Lipoxygenase catalyzed transformation of epoxy fatty acids to hydroxy-endoperoxides: A potential P450 and lipoxygenase interaction

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Running title: Lipoxygenase-epoxygenase route to hydroxy-endoperoxides

Abbreviations

AD1/AD2, 15,16-epoxy-linolenate enantiomers eluted from Chiralpak AD chiral column; EET, epoxyeicosatrieneoic acid; H(P)OTrE, hydro(pero)xyoctadecatrienoic acid; H(P)ETE, hydro(pero)xyeicosatetraenoic acid; mCPBA, meta-chloroperoxybenzoic acid; LOX, lipoxygenase; RP-HPLC, reversed phase high pressure liquid chromatography; S_Ni, substitution nucleophilic intramolecular; SP-HPLC, straight phase high pressure liquid chromatography

ABSTRACT

Herein we characterize a generally applicable transformation of fatty acid epoxides by lipoxygenase (LOX) enzymes that results in the formation of a 5-membered endoperoxide ring in the end product. We demonstrated this transformation using soybean LOX-1 in the metabolism of 15,16-epoxy-α-linolenic acid, and murine platelet-type 12-LOX and human 15-LOX-1 in the metabolism of 14,15-epoxyeicosatrienoic acid (14,15-EET). A detailed examination of the transformation of the two enantiomers of 15,16-epoxy-α-linolenic acid by soybean LOX-1 revealed that the expected primary product, a 13*S*-hydroperoxy-15,16-epoxide, underwent a non-enzymatic transformation in buffer into a new derivative that was purified by HPLC and identified by UV, LC-MS and ^{1}H-NMR as a 13,15-endoperoxy-16-hydroxy-octadeca-9,11-dienoic acid. The configuration of the endoperoxide (*cis* or *trans* side chains) depended on the steric relationship of the new hydroperoxy moiety to the enantiomeric configuration of the fatty acid epoxide. The reaction mechanism involves intramolecular nucleophilic substitution (S_Ni) between the hydroperoxy (nucleophile) and epoxy-group (electrophile). Equivalent transformations were documented in metabolism of the enantiomers of 14,15-EET by the two mammalian LOX enzymes, 15-LOX-1 and platelet-type 12-LOX. We conclude that this type of transformation could occur naturally with the co-occurrence of LOX and cytochrome P450 or peroxygenase enzymes, and it could also contribute to the complexity of products formed in the autoxidation reactions of polyunsaturated fatty acids.

Supplementary key words: fatty acid/physical chemistry, lipids/chemistry, NMR, fatty acid epoxide, endoperoxide, monocyclic peroxide, epoxyeicosatrienoic acid

INTRODUCTION

The oxygenation reactions of polyunsaturated fatty acids include several examples in which different enzymes collaborate in synthesis of the end product. The cyclooxygenases are paired with cytochrome P450s in the transformation of arachidonic acid to prostaglandin endoperoxides and on to thromboxane A_2 and prostacyclin (1, 2). A P450 and cyclooxygenase participate in the opposite order in the metabolism of P450-derived 8,9-epoxyeicosatrienoic acid (8,9-EET) by cyclooxygenase, giving epoxy-hydroxy products (3). The plant pathway of jasmonic acid synthesis involves initial oxygenation by a 13*S*-lipoxygenase (LOX) followed by transformation to an allene oxide by the P450, CYP74 (2, 4). Other examples include the cooperation of LOX and peroxygenase enzymes in the formation of epoxy and hydroxy fatty acids (5-7). The present work describes a new type of interaction, not so far described in Nature, yet with the potential to produce fatty acid hydroxy-endoperoxides. Further transformations could readily form triols or other derivatives.

The impetus for the present work arose from further studies of fatty acid epoxidation by catalase-related enzymes primed with an oxygen donor, analogous to an earlier report of arachidonate and 8-hydroxy-eicosatetraenoic acod (8-HETE) epoxidation by catalase-related allene oxide synthase (8). One example involved enzymatic epoxidation of 13*S*-hydroxy-α-linolenic acid on the 15,16-double bond. To assign the configuration of the new 15,16-epoxide group, we proposed a scheme using soybean lipoxygenase (LOX) to introduce a 13*S*-hydroperoxide into the individual 15*R*,16*S* and 15*S*,16*R* enantiomers of 15,16-epoxy-linolenic acid. Following reduction to the 13*S*-hydroxy derivatives, this would give authentic standards to compare with the original product. While the desired primary products were recovered from the LOX-catalyzed transformation, it was obvious that additional conjugated diene-containing derivatives were equally abundant in the extracts. This report describes the characterization of these novel products and the mechanism of their synthesis. Subsequent work with mammalian LOX enzymes and an arachidonic acid epoxide (14,15-epoxyeicosatrienoic acd, 14,15-EET) demonstrate the generality of these reactions.

MATERIALS AND METHODS

Materials

α-Linolenic acid and arachidonic acid were purchased from Nu-Check Prep. mCPBA, stannous chloride, and soybean (*Glycine max*) lipoxidase type V were from Sigma-Aldrich. Human 15-lipoxygenase-1 was prepared exactly as described by expression of the recombinant enzyme in *E. coli* (9), and a baculovirus construct of the murine platelet-type 12-LOX was expressed in Sf9 cells as described (10).

Preparation of epoxy fatty acids

Preparation of α-linolenic acid 15,16-epoxide enantiomers – α-Linolenic acid (25 mg) was epoxidized using mCPBA, the 15,16-epoxide purified by RP-HPLC then SP-HPLC (11) and the enantiomers resolved using a Chiralpak AD column (12) and designated as AD1 and AD2 in order of elution: these were identified as the 15*S*,16*R*- and 15*R*,16*S*-epoxides, respectively, by CD analysis of the methyl ester O-benzoyl derivatives as described in detail for related linoleate epoxides (13).

Preparation of 14,15-EET enantiomers – Arachidonic acid (100 mg) in 2 ml dichloromethane was incubated with a 1.5-fold molar excess of mCPBA at room temperature for 30 min. The formed products were taken to dryness and dissolved in MeOH prior to HPLC analysis. The arachidonate monoepoxides are well precedented to elute in RP-HPLC in the order 14,15-, 11,12-, 8,9-, and 5,6-EET (14); 14,15-EET was isolated by RP-HPLC using MeOH/H_2O/HAc 85:15:0.01 (v/v/v) on a Waters Symmetry C18 column (4.6 x 250 mm; 5 μ). The enantiomers of 14,15-EET were resolved using a Chiralcel OJ column (4.6 x 250 mm; 5 μ) and a solvent of Hexane/MeOH/HAc (100:0.5:0.1, v/v/v, run at 2 mL/min), eluting in the order 14*S*,15*R* as peak 1 and 14*R*,15*S* as peak 2 as reported (15).

