13E,15Z geometry of the double bonds. Thus, the mechanism can be verified by determining the geometry of the double bonds of the final product and/or by checking the role of the hydroperoxy group at C(17), as its substitution with the hydroxyl group should completely prevent the formation of 16,17-epoxy-DHA, the key intermediate of the rearrangement.

Interestingly, the compound was reported to be produced from DHA by plant LOXs in vitro (2–9). In recent studies, 10,17(S)-docosatriene (along with some other oxidation products of DHA) was synthesized by soybean lipoxynegase (sLOX) (3, 8) and/or by a combination of sLOX and potato tuber lipoxynegase (ptLOX) (6, 7, 9), although no details of its making were provided. At present, the compound is not available commercially.

Apparently, 10,17(S)-docosatriene generated by the plant enzymes was considered to be similar to its mammalian counterpart, as the former was repeatedly used in biological studies as a physiologically active compound and an analytical standard (2–9).

Previously, my colleagues and I presented the results of our study of DHA and 17(S)-hydro(pero)xydocosahexa-4Z,7Z,11Z,13Z,15E,19Z-enoic acid [17(S)-H(P)DHA] oxidation by ptLOX (10, 11). It was found that one of the two major products of 17(S)-H(P)DHA oxidation by ptLOX was 10,17(S)-dihydro(pero)xydocosahexa-4Z,7Z,11E,13Z,15E,19Z-enoic acid [10,17(S)-dih(H(P))DHA], and the second largest metabolite was 7,17(S)-dihydroxydocosahexaenoic acid [7,17(S)-dih(D(P))H(D)HA]. Taking into account that both 17(S)-HDHA and 17(S)-HPDHA were effectively transformed into 10,17(S)-dih(H(P))DHA by ptLOX, we speculated that epoxidation/isomerization was not involved in those reactions and that 10,17(S)-dih(H(P))DHA was clearly a product of double lipoxygenation with 11E,13Z,15E arrangement of the conjugated triene. Corroborating this hypothesis was the fact that while oxidizing DHA, ptLOX produced 10(S)-HPDHA as the major monoxygenated product. Later, new direct evidence emerged that confirmed our prediction that the product synthesized by ptLOX from 17(S)-H(P)DHA indeed had 11E,13Z,15E configuration of the conjugated triene fragment (11).

At the same time, direct evaluation of the cis,trans geometry of the sLOX-derived DHA oxidation products has never been performed before. Therefore, the goals of this study were as follows: 1) to design and compare various (chemo)enzymatic methods to propose a simple and scalable biosynthetic procedure of making 10,17(S)-dih(H(P))DHA; and 2) to validate the stereochemistry of the target product of DHA oxidation.

**MATERIALS AND METHODS**

**Materials**

The following equipment, reagents, and supplies were used in this study: DHA (Nu-Chek Prep, Inc., Elysian, MN); CD$_3$OD (99.8%), PtO$_2$, NaBH$_4$, and bis(trimethylsilyl)trifluoroacetamide (BSTFA) (Aldrich, Milwaukee, WI); sLOX preparations type I-B and V and monododecyl ether of decaoxoethylene glycol (C$_{12}$E$_{10}$) (Sigma Chemical Co., St. Louis, MO); octadecyl (C$_{18}$) solid phase extraction cartridges (J. T. Baker, Philipsburg, NJ); and type 528-P NMR tubes (Wilmad Glass Co., Inc., Buena, NJ). A 5980 series II gas chromatograph equipped with a 5971 series electron-impact (EI) mass selective detector was manufactured by Hewlett-Packard. Ultraviolet (UV) light spectra of the reaction mixtures and purified products were recorded on a Beckman DU800 spectrophotometer with a temperature-controlled unit. An Agilent DB-17HT column (30 m × 0.25 mm column with 0.15 μm polymer layer) was used for GC-MS analysis of the reaction products. Mass spectra of the compounds were obtained with an LCQ Deca XP Max MS$^*$ spectrometer (Thermo Electron Corp., San Jose, CA) equipped with an electrospray ionization (ESI) ion source. A Waters Alliance 2695 HPLC separations module equipped with a Waters 2996 diode-array detector was used to analyze and purify the DHA products. Proton NMR spectra were taken on a 400 MHz Varian spectrometer in CD$_3$OD at room temperature. 17(S)-HPDHA and 17(S)-HDHA were synthesized and analyzed as described previously (10, 12).

