Dihydroxydocosahexaenoic acids of the neuroprotectin D family: synthesis, structure, and inhibition of human 5-lipoxygenase

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Abstract **During aerobic oxidation of docosahexaenoic acid (DHA), soybean lipoxygenase (sLOX) has been shown to form 7,17(*S*)-dihydro(pero)xydocosahexaenoic acid [7,17(*S*)-diH(P)DHA] along with its previously described positional isomer, 10,17(*S*)-dihydro(pero)xydocosahexa-4*Z*,7*Z*,11*E*,13*Z*,15*E*,19*Z*-enoic acid. 7,17(*S*)-diH(P)DHA was also obtained via sLOX-catalyzed oxidation of either 17(*S*)-hydroperoxydocosahexaenoic acid [17(*S*)-HPDHA] or 17(*S*)-hydroxydocosahexaenoic acid [17(*S*)-HDHA]. The structures of the products were elucidated by normal-phase, reverse-phase, and chiral-phase HPLC analyses and by ultraviolet, NMR, and tandem mass spectroscopy and GC-MS. 7,17(*S*)-diH(P)DHA was shown to have 4*Z*,8*E*,10*Z*,13*Z*,15*E*,19*Z* geometry of the double bonds. In addition, a compound apparently identical to the sLOX-derived 7,17(*S*)-diH(P)DHA was produced by another enzyme, potato tuber LOX, in the reactions of oxygenation of either 17(*S*)-HPDHA or 17(*S*)-HDHA. All of the dihydroxydocosahexaenoic acids (diHDHAs) formed by either of the enzymes were clearly produced through double lipoxygenation of the corresponding substrate. 7,17(*S*)-diHDHA inhibited human recombinant 5-lipoxygenase in the reaction of arachidonic acid (AA) oxidation. In standard conditions with 100 μM AA as substrate, the IC_{50} value for 7,17(*S*)-diHDHA was found to be 7 μM, whereas IC_{50} for 10,17(*S*)-DiHDHA was 15 μM. Similar inhibition by the diHDHAs was observed with sLOX, a quintessential 15LOX, although the strongest inhibition was produced by 10,17(*S*)-diHDHA (IC_{50} = 4 μM). Inhibition of sLOX by 7,17(*S*)-diHDHA was slightly less potent, with an IC_{50} value of 9 μM. These findings suggest that 7,17(*S*)-diHDHA along with its 10,17(*S*) counterpart might have anti-inflammatory and anticancer activities, which could be exerted, at least in part, through direct inhibition of 5LOX and 15LOX.**—Butovich, I. A., S. M. Lukyanova, and C. Bachmann. **Dihydroxydocosahexaenoic acids of the neuroprotectin D family: synthesis, structure, and inhibition of human 5-lipoxygenase.** ***J. Lipid Res.*** **2006.** 47: **2462–2474.**

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ω-3 fatty acids [e.g., docosahexaenoic acid (DHA)] have long been considered a vital diet/food supplement that is critical for brain (1) and sight (2) development in children as well as for maintaining the health of adults (3). Being an essential ingredient of several national diets (4), ω-3 fatty acids were recognized as the compounds that reduce the risk of certain cardiovascular diseases (5, 6) and asthma (7).

The most recent advancement in this area was a series of publications in which a group of novel oxygenated DHA metabolites was described (8, 9 and references cited therein). One of the metabolites, namely, 10,17(s)-dihydroxydocosahexaenoic acid with postulated 4Z, 7Z, 11E, 13E, 15Z, 19Z geometry of the double bonds, which has several names—10,17(*S*)-docosatriene (10), neuroprotectin D1 (11), and, most recently, protectin D1 (PD1) (9)—was found to have potent anti-inflammatory properties. In addition, this compound showed antiapoptotic neuroprotective activity in brain (12), promoted the apoptosis of T-cells (13), and facilitated corneal wound healing by mechanisms that differed from its anti-inflammatory activity (14). For simplicity, this compound will be referred to here as PD1. It was proposed that PD1 was produced in vivo from 17(*S*)-hydroperoxydocosahexaenoic acid [17(*S*)-HPDHA] by a lipoxygenase-like enzyme via an epoxidation/isomerization pathway (8–11 and references cited therein). Preliminary structural analysis of PD1 suggested that it was 10,17(*S*)-dihydroxy-

Abbreviations: AA, arachidonic acid; BSTFA, bis(trimethylsilyl)trifluoroacetamide; DHA, docosahexaenoic acid; diHDA, dihydroxydocosanoic acid; diHDHA, dihydroxydocosahexaenoic acid; hr5LOX, human recombinant 5-lipoxygenase; NP, normal-phase; PD1, protectin D1; ptLOX, potato tuber lipoxygenase; RP, reverse-phase; RT, retention time; sLOX, soybean lipoxygenase; 10,17(*S*)-diH(P)DHA, 10,17(*S*)-dihydro(pero)xydocosahexa-4*Z*,7*Z*,11*E*,13*Z*,15*E*,19*Z*-enoic acid; 7,17(*S*)-diH(P)DHA, 7,17(*S*)-dihydro(pero)xydocosahexaenoic acid; 17(*S*)-H(P)DHA, 17(*S*)-hydro(pero)xydocosahexa-4*Z*,7*Z*,11*Z*,13*Z*,15*E*,19*Z*-enoic acid; 5(*S*)-HPETE, 5(*S*)-hydroperoxyeicosatetraenoic acid; 13(*S*)-HPODE, 13(*S*)-hydroperoxyoctadecadi-9*Z*,11*E*-enoic acid; UV, ultraviolet.

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docosahexa-4*E*,7*Z*,11*E*,13*E*,15*Z*,19*Z*-enoic acid (15). Interestingly, the compound was also reported to be produced by certain plant lipoxygenases, such as soybean lipoxygenase (sLOX; which is a typical 15LOX) (11) and/or its potato tuber counterpart [potato tuber lipoxygenase (ptLOX); which is 5LOX] (15). The plant enzyme-generated PD1 was used in physiological studies and, apparently, was considered to be identical to the compound formed by mammals (8–15).

Further progress in this area was hampered by the following facts: *1*) at the time, the precise stereochemistry of the DHA derivatives produced by either living cells or isolated plant enzymes had not been established, whereas many geometrical isomers and stereoisomers of the compound might exist; *2*) no detailed procedure for PD1 preparation had been published; and *3*) the compound was not (and still is not) commercially available. These factors urged us to systematically investigate the oxidation of DHA and related compounds by ptLOX (16, 17) and sLOX (18). When studying the reaction products, four major oxidized derivatives were found: 10(*S*)-hydroperoxydocosahexaenoic acid [10(*S*)-HPDHA], 10(*S*),20-dihydroperoxydocosahexaenoic acid [10(*S*),20-diHPDHA], 7,17(*S*)-dihydro(pero)xydocosahexaenoic acid [7,17(*S*)-diH(P)DHA], and 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] (16–18). The former two compounds were produced by incubating ptLOX with DHA, and the latter two were formed by the same enzyme from 17(*S*)-HDHA and 17(*S*)-HPDHA. sLOX, a quintessential 15LOX that is better known for making 17(*S*)-HPDHA from DHA (19), was capable of synthesizing 10,17(*S*)-diH(P)DHA as well (18). Contrary to the previously suggested structure of sLOX-derived PD1, which was thought to have a 4*E*,7*Z*,11*E*,13*E*,15*Z*,19*Z* configuration (11, 20), our product had solely a 4*Z*,7*Z*,11*E*,13*Z*,15*E*,19*Z* arrangement of the double bonds (18), identical with the compound made by ptLOX (16, 17). No appreciable formation of 10,17(*S*)-dihydroxydocosahexa-4*E*,7*Z*,11*E*,13*E*,15*Z*,19*Z*-enoic acid was detected in either of these reactions. In a recent paper, several stereoisomers and geometrical isomers of 10,17-dihydroxydocosahexaenoic acid (10,17-diHDHA) were compared (9). The compounds were reported to be produced either enzymatically or via total organic synthesis. However, neither details of the synthetic procedures nor the absolute or relative yields of the major reaction products were provided. It remains unclear which particular isomer of 10,17-diHDHA was used in the original papers (8–15) for biological studies, because, as mentioned above, ptLOX and sLOX, which had been used to make the compound(s), did not produce noticeable amounts of a compound with 4*E*,7*Z*,11*E*,13*E*,15*Z*,19*Z* configuration of the double bonds (16–18).