Incubations with soybean LOX-1

The individual enantiomers of 15,16-epoxy-linolenate were reacted at room temperature with soybean LOX-1 (76 nM) in 0.5 mL 0.1 M K_2HPO_4 (pH 8.7) buffer in a quartz cuvette and the change in the absorbance recorded by repetitive scanning (200 – 300 nm) using a Lambda-35 UV/Vis Spectrometer (Perkin-Elmer). Addition of enzyme led to the appearance of a conjugated diene chromophore (ε ~ 25,000 M^{-1} cm^{-1}) over the course of 10 min, with a faster reaction rate for the AD2 enantiomer (14*R*,15*S*-epoxy). To reduce hydroperoxide products *in situ*, incubations were conducted in the presence of 0.5 mM $SnCl_2$. All the formed products were acidified with 1M KH_2PO_4 and 1N HCl down to pH 5 and extracted on 1 cc HLB Oasis cartridges and eluted with MeOH prior to HPLC analyses. To isolate sufficient products for NMR analyses, the incubations were scaled up to 20 ml 0.1 M K_2HPO_4 buffer containing 152 nM soybean LOX-1. The methyl ester derivatives of isolated products were prepared using diazomethane.

Incubations with platelet 12-LOX and 15-LOX-1

The individual enantiomers of 14,15-EET were incubated with platelet 12-LOX (0.42 μM) in 0.5 ml 50 mM Tris pH 7.5 buffer containing 150 mM NaCl and the changes in the absorbance recorded by repetitive scanning (200 – 300 nm) using a Lambda-35 UV/Vis Spectrometer (Perkin-Elmer). Addition of enzyme led to the appearance of a conjugated diene chromophore over the course of 5 min (ε ~ 25,000), with similar reaction rates for both enantiomers of 14,15-EET. To reduce hydroperoxide products *in situ*, incubations with platelet 12-LOX were conducted in the presence of 0.5 mM $SnCl_2$. All the formed products were extracted on 1 cc HLB Oasis cartridges as previously described. Subsequent incubations

with human 15-LOX-1 were carried out in a similar manner. The methyl esters of products formed by platelet 12-LOX and human 15-LOX-1 were prepared using diazomethane. For the NMR analyses, the incubations with platelet 12-LOX were scaled up to 10 ml buffer and use of 0.4 μM enzyme. The methyl ester derivatives of the human 15-LOX-1 products were reacted with 1.5 equivalents of TPP in MeOH for 30 min at room temperature for 10 min prior to HPLC and GC-MS analyses.

HPLC analyses

The formed products were conducted on SP-HPLC (Hex/IPA/HAc, 100:3:0.1, by volume for free acids and Hex/IPA, 100:2 for methyl esters) using a Thomson silica column (4.6 x 250 mm, 5μ). A flow rate of 1 ml/min was used and UV signals at 205, 220, 235, 270 nm recorded using a Agilent 1100 series diode array detector. To prepare sufficient products for ^{1}H-NMR, about 0.5 mg of fatty acids were injected for the collection. Each collected product was dissolved in MeOH and kept at -20°C until further analysis.

LC- and GC-MS analysis

LC-MS analysis of soybean LOX-1 catalyzed products - The products formed from the enantiomers of 15,16-epox-linolenate on incubation with soybean LOX-1 and LOX-1/$SnCl_2$ were eluted on RP-HPLC (CH_3CN/H_2O/HAc, 45:55:0.01, by volume; 0.4 mL/min) using a Kinetex C18 column (3 x 100 mm, 2.6μ) and molecular weights were established from the M-H anions measured by negative ion electrospray LTQ1 ion trap LC-MS instrument.

GC-MS analysis of human 15-LOX-1 catalyzed products – The methyl esters of hydroperoxy-EETs were treated with TPP to prepare the corresponding hydroxy-EETs and repurified by SP-HPLC. The methyl esters of hydroxy-EETs dissolved in 200 μL ethyl acetate were hydrogenated using H_2 with ~1 mg 5% Pd on alumina for 3 minutes at room temperature. The catalyst was removed by extraction with water and centrifugation and the organic phase containing the saturated hydroxy-C20 fatty acids was transfered for further derivatization. GC-MS analyses of the methyl ester trimethylsilyl derivatives of the reduced and hydrogenated hydroperoxy-EETs was carried out in the positive ion electron impact mode (70 eV) using a ThermoFinnigan DSQ mass spectrometer. The initial temperature was set for 180°C and then increased to 300°C at 20°C/min and then held at 300°C for 4 min.

NMR analysis

^{1}H NMR and ^{1}H,^{1}H COSY NMR spectra were recorded on a Bruker 600-MHz spectrometer at 298 K. The parts/million values are reported relative to residual non-deuterated benzene (C_6D_6; δ = 7.15 ppm). All spectra were analyzed on Bruker TopSpin 3.0 software. In addition to NMR analyses, 3D models of the formed products were created using ACD-Labs software (Supplementary data).

RESULTS

Reaction with soybean LOX-1 with the 15,16-epoxides of α-linolenic acid

The 15,16-epoxide enantiomers of α-linolenic acid were prepared as outlined in Methods and for convenience here are designated as AD1 (15*S*,16*R*) and AD2 (15*R*,16*S*), in order of their elution from the Chiralpak AD column. The 15,16-epoxides retain a 9*cis*,12*cis* pentadiene in the structure and are potential substrates for soybean LOX-1. Figures 1A and 1B illustrate transformation of the two mirror image 15,16-epoxides with the LOX-1 enzyme as observed by UV spectroscopy. Both enantiomers give rise to a typical conjugated diene chromophore, with a much slower reaction occurring with AD1, the 15*S*,16*R* isomer, (cf. Fig. 2A, 2B); under the conditions used here, AD1 has an initial turnover of 2 s^{-1} and AD2 of 13 s^{-1}, in the order of 1% and 5% of the rates with α-linolenic acid itself (16).

Subsequent SP-HPLC analyses revealed a more complex pattern of products than initially expected, with three products recovered from a 10 min reaction of AD1 (Fig. 2A, top chromatogram), and two products from AD2 (Fig. 2B, top). All these products showed UV absorbance at 235 nm and contained a conjugated diene chromophore (Supplementary Fig. S1). The product pattern was time-dependent, such that upon 20 min incubation in the reaction buffer with soybean LOX-1, there was an increase in the relative abundance of the earliest-eluting product (Fig. 2AB, lower chromatograms, products **1** and **4**).

