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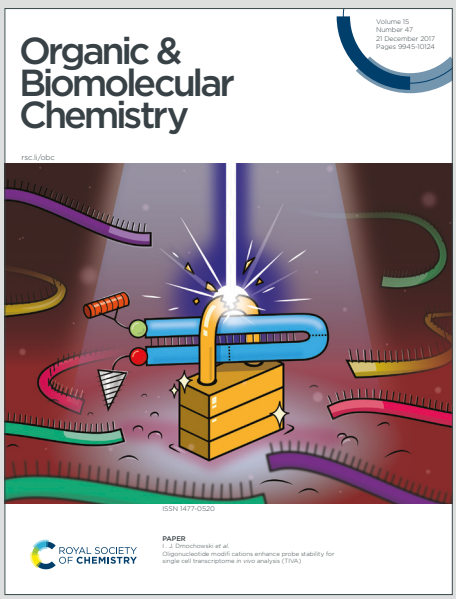

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Macrocyclic oxygen transfer in conversion of fatty acid hydroperoxide to a single species of triol in physiological saline

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ABSTRACT

We analyzed the autoxidation and non-enzymic reactions of the 17*S*-hydroperoxide of docosahexaenoic acid **1**, a common lipoxygenase product in mammalian cells and with its six double bonds presenting opportunities for reactions not available to all polyunsaturated fatty acids. Incubations in phosphate-buffered saline for one or more days at 37 °C revealed a dominant product identified by comparison to synthetic standard as diastereomers of the γ-lactone **2** of 4,5,17-trihydroxy-docosapentaenoic acid. Over several days in PBS at 37 °C the γ-lactone hydrolyzed to the more polar 4,5,17-trihydroxy derivative **3**. The same γ-lactone is formed by acid hydrolysis of synthetic 4,5-*cis*-epoxy-17-hydroxy-22:5ω3 **4**, but this epoxide is stable in PBS at pH 7.4, indicating the γ-lactone is a primary product and not secondary to hydrolysis of the 4,5-epoxide. Mass spectrometric analysis of γ-lactone and trihydroxy derivatives from incubation with [17-$^{18}O_2$H]-hydroperoxide demonstrated intramolecular oxygen transfer with retention of both hydroperoxy oxygens on the 5- and 17-carbons. Direct involvement of the 17-hydroperoxide group in the oxygen transfer with participation of the C1 carboxyl in the mechanism with high "effective molarity" at the 4,5-double bond can account for the findings. Other fatty acid hydroperoxides with similar spatial relationship of peroxide to the double bonds could also undergo this intramolecular oxygen transfer, a novel pathway in lipid peroxidation.

Introduction

The primary reactions in the autoxidation of unsaturated fatty acids were first studied with oleic and linoleic acids and later extended to trienoic and tetraenoic octadecanoids and eicosanoids.[1-4] From one or two CH_2-interrupted *cis*-double bonds in the starting fatty acid, the primary hydroperoxide can give rise to alkoxyl or peroxyl radicals and a complex mixture of epoxy, hydroxy, keto or aldehydic products.[5] The presence of 3 or 4 methylene-interrupted double bonds gives further opportunities for complexity of the product outcomes including secondary transformations giving cyclized carbon rings and endoperoxides (the mechanistic basis of prostaglandin biosynthesis).[6-13]

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The six double bonds in docosahexaenoic acid (DHA) provide yet further opportunities for complexity and the present work introduces an unprecedented rearrangement of the DHA hydroperoxide **1**. The primary autoxidation products of DHA are well defined as ten *cis*/*trans*-conjugated mono-hydroperoxides.[14, 15] In biology, much attention is directed to the pathways arising from the primary DHA hydroperoxides synthesized by lipoxygenase (LOX) enzymes.[16] DHA is a good substrate for (arachidonate) 15-LOX enzymes[17-19] and the 17*S*-hydroperoxy-DHA product (17-HPDHA, **1**) is the precursor of a family of dihydroxy and trihydroxy derivatives.[16, 20, 21] For the most part the proposed enzymic pathways of 17-HPDHA transformation are modeled on the equivalent reactions of the arachidonate analogue, 15-hydroperoxy-eicosatetraenoic acid (15-HPETE).[22-25], which in turn are analogous to the 5-LOX metabolism to dihydroxy leukotrienes.[26-29] Most of the enzymic transformations parallel the autoxidative oxygenations of mono-hydroperoxides, although the enzymic reactions tend to be stereospecific and the autoxidations give pairs of diastereomers.

The original impetus for the present study was to model the autoxidative oxygenations of 17*S*-HPDHA (1), and we made some headway with the α-tocopherol-controlled formation of

dihydro(pero)xides conducted as the neat oil (detailed in the Supplement). We detected the predicted dihydro(pero)xides and noted the absence of the structural analogue related to leukotriene B_4 and known by the acronym Protectin D1 (cf. refs[20, 21]). The project took a different turn when we carried out the incubations in aqueous solution, as a 30 - 40 μM solution of 17*S*-HPDHA in phosphate-buffered saline (PBS). Initial reversed-phase HPLC (RP-HPLC) with UV analyses showed two prominent new peaks dominating the chromatogram, with absence of the mixture of dihydroperoxides known from enzyme reactions and that also appear in the autoxidations with α-tocopherol. One of the new products was quickly identified as a C17 chain cleavage fatty acid-aldehyde, a well-known transformation of fatty acid hydroperoxides.[3-5] Of more interest and novelty is the identity of the second prominent peak, a single species of trihydroxy-docosanoid, and its structural analysis and the mechanistic basis of its synthesis are described herein.