Along with 10,17(*S*)-diH(P)DHA, another major DHA derivative generated by sLOX, 7,17(*S*)-diH(P)DHA, was detected among the reaction products (18). 7,17(*S*)-diH(P)DHA [also termed protectin D5, or PD5 (9)], being a second major product of sLOX-catalyzed oxidation of DHA and 17(*S*)-H(P)DHA (18) as well as a product of ptLOX-catalyzed oxidation of 17(*S*)-hydro(pero)xydocosahexa-4*Z*,7*Z*,11*Z*,13*Z*,15*E*,19*Z*-enoic acid [17(*S*)-H(P)DHA] (16, 17), remains a lesser studied compound compared with 10,17(*S*)-diHDHA in terms of its structure and biochemical properties. During our ptLOX studies, we noticed that while oxidizing DHA, ptLOX quickly lost its activity, which was indicative of enzyme inhibition and/or inactivation during the reaction (16). We also reported that it required relatively large amounts of sLOX to make 10,17(*S*)- and 7,17(*S*)-diH(P)DHA from either DHA or 17(*S*)-H(P)DHA (18). Therefore, we speculated that one or both of the diH(P)DHAs could have an inhibitory/inactivatory effect on the lipoxygenases themselves. Inasmuch as PD1 was shown to have anti-inflammatory properties in vivo, we also speculated that 10,17(*S*)-diHDHA and 7,17(*S*)-diHDHA could directly inhibit mammalian enzymes [e.g. 5-lipoxygenase (5LOX)] that are involved in oxidative transformations of polyunsaturated fatty acids.

Thus, the goals of this study were *1*) to develop a simple and scalable procedure for making authentic 7,17(*S*)-diHDHA; 2) to evaluate its stereochemistry; *3*) to estimate its in vitro inhibitory activity toward human recombinant 5-lipoxygenase (hr5LOX); and *4*) to compare its biochemical properties with those of 10,17(*S*)-diHDHA.

MATERIALS AND METHODS

Materials

hr5LOX-1 (product 60402) was purchased from Cayman Chemical (Ann Arbor, MI), whereas hr5LOX-2 was expressed in *Escherichia coli* and affinity-purified on ATP-agarose (O. Rådmark, M. Rakonjac, and I. Butovich, unpublished results). DHA was from Nu-Chek Prep, Inc. (Elysian, MN). Methanol-d_4 (99.8%), platinum (IV) oxide, sodium borohydride, and bis(trimethylsilyl)trifluoroacetamide (BSTFA) were products of Aldrich (Milwaukee, WI). sLOX preparation type I-B and monododecyl ether of decaoxyethylene glycol were supplied by Sigma Chemical Co. (St. Louis, MO). Octadecyl (C_{18}) solid-phase extraction cartridges were from J. T. Baker (Philipsburg, NJ). A 5980 series II gas chromatograph equipped with a 5971 series electron-impact 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 equipped 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 recorded with an LCQ Deca XP Max MS^n mass spectrometer (Thermo Electron Corp., San Jose, CA) equipped with an electrospray ionization ion source. A Waters Alliance 2695 HPLC separations module equipped with a Waters 2996 diode-array detector was used for analysis and purification of the DHA oxidation products. Proton NMR spectra were taken on a 400 MHz Varian spectrometer in methanol-d_4. 13(*S*)-Hydroperoxyoctadecadi-9*Z*,11*E*-enoic acid [13(*S*)-HPODE], 17(*S*)-HPDHA, and 17(*S*)-HDHA were synthesized by sLOX from linoleic acid and DHA using methods described previously (16, 18, 19).

Enzymatic synthesis of the DHA oxidation products

The preparative scale reactions were conducted in 100 ml flasks. Two synthetic procedures were used. The first method used a two-enzyme approach in which both sLOX and ptLOX were used in sequence to catalyze the chain of reactions DHA →

17(*S*)-H(P)DHA → 7,17(*S*)- and 10,17(*S*)-diH(P)DHAs (16). The second method, which is briefly described below, implemented only sLOX to catalyze both steps (18). The reaction mixtures, composed of 50 ml of 0.05 M sodium borate buffer, pH 10, with 0.1 mM DHA as substrate, were saturated with air and preequilibrated at 4°C. The reactions were started by adding a stock solution of sLOX (10 mg of the enzyme preparation type I-B) and were left to proceed on ice for 15 min under constant stirring. The reactions were terminated by adding glacial acetic acid to the reaction mixture to bring its pH to ≤4. Then, the products were immediately passed through a Bakerbond C_{18} solid-phase extraction cartridge (500 mg) preequilibrated with deionized water. Oxidation products and the unreacted substrate were retained by the cartridge, which was then washed with 10 ml of cold water. Excess water was removed from the cartridge by vacuum suction, and the absorbed components were eluted with 2 ml of ethanol. The eluted products were dried under a stream of nitrogen. If necessary, trace water was removed by azeotropic distillation with dry ethanol. Then, the product mixture was dissolved in nitrogen-saturated ethanol and stored at −80°C. The oxidized products were quantified spectrophotometrically by measuring their optical density at 237 nm [17(*S*)-H(P)DHA], 242 nm [7,17(*S*)-diH(P)DHA], and 270 nm [10,17(*S*)-diH(P)DHA] as described previously (16–18). The hydroperoxides of DHA were stored in the same solvent under nitrogen at −80°C for at least 4 weeks without any noticeable deterioration of the samples. Prolonged storage of the hydroperoxides caused some decomposition of the products, leading to visible yellowing of the solutions even at −80°C.

GC-MS analysis of the products

The entire DHA product mixture was subjected to catalytic hydrogenation with H_2 over PtO_2, trimethylsilylated with BSTFA, and analyzed by GC-MS as described previously (16, 18, 19).

HPLC separation and purification of the products

The main reaction products were separated by gradient normal-phase (NP) HPLC on a 5 μm Waters Spherisorb silica gel column (4.6 × 250 mm) at 30°C at a flow rate of 1 ml/min (18) or isocratically in hexane-propan-2-ol-acetic acid (950:50:1, v/v/v) solvent mixture (16). The product samples were dissolved and injected in propan-2-ol. The eluate was monitored spectrophotometrically at 250 nm, at which all of the major reaction products were visible (see below). Under these conditions, baseline separation of 17(*S*)-H(P)DHA, 7,17(*S*)-diH(P)DHA and 10,17(*S*)-diH(P)DHA was easily achieved in both the gradient and the isocratic experiments. *n*-Hexane was interchangeable with *n*-heptane with minor effect on separation of the analytes.

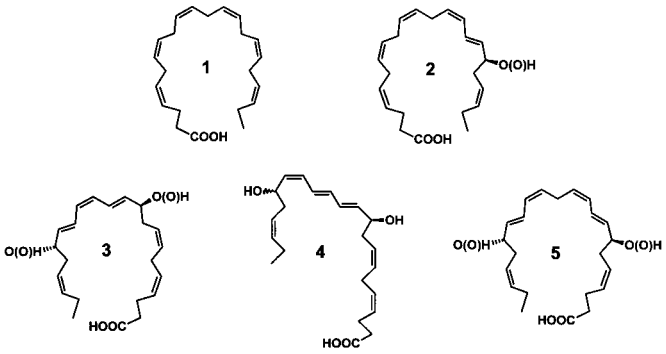
NMR analysis of 7,17(*S*)-diHDHA

Further structural characterization of 7,17(*S*)-diHDHA was performed by conducting 400 MHz one-dimensional and two-dimensional NMR experiments with subsequent modeling of the NMR spectra with MesTre-C software version 4.7.1.1 (purchased through MestreLab Research, Santiago de Compostela, Spain). The samples (0.1 mg or more) were dissolved in 0.5 ml of CD_3OD and transferred into a standard NMR tube (5 mm inner diameter), and their spectra were recorded at 29°C.

Inhibition of hr5LOX by 7,17(*S*)- and 10,17(*S*)-diHDHAs

For the inhibitory studies, 17(*S*)-HDHA, 7,17(*S*)-diHDHA, and 10,17(*S*)-diHDHA were generated enzymatically, purified by NP HPLC, and characterized spectroscopically as described above. Stock solutions of the compounds (between 1 and 2 mM, in methanol) were prepared and stored at −80°C in a nitrogen atmosphere. At low temperatures (−20°C and below), precipitation of the samples typically occurred in concentrated stock solutions (~1 mM and above), which was easily reversed by warming the samples to body temperature. Between the experiments, the samples were stored on ice.

Effects of the diHDHAs on the hr5LOX-catalyzed oxidation of arachidonic acid (AA) were studied as follows. The activity assay used in this study was based on a previously developed protocol of AA oxidation by hr5LOX (21) with only minor modifications. Two different enzyme preparations were used: a commercially available hr5LOX from Cayman Chemical (product 60402; hr5LOX-1) and a hr5LOX expressed in *E. coli* and purified on ATP agarose (hr5LOX-2) (21, 22). In the preliminary experiments with hr5LOX-1, 10^{-4} M of AA was found to be saturating. Therefore, it was used throughout the experiments as the standard substrate concentration for the hr5LOX activity determinations. The other components of the reaction mixture included 0.05 M Tris-HCl buffer, pH 7.6, 0.15 M NaCl, 1.2 mM disodium salt of EDTA, 2 mM $CaCl_2$, 2.3 mM ATP, 0.02 mM β-mercaptoethanol, 0.02 mg/ml egg yolk phosphatidylcholine, and 10 μM 13(*S*)-HPODE in a final volume of 150 μl. The reactions were started by adding AA and 13(*S*)-HPODE to the mixture, which already had hr5LOX-1 in it. After brief vortexing, the reaction was allowed to proceed at 23 ± 1°C for 20 min. Then, 350 μl of ice-cold acetonitrile containing 2.5% glacial acetic acid (v/v) was added to the mixture to stop the reaction and precipitate the proteins. The mixture was vortexed vigorously, left on ice for 10 min, and then centrifuged at 10,000 *g* at 4°C for 15 min. The clear supernatant was transferred into a glass HPLC vial and stored at −20°C until the analysis. Typically, the analyses were performed the same day the enzymatic reactions were run, although no changes in the elution profile were detected if the samples were stored overnight.