To help understand the basis for the appearance of multiple products containing a conjugated diene chromophore, the incubations with soybean LOX-1 were repeated in the presence of stannous chloride ($SnCl_2$) that will reduce hydroperoxides to the hydroxy derivative *in situ*. This simplified the product pattern such that only two conjugated dienes appeared from AD1 and only one product from AD2 (Fig. 2C). It appeared that the $SnCl_2$-catalyzed reduction of the hydroperoxy products of the soybean LOX-1 reaction prevented the time-dependent transformation that gives rise to the earliest-eluting products (**1** and **4** in Fig. 2A and 2B).

Structural analysis of soybean LOX-1 products 1 – 8

LC-MS analyses (negative ion mode, Supplementary Fig. S2) showed that all of the products formed in the initial incubations with soybean LOX-1 (products **1** – **5**) have a M-1 ion at m/z 325, corresponding to the addition of molecular oxygen to the 15,16-epoxy-linolenate. All the $SnCl_2$-reduced products each have a M-1 ion at m/z 309, corresponding to retention of one of the oxygens from O_2.

Fragmentation of the peroxide products provided the first indications of the presence of an endoperoxide moiety in the structures of products **1** and **4**. Whereas the other peroxide products showed only a prominent M-1 ion at m/z 325 in the negative ion mass spectrum, **1** and **4** contained an equally prominent

ion at m/z 237, interpreted as loss of C15-C18 (88 a.m.u.) and compatible with the presence of an oxygen attached to C15 in addition to the oxygen at C16.

All the products described so far were prepared in sufficient amounts for NMR analysis, which revealed their covalent structures, and based on literature precedents, the relative stereochemistry of the carbon chain substituents of the endoperoxides in products **1** and **4** (17, 18) (Table 1). The NMR spectra and COSY analyses are given in Supplementary data, pages 10 - 18. Scheme 1 shows the structures of the peroxide products **1** – **5** and the deduced pathway to the endoperoxides (products **1** and **4**). The AD1 enantiomer (15*S*,16*R*-epoxy) reacts relatively slowly with soybean LOX-1 and is converted to three products: one is the 13*S*-hydroperoxide (product **3**), which is expected, and this is further transformed to the endoperoxide (**1**); in addition, the slowly-reacting AD1 enantiomer also gives the 9-hydroperoxide (**2**) as an additional product. The faster reacting AD2 enantiomer only gives the 13*S*-hydroperoxide (**5**) and the corresponding endoperoxide (**4**). The $SnCl_2$ reduced products are the corresponding 13-hydroxy-15,16-epoxy derivatives (**7** and **8**), plus the 9-hydroxy-15,16-epoxide (**6**) formed from AD1.

Non-enzymatic formation of the endoperoxides

The 13-hydroperoxy-15,16-epoxides (**2** and **5**) were stable in organic solvents and could be purified with conventional HPLC methods at room temperature. Re-incubation of the purified hydroperoxy-epoxides in the original pH 8.5 buffer in the absence of enzyme led to a perceptible change in the UV profile (Fig. 3A); this is attributed to their non-enzymatic transformation to the corresponding endoperoxides, which have a higher lambda max (239 nm, versus 237 nm of the hydroperoxy derivatives) (Fig. 3B). This transformation in buffer alone matches the time-dependent changes noted earlier upon extending the 10 min incubations to 20 min (Fig. 2AB, upper and lower chromatograms). Formation of the endoperoxides in the absence of enzyme was confirmed by extraction and HPLC-UV analysis of the products (data not shown).

There is a noticeable difference in the rate of endoperoxide formation from the 13*S*-hydroperoxy derivatives of the linolenic acid AD1 and AD2 15,16-epoxides. Whereas AD1 reacts slower with soybean LOX-1 (Fig. 1A, 1B), its product (13*S*-hydroperoxy-15*S*,16*R*-epoxy) shows the faster non-enzymic transformation to the endoperoxide. This is understandable in that the newly formed hydroperoxy moiety is better positioned for S_Ni attack on the 15,16-epoxide (as explained in Discussion).

Testing the generality of the endoperoxide formation

To test the generality of the endoperoxide formation from fatty acid hydroperoxy-epoxides we used two mammalian LOX enzymes and the enantiomers of 14,15-EET as substrate. Reactions of the platelet-type 12-LOX with 14,15-EET are analogous to the linolenic acid transformations described above. On the

other hand, the primary oxygenation activity of 15-LOX-1 is not possible using 14,15-EET as substrate; in this case it is the minor 12 dioxygenase activity of 15-LOX-1 that comes into play, as well recognized before in the 15-LOX-1-catalyzed transformations of 15*S*-HPETE to leukotriene epoxides (19) and the related transformations catalyzed by the leukocyte-type 12-LOX (20).

Reactions with mouse platelet-type 12-LOX

Reaction of recombinant platelet-type 12-LOX with the enantiomers of 14,15-EET was observed by repetitive UV scanning, which showed the appearance of a conjugated diene chromophore (cf. Fig. 1A, 1B) and rates similar for the two enantiomers and comparable to the rate of reaction of arachidonic acid. The turnover values (k_{cat}) for 14*S*,15*R*-EET and 14*R*,15*S*-EET were 1.3 s^{-1} and 0.95 s^{-1}, respectively, and 1.8 s^{-1} for arachidonic acid. SP-HPLC analysis of the products revealed the formation of two major products from each 14,15-EET enantiomer, Fig. 4A, 4B. As found before with the α-linolenate epoxide products, there is a time-dependent transformation of the more polar **10** and **13** to the earlier-eluting products **9** and **12**. NMR analysis (Supplementary data, pages 14 - 17) of all four main compounds confirmed what would be predicted, namely that the more polar **10** and **13** are 12-hydroperoxy-14,15-epoxides, and the time-dependent secondary products **9** and **12** are 12,14-endoperoxy-15-hydroxy derivatives from 14*S*,15*R*-EET and 14*R*,15*S*-EET, respectively. Whereas the two 14,15-EET isomers react similarly with the 12-LOX, the secondary transformation to the endoperoxides is faster with the 14*S*,15*R* enantiomer (cf. upper and lower chromatograms of Fig. 4A and 4B), compatible with its more favorable stereochemistry for the S_Ni attack of the hydroperoxide on the epoxide carbon at C14 (see Discussion).