**Measurement of enzymatic activity**

The activity of ptLOX was measured spectrophotometrically by monitoring the product formation at 290 nm (maximum of absorption of conjugated dienes) and 270 nm (conjugated trienes) in a 1 ml reaction mixture composed of 100 μM DHA, 100 μM SDS, and 0.02% C$_{12}$E$_{10}$ in 0.05 M sodium phosphate buffer, pH 6.5, at 4°C (10, 11, 13–15). Preparative scale synthesis of DHA metabolites with ptLOX and their normal phase HPLC purification were performed according to previously published protocols (10, 11). To convert hydroperoxides of DHA to the corresponding hydroxides, the final product mixture was brought to pH ~10 with concentrated NaOH and treated with ~10 M excess of NaBH$_4$ (30 min on ice).

To monitor the course of sLOX-catalyzed reactions, the following method was used. A 1 ml aliquot of the reaction mixture (100 μM in 50 mM sodium borate buffer, pH 9) was placed in a temperature-controlled spectrophotometric quartz cuvette, the reaction was initiated by adding the enzyme, and the progress of the reaction was observed by recording sequential absorption spectra (Δt 1–5 min; range, 200–400 nm) at 4°C. The conjugated diene products produced the spectra with $\lambda_{\text{max}}$ 234–238 nm ($\epsilon_m$ 23,000 M$^{-1}$ × cm$^{-1}$), whereas the conjugated triene(s) gave a characteristic triplet at 260, 270, and 280 nm ($\epsilon_m$ 40,000 M$^{-1}$ × cm$^{-1}$ at 270 nm, estimated).

**Synthesis of the oxygenated derivatives of DHA**

For preparative scale synthesis of the sLOX DHA oxidation products, a 50 ml reaction mixture that contained 100 μM solutions of DHA, 17(S)-HPDHA, or 17(S)-HDHA dissolved in 20 mM sodium borate buffer, pH 9.0, was used. The reaction was conducted on ice to minimize chances of the formation of non-specific oxidation and isomerization products (14, 15). Commercially available sLOX preparations [type I-B from Sigma Chemical Co., or sLOX from Fluka (product 62340)] were used throughout the experiments, although in some cases an affinity-purified type V enzyme from Sigma was tested. All of the preparations were shown to be effective as catalysts of 10,17(S)-dih(H(P))DHA formation. The reactions were initiated by adding a sLOX stock solution (2 mg of sLOX type V, 12.5 mg of sLOX type I-B, or the same quantity of LOX from Fluka dissolved in ~1 ml of the same buffer) and were allowed to proceed for ~30 min. The indicated amounts of sLOX were determined in preliminary experiments to achieve maximal conversion of DHA in the target.
HPLC analysis and purification of the products

The DHA oxidation products were separated by normal phase high performance liquid chromatography (NP HPLC) on a Waters µ-Bondapak silica gel column (4.6 × 300 mm, 5 µm silica) at 30°C in a heptane-2-propanol-acetic acid (94:5:0.1, v/v/v) mobile phase at a flow rate of 2 ml/min essentially as described previously (10). Analytical separations were conducted on a 5 µm Waters Spherisorb silica gel column (3.2 × 250 mm) either isotopically as described above for the preparative HPLC (the flow rate was reduced to 1 ml/min) or in a hexane-2-propanol-acetic acid gradient mixture as follows. Two solvents were prepared: 989 ml of n-hexane, 10 ml of 2-propanol, and 1 ml of glacial acetic acid (1 liter total; solvent A) and 949 ml of n-hexane, 50 ml of 2-propanol, and 1 ml of acetic acid (1 liter total; solvent B). The flow rate was maintained at 2 ml/min throughout the experiment. The elution profile was monitored spectrophotometrically with the help of the diode-array detector operating in scan mode (210–400 nm). The column was equilibrated at 30°C with solvent A until UV light absorbance of the eluent at 236 and 270 nm stabilized, and a sample of the product(s) dissolved in 2-propanol was injected. Then, solvent A was pumped through the column for 3 min, after which a linear gradient from 100% solvent A to a solvent A/solvent B mixture of 50:50 (v/v) was run over the next 10 min. Then, a 5 min linear gradient to 100% solvent B was started, followed by a 5 min isocratic elution with the same solvent. In the next 1 min, the eluent was changed to 100% solvent A, and the column was reequilibrated with 100% solvent A for 6 min. The overall duration of the experiment was 30 min. In both experiments, the fractions that contained target compounds were collected, the solvent was evaporated under a stream of nitrogen, and the individual diH(P)DHAs were stored in nitrogen-saturated ethanol at −80°C.

