methods

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

Igor A. Butovich[1]

Department of Ophthalmology, University of Texas Southwestern Medical Center, Dallas, TX 75390-9057

Abstract **A product of lipoxygenase (LOX) oxidation of docosahexaenoic acid (DHA), 10,17-dihydro(pero)xydocosahexa-4*Z*,7*Z*,11*E*,13*Z*,15*E*,19*Z*-enoic acid [10,17(*S*)-diH(P)DHA] was obtained through various reaction pathways that involved DHA, 17(*S*)-hydro(pero)xydocosahexa-4*Z*,7*Z*,11*Z*,13*Z*,15*E*,19*Z*-enoic acid [17(*S*)-H(P)DHA], soybean lipoxygenase (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*)-diH(P)DHA formed by sLOX through direct oxidation of either DHA or 17(*S*)-H(P)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-diHDHA formed by sLOX had solely 11*E*,13*Z*,15*E* configuration of the conjugated triene fragment, which was 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.**—Butovich, I. A. **A one-step method of 10,17-dihydro(pero)xydocosahexa-4*Z*,7*Z*,11*E*,13*Z*,15*E*,19*Z*-enoic acid synthesis by soybean lipoxygenase. *J. Lipid Res.* 2006. 47: 854–863.**

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 (acetylated) 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 bioregulatory compounds and potential drug candidates. Among those, 10,17(*S*)-dihydroxydocosahexaenoic acid with postulated 4*Z*,7*Z*,11*E*,13*E*,15*Z*,19*Z* 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-x_l 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 enzymatic 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-4*Z*,7*Z*,11*Z*,13*Z*,15*E*,19*Z*-enoic acid [17(*S*)-HPDHA] in a 16,17-epoxy-DHA intermediate, with subsequent hydrolysis of the

Manuscript received 14 December 2005 and in revised form 3 January 2006.
Published, JLR Papers in Press, January 3, 2006.
DOI 10.1194/jlr.D500042-JLR200

Abbreviations: BSTFA, bis(trimethylsilyl)trifluoroacetamide; $C_{12}E_{10}$, monododecyl ether of decaoxyethylene glycol; 1D, one-dimensional; 2D, two-dimensional; DHA, docosahexaenoic acid (ω-3 C22:6); 10,17-diH(P)DHA, 10,17-dihydro(pero)xydocosahexa-4*Z*,7*Z*,11*E*,13*Z*,15*E*,19*Z*-enoic acid; EI, electron-impact; ESI, electrospray ionization; ^{1}H, ^{1}H-DQCOSY, double quantum correlation spectroscopy; LOX, lipoxygenase; neuroprotectin D1 and 10,17(*S*)-docosatriene, 10,17(*S*)-dihydroxydocosahexaenoic acid with postulated 4*Z*,7*Z*,11*E*,13*E*,15*Z*,19*Z* geometry of the double bonds; NP HPLC, normal phase high performance liquid chromatography; ptLOX, potato tuber lipoxygenase; 17(*S*)-H(P)DHA, 17(*S*)-hydro(pero)xydocosahexa-4*Z*,7*Z*,11*Z*,13*Z*,15*E*,19*Z*-enoic acid; sLOX, soybean lipoxygenase; UV, ultraviolet.

[1] To whom correspondence should be addressed.
e-mail: igor.butovich@utsouthwestern.edu

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epoxide and its rearrangement in a conjugated triene with 11*E*,13*E*,15*Z* 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 lipoxygenase (sLOX) (3, 8) and/or by a combination of sLOX and potato tuber lipoxygenase (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-4*Z*,7*Z*,11*Z*,13*Z*,15*E*,19*Z*-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-4*Z*,7*Z*,11*E*,13*Z*,15*E*,19*Z*-enoic acid [10,17(*S*)-diH(P)DHA], and the second largest metabolite was 7,17(*S*)-dihydroxydocosahexaenoic acid [7,17(*S*)-diH(P)DHA]. Taking into account that both 17(*S*)-HDHA and 17(*S*)-HPDHA were effectively transformed into 10,17(*S*)-diH(P)DHA by ptLOX, we speculated that epoxidation/isomerization was not involved in those reactions and that 10,17(*S*)-diH(P)DHA was clearly a product of double lipoxygenation with 11*E*,13*Z*,15*E* arrangement of the conjugated triene. Corroborating this hypothesis was the fact that while oxidizing DHA, ptLOX produced 10(*S*)-HPDHA as the major monooxygenated product. Later, new direct evidence emerged that confirmed our prediction that the product synthesized by ptLOX from 17(*S*)-H(P)DHA indeed had 11*E*,13*Z*,15*E* 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(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_3OD (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 decaoxyethylene 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-PP 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^n 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_3OD 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 236 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 λ_{max} 234–238 nm (ϵ_m 23,000 $M^{-1} \times cm^{-1}$), whereas the conjugated triene(s) gave a characteristic triplet at 260, 270, and 280 nm (ϵ_m 40,000 $M^{-1} \times cm^{-1}$ 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 nonspecific 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(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

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product(s). When the target products were hydroxides of DHA, ~10 M excess of freshly prepared $NaBH_4$ solution in the same buffer was added drop-wise to the mixture of the hydroperoxides, and the reaction vessel was placed on ice for 15 min. The unreacted $NaBH_4$ was then decomposed with an excess of glacial acetic acid (~0.25 ml) added drop-wise (foaming), and the mixture was left on ice until the bubbling stopped. Then, the lipid mixture was slowly loaded on a 500 mg C_{18} solid phase extraction cartridge and washed with 25 ml of deionized water, the excess of water from the cartridge was removed by vacuuming, and the product was eluted with 2 ml of absolute ethanol. The solvent was then evaporated to dryness under a stream of N_2 at room temperature; the products were redissolved in nitrogen-flushed ethanol (1 ml) and stored at −80°C in a glass vial with a Teflon® cup. No decomposition of 10,17(*S*)-diHDHA occurred within at least 2 months of storage. When the hydroperoxides of DHA were to be made, the $NaBH_4$ treatment step was omitted and the initial product acidified with the same amount of acetic acid was loaded directly onto the extraction cartridge. The hydroperoxides of DHA stored as described above were stable for at least 2 weeks. Slow accumulation of decomposition products was observed upon prolonged storage of the compounds.