Scheme 1. Structures of the major reaction products of docosahexaenoic acid (DHA) oxidation: *1*) DHA; *2*) 17(*S*)-dihydro(pero)xy-DHA; *3*) 10,17(*S*)-dihydro(pero)xy-DHA; *4*) 10,17(*S*)-diHDHA with postulated 4*Z*,7*Z*,11*E*,13*E*,15*Z*,19*Z* geometry of the double bonds (neuroprotectin D1 or protectin D1); and *5*) 7,17(*S*)-dihydro(pero)xy-DHA (protectin D5). The primary products of double lipoxygenation (compounds 1, 2, 3, and 5) are initially formed as monohydroperoxides {17(*S*)-hydroperoxydocosahexaenoic acid [17(*S*)-HPDHA]} and dihydroperoxides [10,17(*S*)-diHPDHA and 7,17(*S*)-diHPDHA], which then can be reduced either enzymatically or nonenzymatically in the corresponding mixed hydroxy,peroxy derivatives or dihydroxy derivatives. Compound 4, if formed through the epoxidation/isomerization mechanism (14), may exist only as a dihydroxy derivative.

To analyze the main products of AA oxidation, 5(*S*)-hydroperoxyeicosatetraenoic acid [5(*S*)-HPETE] and 5(*S*)-hydroxyeicosatetraenoic acid [5(*S*)-HETE], the final product mixture was separated by reverse-phase (RP) HPLC on a 4.6 × 125 mm, 5 μm C_8 Zorbax Eclipse XDB-C_8 column. The separation was performed at 30°C at a flow rate of 0.5 ml/min. The compounds were eluted in a water-acetonitrile solvent mixture as follows. Elution started with an acetonitrile-water (50:50, v/v) solvent mixture for 5 min. Then, a linear gradient was applied from that solvent mixture to 100% acetonitrile over the next 7 min and the column was washed with 100% acetonitrile for another 5 min. Finally, the solvent was changed to the initial acetonitrile-water (50:50, v/v) composition within 1 min and the column was re-equilibrated with this solvent mixture for 7 min. The entire analysis took 25 min. The elution profile was monitored at 236 ± 2 nm [the maximum of adsorption of 13(*S*)-HPODE, 5(*S*)-HPETE, and 5(*S*)-HETE; molar extinction coefficient ϵ_m = 34,000 $M^{-1} \times cm^{-1}$, (23)]. 13(*S*)-HPODE (10 μM) was used as an internal standard for quantification purposes.

RESULTS

For the reader's convenience, all of the DHA products that are discussed here are summarized in **Scheme 1**.

Oxidation of DHA catalyzed by sLOX

A typical change in the UV light absorbance of the reaction mixture composed of 10^{-4} M DHA and 0.5 × 10^{-6} M sLOX during the course of reaction is presented in **Fig. 1A**. Under these conditions, an almost instantaneous conversion of DHA into the initial product, 17(*S*)-HPDHA, was observed, followed by a gradual accumulation of secondary reaction products, most noticeably a conjugated triene with λ_{max} of ~260, 270, and 280 nm. Depending on the amount of sLOX added, the reaction proceeded for several minutes and then stopped long

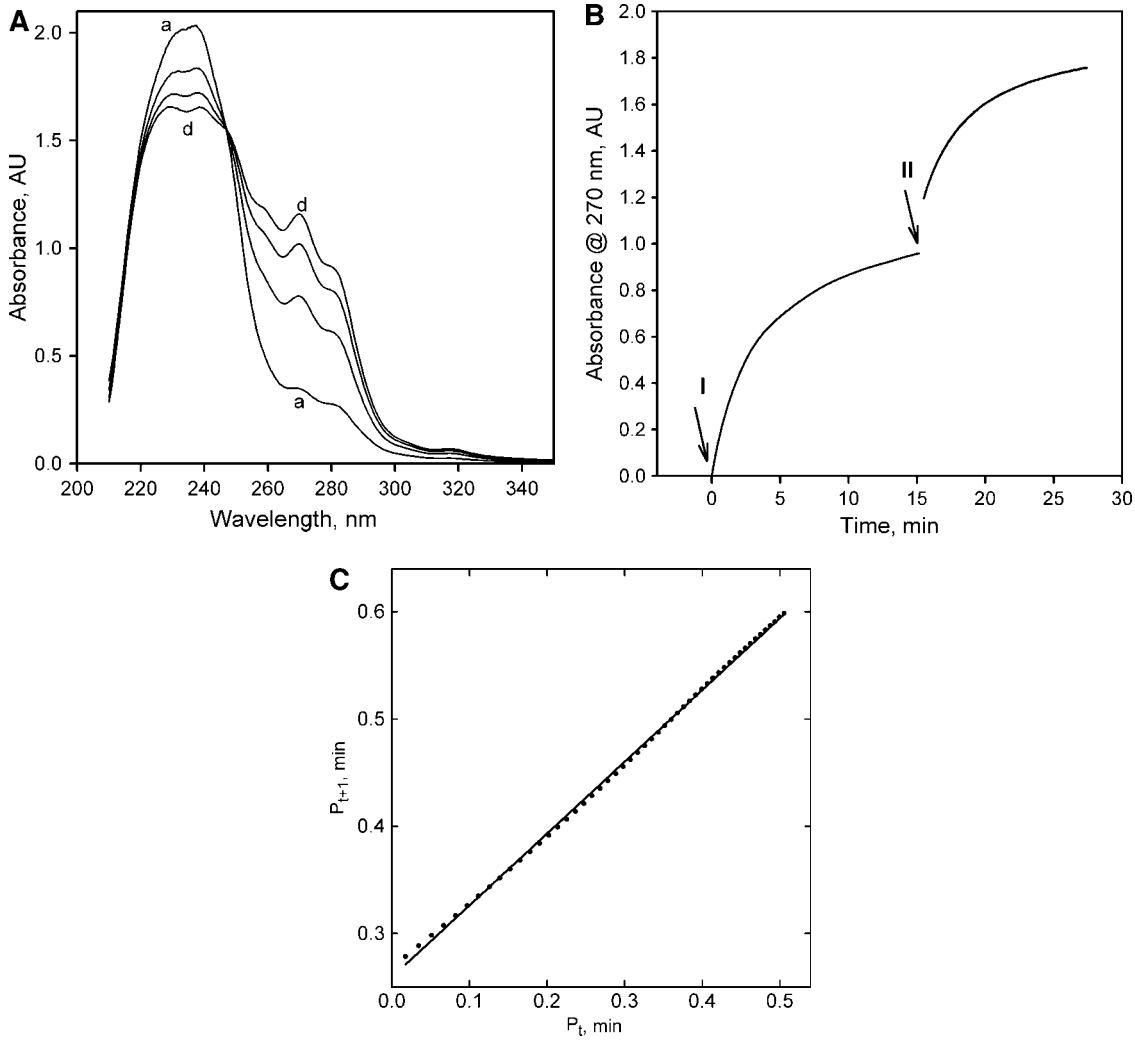


Fig. 1. Spectrophotometric analysis of soybean lipoxygenase (sLOX)-catalyzed oxidation of docosahexaenoic acid (DHA). A: Spectral changes of the reaction mixture during sLOX-catalyzed oxidation of DHA. The reaction proceeded from a to d. The spectra were recorded every 2 min. The reaction conditions were as presented in Materials and Methods. AU, absorbance units. B: Kinetic curves of DHA oxidation by sLOX. The enzyme was added in two equal increments, as indicated by the arrows. The reaction conditions were as presented in Materials and Methods. C: Swinbourne-Jenkins (24, 25) transformation of kinetic curve I presented in B. Dots, experimental values; solid line, theoretical curve calculated using the following parameters: initial reaction rate (V_0) = 0.26 AU × min^{-1}; (pseudo)first-order kinetic inactivation constant (k_{in}) = 0.4 min^{-1}.