Results

Autoxidation of 17*S*-HPDHA in phosphate buffered saline

Upon incubation of approximately 30 μM of 17*S*-HPDHA **1** in PBS at 37 °C, over the course of several days the UV spectrum showed reductions in signal of the conjugated diene chromophore with increases in absorbance around 280 nm, Figure 1. RP-HPLC chromatograms of extracts from day-1 and day-6 of an autoxidation are illustrated in Figure 2; they were run at a relatively slow solvent flow rate (0.5 ml/min) to help optimize resolution. Two single product peaks dominate the day-1 chromatogram, Figure 2A. The first, eluting just after 50 min was recorded mainly in the 270 nm channel and its UV spectrum has a λmax at 282 nm in the RP-HPLC column solvent (inset, Figure 2A). It was readily identified by LC-MS ($[M-H]^-$ m/z 273) as a C17 aldehyde fatty acid. Its formation during the autoxidation is not unexpected and well precedented.[5, 30]

Fig. 1: UV spectra recorded during the course of 17*S*-HPDHA autoxidation in PBS**.** UV spectra of the autoxidation mixture were recorded in PBS without dilution in Days 0, 1, 2 and 6.

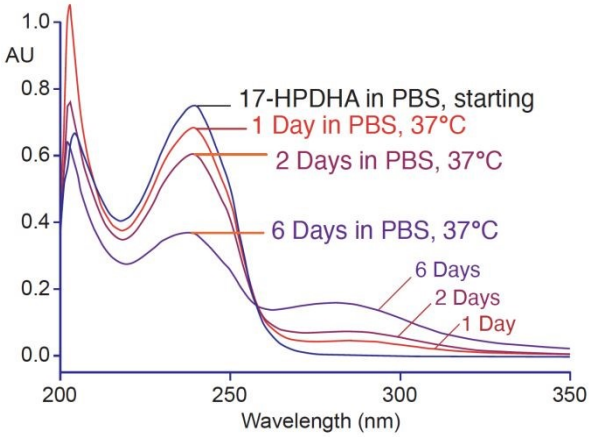
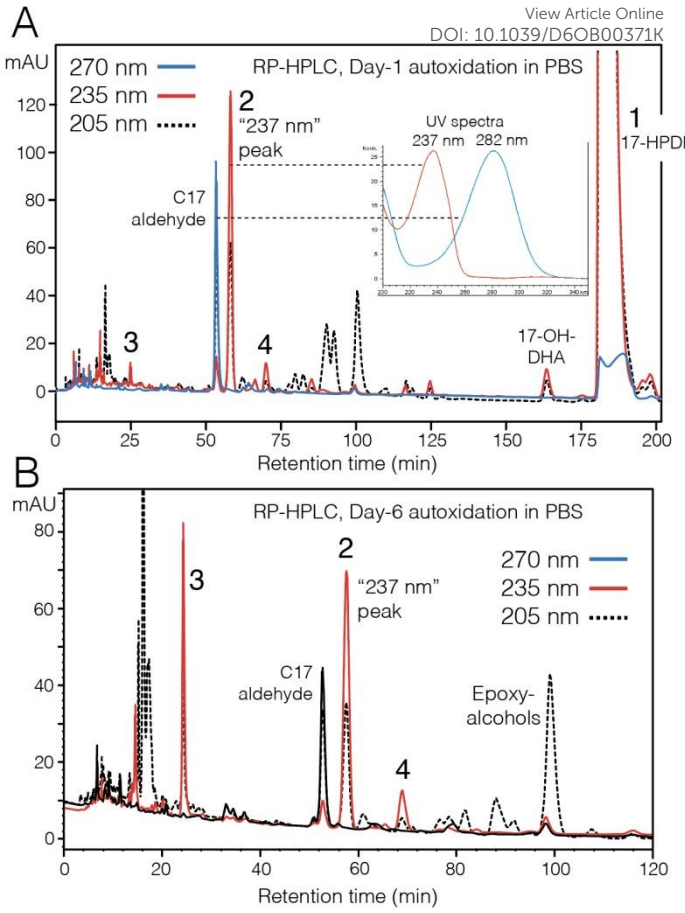


Fig. 2: RP-HPLC analyses of 17*S*-HPDHA autoxidation after 1 day and 6 days in PBS. A: RP-HPLC chromatogram of an aliquot after 1 day in PBS at 37 °C. B: Chromatogram (0 – 120 min) after 6 days in PBS at 37 °C. The major products are the C17 aldehyde (λmax 282 nm), and compound **2** (λmax 237 nm). In panel A, the small peak **3** at 25 min, after several days autoxidation becomes prominent in panel B. The small peak at ~70 min in both chromatograms, designated **4**, is of mechanistic significance and is identified later in Results. Autoxidation products were analyzed using a Waters Symmetry C18 column (25 x 0.46 cm), a solvent of acetonitrile/water/glacial acetic acid (45:55:0.01 by volume) with isocratic elution at a flow rate of 0.5 ml/min with diode array detection at 205, 235 and 270 nm.



The second main peak, eluting around 57 min, is prominent in the 235 nm channel recording and has a λmax at 237 nm and is designated as **2** on the chromatogram. As the autoxidation proceeded at 37 °C for additional days a tiny peak eluting just before 25 min on the day-1 chromatograms became a prominent 235 nm-absorbing product and it was designated the polar product **3** (Figure 2B). All the RP-HPLC chromatograms include another small 235 nm absorbing peak eluting at 70 min and designated as compound **4** in Figure 2A, 2B. Its significance developed later.

Close inspection of the UV spectra recorded by diode array detection on HPLC is revealing, Figure 3. Notably, the conjugated

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diene chromophores of the starting material 17-hydroperoxy-DHA and its 17-hydroxy analog clearly differ (Figure 3A). By contrast, the conjugated diene chromophores of the 237 nm product **2** and 17-hydroxy-DHA are an exact match, except at the lowest wavelengths towards 200 nm (Figure 3B). This strongly suggests that the structures of **2** and 17-HDHA near the conjugated diene are indistinguishable and the lower absorbance of **2** near 200 nm suggests it is lacking in the number of non-conjugated double bonds of 17-HDHA. Figure 3C shows that the UV spectra of the **2** and polar product **3** are identical. By a similar line of deduction, this suggests the two have an identical environment around the conjugated diene and identical non-conjugated double bonds.