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 (949:50:1, 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 isocratically 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^n 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.

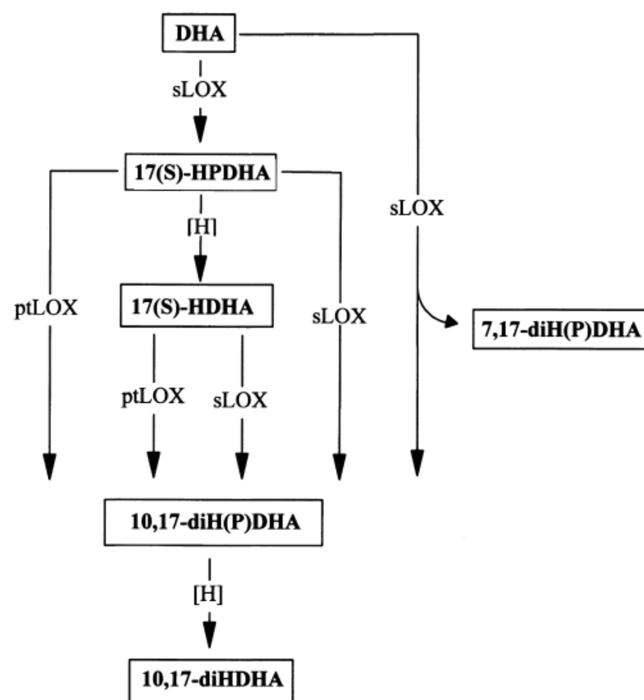
The individual products and/or product mixtures were subjected to EI GC-MS analysis after catalytic hydrogenation with H_2/PtO_2 and trimethylsilylation with BSTFA (10). Briefly, helium was used to elute the compounds from the DB-17HT capillary column. The following elution program was used. The column was preequilibrated 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) ^{1}H-NMR spectra of the $NaBH_4$-reduced products were recorded in CD_3OD at room temperature. Analysis of the spectra was performed with the help of MesTre-C version 4.5.1 software (purchased through www.mestrec.com). Terminal [omega, $C_{(22)}$, 3H] methylene and $C_{(10)}$ and/or $C_{(17)}$ 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-dihydro(pero)xydocosa-



Scheme 1. Biosynthetic pathways leading to 10,17-dihydroxydocosahexa-4*Z*,7*Z*,11*E*,13*Z*,15*E*,19*Z*-enoic acid [10,17-(*S*)-diHDHA].

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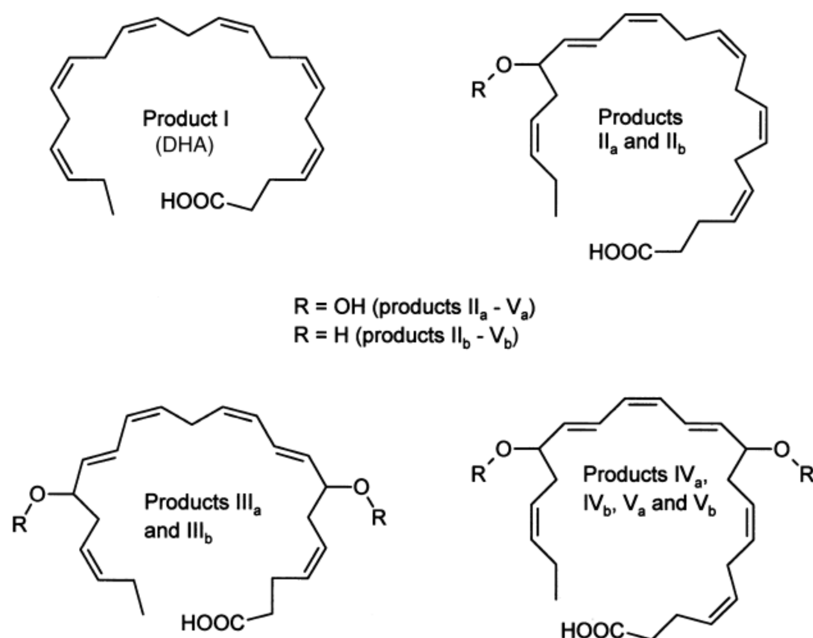
hexa-4*Z*,7*Z*,11*E*,13*Z*,15*E*,19*Z*-enoic acid [10,17-diH(P)DHA] and, consequently, 10,17-diHDHA.

sLOX-catalyzed reactions

In agreement with earlier publications (10, 12), low concentrations of sLOX (5×10^{-8} M to 1×10^{-7} M) caused rapid and virtually quantitative conversion of 10^{-4} M DHA into 17(*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 II_a had the UV light absorbance spectrum of a typical conjugated diene, with λ_{max} of 238 nm (**Fig. 1A**) and molecular weight of 360.3 (molecular formula, $C_{22}H_{32}O_4$; theoretical isotopic mass, 360.2). The molecular weight of its $NaBH_4$-reduced derivative was 344.3 ($C_{22}H_{32}O_3$; product II_b; theoretical isotopic mass, 344.2) (Fig. 1B). The structure of product II_b was elucidated by EI GC-MS and by ^{1}H-NMR. After catalytic hydrogenation of product II_b 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}H-NMR spectrum of product II_b is shown in Fig. 1C. The spectrum displayed resonances that were similar to those published earlier for the *cis,trans* isomers of 9/13-hydroxy linoleyl alcohol and 9/13-hydroxy monolinoleoyl glycerol (14, 15). No traces of *trans,trans* isomers were detected [the latter would have been seen as a quartet with $\delta_{CH} \sim 6.1–6.2$ 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}H, ^{1}H-DQCOSY) scan of product II_b (Fig. 1D) along with the 1D ^{1}H-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 δ (**Table 1**). Importantly, the protons with $\delta \sim 4.15–4.17$ ppm [believed to be $=C_{(17)}$**H**-OH] produced cross-peaks with protons with δ ~2.3 ppm [$=C_{(18)}\mathbf{H}_2$] and 5.75 [$=C_{(16)}$**H**-], but not with the protons at $C_{(13)}$ and $C_{(14)}$. Therefore, product II_a was identified as 17-hydroperoxydocosahexa-4*Z*,7*Z*,10*Z*, 13*Z*,15*E*,19*Z*-enoic acid, with the hydroxyl group at $C_{(17)}$ being in the *S* configuration (10, 12).