before the depletion of 17(*S*)-HPDHA. A fresh portion of the enzyme added to the mixture restored the reaction, although at a slower pace (Fig. 1B). Therefore, the enzyme showed clear signs of product inhibition and/or reaction inactivation, the rate of which depended on the initial conditions of the reaction. Because of the strong overlap of the UV absorption spectra of 17(*S*)-HPDHA and 7,17(*S*)-diH(P)DHA, direct monitoring of the 17(*S*)-HPDHA → 7,17(*S*)-diH(P)DHA transformation was not possible. Therefore, an apparent (pseudo)first-order kinetic constant of inactivation (k_{in}) was determined by monitoring 10,17(*S*)-diH(P)DHA formation at 270 nm. The k_{in} value of 0.4 min^{-1} was numerically computed from the slope of the kinetic curve in Swinbourne-Jenkins coordinates (24–26) using a linear regression routine of the SigmaPlot (version 9.01) software package (Systat Software,

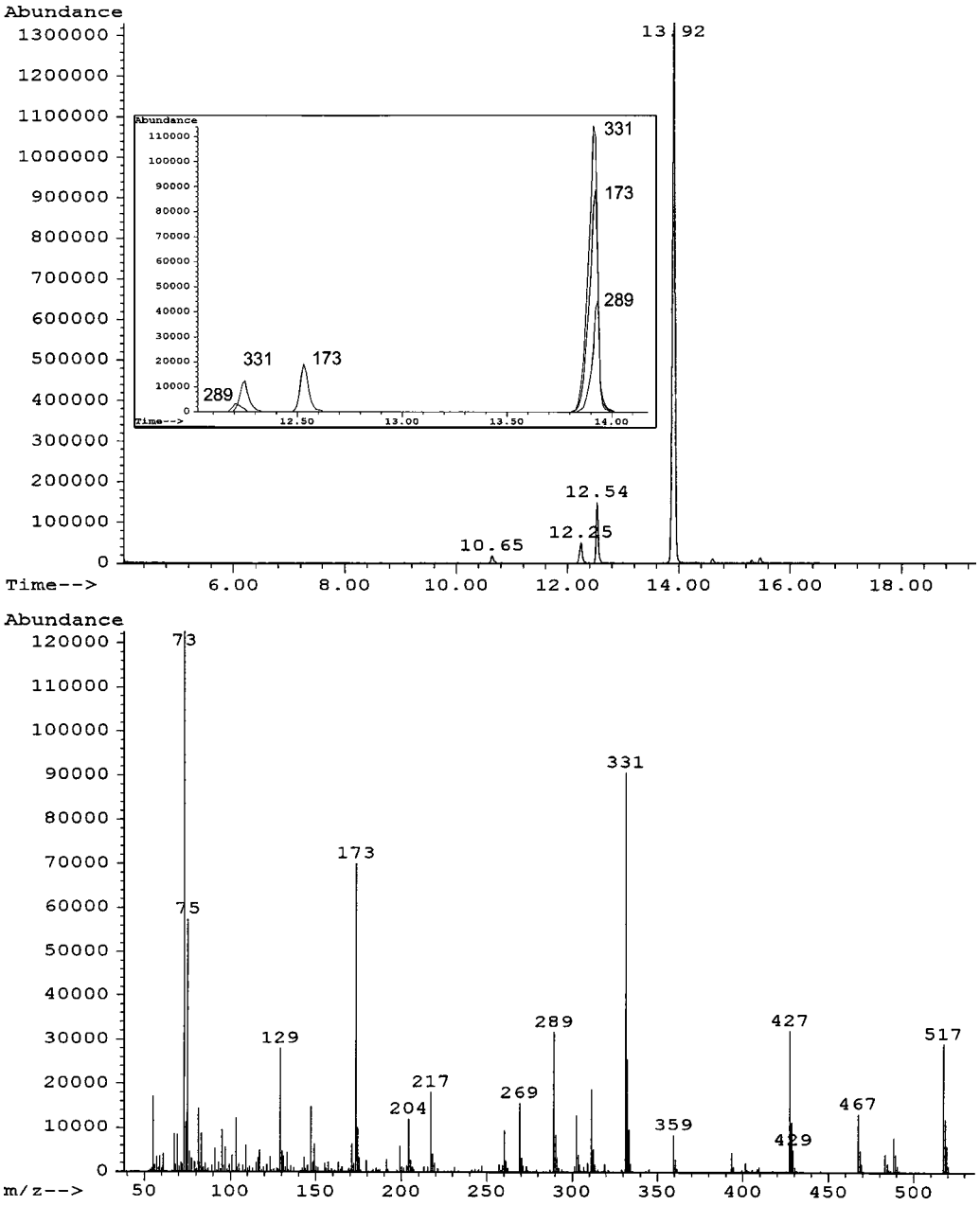


Fig. 2. Gas liquid chromatography-electron impact-mass spectrometry analysis of the reaction products of DHA oxidation by sLOX. The enzymatic products were subsequently hydrogenated with H_2 over PtO_2 and trimethylsilylated with bis(trimethylsilyl)trifluoroacetamide. Upper panel: Total ion chromatogram of the reaction products and single ion chromatogram (inset) of the fragments with *m/z* values of 289, 331, and 173. The ion *m/z* 289 is indicative of a fragment with a hydroxyl group at C7 (C7-OH), whereas fragments 331 and 173 belong to C10-OH and C17-OH. Lower panel: Mass spectrum of the main chromatographic peak with retention time (RT) of 13.92 min. Note that all three fragments are present in the spectrum of the major product (upper and lower panels), which indicates that the peak is composed of a mixture of 7,17-dihydroxydocosanoic acid (7,17-diHDA) and 10,17-diHDA.

Inc., Point Richmond, CA), as depicted in Fig. 1C. The correlation coefficient (r) exceeded 0.999.

GC-MS analysis of the products

Preliminary analysis of the trimethylsilyl derivatives of the fully hydrogenated reaction products was performed by means of GC-MS. Several peaks were observed under the conditions of a total ion chromatogram experiment (**Fig. 2A, B**), of which the major component with retention time (RT) of 13.9 min displayed the fragmentation pattern that would have been expected of a coeluting mixture of tri-trimethylsilyl derivatives of 7,17- and 10,17-dihydroxydocosanoic acids (7,17- and 10,17-diHDAs). We observed prominent fragments with m/z ratios of 73, 129, 173, 217, 269, 289, 311, 331, 359, 427, 467, and 517. Based on the well-known fragmentation patterns of similar hydroxylated saturated fatty acids, fragments 173 and 289 uniquely identified tri-trimethylsilyl-7,17-diHDA, whereas fragments 173, 331, and 359 were indicative of tri-trimethylsilyl-10,17-diHDA. From a single-ion monitoring experiment, it became clear that this peak (RT, 13.9 min) was, in fact, composed of a pair of coeluting compounds, 10,17- and 7,17-diHDAs (Fig. 2). It was observed that the 10,17-dihydroxy derivative eluted slightly faster than the 7,17-dihydroxy derivative. The difference in the RTs was visible under the conditions of single-ion monitoring experiments, but there was only one elution peak observed on the total ion chromatogram. Changes in the elution parameters (temperature gradient and flow rate) did not improve the product separation. In addition to the doubly oxygenated products, a small amount of monooxygenated fatty acids with hydroxyls at C7, C10, and C17 was observed.

HPLC analysis and separation of the products

Because biochemical experiments that were to be conducted with the products required pure samples of 7,17(*S*)- and 10,17(*S*)-diHDHAs, several HPLC techniques have been tried to fractionate the products. We confirm earlier observations (14) that under the conditions of the traditional RP HPLC analysis, 7,17(*S*)-diHDHA coeluted with its 10,17-dihydroxy counterpart (**Fig. 3**). Neither isocratic nor gradient elution separated the two compounds. 10,17(*S*)-diHDHA typically led the pair by constituting the front of the elution peak, whereas 7,17(*S*)-diHDHA trailed, being the second to elute (Fig. 3). None of the standard RP HPLC columns tried provided separation efficiency high enough to separate the analytes. The separation has been equally poor in methanol- and acetonitrile-based eluents. As will be discussed somewhat later, the inability of RP HPLC to separate 7,17(*S*)- and 10,17(*S*)-diHDHAs may have complicated their quantification and the analysis of their biological properties in the previous studies.

Among the tested protocols, the best HPLC procedure for separating the reaction products was found to be NP HPLC on a silica gel or cyanonitrile column in a *n*-hexane (or *n*-heptane)-propan-2-ol-acetic acid mixture (**Fig. 4**). A mixture of the DHA oxidation products showed the presence of 7,17(*S*)-diHDHA (RT, 12.66 min) and 10,17(*S*)-diHDHA (15.28 min). The latter compound has been characterized previously (16–18), but the former one has not.