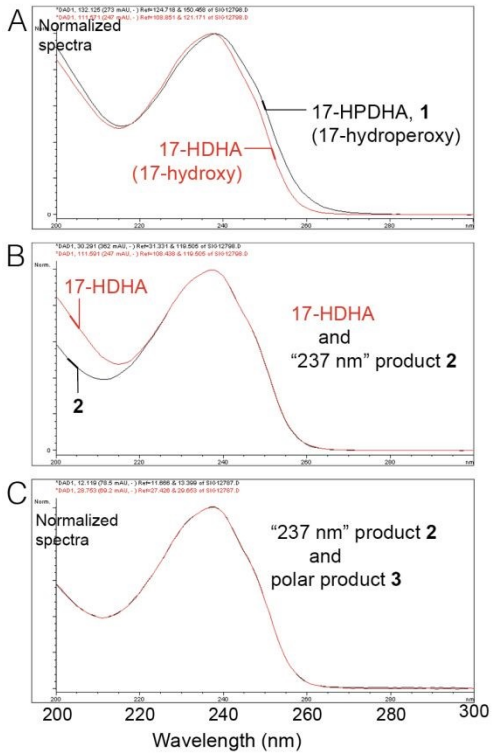

Fig. 3: Overlaid UV spectra from RP-HPLC of the 17*S*-HPDHA autoxidation. Spectra of the starting 17-hydroperoxide **1** and autoxidation products were recorded on-line in RP-HPLC solvent (CH_3CN/H_2O/HAc 45/55/0.01 by volume, cf. Fig. 2), and are normalized to absorbance maxima. A: 17-HPDHA and 17-HDHA. B: 17-HDHA and the "237 nm" **2**. C: Spectra of **2** and **3** from RP-HPLC (retention times ~57 and ~25 min, respectively).

Autoxidation should produce diastereomers from 17-HPDHA so the single clean peak of the 237 nm product **2** on RP-HPLC was anticipated to be resolved on a different HPLC system. NP-HPLC separated two diastereomers with identical UV spectra, and in proportions of 57:43, Figure 4. LC-MS and GC-MS (illustrated later) established molecular weights of compounds **2** and **3** as 360 and 378, respectively; 360 matches the molecular weight of the starting 17-HPDHA **1** and 378 represents the addition of 18 a.m.u, likely by hydrolysis with H_2O. Chemo-enzymatic synthesis of derivatives of 17-hydroxy-DHA helped eliminate potential candidates and establish the correct structural assignments.

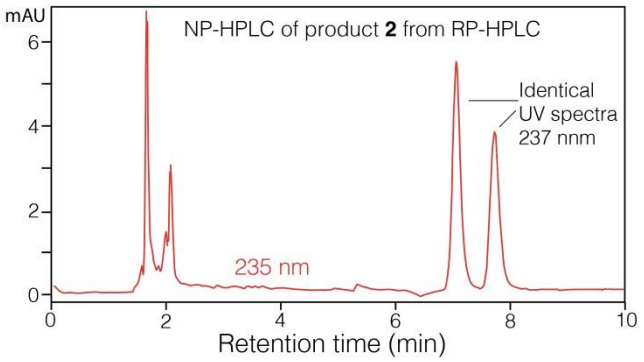

Fig. 4: NP-HPLC analysis of product 2 from 17*S*-HPDHA autoxidation. The 237 nm product **2** from RP-HPLC was analyzed using an Apollo 5 µ silica column (25 x 0.46 cm) with an isocratic solvent of hexane/isopropanol/glacial acetic acid (100:5:0.02 by volume) at a flow rate of 2 ml/min and showing the UV recording at 235 nm.

Candidates considered included 4,17-dihydroxy-22:6ω3 and 4,5-*cis*-epoxy-17-hydroxy-22:5ω3 **4** (both with a molecular weight of 360). The 4,17-dihydroxy derivative, produced via soybean LOX oxygenation of 4-hydroxy-DHA, was quickly excluded on account of its slightly broad conjugated diene chromophore and higher polarity on HPLC. Synthesis of the methyl ester of 4,5-*cis*-epoxy-17-hydroxy-22:5ω3 gave ^{1}H-NMR results consistent with its proposed structure (Supplement Figure S2) and clearly not matching key chemical shifts in the spectrum of **2** (Figure 5, text in next paragraph). Significantly, our attempted synthesis of the free acid of the 4,5-epoxy-17-alcohol gave an exact match to NMR of **2** ! The explanation turned out to be hydrolysis of the 4,5-epoxy moiety during the mildly acidic conditions used for extraction after the soybean lipoxygenase oxygenation at C17. Alkaline hydrolysis of the methyl ester with Oasis cartridge extraction of the KOH solution (acidification not required for efficient extraction) gave the free acid of the authentic 4,5-epoxy-17-alcohol **4** as confirmed by NMR (Supplemental Figure S3). This suggested that product **2** with molecular weight of 360 was the γ-lactone of 4,5,17-trihydroxy-docosa-7*Z*,10*Z*,13*Z*,15*E*,19*Z*-pentaenoic acid, a deduction confirmed by LC-MS, GC-MS and ^{1}H-NMR analyses. As shown below in the GC-MS analyses, the polar product **3** is the open form of the γ-lactone and thus 4,5,17-trihydroxy.