A dramatically different result was obtained when the concentration of sLOX in the reaction mixture was increased to 0.4×10^{-6} 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 λ_{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 $C_{22}H_{32}O_6$ 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}H-NMR. The molecular mass of product IV_b was found to be 360.1 Da (**Fig. 3A**), consistent with the molecular formula $C_{22}H_{32}O_4$ (isotopic mass, 360.2). The fragmenta-



Scheme 2. Reaction products of lipoxygenase (LOX)-catalyzed oxidative transformations of docosahexaenoic acid (DHA).

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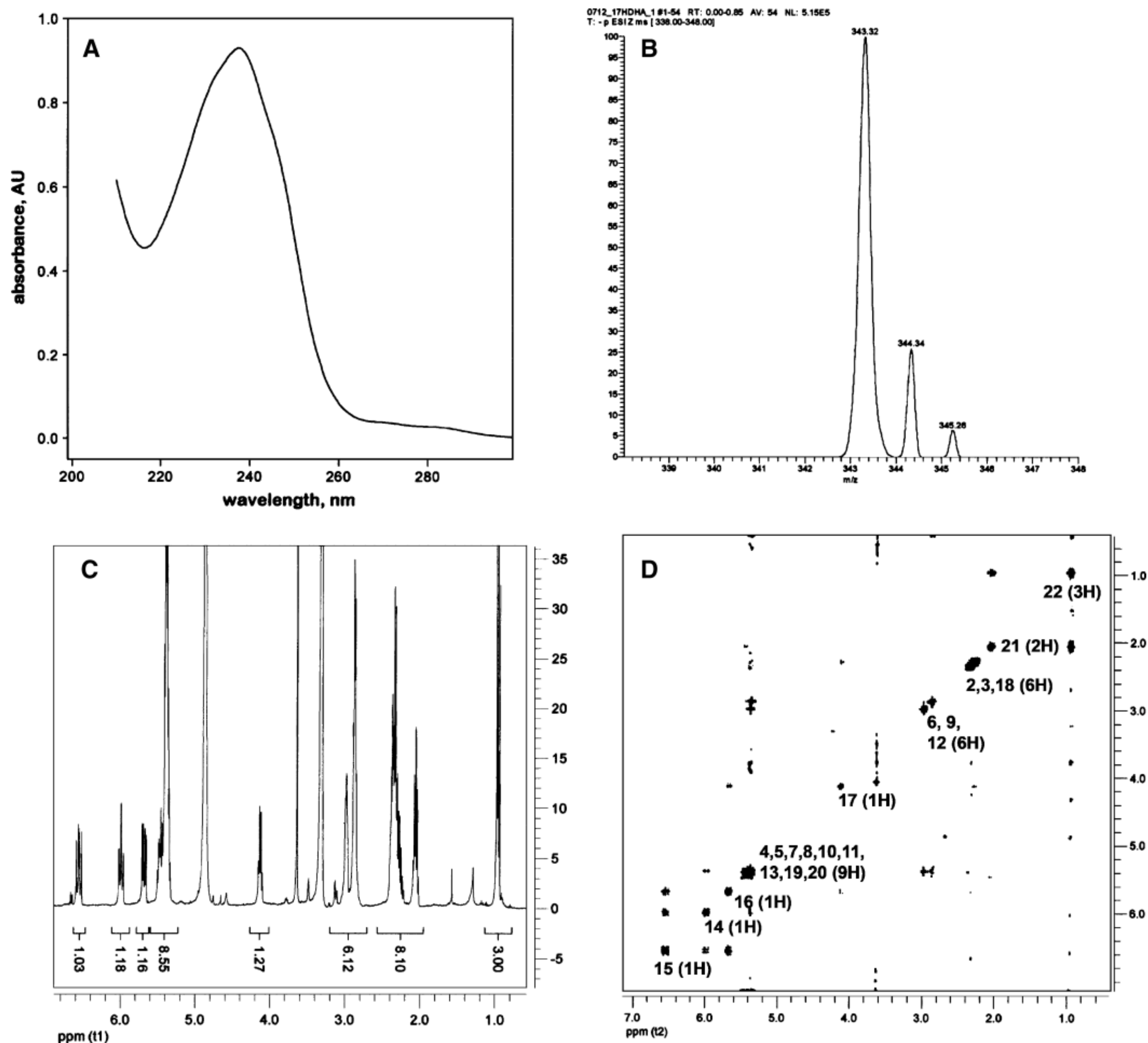

Fig. 1. Structural analysis of product II_b. A: Ultraviolet (UV) light spectrum of the product in methanol. Maximum absorption is at 237 nm. AU, absorbance units. B: Mass spectrum of the compound taken in the negative ion zoom scan mode (methanol as solvent). C: A 400 MHz one-dimensional (1D) ^{1}H-NMR 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 II_b (CD_3OD as solvent).

tion pattern of its catalytically hydrogenated and fully trimethylsilylated derivative (*m/z* of 73, 173, 331, 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 IV_b (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 δ 2.84 ppm [=$C_{(6)}$**H_2**, 2H, triplet] and δ 5.33–5.39 ppm (-C**H**=C**H**-, 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 δ ~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 δ 4.17 ppm (2H, quintet), believed to be $C_{(10)}$**H**-OH and $C_{(17)}$**H**-OH, produced cross-peaks with proton δ 5.73 and 5.75 ppm (triplets). No such cross-peaks with *cis* protons of the $C_{(13)}/C_{(14)}$ vinyl group (δ 5.95–5.99 ppm) were discovered. A full 400 MHz 2D ^{1}H, ^{1}H-DQCOSY spectrum of product IV_b is presented in **Fig. 4**. The spectrum was found to be essentially iden-