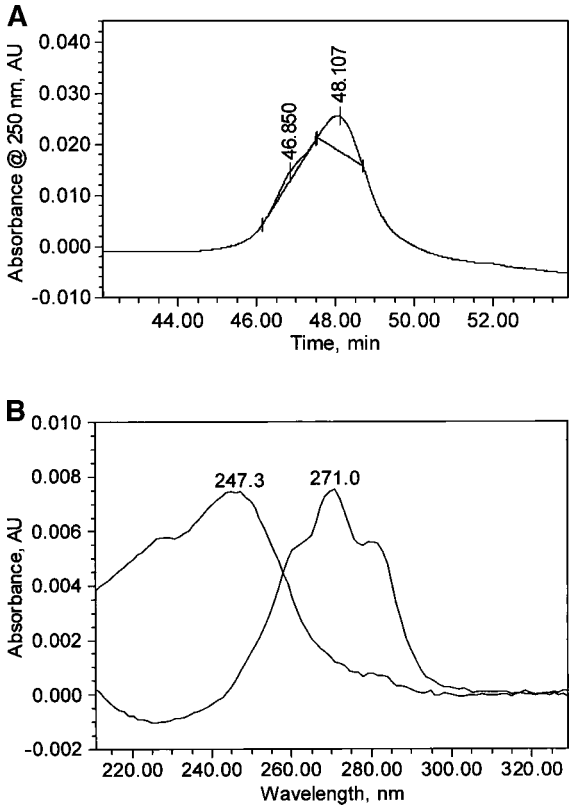


Fig. 3. Reverse-phase HPLC analysis of dihydroxydocosahexaenoic acids (diHDHAs). A: Fragment of a sample chromatogram of a mixture of 10,17(*S*)-diHDHA and 7,17(*S*)-diHDHA [acetonitrile-0.1% H_3PO_4 in water (70:30, v/v)] at 2 ml/min and 30°C. AU, absorbance units. B: Ultraviolet (UV) light spectra of the conjugated triene [RT, 46.85 min (solid line); λ_{max}, 271 nm; 10,17(*S*)-diHDHA] and the conjugated diene [RT, 48.2 min (broken line); λ_{max}, 245 nm; 7,17(*S*)-diHDHA].

To generate 7,17(*S*)-diH(P)DHA in quantities sufficient for structural analysis, a preparative-scale synthesis was performed by oxidizing DHA by sLOX as described in Materials and Methods. The crude product was fractionated by silica gel NP HPLC, and fractions that contained 7,17(*S*)-diH(P)DHA were pooled. The pooled fraction was reanalyzed by analytical NP HPLC to check its purity. In a separate experiment, a ptLOX-catalyzed reaction of 17(*S*)-H(P)DHA oxidation and the product purification were performed in accordance with previously published protocols (16, 17).

Chiral HPLC of 7,17(*S*)-diHDHA

The sLOX product was apparently composed of a single stereoisomer, as chiral-phase HPLC on a 250 mm × 4.6 mm Chiralcel OD-H column (Daicel USA, Inc., Fort Lee, NJ) in various *n*-hexane-propan-2-ol solvent mixtures produced a single peak regardless of the eluent (**Fig. 5**, peak with RT of ~26 min). The chiral HPLC effectively separated 7,17(*S*)- and 10,17(*S*)-diHDHAs (RT, ~58 min), providing an additional analytical and preparative tool for these compounds.

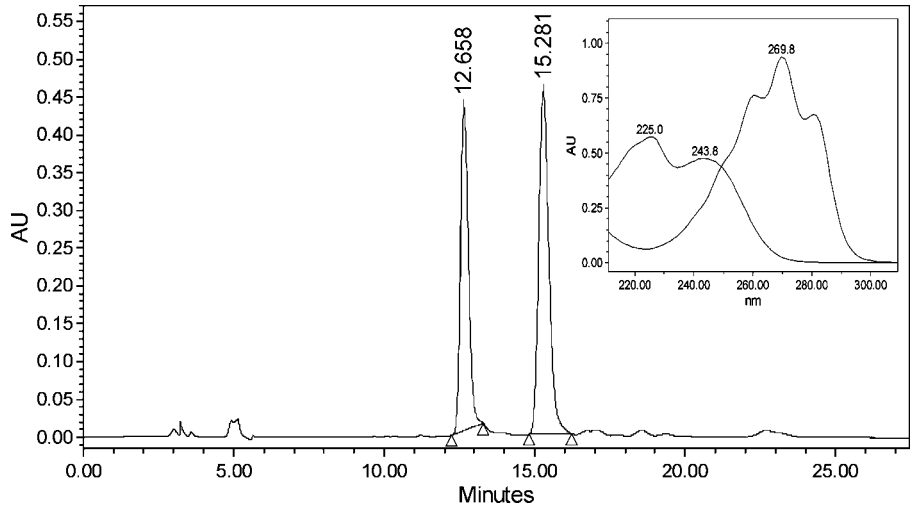


Fig. 4. Normal-phase (NP) HPLC separation of 7,17(*S*)-diHDHA (peak RT, 12.658 min) and 10,17(*S*)-diHDHA (RT, 15.281 min). The chromatogram was recorded at 250 nm. AU, absorbance units. Inset: UV spectra of 7,17(*S*)-diHDHA (λ_{max}, 225.0 and 243.8 nm) and 10,17(*S*)-diHDHA (λ_{max}, 269.8 nm).

UV light spectra of the products

In nonpolar solvents, 7,17(*S*)-diHDHA showed a split UV light absorption spectrum with two maxima at 225 and ~245 nm, identical to the UV light absorption spectrum of 10(*S*),20-diHDHA (16). An interesting change in the spectrum of the pure 7,17(*S*)-diHDHA was observed once the solvent in which it had been eluted from the HPLC column was replaced with methanol, ethanol, or propan-2-ol. In ethanol, for example, the UV light spectrum acquired a shape more typical of a conjugated diene chromophore with λ_{max} of 244.8 nm, whereas in hexane, it had a maximum at 225 nm and a shoulder at 245 nm (**Fig. 6**). The isosbestic point of the spectra was at ~231 nm. Alternate changing of the solvents in which 7,17(*S*)-diHDHA was dissolved by repetitive evaporation/reconstitution of the same sample showed that the transformation of the spectrum was reversible. When the ratio of hexane to ethanol changed, the spectrum of the compound showed a gradual change from the split pattern with two maxima to the single maximum spectrum (Fig. 6). As the 7,17(*S*)-diHDHA molecule has two identical diene chromophores, its molar absorptivity at either of 225 and

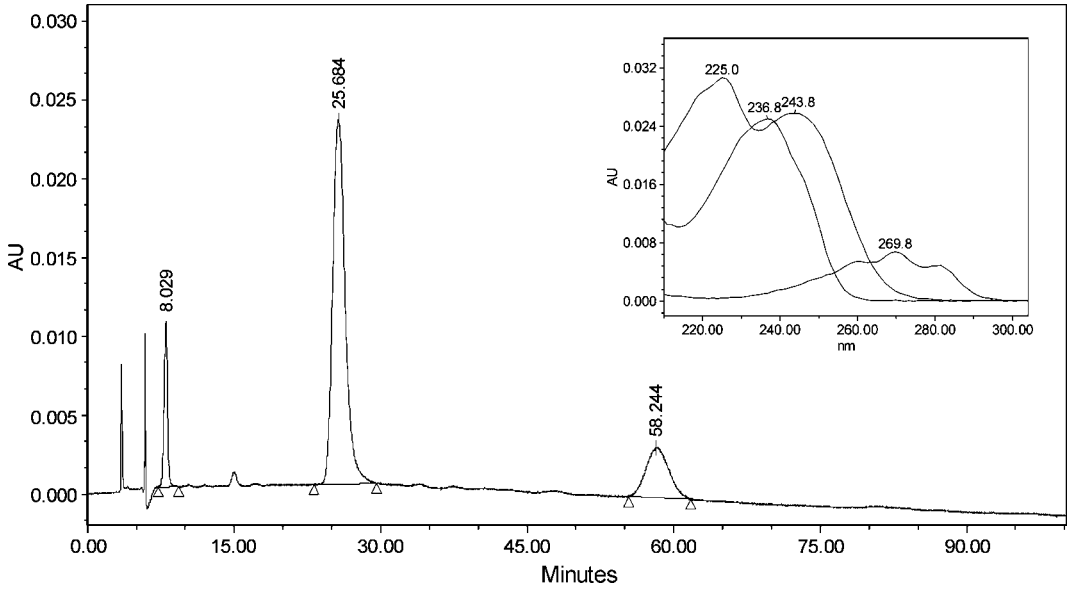


Fig. 5. Chiral-phase HPLC analysis of a mixture of 17(*S*)-HDHA (peak RT, 8.029 min), 7,17(*S*)-diHDHA (25.684 min), and 10,17(*S*)-diHDHA (58.244 min). The eluent was *n*-hexane-propan-2-ol-acetic acid (950:50:1, v/v/v) at a flow rate of 1 ml/min and 30°C. AU, absorbance units. Inset: UV spectra of the products. 17(*S*)-HDHA had an absorption maximum at 237 nm; 7,10(*S*)-diHDHA showed a split spectrum with λ_{max} at 225 and 244 nm; 10,17(*S*)-diHDHA showed a typical absorption spectrum of a conjugated triene with λ_{max} at 260, 270, and 280 nm. The elution profile was monitored at 250 nm, at which all three products had sufficient UV absorptivity.