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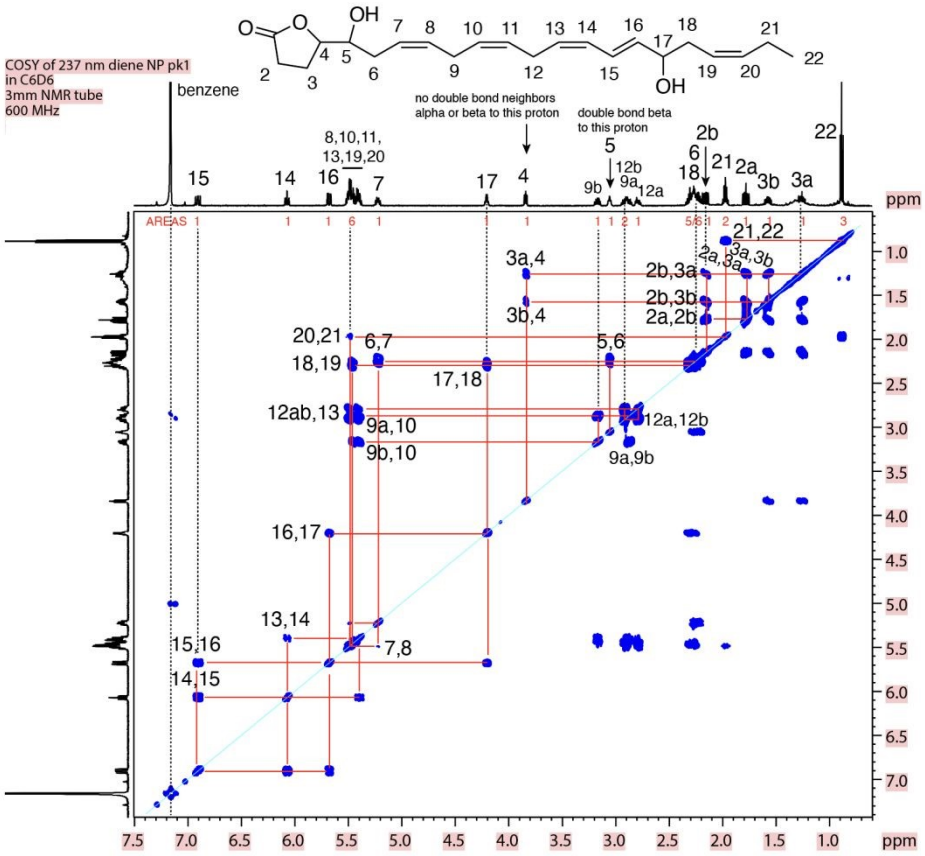

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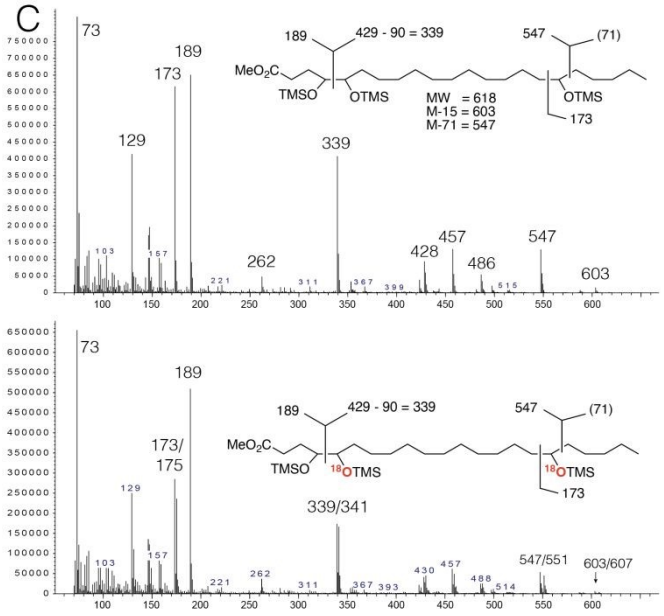
Fig. 5: Proton NMR spectrum and COSY analysis of the 237 nm product 2 in d_6-benzene . The spectrum and COSY were recorded on the first eluting diastereomer from NP-HPLC (cf. Fig. 4).

The proton NMR spectrum with COSY analysis of **2** shows downfield signals from the *cis-trans* conjugated diene between 5 – 7 ppm (cf. ref. [31]), with two signals near 4 ppm representing H17 and H4, and the H5 proton further upfield at 3.07 ppm, Figure 5. The existence of the γ-lactone is strongly supported by protons on the lactone ring being split into H2a, H2b and H3a, H3b on account of the chiral environment imposed by the cyclic structure and the lactone oxygen at C4. The CH_2 protons between double bonds at C9 and C12 are also split into separate signals for 9a/9b and 12a/12b; although these do not have chiral neighbors, the environment has chirality due to folding over of the carbon chain as originally deduced and illustrated in the proton NMR spectrum of 12-HETE in d_6-benzene.[32] ^{1}H-NMR (600 MHz, C_6D_6), Chemical shift, number of protons, multiplicity, *J*, and proton number: δ (ppm), 6.91 (1H, dd, J = 15, 11 Hz, H15); 6.07 (1H, t, J = 11 Hz, H14); 5.67 (1H, dd, J = 15.2, 5.3 Hz, H16); 5.38 – 5.52 (6H, m, H8, H10, H11, H13, H19, H20); 5.22 (1H, m, H7); 4.20 (1H, q, H17); 3.84 (1H, m, H4); 3.16 (1H, m, H9b); 3.05 (1H, m, H5); 2.84 – 2.95 (2H, m, H9a, H12b); 2.79 1H, m, H12a); 2.34 - 2.19 (4H, m, H6, H18); 2.19 - 2.13 (1H, m, H2b); 1.97 (2H, quintet, J = 7 Hz, H21); 1.78 (1H, m, H2a); 1.57 (1H, m, H3b); 1.26 (1H, m, H3a); 0.88 (3H, t, J = 7 Hz, H22).



Autoxidation of ^{18}O-labeled 17-HPDHA

In order to distinguish *intra*molecular transfer of hydroperoxy oxygens from *inter*molecular exchange, autoxidation was conducted using 17-HPDHA labeled with two ^{18}O in the hydroperoxy group mixed with a similar proportion of unlabeled 17-HPDHA ([^{16}O^{16}O]17-HPDHA); any cross-labeling or oxygens from outside sources would give ^{18}O^{16}O mixtures, readily discernible by mass spectrometry. GC-MS of hydrogenated 17-HDHA and the γ-lactone and trihydroxy products **2** and **3** gave easily visualized results, including a definitive look at the positions of the hydroxyls and their ^{16}O/^{18}O labels. The mass spectrum of the 17-HDHA derivative shows an ion fragment at m/z 173/175 representing the fatty acid tail including the 17-hydroxyl, Figure 6A, (see over). The mass spectrum of the γ-lactone product **2** shows the m/z 173/175 ions with their ratio suggesting slightly less than complete retention of ^{18}O although the higher mass ions (m/z 443/447) show that mainly two ^{18}O are retained (Figure 6B). The corresponding mass spectrum of the polar product **3** is of particular interest because the m/z 189 ion, a fragment representing carbons 1 – 4, demonstrates there is no oxygen-18 on the C4 hydroxyl, while the high mass ions (e.g. m/z 547/551) confirm there are two ^{18}O atoms in the molecule, with ion fragments establishing these are at C5 and C17 (Figure 6C).