Characterization of the products

Molecular masses of the DHA oxidation products were determined on an LCQ Deca XP Max MS® mass spectrometer with an ESI ion source operating in either negative (M – H+, free fatty acids, and/or M + Cl− adducts) or positive (M + H+ and/or M + Na+ adducts) mode. The following parameters were used in the direct infusion experiments with the samples dissolved in methanol: infusion rate of 2–10 µl/min; nitrogen as sheath gas (10 arbitrary units); capillary temperature of 325°C; data collection for at least 1 min at 5 × 5 ms microscans; spray voltage of 5 kV; capillary voltage of −14 V; tube lens offset of −5 V; and scan range of 50–1,000 mass units.

The individual products and/or product mixtures were subjected to EI GCMS analysis after catalytic hydrogenation with H2/PtO2 and trimethylsilylation with BSTFA (10). Briefly, helium was used to elute the compounds from the DB-17HT capillary column. The following elution program was used. The column was reequilibrated at 150°C. Then, the sample (~2 µl of solution in BSTFA) was injected. The column was washed for 3.5 min at the initial temperature, then the oven temperature was increased at 1.5°C/min until it reached 210°C. Total ion chromatograms were recorded with a sampling rate of two per second. Later, single ion monitoring chromatograms were extracted, plotted, and integrated using a Hewlett-Packard Chemstation’s built-in routine.

The 400 MHz one-dimensional (1D) and two-dimensional (2D) 1H-NMR spectra of the NaBH4-reduced products were recorded in CD3OD at room temperature. Analysis of the spectra was performed with the help of MesTrec version 4.5.1 software (purchased through www.mestrec.com). Terminal [omega, C(22), Cl]-adducts) or positive (M + H2 and/or M + Cl− adducts) methine protons of the compounds were used as internal standards for integration.

Chiral analysis of dihydroxylated compounds was performed on a Chiralcel OD-H column (4.6 × 250 mm; Daicel USA, Inc., Fort Lee, NJ) in hexane-2-propanol-acetic acid (95:5:0.1, v/v/v) with an elution rate of 1 ml/min at 30°C.

RESULTS

All of the enzymatic reactions presented in Scheme 1 led to effective formation of 10,17-dihydroxy(pero)xydocosa-
hexa-4Z,7Z,11E,13Z,15E,19Z-enolic acid [10,17-diH(P) DHA] and, consequently, 10,17-diHDHA.