The Supplementary data also illustrates that the UV spectra of the four conjugated dienes show distinct differences with a higher lambda-max and smoother profile for the earlier eluting endoperoxide derivatives (Supplementary Fig. S3A), and that in the presence of $SnCl_2$ only a single product, the corresponding hydroxy-epoxide (**11** and **14**) is formed from each 14,15-EET enantiomer (Supplementary Fig. S4).

Reactions with human 15-LOX-1

Using arachidonic acid as substrate, 15-LOX-1 displays a number of reactions and secondary transformations. The primary dioxygenase activities give 15*S*-HPETE and 12*S*-HPETE in ~10:1 proportions (21) and secondary reactions give 5,15- and 8,15-diHPETEs as well as 14,15-LTA_4 and its hydrolysis products (19). Understanding these properties help in explaining a slightly more complex profile of products formed from the enantiomers of 14,15-EET. Lipoxygenase catalysis of 14,15-EET to the 12-hydroperoxide requires an initial hydrogen abstraction from C10, which is a secondary property of 15-LOX-1, giving rise normally to the minor proportion of 12-HPETE from arachidonate. Therefore it

was not unexpected that the two 14,15-EET enantiomers are transformed by recombinant human 15-LOX-1, albeit at a rate about an order of magnitude slower than using arachidonate as substrate. The products were separated by SP-HPLC and the structures determined by GC-MS of the methyl ester TMS derivative after reduction and hydrogenation of the double bonds (Supplementary Fig. S5).

The 14*S*,15*R* enantiomer is the slightly faster reacting (1.0 s^{-1} versus 0.3 s^{-1} for 14*R*,15*S*-EET, and 10 s^{-1} for arachidonic acid), and it forms three main products (Fig 4C). The tallest peak (**10**) is the 12-hydroperoxy-14,15-epoxide and it shows a time-dependent transformation to the earlier-eluting compound **9**, which was shown by UV analysis and HPLC mobility to be identical to the 12*S*,14*R*-endoperoxy-15*R*-hydroxy product of 12-LOX (compound **9**). The "extra" peak in Fig. 4C, compound **15**, was identified by GC-MS (after reduction of the hydroperoxide) as an 8-hydroxy-14,15-epoxide (Scheme 2, upper half).

The second 14,15-EET enantiomer (14*R*,15*S*) was converted by 15-LOX-1 to **13**, identical to the 12*S*,14*S*-endoperoxy-15*S*-hydroxy product of 12-LOX, and which showed very little further transformation to an endoperoxide (**12**, a very small peak in Supplementary Fig. S6, lower chromatogram). The major peak (**16**) was identified by GC-MS (after reduction) as a 5-hydroxy-14,15-epoxide (Scheme 2, lower half).

DISCUSSION

Routes to hydroxy-endoperoxides - Fatty acid epoxides are typical products of cytochrome P450 enzymes (22-24) and we describe a generally applicable reaction in which the fatty acid epoxide is further oxygenated by a LOX enzyme followed by the non-enzymatic formation of a mono-cyclic endoperoxide (Scheme 3). In principle the order of the first two reactions could be reversed, with initial formation of the hydroperoxide and secondary epoxidation (Scheme 3, lower half). Peroxygenase enzymes are an alternative source of the fatty acid epoxide: typical plant peroxygenases use a fatty acid hydroperoxide to "charge" the enzyme and effect intermolecular oxygen transfer and epoxidation (5-7, 25). This co-occurrence of the LOX and epoxygenase activities together presents favorable circumstances for the further transformation to hydroxy-endoperoxides. It is interesting to consider that the cyclooxygenase-catalyzed oxygenation of 8,9-epoxyeicosatrienoic acid referred to in the Introduction sets up the potential for the synthesis of an 8-hydroxy-9,11-endoperoxide synthesis – however the concomitant peroxidase activity of PGH synthase rapidly reduces the initial 11-hydroperoxy moiety and the product recovered is the 8,9-epoxy-11-hydroxy derivative (3).

Mechanism of formation of the endoperoxides - The step of endoperoxide synthesis is understandable as an intramolecular nucleophilic substitution (S_Ni-type) between the hydroperoxy nucleophile and the epoxy electrophile (Scheme 4). Differences in the rate of endoperoxide formation depend on the relative configuration of the hydroperoxy group to the epoxide moiety. The rate is faster when the hydroperoxide is more favorably position for S_Ni attack on the epoxide (Scheme 4, left side). For example, although the 15*S*,16*R*-epoxy isomer of α-linolenate (AD1) reacts slower with soybean LOX-1 (Fig. 1A, 1B), the oxygenation product (13*S*-hydroperoxy-15*S*,16*R*-epoxy) shows the faster non-enzymic transformation to the endoperoxide (Scheme 4, left side). The same observations applied to the step of endoperoxide synthesis involving the 12-hydroperoxy-14,15-epoxy products of 12-LOX and 15-LOX-1.

There is a precedent for this fatty acid endoperoxide synthesis in the chemical literature. Porter and coworkers used mCPBA and a catalytic amount of trichloroacetic acid in methylene chloride to convert 9-hydroperoxy-octadeca-6,10,12-trienoic acid (9-HPOTrE-ω6) to multiple products including a 6-hydroxy-7,9-endoperoxide (26). The mechanism clearly involved the intermediacy of the direct mCPBA-catalyzed formation of the 6,7-epoxide of the 9-hydroperoxide which was converted by the trace of acid to the corresponding hydroxy-endoperoxide. Earlier, Corey had suggested a related theoretical pathway of prostaglandin H_2 synthesis is which an arachidonate 14,15-epoxide could be oxygenated and cyclized to an endoperoxide with final opening of the epoxide ring to give the 9,11-endoperoxy-15-hydroxy product (27). Another related transformation involves formation of 5-hydroxy-prostaglandin I_1 from 5,6-EET via COX-1 catalysis: synthesis of the corresponding endoperoxide and its reduction to a $PGF_{2\alpha}$ analog permits

nucleophilic attack of the C9 hydroxyl on the 6-carbon of the 5,6-epoxide, giving the 5-hydroxy-PGI_1 isomers as final products (28).