Fig. 6: Mass spectra from GC-MS analysis of unlabeled and ^{16}O/^{18}O-labeled 17*S*-HDHA 1, γ-lactone 2, and trihydroxy 3. The compounds were hydrogenated and derivatized then analyzed by GC-MS in the electron impact mode. A: Unlabeled and ^{16}O/^{16}O-labeled 17-HDHA prepared from the 1:1 mixture of ^{16}O-^{16}O and ^{18}O-^{18}O 17-hydroperoxide used in the incubation in PBS. B: unlabeled and labeled product **2** from the $^{16}O_2$/$^{16}O_2$ 17-HPDHA incubation. C: unlabeled and labeled product **3** from the $^{16}O_2$/$^{16}O_2$ 17-HPDHA incubation.

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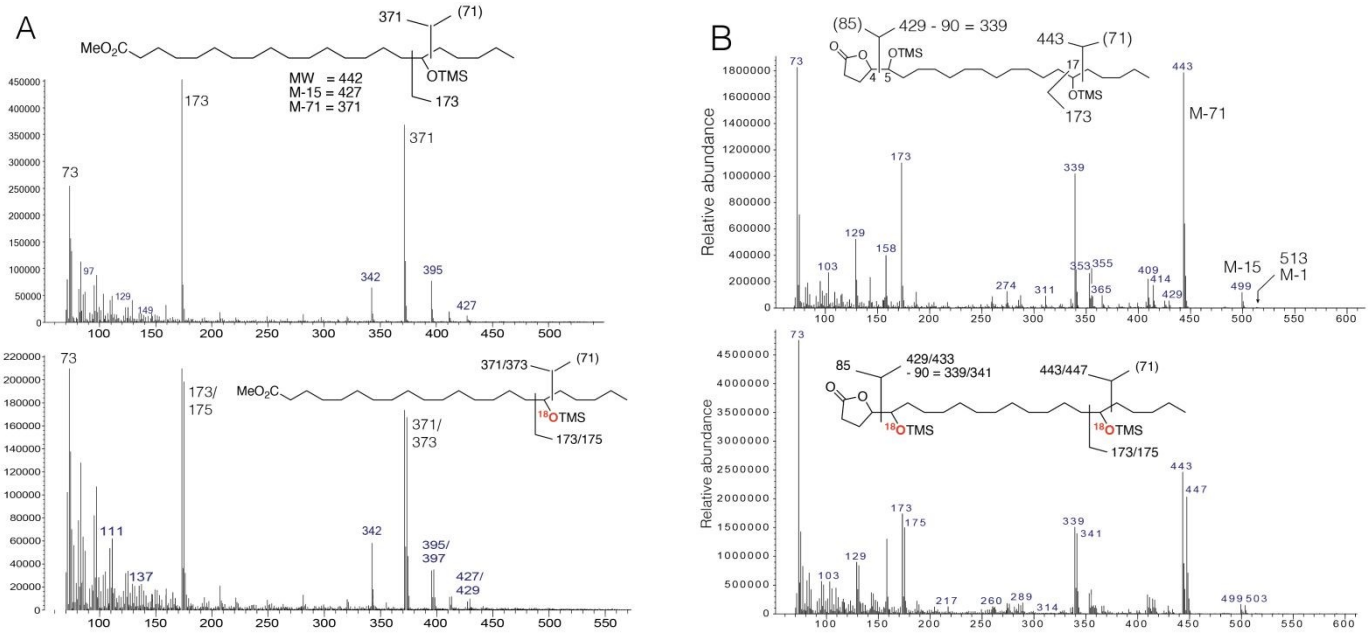

The percent retention of $^{16}O/^{18}O$ labels in the products was quantified by LC-MS as this gave superior signal-to-noise which was helpful for the M+2 signals representing mixed ^{16}O-^{18}O composition. The results for the product 2 measured at [M-H + acetate]$^-$ at m/z 419/423 were 96.5 % retention of two ^{18}O labels with the measured abundance of the mixed signal for ^{16}O-^{18}O cancelling to zero based on the 3 % content in the 17-HPDHA substrate. In the polar product **3**, the LC-MS results indicated 90.0 % retention of two ^{18}O based on abundances of the [M-H]$^-$ ion at m/z 379/383.

Thus, the structure of the parent non-hydrogenated product **3** is 4,5,17-trihydroxy-docosa-7*Z*,10*Z*,13*Z*,15*E*,19*Z*-pentaenoic acid **3**. It appears as a major product after several days in PBS at 37 °C due to slow hydrolysis of the γ-lactone product. This was confirmed by incubation of the purified γ-lactone product for 5 days in PBS, resulting in 65% hydrolysis to its open form, the trihydroxy derivative **3** (Supplement Figure S4).

Identification of 4,5-epoxy-17S-hydroxy-22:5ω3 in the autoxidation

In Figure 2, the small 70 min peak eluting after the γ-lactone and designated as 4 was found to match in UV spectrum, retention time on both RP-HPLC and NP-HPLC, and mass spectrum on LC-MS to synthetic 4,5-*cis*-epoxy-17*S*-hydroxy-22:5ω3. A synthetic standard for the corresponding *trans*-4,5-epoxy-17-hydroxy-22:5ω3 was not available although its retention time is expected to be close to the *cis*-epoxide (cf.[33, 34]) and there are no nearby peaks with the required 235 nm absorbance and with an appropriate UV spectrum, strongly suggesting the product is exclusively the *cis*-epoxide. The synthetic product (prepared from racemic 4,5-*cis*-epoxide) gave two equal-sized peaks on NP-HPLC whereas the proportions of the diastereomers from the autoxidation were 1:3 in order of elution, indicating some retention of chirality in its formation from pure 17*S*-HPDHA.