sLOX-catalyzed reactions

In agreement with earlier publications (10, 12), low concentrations of sLOX (5 \times 10^{-8} \text{ M to } 1 \times 10^{-7} \text{ M}) caused rapid and virtually quantitative conversion of 10^{-4} \text{ M DHA into } 17(\text{S})-HPDHA. There were two peaks detected under the conditions of NP HPLC. The first very hydrophobic compound (product I, Scheme 2) with a short retention time was positively identified as unreacted DHA. The major reaction product IIa had the UV light absorbance spectrum of a typical conjugated diene, with \( \lambda_{\text{max}} \) of 258 nm (Fig. 1A) and molecular weight of 360.3 (molecular formula, \text{C}_{22}\text{H}_{32}\text{O}_4; theoretical isotopic mass, 360.2). The molecular weight of its NaBH\(_4\)-reduced derivative was 344.3 (\text{C}_{22}\text{H}_{32}\text{O}_3; product IIb; theoretical isotopic mass, 344.2) (Fig. 1B). The structure of product IIb was elucidated by EI GC-MS and by \(^1\text{H}-\text{NMR}.\) After catalytic hydrogenation of product IIb over PtO\(_2\) in methanol followed by full trimethylsilylation of its hydroxyl and carboxyl groups, the compound produced EI MS fragments with \( m/z \) of 73, 173, 429, and 485 that positively identified it as di-trimethylsilyl-17-hydroxydocosanoic acid. A full 400 MHz 1D \(^1\text{H}-\text{NMR}\) spectrum of product IIb is shown in Fig. 1C. The spectrum displayed resonances that were similar to those published earlier for the \( \text{cis,trans} \) isomers of 9/13-hydroxy linoleyl alcohol and 9/13-hydroxy monolinoleoyl glycerol (14, 15). No traces of \( \text{trans,trans} \) isomers were detected [the latter would have been seen as a quartet with \( \delta_{\text{CH}} \approx 6.1–6.2 \text{ ppm} \) that belongs to the C(3) hydrogen atom of 1-hydroxy-2\( E,4\)E-pentadiene fragment (11, and references therein)]. A 2D double quantum correlation spectroscopy (\(^1\text{H}, \text{H-DQCOSY}\)) scan of product IIb (Fig. 1D) along with the 1D \(^1\text{H}-\text{NMR}\) spectrum allowed us to unambiguously deduce the structure of the compound and assign the observed resonances to particular protons of the compound, except for those that had close or equivalent values of \( \delta \) (Table 1). Importantly, the protons with \( \delta \approx 4.15–4.17 \text{ ppm} \) [believed to be \( =\text{C}_{(17)}\text{H}-\text{OH} \)] produced cross-peaks with protons with \( \delta \approx 2.3 \text{ ppm} \) [=C\(_{(18)}\)H\(_2\)] and 5.75 [=C\(_{(16)}\)H\(_2\)], but not with the protons at C\(_{(13)}\) and C\(_{(14)}\). Therefore, product IIa was identified as 17-hydroperoxydocosahexa-4Z,7Z,10Z,13Z,15E,19Z-enolic acid, with the hydroxyl group at C\(_{(17)}\) being in the \( \text{S} \) configuration (10, 12).

A dramatically different result was obtained when the concentration of sLOX in the reaction mixture was increased to 0.4 \times 10^{-6} \text{ M and greater} (Fig. 2). DHA was rapidly converted to a mixture of two major products, III\(_a\) and IV\(_a\), one of which had a UV light absorption spectrum of a typical conjugated triene, with \( \lambda_{\text{max}} \) of 260.5, 270.0, and 280.3 nm (product IV\(_a\); Fig. 2A), whereas the other (product III\(_a\)) showed a split spectrum, with two maxima at 225.6 and 243.4 nm, similar to the spectra of 7,17-diHDHA and 10,20-diHDHA (10). The molecular masses of both products III\(_a\) and IV\(_a\) were estimated to be 392.1 Da, as negative mode ESI MS analysis of the products produced strong parent ions with \( m/z \) 391.1 and 427.2 (M – H\(^+\) and M + Cl\(^–\), correspondingly) (Fig. 2B). These masses are indicative of isobaric compounds with the molecular formula \text{C}_{22}\text{H}_{32}\text{O}_6 and isotopic mass of 392.2.

After treatment of product IV\(_a\) with NaBH\(_4\), its reduction product IV\(_b\) was isolated by NP HPLC and its structure was determined by ESI MS, GC-MS, and 1D and 2D \(^1\text{H}-\text{NMR}.\) The molecular mass of product IV\(_b\) was found to be 360.1 Da (Fig. 3A), consistent with the molecular formula \text{C}_{22}\text{H}_{32}\text{O}_4 (isotopic mass, 360.2). The fragmenta-
tion pattern of its catalytically hydrogenated and fully trimethylsilylated derivative (m/z of 73, 173, 359, 393, 427, 517, and 574) confirmed that it was tri-trimethylsilyl-10,17-dihydroxydocosanoic acid.