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TABLE 1. One-dimensional 400 MHz ^{1}H-NMR analysis of the DHA oxidation products

Carbon atom number	DHA[a]		Product II_b[b] 17(*S*)-HDHA		Products IV_b and V_b[c] 10,17(*S*)-diHDHA	
	δ, ppm	Features	δ, ppm	Features	δ, ppm	Features
1	N/A		N/A		N/A	
2	2.3–2.4	m	2.2–2.4		2.3–2.4	
3	2.3–2.4	m	2.2–2.4		2.3–2.4	
4	5.3–5.4	m	5.3–5.4		5.4	
5	5.3–5.4	m	5.3–5.4		5.4	
6	2.8–2.9	m	2.87	2H	2.84	2H
7	5.3–5.4	m	5.3–5.5		5.4	
8	5.3–5.4	m	5.3–5.5		5.4	
9	2.8–2.9	m	2.87	2H	2.3–2.4	
10	5.3–5.4	m	5.3–5.5		4.17	1H
11	5.3–5.4	m	5.3–5.5		5.71	1H
12	2.8–2.9	m	2.99	2H	6.72	1H
13	5.3–5.4	m	5.38	1H	5.97	1H
14	5.3–5.4	m	5.99	1H	5.97	1H
15	2.8–2.9	m	6.55	1H	6.72	1H
16	5.3–5.4	m	5.75	1H	5.75	1H
17	5.3–5.4	m	4.13	1H	4.17	1H
18	2.8–2.9	m	2.29	2H	2.3–2.4	
19	5.3–5.4	m	5.45	1H	5.4	
20	5.3–5.4	m	5.40	1H	5.45	
21	2.09	2H, p	2.05	2H, p	2.06	2H, p
22	0.97	3H, t	0.96	3H, t	0.96	3H, t

DHA, docosahexaenoic acid; HDHA, dihydroxydocosahexaenoic acid; N/A, not applicable. All of the spectra were taken in CD_3OD at room temperature.

[a] Total number of protons with δ 2.3–2.4, 2.8–2.9, and 5.3–5.4 ppm were 4.4 (n = 4), 10.4 (n = 10), and 12.1 (n = 12), respectively.

[b] Total number of protons with δ 2.2–2.4 and 5.3–5.5 ppm was 6 and 7.55 (n = 8), respectively.

[c] Total number of protons with δ 2.3–2.4 and 5.3–5.45 ppm were 7.7 (n = 8) and 6 (n = 6), respectively.

tical to a spectrum of the ptLOX-derived 10,17(*S*)-diHDHA published previously (11). Interestingly, product IV_b gave a single symmetrical peak on a Chiralcel OD-H column with a retention time of 58 min (**Fig. 5**). A similar product was formed by sLOX from 17(*S*)-HDHA or 17(*S*)-HPDHA (data not shown; see Scheme 1). It had the same retention time as product III_b in all tested conditions, including chiral, reverse phase, and NP HPLC. Thus, product IV_a was identified as 10,17(*S*)-dihydroperoxydocosahexa-4*Z*,7*Z*,11*E*,13*Z*,15*E*,19*Z*-enoic acid, whereas product IV_b was identified as the corresponding hydroxide (Scheme 2).

Using the analytical procedures described above, product III_a was found to be a DHA derivative with hydroperoxy groups at $C_{(7)}$ and $C_{(17)}$, similar to the compound described previously (10) as one of the two major products of ptLOX-catalyzed oxygenation of 17(*S*)-HDHA and 17(*S*)-HPDHA [a detailed report on 7,17-dihydro(pero)xydocosahexaenoic acid synthesis and characterization will be published elsewhere]. Minor amounts of various H(P)DHAs were detected among the reaction products by NP HPLC (Fig. 2) and in a single ion monitoring GC-MS experiment (**Fig. 6**). Among those, 17-HDHA was positively identified by its coeluting fragments with *m/z* 173 and 429, whereas 10-HDHA produced fragments with *m/z* 271 and 331. The single ion monitoring elution peaks were integrated, and the apparent ratio of 10-HDHA to 17-HDHA of 1:2 was calculated.

The elution peaks with UV light absorbance maxima at 236 ± 2 nm that were observed during NP HPLC had retention times similar to those of authentic 10(*S*)- and 17(*S*)-HDHAs, although no attempts were made to isolate and further characterize individual monohydroxylated products because of their relatively low abundance.

Interestingly, at high enzyme concentrations, the sLOX-catalyzed reaction of DHA oxidation showed clear signs of autoinactivation/product inhibition. Under the implemented conditions, 1 molecule of sLOX was able to make ~97 molecules of 10,17-diH(P)DHA before the reaction stopped as a result of the apparent inactivation/product inhibition of the enzyme.

ptLOX-catalyzed conversion of 17(*S*)-HDHA and 17(*S*)-HPDHA

In agreement with our earlier findings, ptLOX converted 17(*S*)-HDHA and 17(*S*)-HPDHA to the conjugated triene product V_a, whose structure was studied by HPLC, UV light spectrometry, ESI MS, EI GC-MS, and proton NMR as described above for products II–IV. The molecular mass of product V_a was found to be identical to that of product IV_a (392.3 Da; molecular formula, $C_{22}H_{32}O_6$). The UV light spectrum of product V_a taken in methanol was indistinguishable from the spectrum of product IV_a. Based on the EI GC-MS fragmentation of its fully hydrogenated and trimethylsilylated derivative, product V_a was identified as 10,17(*S*)-diHPDHA, with the hydroperoxy group at $C_{(10)}$ being, most likely, in the *S* configuration as well.