The reactions we characterize take place in an aqueous environment and being enzyme-catalyzed, the products have a specific stereochemistry. Even though the endoperoxide formation is non-enzymatic, the reaction is stereoselective – a chiral epoxide and hydroperoxide giving a single endoperoxide diastereomer via intramolecular nucleophilic substitution. The reaction occurs most readily when the epoxide oxygen is on the opposite face of the carbon chain to the attacking hydroperoxide, allowing ready S_N substitution and producing an endoperoxide with side-chains in the *cis* configuration (Scheme 4, left side). When the epoxide oxygen is on the same face as the hydroperoxide, the reaction is slower and the product is a *trans* endoperoxide (Scheme 4, right side). Although the transformation is slightly favoured in more alkaline solution which we detected using incubation buffers (pH 7.5 vs 8.5) for corresponding soybean LOX-1 and mammalian LOXs, the water- and time-dependent interaction is primarily determined by the stereochemistries of the epoxy and hydroperoxy moieties.

Comparison and contrast with autoxidative formation of endoperoxides - It is well recognized that non-enzymatic peroxidation can give rise to hydroperoxy-monocyclic endoperoxides (29, 30). Reduction of such hydroperoxy-endoperoxides will give a racemic version of the hydroxy-endoperoxide products we describe. In addition, several authors have used the initial LOX-catalyzed formation of 13-hydroperoxy-linolenic acid to study its further non-enzymatic transformation to hydroperoxy-endoperoxides (31, 32). The direct formation of hydroxy-endoperoxides is also theoretically feasible via lipid peroxidation because autoxidative reactions can include the production of fatty acid epoxides (33, 34), which are then open to a non-enzymatic peroxidation to the hydroxy endoperoxides as we have characterized.

Trihydroxy fatty acids and other predicted derivatives – The fatty acid monocyclic endoperoxides are relatively stable under mild conditions (e.g. HPLC at room temperature). Also, unlike hydroperoxides, the monocyclic endoperoxide moiety is resistant to reduction using mild reducing agents such as triphenylphosphine and even sodium borohydride (31). Nonetheless, in biological systems opening of the epoxide ring is likely to occur on exposure to peroxidases, in which case the products will be fatty acid triols. The same triols could arise also from hydrolysis of the precursor hydro(pero)xy-epoxides, which are likely substrates for epoxide hydrolase (35).

Epoxy fatty acids as LOX substrates – A side issue of some interest in relation to our results is the suitability of specific fatty acid epoxides as substrates for LOX catalysis. One of the central concepts of substrate binding in lipoxygenases is the head-to-tail orientation of the fatty acid, which in the case of the soybean LOX-1, human 15-LOX-1 and the platelet-type 12-LOX involves a "tail-first" entry into the

LOX active site (36). Our results indicate that the presence of an epoxide on the tail carbons slows the rate of reaction but does not interfere with the specificity of the oxygenation. As the reaction rate of the platelet-type 12-LOX with 14,15-EET is comparable to the rate with arachidonic acid, this raises the possibility of their co-occurrence and interaction in the cardiovascular system.

Conclusion

The dioxygenation of epoxy fatty acids by lipoxygenases give rise to hydroperoxy- and hydroxy-epoxy derivatives. Subsequent transformation of hydroperoxy-epoxides to hydroxy-endoperoxides as a result of intramolecular nucleophilic substitutions between hydroperoxy and epoxy moieties is an alternative way of synthesizing monocyclic peroxides *in vitro*. *In vivo*, the crosstalk between epoxygenase and lipoxygenase pathways might result in hydroperoxy-epoxy and corresponding endoperoxy derivatives that might undergo to reduction, giving variety of hydroxy fatty acids. The presence and biological significance of described fatty acid derivatives might be a matter of future research in lipidomics.

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Supplemental Information (Separate file)

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Table 1. [1]H-NMR chemical shifts (δ) and coupling constants (J) of *cis*- and *trans*-endoperoxides formed in the soybean LOX-1 (left side) and platelet-type 12*S*-LOX (right side) catalyzed metabolism with 15,16-epoxy-α-linolenic acid and 14,15-EET, respectively. Characteristically different values of *cis* and *trans* are shown in bold.

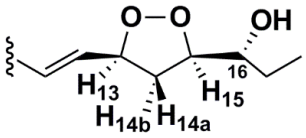
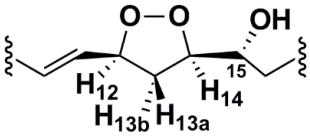
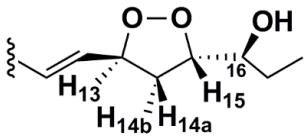
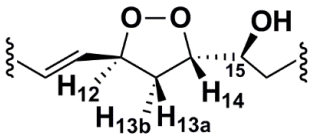
Soybean LOX-1/15,16-epoxide		**Platelet-type 12S-LOX/14,15-EET**	
cis-13,15-endoperoxide 		*cis*-12,14-endoperoxide 	
trans-13,15-endoperoxide 		*trans*-12,14-endoperoxide 	
δ (ppm)	**J (Hz)**	**δ (ppm)**	**J (Hz)**
cis / trans	**cis / trans**	**cis / trans**	**cis / trans**
H13	$J_{13,12}$ = 7.6 / 7.4	**H12**	$J_{12,11}$ = 7.7 / 7.4
4.56 / 4.64	$J_{13,14a}$ = 7.5 / 7.0	4.58 / 4.59	$J_{12,13a}$ = 7.6 / 5.5
	$J_{13,14b}$ = 7.6 / 6.8		$J_{12,13b}$ = 7.6 / 5.5
H14a	$J_{14a,13}$ = 7.5 / 7.4	**H13a**	$J_{13a,12}$ = 7.6 / 7.6
2.17 / 2.37	**$J_{14a,15}$ = 7.7 / 4.6**	2.24 / 2.27	**$J_{13a,14}$ = 7.6 / 4.8**
	$J_{14a,14b}$ = 12 / 11.9		$J_{13a,13b}$ = 11.9 / 12
H14b	**$J_{14b,13}$ = 8 / 5.9**	**H13b**	**$J_{13b,12}$ = 8 / 5.2**
1.87 / 1.97	**$J_{14b,15}$ = 5.2 / 8.3**	1.91 / 1.98	**$J_{13b,14}$ = 5.2 / 8.1**
	$J_{14a,14b}$ = 12 / 11.9		$J_{13a,13b}$ = 12.1 / 11.9
H15	**$J_{15,14a}$ = 7.9 / 4.7**	**H14**	**$J_{14,13a}$ = 7.9 / 5.2**
3.88 / 3.94	**$J_{15,14b}$ = 5.5 / 8.3**	3.91 / 3.96	**$J_{14,13b}$ = 5.3 / 8.1**
	$J_{15,16}$ = 5.6 / 4.7		$J_{14,15}$ = 5.3 / 5.2

Figure legends

Figure 1. Repetitive UV scans of the formation of products from the α-linolenate 15,16-epoxides AD1 (**A**) and AD2 (**B**) by soybean LOX-1. Turnover numbers are presented in the text.