Finally, from the structural analyses and the ^{18}O experiments it is apparent that a 17-hydroperoxide oxygen is transferred internally within the molecule onto the 4,5-double bond. There remains a significant mechanistic question: is the product of oxygen transfer the 4,5-epoxide which then opens to the γ-lactone during the incubation in PBS, or is formation of the γ-lactone intrinsic to the mechanism? RP-HPLC analysis of synthetic 4,5-*cis*-epoxy-17-hydroxy-22:5ω3 incubated for 24 h at 37 °C in PBS (pH 7.4) gave a definitive answer of no detectable lactonization (Supplement Figure S5), suggesting that the γ-lactone is a primary product of the oxygen transfer reaction.

Discussion

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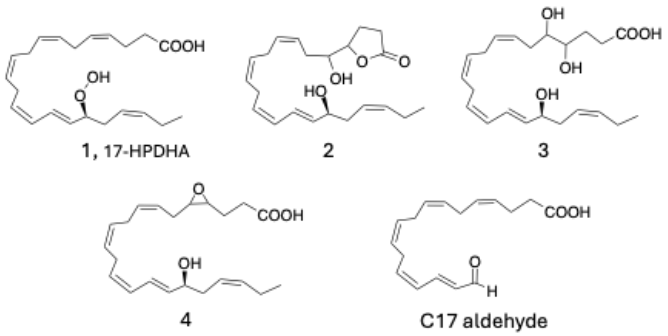
Herein we found an unusual, unexpected and unique major product from the incubation of 17*S*-HPDHA **1** in PBS at 37 °C and identified it as stereoisomers of **2**, the γ-lactone of 4,5,17-trihydroxy-docosa-7*Z*,10*Z*,13*Z*,15*E*,19*Z*-pentaenoic acid, Scheme 1 with associated products.



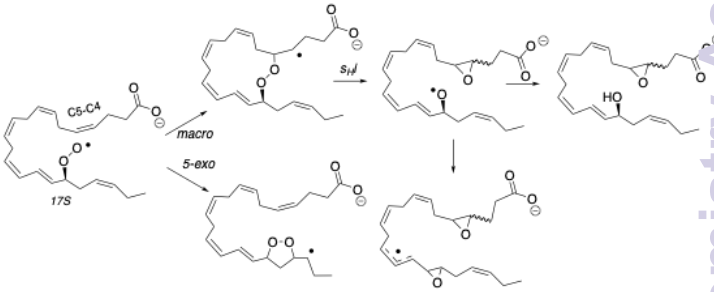
Scheme 1: Structures of 17*S*-HPDHA and identified products

As established by the oxygen-18 experiment, the two diastereomers of the γ-lactone **2** are formed via an internal oxygen transfer from the 17-hydroperoxide. Almost certainly this oxygen transfer is facile on account of an optimal length of the carbon chain from the C17 hydroperoxide to the 4,5-double bond. Such a requirement constrains the number of naturally occurring hydroperoxy fatty acids on which this reaction could occur, most probably limited to several species of docosanoid hydroperoxide (in addition to 17-HPDHA, potentially 4-, 7-, or 20-hydroperoxides) and perhaps an 18-hydroperoxide of eicosapentaenoic acid. Interestingly, these DHA-related candidates might form an epoxide as the major product as, unlike in the case with transfer from 17-HPDHA, there is no likely aptitude for involvement of the fatty acid carboxyl. For 18-hydroperoxy-EPA reacting on the 5,6-double bond, there is the potential for formation of a δ-lactone with the C1 carboxyl and direct involvement of the carboxyl group may also facilitate this overall transformation.

Formation of these molecules in cells or tissues will depend on burgeoning lipid peroxidation or lipoxygenase activity, and perhaps with the hydroperoxide in a protected environment, as alternatives are reduction to the hydroxy derivative, further lipoxygenase metabolism, or competing radical reactions. A free carboxyl is a prerequisite for γ-lactone synthesis, although oxygen transfer to epoxide may occur from esterified hydroperoxide in membranes. Notably, as a neutral molecule the γ-lactone is poorly ionizable for LC-MS analysis, compromising its detectability. In our experiments with HPLC-UV the γ-lactone was prominent, roughly matching the appearance of aldehyde and epoxy alcohols that do have a natural occurrence (e.g. refs[5, 35-38]).

The evidence points to the γ-lactone being a primary product of the 17-HPDHA reaction and not a secondary product from a 4,5-epoxide. There is a small peak of 4,5-*cis*-epoxy-17-alcohol **4** on the HPLC chromatograms but a control experiment established it is stable at pH 7.4 and does not lactonize under the conditions of the incubations in PBS. Nonetheless, as expected, the synthetic 4,5-*cis*-epoxy-5,17-dihydroxy docosanoid is acid labile, as we discovered when using acidic conditions for its extraction (described in Results). This acid hydrolysis gave two γ-lactone diastereomers of 4,5,17-trihydroxy-22:5ω3 identical to the product from PBS. This consolidates the structural assignment of the product from 17-HPDHA as matching the hydrolysis and lactonization derivatives of a 4,5-*cis*-epoxide (and not a 4,5-*trans*-epoxide).

We considered two possible mechanisms to account for the oxygen transfer and including formation of the γ-lactone moiety. A peroxyl radical mechanism would involve a 15-*exo* macrocyclization, followed by an intramolecular homolytic substitution (s_Hi) of the carbon radical on the peroxide bond to form 4,5 epoxides, as shown in Scheme 2.[39]



Scheme 2: Potential peroxyl radical reactions of 17-hydroperoxide **1**

16-*endo* macrocyclization of the peroxyl would provide a parallel mechanistic pathway to the product epoxides. In support of this mechanism, free radical macrocyclizations are well-known[40] and this mechanism would account for the ^{18}O labelling results as well, with one oxygen from the hydroperoxide transferred to C5 of the product lactones.