Product V_a was then reduced with $NaBH_4$ to yield product V_b. The chromatographic properties of products IV_b and V_b were compared in various conditions, and the compound appeared to be coeluting in the following HPLC systems: *1*) on a silica gel column in several isocratic and gradient hexane-2-propanol-acetic acid mixtures (between 989:10:1 and 949:50:1, v/v/v), as described in Materials and Methods; *2*) on a cyanopropyl-silica gel column in a hexane-2-propanol-acetic acid mixture (949:50:1, v/v/v); *3*) on a C_{18} silica gel column in isocratic and gradient methanol-water-phosphoric acid and acetonitrile-water-phosphoric acid mixtures; and *4*) on the Chiralcel OD-H column in a hexane-2-propanol-acetic acid mixture (949:50:1, v/v/v).

A full 1D ^{1}H-NMR spectrum of the isolated product V_b was identical to that of product IV_a, confirming our earlier prediction that 10,17(*S*)-diHDHA formed from 17(*S*)-H(P)DHA by ptLOX had the 11*E*,13*Z*,15*E* fragment in it. A full 2D ^{1}H, ^{1}H-COSY spectrum of product V_b also showed no differences from product III_b.

DISCUSSION

There is a controversy regarding the *cis/trans* geometry of the conjugated triene fragment of the two possible

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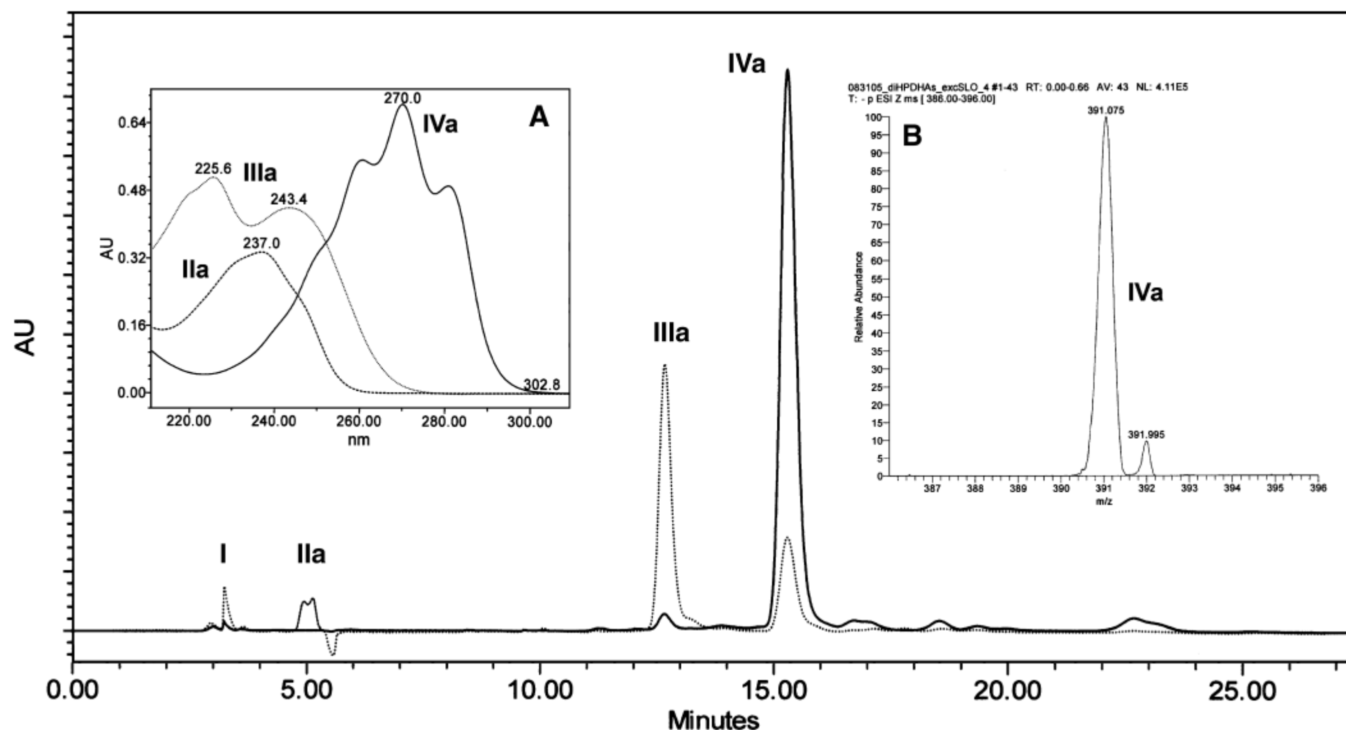

Fig. 2. Normal phase HPLC separation of the major products of DHA oxidation by soybean lipoxygenase (sLOX). The chromatograms were recorded at 270 nm (solid line) and 237 nm (dotted line). The conditions of the HPLC separation are described in Materials and Methods for isocratic elution (1 ml/min, 30°C,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

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 11*E*,13*E*,15*Z* 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 11*E*,13*E*,15*Z* 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.