Figure 2. SP-HPLC analyses of products (analyzed as methyl esters) from 15,16-epoxides AD1 (**A**) and AD2 (**B**) by soybean LOX-1, and (**C**) from identical incubations with soybean LOX-1 carried out in the presence of $SnCl_2$. The top chromatogram in **A** is from a 10 min incubation with enzyme and the lower chromatogram from a 20 min incubation. In **B**, the incubation times were 3 min and 10 min. In panel (**C**), the hydroxy products **6**, **7**, and **8** are analogues of hydroperoxy derivatives **2**, **3**, and **4**, respectively. Samples were run on a Thomson 5μ silica column (250 x 4.6 mm) with a solvent of hexane/IPA (100:2 by volume) at a flow rate of 1 ml/min with UV detection at 235 nm. The structures of products **1** – **5** are shown in Scheme 1.

Figure 3. The non-enzymic formation of endoperoxides from 15,16-epoxide AD2 (**A**). Formation of endoperoxides from other hydroperoxy-epoxides can be detected in a similar manner due to the characteristic UV spectra of endoperoxides (**B**).

Figure 4. SP-HPLC analyses for products (analyzed as methyl esters) from incubation of platelet 12-LOX with 14*S*,15*R*- (**A**) and 14*R*,15*S*-EETs (**B**) after 10 and 30 min, and (**C**) the products from incubation of human 15-LOX-1 with 14*S*,15*R*-EET. (Incubation of 15-LOX-1 with 14*R*,15*S*-EET is given in the Supplement, Fig. S5). Chromatographic conditions were as in Fig. 2. The structures of the products are shown in Scheme 2.

Scheme 1. Products formed from α-linoleate 15,16-epoxides AD1 and AD2 by soybean LOX-1.

Scheme 2. Products formed from 14,15-EETs by platelet 12-LOX and human 15-LOX-1.

Scheme 3. The potential interaction between epoxygenase and lipoxygenase pathways.

Scheme 4. The reaction mechanism for the formation of endoperoxides from epoxy fatty acids.