There are, however, reasons to cast doubt on the peroxyl radical mechanism. First, the 17*S* peroxyl radical has a favorable competing pathway to the proposed macrocyclization, namely the 5-*exo* cyclization shown in Scheme 2. This cyclization to endoperoxide is a cornerstone reaction in lipid peroxidation chemistry, with dozens of examples reported.[41] In this case the endoperoxide products would retain the conjugated diene chromophore of 17-HPDHA and would be readily detected by HPLC-UV but were not apparent on the RP-HPLC-UV chromatograms. Second, the isolated products from the 17*S* hydroperoxide reaction at 37°C in pH 7.4 buffer are diastereomeric γ-lactones but the epoxide products of the radical mechanism are not converted to diastereomeric γ-lactones in pH 7.4 buffer. Third, epoxide formation by the s_Hi mechanism generally favors formation of *trans* substituted epoxides but the two

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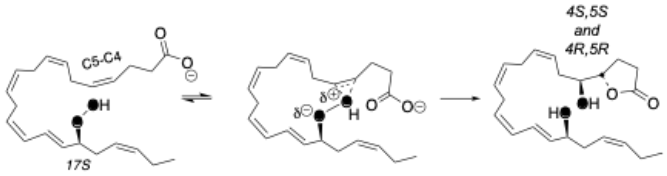
diasteromeric lactone products are selectively formed from independently prepared *cis* epoxides. And fourth, there is ample precedent for the formation of the intermediate alkoxyl radical in the Scheme 2[5, 42], a transformation that competes favorably with the H atom transfer required to give the 17-hydroxyl of the major product **2** in PBS.

An alternative mechanism that accounts for the observations, on balance with more points in its favor, is presented in Scheme 3.



Scheme 3: Hydroperoxide transfer in conversion of hydroperoxide **1** to γ-lactone **2**

This suggests involvement of both the C17-hydroperoxide and the C1-carboxylate in the oxygen transfer process to C4-C5. Such a mechanism is consistent with the observations that ^{18}O is transferred to C5 from the hydroperoxide, that the structures of the diastereomeric γ-lactones formed from the hydroperoxide at pH 7.4 are identical to those we formed (initially unintentionally, see early results above) by acid hydrolysis of the 4,5-*cis*-epoxy-17-hydroxy-22:5ω3.

There are other examples of oxygen transfers to remote centers in the chemistry of polyunsaturated fatty peracids. Peroxyarachidonic acid for example, selectively reacts to give the 14,15-*cis*-epoxy arachidonate (14,15-EET) in >98% yield, in this case the reaction occurring at the double bond in the molecule that is most remote from the peroxyacid [43]. This remote site reactivity can be understood by consideration of the "effective molarity" of reaction centers in ring-forming reactions.[44, 45] Cyclization reactions forming three to six membered rings are favored since the effective molarity of one reactive center with its partner reactant is high. Seven to twelve-membered ring formation is not favored since these rings sizes have destabilizing cross ring steric interactions and the effective molarity of reactant centers forming mid-size rings is lower than the smaller ring sizes. Cross-ring destabilization is reduced in fourteen to eighteen-membered ring transition states and the effective molarity of reactive centers increases for these ring sizes.

The arachidonic peroxyacid direct oxygen transfer [43] occurs *via* a sixteen-membered ring in which cross-ring steric effects are reduced and the effective molarity of the C14-C15 double bond is favorable relative to the transfer oxygen center. In comparison, the oxygen transfer reaction proposed in Scheme 3 occurs by a fifteen-membered ring. While this ring size is favorable, the transformation is nevertheless surprising. In contrast to peroxyacids, the reaction of hydroperoxides with alkenes generally requires metal catalysis or occurs by free radical mechanisms.

To account for formation of the γ-lactone products in this system, we suggest that the C1 carboxylate of 17*S*-HPDHA participates in the oxygen transfer process in a way that parallels carboxylate participation in well-known iodo-lactonization reactions of polyunsaturated fatty acids, see Scheme 3.[46, 47] Iodo-lactonizations of DHA and EPA for example, give high yields of the corresponding γ- and δ-lactones.[47] In these reactions, a presumed reversible alkene-iodine complex is trapped by the nearby carboxylate, a sequence that is reproduced in Scheme 3 for the hydroperoxide transfer.

A final *caveat* for any discussion of mechanism for the conversion of 17*S* HPDHA to the γ-lactone products is the fact that the reaction is carried out in pH 7.4 buffer while the precedents for free radical reactions described come for the most part, from those carried out in isotropic organic solvents. The medium for the reactions described here is likely micellar in nature and this could well affect the molecular reorganizations required for selectivity and reactivity of peroxyl radical species.

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Experimental

Materials

Docosahexaenoic acid was purchased from Nu-Check Prep (Elysian, MN) and protectin D1 from Cayman Chemical (Ann Arbor, MI). Soybean LOX (lipoxidase, type V) and 10 x PBS (P7059), were from Sigma. HPLC hexanes were purchased from Burdick and Jackson (Muskegon, MI). Other reagents were purchased from Aldrich (Milwaukee, WI) or Acros Organics (Morris Plains, NJ), and oxygen gas (^{18}O 98 %, 100 ml) from Icon stable isotopes (Summit, NJ).

Synthesis and purification of 17*S*-hydroperoxy-docosahexaenoic acid, 1

The stereospecific 17*S*-hydroperoxydocosahexaenoic acid **1** was synthesized by soybean lipoxygenase catalyzed reactions following methods described for linolenate with minor modifications.[48] The DHA substrate (50 mg, redissolved in 200 μl ethanol) was prepared in dipotassium phosphate buffer (100 ml, 0.1M, pH 8.7) from a stock solution (20 mg/ml). The mixture was saturated with oxygen and pre-equilibrated in an ice bath at 4 °C, followed by the addition of soybean LOX in aliquots of 300 μg in 100 μl of buffer under constant stirring and oxygenation in an ice bath. The activity was measured spectrophotometrically by monitoring the product formation at 238 nm (maximum of absorption of conjugated dienes) with 10 μl aliquot in 490 μl buffer. After the activity was observed to have stalled after ~30 minutes while avoiding formation of undesired products observable at around 270 nm (of conjugated trienes), the reaction was terminated by adding hydrochloric acid dropwise to bring the pH to <5. Products were extracted with dichloromethane, the extract washed with a small volume of water, and evaporated under a stream of N_2, and purified by preparative SP-HPLC using a TLC Advantage semi-preparative silica column (25 x 1 cm) with a solvent of hexane/isopropanol/glacial acetic acid (100:3:0.02 by volume) at a flow rate of 4 ml/min. Injections of 3 – 5 mg were chromatographed and the purified 17*S*-HPDHA quantified by UV in ethanol (λmax 237 nm, ε = 25,000 M^{-1} cm^{-1}).