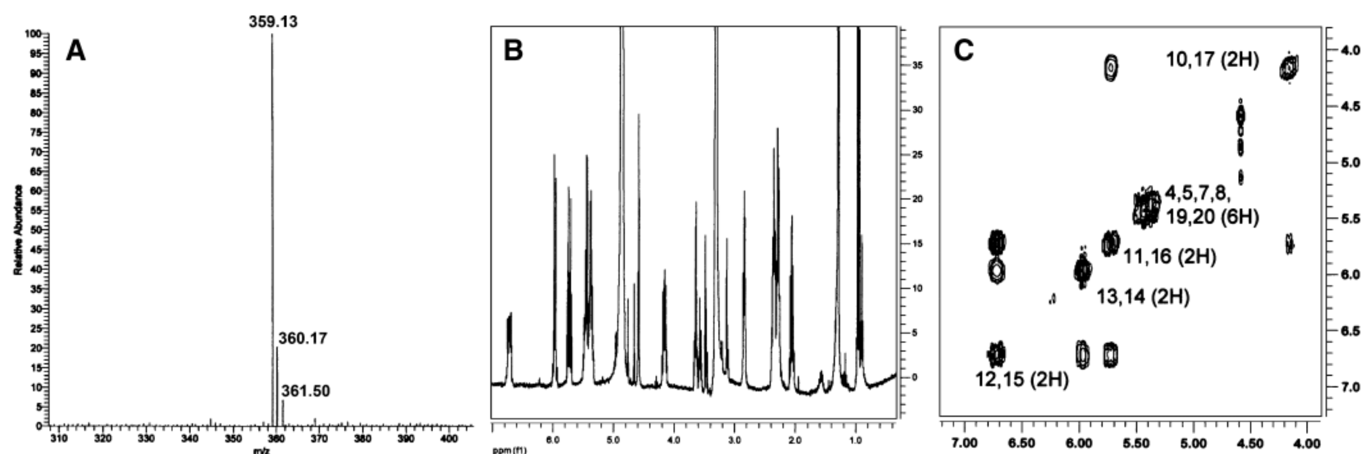

Fig. 3. Structural analysis of product IV_b. A: Mass spectrum of product IV_b taken in the negative ion zoom scan mode (methanol as solvent). B: A 400 MHz 1D ^{1}H-NMR spectrum of the compound recorded in CD_3OD. C: A 400 MHz 2D ^{1}H, ^{1}H-DQCOSY NMR spectrum of product IV_b (expanded vinyl region of the spectrum; CD_3OD as solvent).

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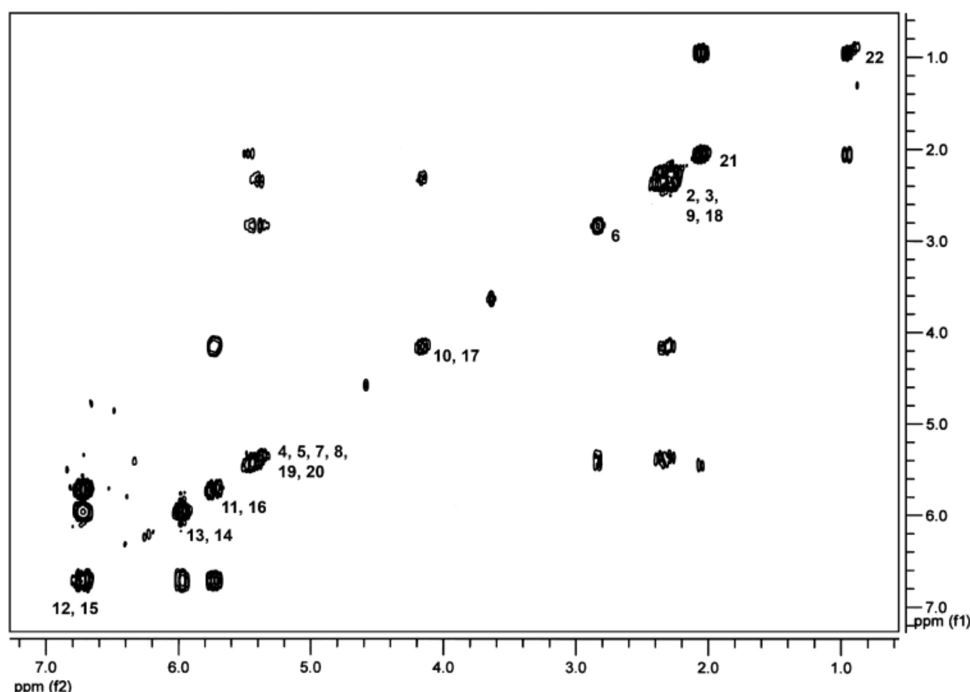

Fig. 4. A full 400 MHz 2D ^{1}H, ^{1}H-DQCOSY NMR spectrum of product IV_b and the corresponding proton assignments.

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 11*E*,13*Z*,15*E* 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).