Figure 1

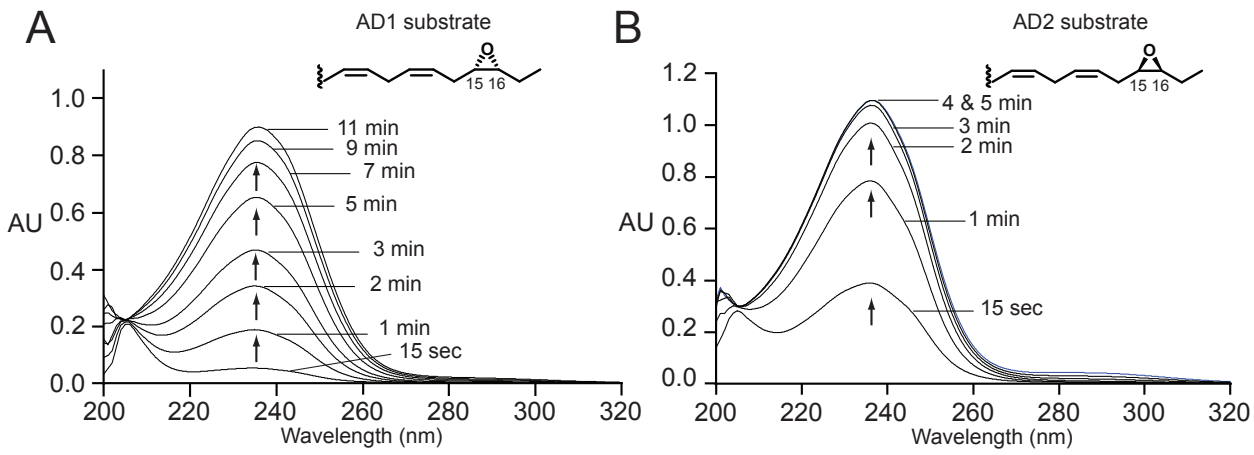


Figure 2

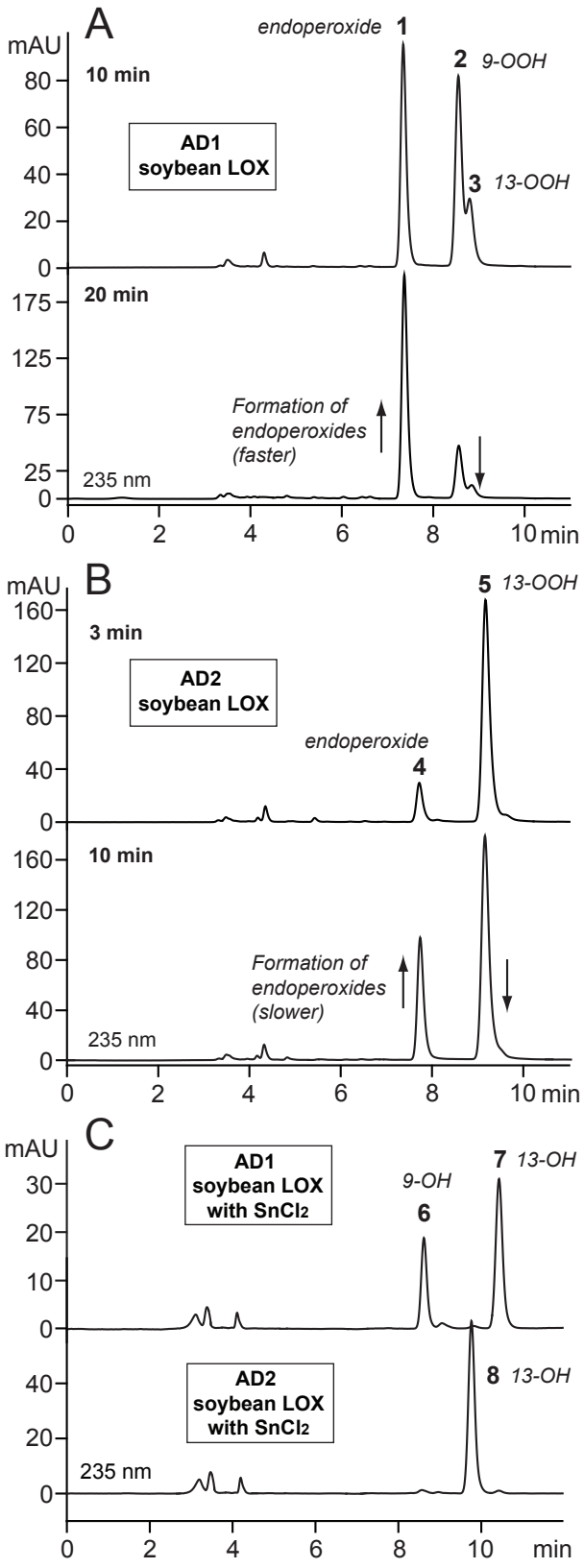


Figure 3

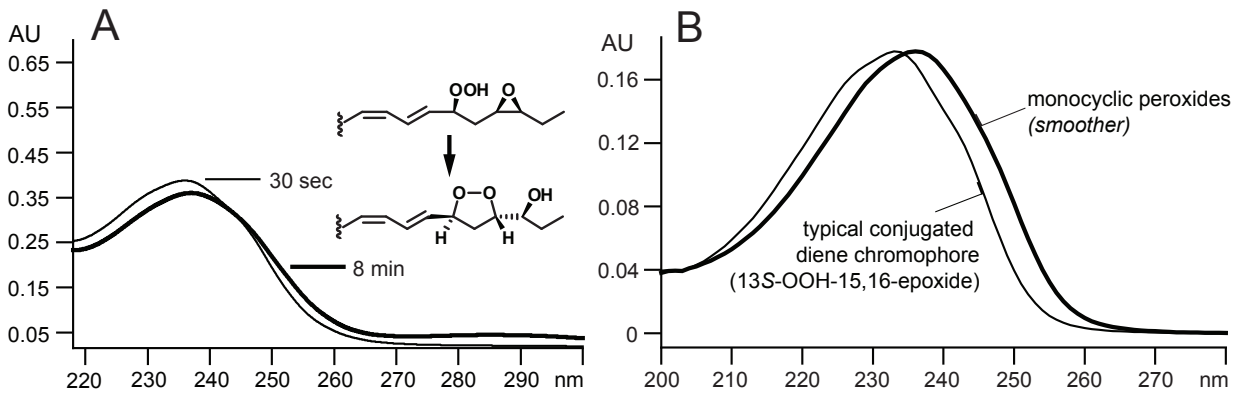
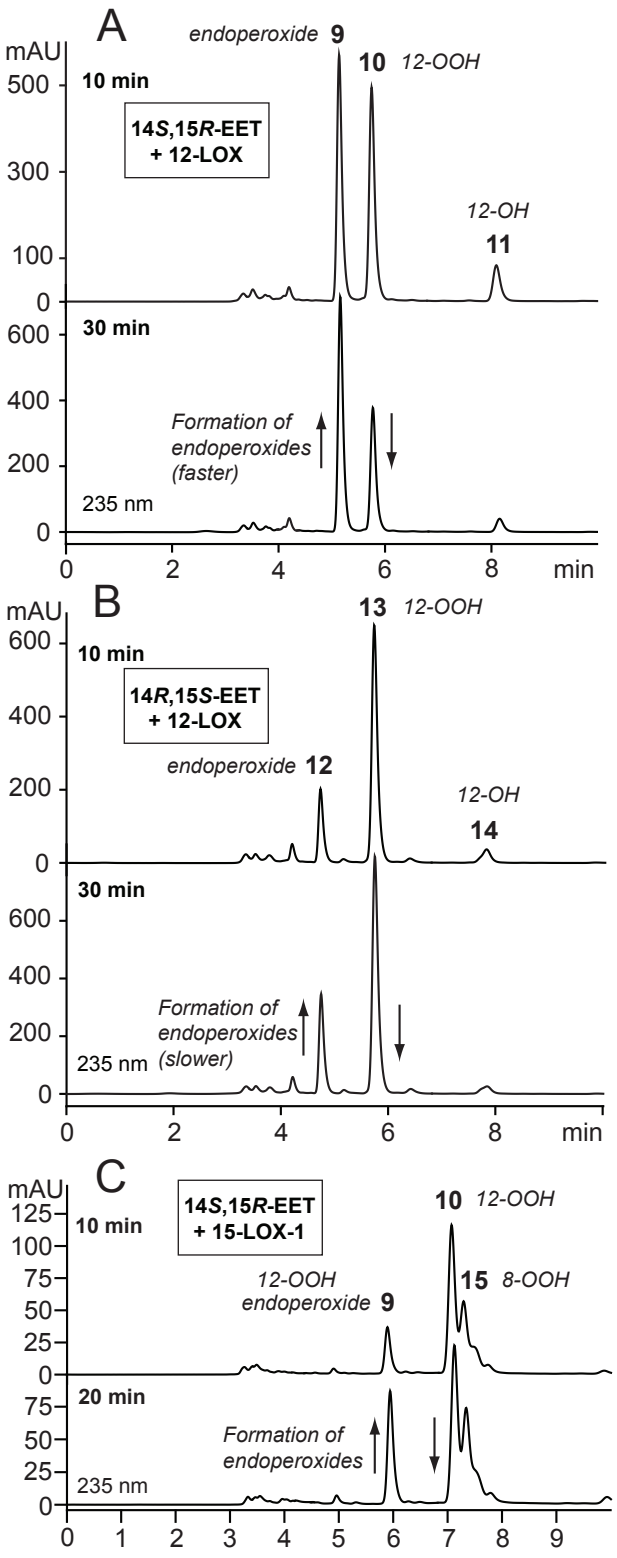
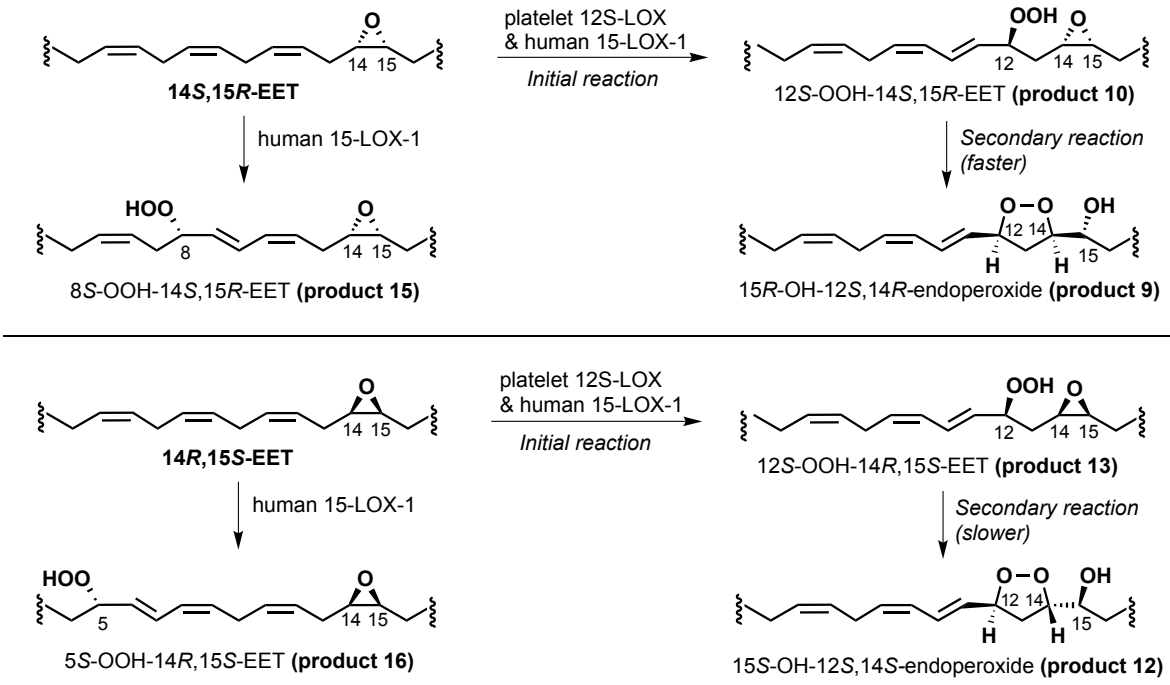