The 1D $^1$H-NMR and 2D $^1$H, $^1$H-DQCOSY spectra of product IVb (Fig. 3B, C) revealed that the geometry of the conjugated triene fragment was of the 11$^E$,13$^Z$,15$^E$ type (for detailed information on the spectra, see Table 1). The rest of the double bonds of the molecule remained unchanged during the enzyme-catalyzed oxygenation, which was confirmed by the presence of two methylene-interrupted double bonds in its structure, with $\delta$ 2.84 ppm (H$_2$, 2H, triplet) and $\delta$ 5.33–5.39 ppm (C=CH, 6H, multiplet). The spectrum lacked features that would have been present if the product had a trans,trans fragment, being, for example, an 11$E$,13$E$,15$Z$ or 11$Z$,13$E$,15$E$ isomer. For instance, resonances with $\delta$ ~6.2 ppm, characteristic of a trans,trans conjugated double bond, were not detected. Instead, in a $^1$H, $^1$H-COSY experiment, it was revealed that protons with $\delta$ 4.17 ppm (2H, quintet), believed to be C(10)H$_2$OH and C(17)H$_2$OH, produced cross-peaks with proton $\delta$ 5.73 and 5.75 ppm (triplets). No such cross-peaks with cis protons of the C(13)/C(14) vinyl group ($\delta$ 5.95–5.99 ppm) were discovered. A full 400 MHz 2D $^1$H, $^1$H-DQCOSY spectrum of product IVb is presented in Fig. 4. The spectrum was found to be essentially iden-

![Fig. 1. Structural analysis of product IIb. A: Ultraviolet (UV) light spectrum of the product in methanol. Maximum absorption is at 237 nm. AU, absorbance units. B: Mass spectrum of the compound taken in the negative ion zoom scan mode (methanol as solvent). C: A 400 MHz one-dimensional (1D) $^1$H-NMR spectrum of the compound recorded in deuteromethanol at room temperature. D: A 400 MHz two-dimensional (2D) double quantum correlation spectroscopy ($^1$H, $^1$H-DQCOSY) NMR spectrum of product IIb (CD$_3$OD as solvent).](image-url)
### DISCUSSION

There is a controversy regarding the cis/trans geometry of the conjugated triene fragment of the two possible products from the enzymatic synthesis of 10,17-dihydro(pero)xy-DHA.
isomers of 10,17(S)-diHDHA described recently (2–11). In pioneering papers, Serhan and colleagues (6, 7) proposed that the compound was formed through a LOX-like epoxidation-isomerization of 17(S)-HPDHA, which should yield an 11E,13E,15Z geometric isomer of the product. That mechanism is based on an epoxidation reaction of the C(17) hydroperoxy group of 17(S)-HPDHA yielding a compound with the 11E,13E,15Z geometry of the conjugated triene. If the C(17) hydroperoxy group is absent (e.g., is substituted with a hydroxy group), the reaction should not occur because the crucial intermediate, C(16)/C(17) epoxide, cannot be formed. Contrary to this assumption, in our recent experiments (10), we were able to obtain 10,17(S)-diHDHA from either 17(S)-HPDHA or 17(S)HDHA.

Fig. 2. Normal phase HPLC separation of the major products of DHA oxidation by soybean lipooxygenase (sLOX). The chromatograms were recorded at 270 nm (solid line) and 237 nm (dotted line). The conditions of the HPLC separation are described in Materials and Methods for isocratic elution (1 ml/min, 30°C, Waters Spherisorb silica gel column, 5 μm, 3.2 × 250 mm). A: UV light spectra of the eluted peaks IIa, IIIa, and IVa. B: Mass spectrum of product IVa taken in the negative ion zoom scan mode (methanol as solvent). AU, absorbance units.