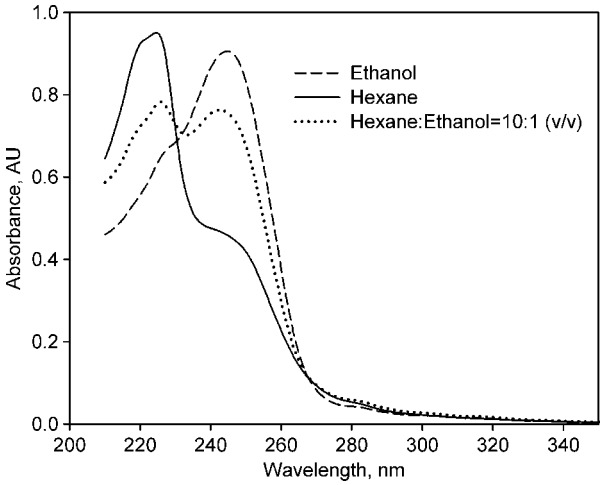


Fig. 6. Reversible changes of the UV light spectra of 7,17(*S*)-diHDHA in solvents with different polarities. AU, absorbance units.

245 nm was assumed to be 46,000 $M^{-1} \times cm^{-1}$, which is double that of a typical diene with just one conjugated double bond.

NMR analysis of the products

A side-by-side comparison of 400 MHz one-dimensional ^{1}H NMR spectra of authentic DHA, 17(*S*)-HDHA, and 7,17(*S*)-diHDHA allowed us to visualize the changes in the structures that occurred during the enzymatic transformation and assign particular resonances to particular protons and proton groups (**Fig. 7**). Corroborating information on the proton assignments was obtained from a 400 MHz two-dimensional double quantum correlation spectroscopy ^{1}H NMR spectrum of 7,17(*S*)-diHDHA (**Fig. 8**). No signals with a chemical shift δ of 6.15–6.25 [which is a signature of conjugated *trans,trans* dienes (17, 18, 27–29)] were observed in either of these spectra.

Inhibition of hr5LOX and sLOX

It is well known that substrates may, to some extent, reduce the inhibitory effects of various effectors on LOXs. Therefore, one can expect that at a lower concentration of AA, the inhibitory effects could be higher than reported below. Nevertheless, 10^{-4} M of AA was chosen as a standard and a practical concentration for the HPLC-based analytical methods. Because preincubation of LOXs with their inhibitors may potentiate their effects (26, 30), the hr5LOX/inhibitor mixtures were preincubated for 5 min at room temperature. Then, the reactions were started by adding a substrate stock solution. Typical inhibition curves for the hr5LOX-1-catalyzed oxidation of 10^{-4} M AA are presented in **Fig. 9A**. The diHDHAs inhibited hr5LOX-1 in a concentration- and time-dependent manner. For 10,17(*S*)-diHDHA, the calculated IC_{50} value of ~17 μM

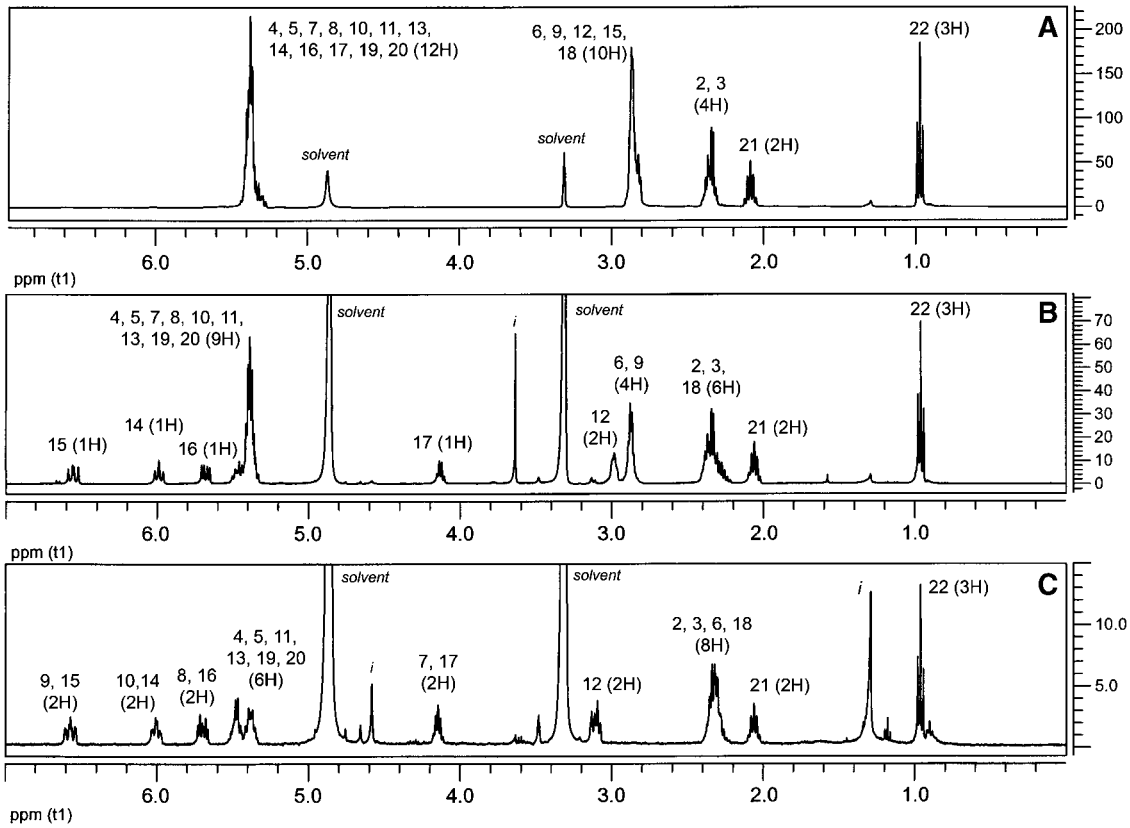


Fig. 7. One-dimensional ^{1}H NMR spectra of DHA (A), 17(*S*)-HDHA (B), and 7,17(*S*)-diHDHA (C). The proton resonances are labeled according to the carbon atom numbers (from 2 to 22). The total number of each type of protons is shown in parentheses. *i*, impurity.

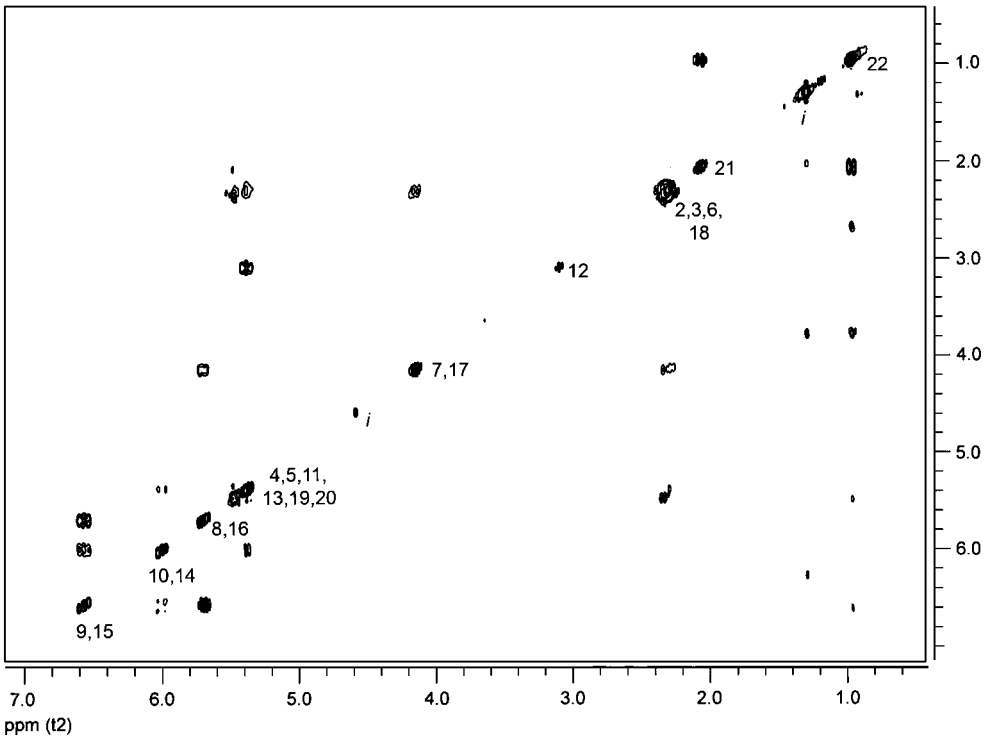


Fig. 8. Two-dimensional 400 MHz double quantum correlation spectroscopy ^{1}H NMR spectra of 7,17(*S*)-diHDHA. The proton resonances are labeled according to the carbon atom numbers (from 2 to 22). The solvent peaks were removed using the MesTre-C High-Pass Filter built-in routine. *i*, impurity.

matched well the same parameter determined previously (I. Butovich, M. Rakonjac, and O. Rådmark, unpublished results) for hr5LOX-2 (12 μM). 7,17(*S*)-diHDHA proved to be twice as effective, with an IC_{50} value of 7 μM for hr5LOX-1. Interestingly, sLOX was inhibited by both of the diHDHAs in the reaction of AA oxidation (Fig. 9B). These experiments were performed exactly as described previously for sLOX and linoleyl hydroxamic acid (26). The IC_{50} values for 7,17(*S*)- and 10,17(*S*)-diHDHAs were 4 and 9 μM, respectively.