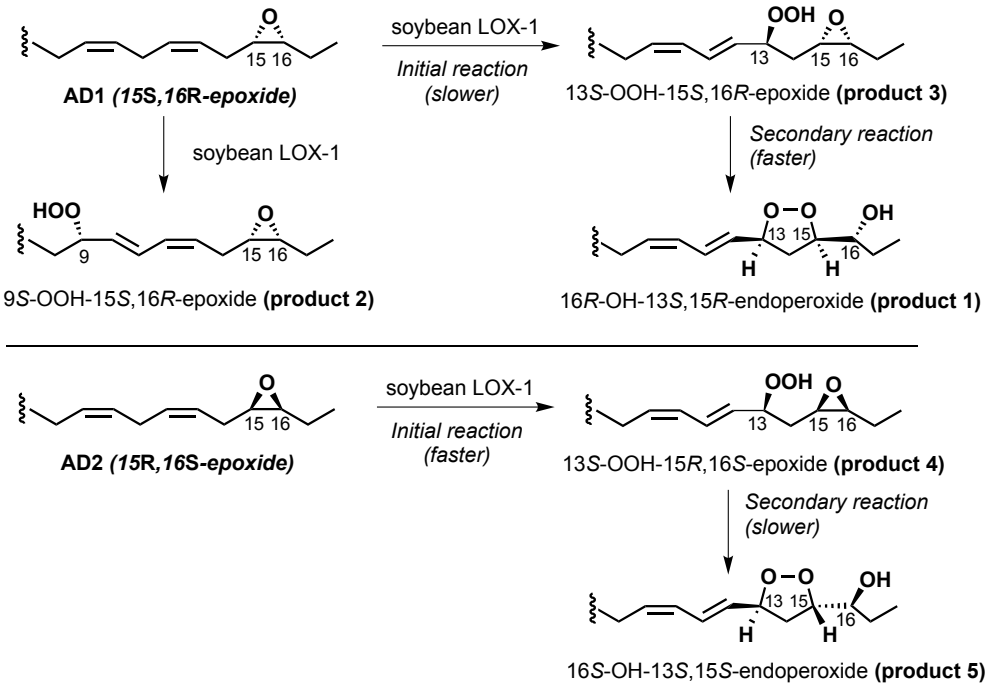
Figure 4



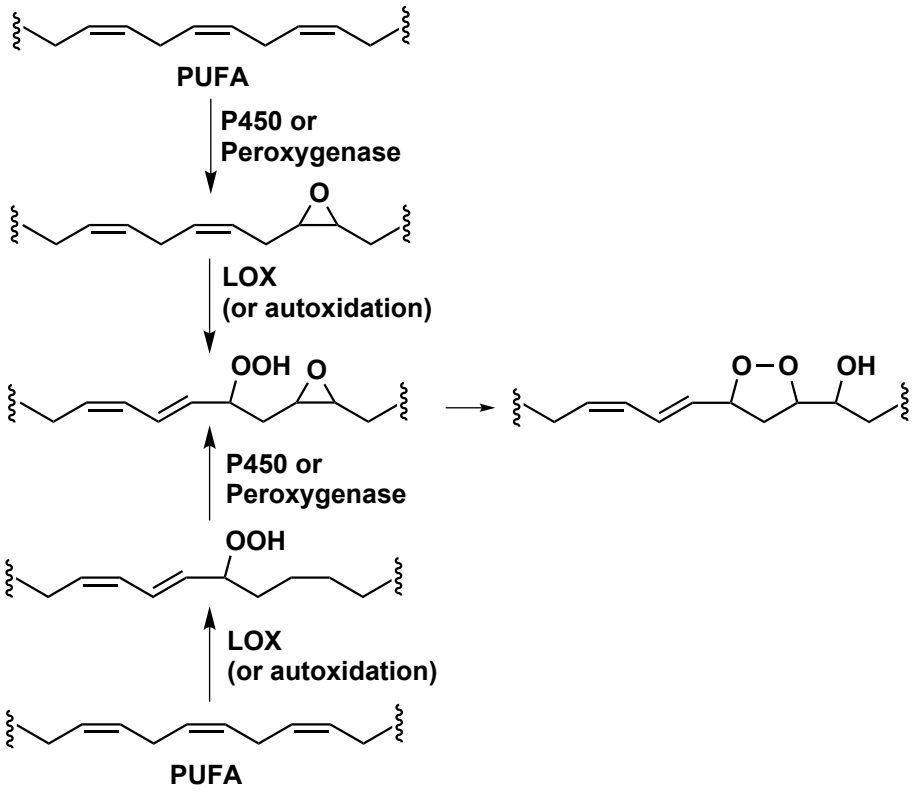
Scheme 2



Scheme 1



Scheme 3



Scheme 4