Fig. 3. Structural analysis of product IVb. A: Mass spectrum of product IVb taken in the negative ion zoom scan mode (methanol as solvent). B: A 400 MHz 1D 1H-NMR spectrum of the compound recorded in CD3OD. C: A 400 MHz 2D 1H,1H-DQCOSY NMR spectrum of product IVb (expanded vinyl region of the spectrum; CD3OD as solvent).
Although at the time the geometries of the resulting trienes were not precisely established, the fact that 10,17(S)-diH(P)DHA could be formed from 17(S)-HDHA ruled out the epoxidation-isomerization mechanism for that reaction and suggested the double lipoxygenation mechanism, with the likely 11E,13Z,15E arrangement of the double bonds in the final product. Later, a more detailed structural analysis of the ptLOX product was published that confirmed our earlier prediction of the geometric features of the compound (11).

**Fig. 4.** A full 400 MHz 2D 1H, 1H-DQCOSY NMR spectrum of product IVb and the corresponding proton assignments.

**Fig. 5.** Chiral high-performance liquid chromatogram of 10,17(S)-diHDHA on a Chiralcel OD-H column. The analysis was performed as described in Materials and Methods. A: UV light spectrum of the elution peak with retention time of 50.503 min. B: Mass spectrum of 10,17(S)-diHDHA. AU, absorbance units.
In this study, for the first time, 1D and 2D $^1$H-NMR techniques were used to address the structure of the sLOX-derived 10,17(S)-diHDHA. Cross-peaks detected in the 2D $^1$H, $^1$H-DQCOSY experiment allowed us to unequivocally assign observed resonances to particular protons of 10,17(S)-diHDHA (Table 1). The spectrum lacked a proton resonance with δ 6.15–6.24 ppm, which would have been present if the compound had a conjugated 11E,13E or 13E,15E fragment. Such resonances have been reported for several compounds with conjugated $E,E$ double bonds, for example, all-trans isomers of 9(S)- and 13(S)-hydroxy linoleyl alcohols (14), 9(S)- and 13(S)-hydroxy monolinoleol glycerols (15), 5, 6-dihydroxyeicosapenta-7E,9E,11Z,14Z,17Zenoic acid (16), 5-ketoicosatetra-7E,9E,11Z,14Zenoic acid (17), 14,15-dihydroxyeicosatetra-5Z,8Z,10E,12Z-enoic acid (18), and 5,12(S)-dihydroxyeicosatetra-6E,8E,10E-enoic acid (19).

Only three types of protons that belonged to the 11E,13Z,15E fragment were observed in the NMR experiments (Table 1, Figs. 1, 3, 4). This was not surprising considering the highly symmetrical nature of 10,17(S)-HDHA (Scheme 2), with four pairs of equivalent protons at C(10)/C(17), C(11)/C(16), C(12)/C(15), and C(13)/C(14). The clear absence of trans,trans vinyl protons was also indicative of a specific (i.e., purely enzymatic) mechanism of formation of the products, as any involvement of a free radical chain reaction similar to those described previously (14, 15) would have produced measurable quantities of thermodynamically favorable trans,trans or all-trans isomers of the products, which was not the case in the current experiments.

Additional evidence that supports the 11E,13Z,15E arrangement of the conjugated triene came from the fact that the methine protons at C(10) and C(17) (δ ~4.15–4.17 ppm) produced cross-peaks with trans vinyl protons at C(11) and C(16) (δ ~5.75 ppm). No such cross-peaks with protons of a cis vinyl group (δ ~5.97 ppm) were observed. These data unambiguously identify the sLOX product of DHA oxidation as 10,17(S)-diHDHA. Interestingly, this product was invariably formed no matter which synthetic procedure was implemented (Scheme 1). Because there were no visible differences in the NMR spectra of the ptLOX-derived 10,17(S)-diHDHA and its sLOX-derived counterpart, the compounds were considered to be identical in all respects except for the possible (S)/ (R) stereoisomerism of the hydroxyl group at C(10) [for a discussion of its putative (S)-stereochemistry, see (10, 11)]. The exact alignment of DHA and 17(S)-H(P)DHA in sLOX and ptLOX catalytic centers during the formation of 10,17-diH(P) DHA remains to be investigated.
The finding that 10,17-diH(P)DHA formed through two convenient and easily reproducible chemoenzymatic pathways consistently had the 11E,13Z,15E arrangement of the conjugated triene fragment needs to be taken into account when making this compound and studying its biological properties.

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