DISCUSSION

sLOX-catalyzed oxidation of DHA and 17(*S*)-H(P)DHA

Our data clearly demonstrate that sLOX is able to simultaneously produce 7,17(*S*)- and 10,17(*S*)-diHDHAs as the major products of two parallel reactions of double lipoxygenation of DHA (Scheme 1). No discernible contribution of the epoxidation/isomerization reaction has been observed. A series of four small peaks with characteristic triene chromophores trailed the major peak of 10,17(*S*)-diHDHA and contributed <7% of the conjugated trienes present among the reaction products. These minor products were likely produced nonenzymatically, as their ratio was close to equimolar (data not shown). Similarly formed positional isomers, geometric isomers, and stereoisomers of the main reaction products were described previously for the ptLOX-catalyzed oxidation of linoleyl alcohol and 1-monolinoleoyl-rac-glycerol (28, 29). Whether the four conjugated trienes were positional or geometric (*cis,trans*) isomers of diHDHAs remains to be investigated. It has also been shown that 7,17(*S*)-diHDHA formed in the reaction of DHA and 17(*S*)-H(P)DHA oxidation by sLOX was chemically identical to the compound produced by ptLOX from 17(*S*)-H(P)DHA. The only remaining question is the stereochemistry of the hydroxyl groups at C7 of both compounds, but in view of their similarity under the conditions of the chiral HPLC analysis, they are expected to be identical.

Although the exact alignment of the substrate molecule in the reaction center of either of the enzymes is currently unknown, the following simplified mechanistic scheme can be envisioned (the "flip-flop" or "pancake" mechanism; **Scheme 2**). The reaction of DHA oxidation starts with antarafacial abstraction of a pro-(*S*) hydrogen at C15 (step A) followed by delocalization of the double bonds and their subsequent attack by molecular oxygen and the formation of 17(*S*)-HPDHA (step B). Because of the large amount of the enzyme used, the reaction results in almost instantaneous transformation of DHA into 17(*S*)-HPDHA. Once the first peroxidation step has been completed, the monohydroperoxide molecule flips horizontally along the indicated line with or without leaving the catalytic center first (step C). Then, the enzyme faces two choices: to antarafacially abstract a pro-(*S*) hydrogen atom from either C9 [which is a priori a more traditional reaction pathway (step D), with subsequent dioxygen attack (step E) and formation of 7(*S*),17(*S*)-diHPDHA] or C12 (steps G through I), as proposed for ptLOX in our previous publications (28, 29). Considering that in the sLOX-catalyzed

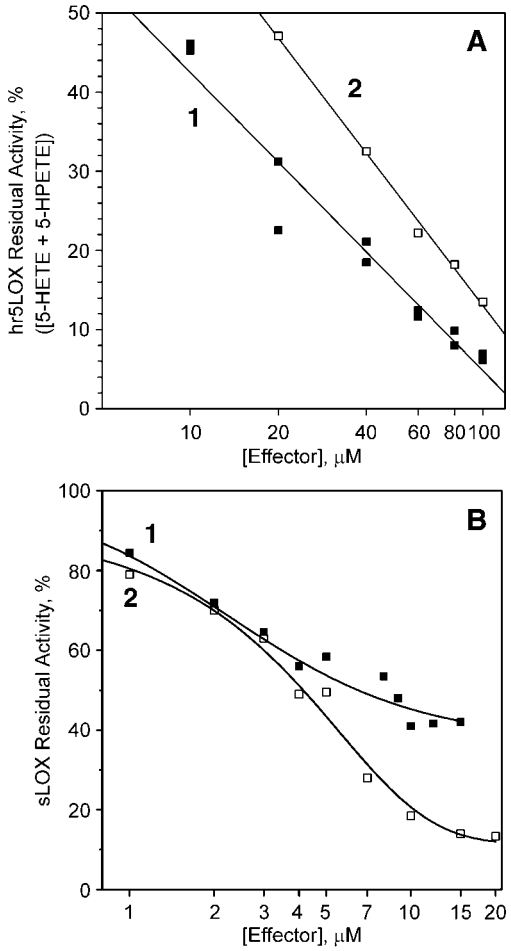


Fig. 9. Inhibition of human recombinant 5-lipoxygenase (hr5LOX-1) (A) and sLOX (B) by 7,17(*S*)-diHDHA (closed squares, line 1) and 10,17(*S*)-diHDHA (open squares, line 2) in the oxidation reaction of 100 μM arachidonic acid (AA). Inhibition of hr5LOX was studied as described in Materials and Methods. Inhibition of sLOX by 7,17(*S*)- and 10,17(*S*)-diHDHAs was studied in accordance with a previously published protocol (26). Briefly, inhibition was measured by monitoring the residual activity of sLOX after a 5 min preincubation of the enzyme with the effectors at 25°C in a borate buffer, pH 9.0, directly in a spectrophotometric cuvette. The reaction was started by adding a small volume of concentrated AA stock solution (final concentration, 100 μM), and the accumulation of AA hydroperoxide(s) was monitored continuously at 236 nm. The reaction rate was computed from the kinetic curve and was used as a measure of the residual enzymatic activity.

reaction two the positional isomers of diHDHAs were formed in comparable amounts, both reactions D and G seem to be equiprobable. Such reactions should lead to the (*S*)-geometry of the hydroxyls at C7 and C10. This mechanism could also explain the same reactions catalyzed by ptLOX. The final assignment of the stereochemistry of the hydroxyl group at C7 requires direct stereochemical analysis of the enzymatically generated 7,17(*S*)-diHPDHA or 7,17(*S*)-diHDHA, which is currently under way.

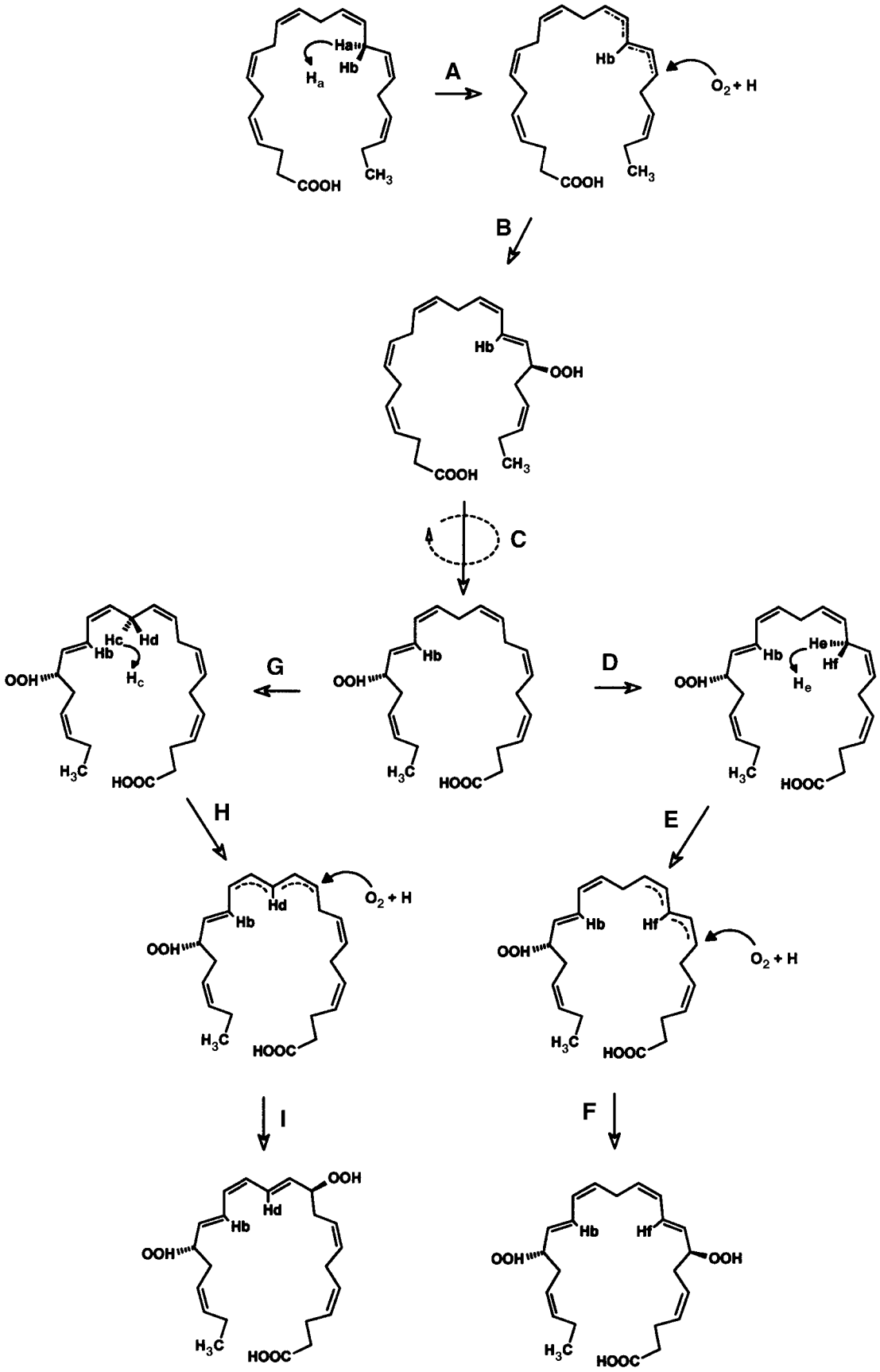
HPLC analysis and separation of the products