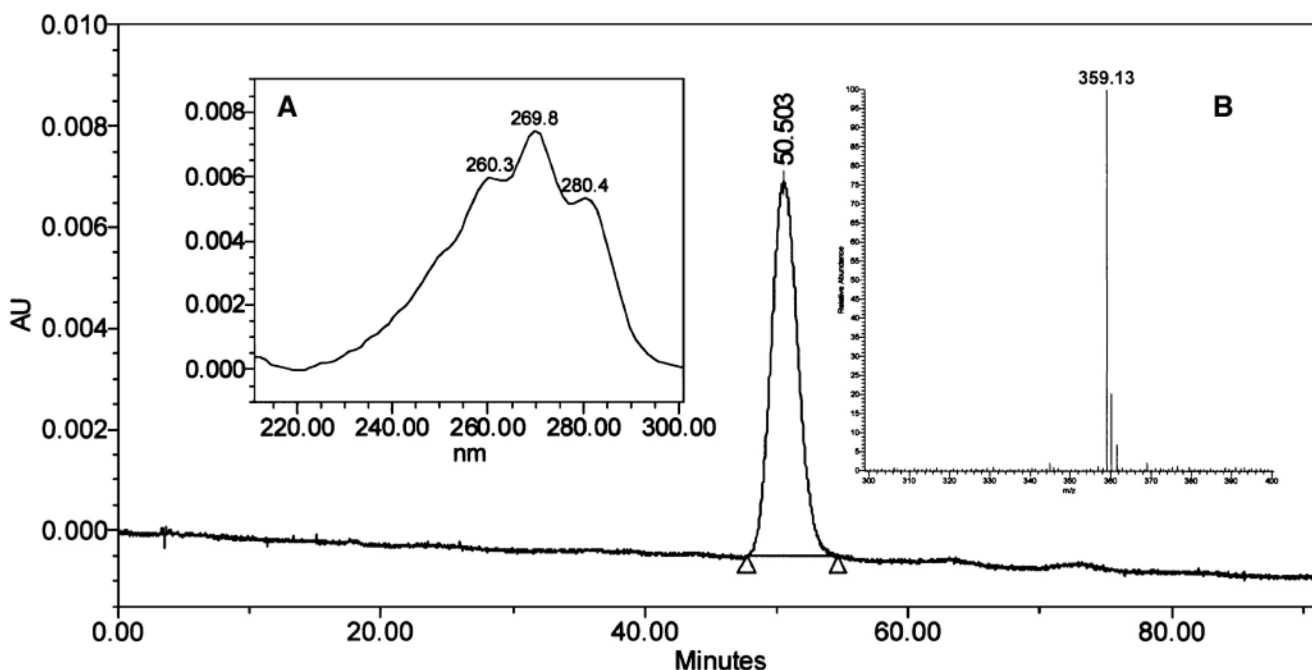

Fig. 5. Chiral high-performance liquid chromatogram of 10,17(*S*)-diHDHA on a Chiralcel OD-H column. The analysis was performed as described in Materials and Methods. A: UV light spectrum of the elution peak with retention time of 50.503 min. B: Mass spectrum of 10,17(*S*)-diHDHA. AU, absorbance units.

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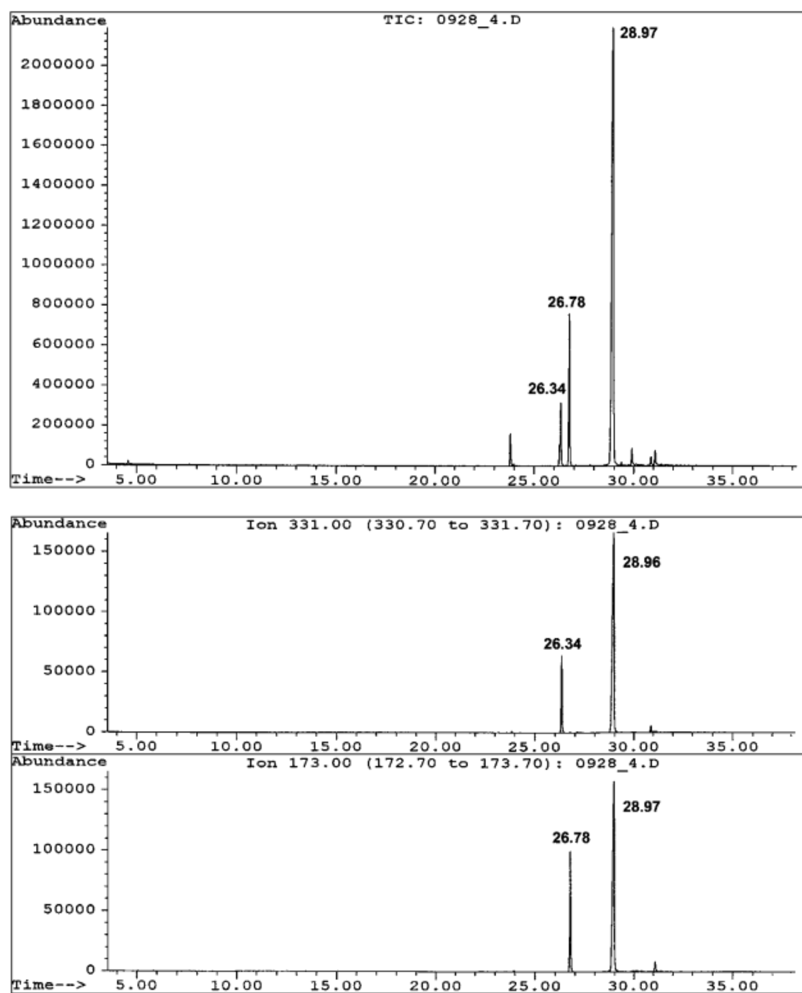

Fig. 6. GC-MS analysis of 10-HDHA, 17-HDHA, and 10,17-diHDHA formed in the reaction of DHA oxidation by sLOX. Catalytic hydrogenation/trimethylsilylation of the sample and GC-MS analysis were performed as described in Materials and Methods. Upper panel: Total ion chromatogram (TIC) of the product mixture. Peak 26.34 min is 10-HDHA, peak 26.78 is 17-HDHA, and peak 28.97 is 10,17-diHDHA. Lower panel: Single ion monitoring experiment. Ions with *m/z* of 331 are indicative of fragments with hydroxyl groups at $C_{(10)}$, whereas ions with *m/z* of 173 reveal compounds with hydroxyls at $C_{(17)}$.

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 11*E*,13*E* or 13*E*,15*E* 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 monolinoleoyl glycerols (15), 5, 6-dihydroxyeicosapenta-7*E*,9*E*,11*Z*,14*Z*,17*Z*-enoic acid (16), 5-ketoeicosatetra-7*E*,9*E*,11*Z*,14*Z*-enoic acid (17), 14,15-dihydroxyeicosatetra-5*Z*,8*Z*,10*E*,12*E*-enoic acid (18), and 5,12 (*S*)-dihydroxyeicosatetra-6*E*,8*E*,10*E*-enoic acid (19).

Only three types of protons that belonged to the 11*E*,13*Z*, 15*E* 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 11*E*,13*Z*,15*E* 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*)-diH(P)DHA. 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.

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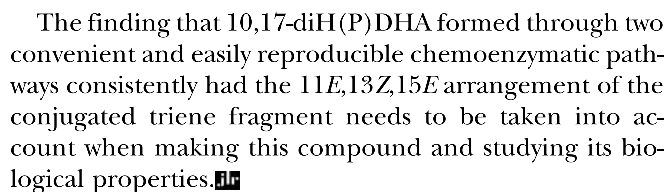

The finding that 10,17-diH(P)DHA formed through two convenient and easily reproducible chemoenzymatic pathways consistently had the 11*E*,13*Z*,15*E* arrangement of the conjugated triene fragment needs to be taken into account when making this compound and studying its biological properties.