In our hands, RP HPLC methods were proven to be unsuitable for making HPLC-pure samples of 7,17(*S*)- and 10,17(*S*)-diHDHAs, as both compounds coeluted under all conditions tested. This corroborates the preceding findings of other groups (14). Currently, the method of choice for reliable separation of 7,17(*S*)-diHDHA and 10,17(*S*)-diHDHA is NP HPLC, because this method is capable of efficient baseline separation of the analytes. Chiral chromatography on, for example, a Chiralcel OD-H column, was also successful in separating the compounds (even more so than NP HPLC), but considering the much higher cost of the chiral media compared with silica gel, and substantially longer RTs on the former, it seems to be a less practical approach. For practical purposes, no deactivation (or "poisoning") of the silica-based columns with water was observed during multiple repetitive runs if care was taken to inject samples dissolved in anhydrous solvents and the column had been reequilibrated with *n*-hexane-propan-2-ol-acetic acid (950:50:1, v/v/v) solvent mixture before the runs. Therefore, this method was found to be adequate for quantification purposes as well.

The proven failure of RP HPLC to separate 7,17(*S*)- and 10,17(*S*)-diHDHAs, under the tested conditions, raises the question of whether the enzymatically generated samples used for biological studies in the previous reports were indeed HPLC-pure compounds. If 7,17-diHDHA and 10,17-diHDHA did coelute during those RP HPLC experiments, the final preparations of both compounds were most likely cross-contaminated with each other, which would have a detrimental effect on the results of biological studies, as the compounds differed somewhat in their biochemical activities (see below).

UV absorption spectra of 7,17(*S*)-diHDHA

An interesting observation of cyclic changes in the UV light absorption spectra of 7,17(*S*)-diHDHA upon repetitive changes of the solvents in which it was dissolved was exactly the same as the transformation reported previously for 10(*S*),20-diHDHA (16). The latter compound was proposed to form an intramolecular hydrogen bond in nonpolar solvents (e.g., hexane) that would be disrupted in a more polar solvent (e.g., ethanol), with a mixture of both forms coexisting in a solvent of intermediate polarity (e.g., *n*-hexane-ethanol mixture). Inasmuch as the distance between the hydroxyl groups (10 carbon atoms) is identical for both 7,17(*S*)- and 10(*S*),20-diHDHAs, the solvatochromic effects should be (and are) quite similar for both compounds. A possible acid-catalyzed formation of a C1-C7 or C1-C10 cyclic ester (or lactones) similar to the 5-HETE lactone was ruled out because *1*) the transformation occurred not only in the HPLC solvent containing acetic acid but in the acid-free solvent mixtures as well; *2*) the distance between the C1 carboxyl group and either C7 or C10 is too great for effective lactonization to occur; and *3*) methyl esters were shown to undergo the same reversible changes. These observations should be taken into account, as the UV light spectra of the very same compound taken during an RP HPLC and an NP HPLC experiment would produce dramatically different results, which could lead a researcher to believe that those were two different compounds.



Scheme 2. Proposed mechanism of 10,17(*S*)- and 7,17(*S*)-diHPDHA formation by soybean lipoxygenase (sLOX; steps A–C and G–I) and by potato tuber lipoxygenase (steps D–F). Step C depicts a change in the orientation of the substrate after the first lipoxygenation step catalyzed by sLOX.

Preliminary analysis of the inhibitory activity of 7,17(*S*)- and 10,17(*S*)-diHDHAs

Previous findings showed that a compound of the 10,17(*S*)-diHDHA family possessed clear anti-inflammatory and antiapoptotic activities manifested in various cells through mechanisms that are currently being investigated (9). The originally used 10,17(*S*)-diHDHA preparations were generated enzymatically by the concerted action of sLOX and ptLOX (8–10, 14, 15) or by sLOX alone (11, 20). According to our data (16–18 and this paper), those preparations, most likely, had the 4*Z*,7*Z*,11*E*,13*Z*,15*E*,19*Z* arrangement of the double bonds of the major (if not sole) 10,17-dioxygenated product. Later, it was confirmed that the major naturally occurring isomer of 10,17(*S*)-diHDHA was indeed the one that we had predicted on the basis of our earlier mechanistic studies (16) and whose structure was originally confirmed by ^{1}H NMR spectroscopy (17, 18). Considering that the previously proposed RP HPLC purification procedure does not efficiently separate 10,17(*S*)-diHDHA and 7,17(*S*)-diHDHA, it was important to develop a suitable method of isolation for the enzymatically generated isomers and evaluate their biological activity separately. The satisfactory (baseline) separation of the isomers was achieved using NP HPLC and/or chiral HPLC, as described in Materials and Methods. Then, the pure compounds were characterized to confirm their chemical structures and were subsequently used in biochemical experiments.

We speculated that both compounds might directly inhibit mammalian 5LOX in the reaction of AA oxidation. The reasoning behind this was the rapid loss of enzymatic activity during the oxidation of 17(*S*)-H(P)DHA, which was noted for both sLOX and ptLOX. The latter enzyme is considered to be functionally similar to human 5LOX (31) and is currently incorporated in a 5LOX inhibitor screening assay (Cayman Chemical; product 60401). Therefore, we tested whether hr5LOX would be inhibited by 7,17(*S*)- and 10,17(*S*)-diHDHAs. The inhibition did occur with both compounds. 7,17(*S*)-diHDHA was slightly more effective an inhibitor than 10,17(*S*)-diHDHA. The IC_{50} values of 7 and 15 μM for 7,17(*S*)- and 10,17(*S*)-diHDHAs, respectively, should be taken as conservative estimates of the compounds' activity in vivo, considering the high concentration of the substrate, AA, that was used in our in vitro experiments.

The dihydroxy compounds were also capable of inhibiting sLOX in the reaction of AA oxidation (Fig. 9B). Unlike the possible suicidal reaction inactivation of sLOX during the oxidation of DHA [or 17(*S*)-HPDHA] into a mixture of 7,17(*S*)- and 10,17(*S*)-diH(P)DHAs (Fig. 1C), the observed effects of the diHDHAs on sLOX and hr5LOX should be considered a form of (competitive) inhibition, as no further conversion of either diHDHA was detected during their preincubation with either enzyme. Apparently, diHDHAs did not contribute much to the progressive loss of activity of sLOX during the reactions of diH(P)DHA synthesis, as the dihydroxy products were not formed in appreciable quantities in the reactions. A more detailed kinetic study of the inhibition of various enzymes by diHDHAs is currently under way.

Unlike other known drugs—inhibitors of lipoxygenases, 7,17(*S*)- and 10,17(*S*)-diHDHAs may be considered natural compounds. From our point of view, this alone justifies continuing interest in these compounds, as they (or their close analogs) formed either in vivo (8–15) or enzymatically in vitro have good chances of being safe, low-toxicity drug candidates. The demonstrated ability of the enzymatically synthesized 7,17(*S*)- and 10,17(*S*)-diHDHAs to directly inhibit 5LOX and 15LOX in vitro is of practical importance, because there is a possibility that these two compounds may also inhibit the biosynthesis of leukotrienes and related compounds in vivo. Thus, the compounds may be of interest not only as potential nonsteroidal anti-inflammatory drugs but also as antitrauma and anticancer agents, because the inhibition of human 5LOX could have beneficial effects in certain types of trauma and cancer (32, 33).

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