This study was supported by grants from the Swedish Foundation for International Cooperation in Research and Higher Education and the National Institutes of Health (Grant 5P30AR-04194014) and by an unrestricted grant from Research to Prevent Blindness, Inc. The author thanks Carl Bachmann (Department of Biochemistry, University of Texas Southwestern Medical Center) for his help in conducting the NMR experiments.

REFERENCES

1. Bannenberg, G. L., N. Chiang, A. Ariel, M. Arita, E. Tjonahen, K. H. Gotlinger, S. Hong, and C. N. Serhan. 2005. Molecular circuits of resolution: formation and action of resolvins and protectins. *J. Immunol.* **174:** 4345–4355.
2. Serhan, C. N., M. Arita, S. Hong, and K. Gotlinger. 2004. Resolvins, docosatrienes, and neuroprotectins, novel omega-3-derived mediators, and their endogenous aspirin-triggered epimers. *Lipids.* **39:** 1125–1132.
3. Mukherjee, P. K., V. L. Marcheselli, C. N. Serhan, and N. G. Bazan. 2004. Neuroprotectin D1: a docosahexaenoic acid-derived docosatriene protects human retinal pigment epithelial cells from oxidative stress. *Proc. Natl. Acad. Sci. USA.* **101:** 8491–8496.
4. Serhan, C. S., K. Gotlinger, S. Hong, and M. Arita. 2004. Resolvins, docosatrienes, and neuroprotectins, novel omega-3-derived mediators, and their aspirin-triggered endogenous epimers: an overview of their protective roles in catabasis. *Prostaglandins Other Lipid Mediat.* **73:** 155–172.
5. Serhan, C. N. 2005. Novel ω-3-derived local mediators in anti-inflammation and resolution. *Pharmacol. Ther.* **105:** 7–21.
6. Marcheselli, V. L., S. Hong, W. Lukiw, X. H. Tian, K. Gronert, A. Musto, M. Hardy, J. M. Gimenez, N. Chiang, C. N. Serhan, et al. 2003. Novel docosanoids inhibit ischemia-reperfusion-mediated leukocyte infiltration and pro-inflammatory gene expression. *J. Biol. Chem.* **278:** 43807–43817.
7. Hong, S., K. Gronert, P. R. Devchand, R. L. Moussignas, and C. N. Serhan. 2003. Novel docosatrienes and 17(S)-resolvins generated from docosahexaenoic acid in murine brain, human blood, and glial cells. *J. Biol. Chem.* **278:** 14677–14687.
8. Lukiw, W. J., J-G. Cui, V. L. Marcheselli, M. Bodker, A. Botkjaer, K. Gotlinger, C. N. Serhan, and N. G. Bazan. 2005. A role for docosahexaenoic acid-derived neuroprotectin D1 in neural cell survival and Alzheimer disease. *J. Clin. Invest.* **115:** 2774–2783.
9. Gronert, K., N. Maheshwari, N. Khan, I. R. Hassan, M. Dunn, and M. L. Schwartzmann. 2005. A role for the mouse 12/15-lipoxygenase pathway in promoting epithelial wound healing and host defense. *J. Biol. Chem.* **280:** 15267–15278.
10. Butovich, I. A., M. Hamberg, and O. Rådmark. 2005. Novel oxylipins formed from docosahexaenoic acid by potato lipoxygenase—10(S)-hydroxydocosahexaenoic acid and 10,20-dihydroxydocosahexaenoic acid. *Lipids.* **40:** 249–257.
11. Butovich, I. A. 2005. On the structure and synthesis of neuroprotectin D1, a novel anti-inflammatory compound of the docosahexaenoic family. *J. Lipid Res.* **46:** 2311–2314.
12. Hamberg, M., and B. Samuelsson. 1967. On the specificity of the oxygenation of unsaturated fatty acids catalyzed by soybean lipoxidase. *J. Biol. Chem.* **242:** 5329–5335.
13. Butovich, I. A., V. A. Soloshonok, and V. P. Kukhar. 1991. The unusual action of (R,S)-2-hydroxy-2-trifluoromethyl-trans-n-octadec-4-enoic acid on 5-lipoxygenase from potato tubers. *Eur. J. Biochem.* **199:** 153–155.
14. Butovich, I. A., S. M. Luk'yanova, and C. C. Reddy. 2000. Oxidation of linoleyl alcohol by potato tuber lipoxygenase: kinetics and positional, stereo, and geometrical (cis,trans) specificity of the reaction. *Arch. Biochem. Biophys.* **378:** 65–77.
15. Butovich, I. A., and C. C. Reddy. 2001. Enzyme-catalyzed and enzyme-triggered pathways in dioxygenation of 1-monolinoleoyl-rac-glycerol by potato tuber lipoxygenase. *Biochim. Biophys. Acta.* **1546:** 379–398.
16. Jiang, Z. D., S. O. Ketchum, and W. H. Gerwick. 2000. 5-Lipoxygenase-derived oxylipins from the red alga *Rhodymenia pertusa*. *Phytochemistry.* **53:** 129–133.
17. Gravel, J., J-P. Falgueyret, J. Yergey, L. Trimble, and D. Riendeau. 1993. Identification of 5-keto-(7E,9E,11,Z,14Z)-eicosatetraenoic acid as a novel nonenzymatic rearrangement product of leukotriene A4. *Arch. Biochem. Biophys.* **306:** 469–475.
18. Rådmark, O., C. Serhan, M. Hamberg, U. Lundberg, M. D. Ennis, G. L. Bundy, T. D. Oglesby, P. A. Aristoff, A. W. Harrison, G. Slomp, et al. 1984. Stereochemistry, total synthesis, and biological activity of 14,15-dihydroxy-5,8,10,12-eicosatetraenoic acid. *J. Biol. Chem.* **259:** 13011–13016.
19. Kitamura, S., T. Shimizu, I. Miki, T. Izumi, T. Kasama, A. Sato, H. Sano, and Y. Seyama. 1988. Synthesis and structural identification of four dihydroxy acids and 11,12-leukotriene C4 derived from 11,12-leukotriene A4. *Eur. J. Biochem.* **176:** 725–731.

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