Synthesis of 4*RS*-*cis*-epoxy,17*S*-hydroxy-docosapentaenoic acid

Of the epoxides formed from DHA by mCPBA, the 4,5-epoxide has the longest lipophilic tail and is the most highly retained on RP-HPLC (cf. 5,6-epoxide of arachidonic acid[49]). Its relative retention is more prolonged than might be anticipated and it is missing from DHA-derived epoxides in several published analyses e.g. refs[50, 51], possibly also due its facile lactonization in acid. DHA (10 mg) was treated with a 2-fold molar excess of mCPBA in DCM on ice for 30 min and the solution then washed twice with a half volume of cold 0.1 M phosphate pH 8 followed by a wash with cold water. The resulting epoxides were separated on a semi-preparative Waters Symmetry 7 μ C18 column (15 x 0.78 cm) with a solvent of MeOH/H_2O/HAc (80:20:0.01 by volume) at a flow rate of 3 ml/min. The last-eluting epoxide, separated by 5 min from the others at a retention time of 29 min, was collected and a small aliquot used for quantitation based on reaction with soybean LOX and assuming ε = 25,000 M^{-1} cm^{-1} for the conjugated diene product. A total of 310 μg was recovered as pure 4,5-*cis*-epoxy-docosapentaenoic acid, and a larger scale soybean LOX reaction in 4 ml 0.1 M K_2HPO_4 pH 8.7 buffer was used to convert 100 μg to the 4,5-epoxy-17-hydroperoxy derivative. After complete reaction as confirmed by UV scanning, the 17-hydroperoxy group was reduced with $NaBH_4$ *in situ* and the alkaline solution applied without acidification to an equilibrated 60 mg Oasis cartridge (Waters). UV scanning of the eluate proved that the lipids were completely retained. The Oasis cartridge was washed with 3 – 4 ml water until the eluate was neutral, and the product collected by elution with 3 ml ethyl acetate. The 4*RS*-epoxy-17*S*-hydroxy diastereomers were resolved by NP-HPLC using a Grace Apollo 5μ silica column (25 x 0.46 cm) with a solvent of hexane/isopropanol/glacial acetic acid (100:3:0.02 by volume) at a flow rate of 1 ml/min with retention times of 8.34 and 8.73 min, and were then subject to NMR in d_6-benzene (illustrated for the first-eluting diastereomer in Supplemental Figure S3).

Synthesis of 4*RS*,17*S*-dihydroxy-docosa-5*E*,7*Z*,10*Z*,13*Z*,15*E*,19*Z*-hexaenoic acid

DHA (10 mg) was converted to the γ-lactone of 4-hydroxy-DHA via the iodolactone [52] and a 5 mg aliquot purified by RP-HPLC (Beckman semi-preparative C18 column, 25 x 1 cm, solvent CH_3CN/H_2O/HAc 80:20:0.01 by volume, retention time 8.7 min at 5 ml/min). To form the 4-hydroxy-17-hydroperoxy derivative, the γ-lactone (200 μg in a 5 ml Reactivial) was opened in 20 μl 1 M KOH in MeOH/H_2O (80:20 by volume) for 15 min at RT, 0.1 M K_2HPO_4 (4 ml) was added followed by 1 μg/ml soybean LOX (Sigma, type V); UV scanning (200 – 350 nm) showed within 30 s the 4-HDHA was converted completely to a new product with double the UV absorbance of the starting 4-HDHA. The K_2HPO_4 solution was applied to a 60 mg Oasis cartridge without acidification, the cartridge washed with water and the product

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eluted with ethyl acetate. Treatment with a molar excess of triphenylphosphine in 50 µl MeOH for 15 min at RT gave the 4,17-dihydroxy derivative that was purified by RP-HPLC (Waters Symmetry C18 column, 25 x 0.46 cm, solvent CH_3CN/H_2O/HAc 55:45:0.01 by volume, retention time 9.8 min at 1 ml/min) and the proton NMR spectrum acquired in d_6-benzene on the two diastereomers together. ^{1}H-NMR (600 MHz, C_6D_6), Chemical shift, number of protons, multiplicity, *J*, and proton number: δ (ppm), 6.73 (1H, dd, J = 15, 11 Hz, H15); 6.61 (1H, dt, J = 15, 10 Hz, H6); 6.03 (1H, t, J = 11 Hz, H14); 5.98 (1H, t, J = 11 Hz, H7); 5.61 (1H, dd, J = 15, 5.5 Hz, H16); 5.49 (1H, br. dt, H20); 5.33 – 5.46 (6H, m, H5, H8, H10, H11, H13, H19); 4.11 (1H, quintet, J = 6 Hz, H17); 4.04 (1H, apparent q, J = 6 Hz, H4); 2.85 – 3.05 (4H, m, H9, H12); 2.17 – 2.32 (4H, m, H12, H18); 1.95 (2H, quintet, J = 7 Hz, H21); 1.68 (2H, br. quintet, H3); 0.88 (3H, t, J = 7 Hz, H22).

Autoxidation of 17*S*-HPDHA 1 in PBS

17*S*-HPDHA was added to 15 – 150 ml of 1 x PBS, pH 7.4, in 0.1% volume of ethanol at a final concentration of approximately 30 - 40 µM (giving ~1.0 AU absorbance at 237 nm) and incubated at 37 °C for up to 6 days. The UV spectrum of the PBS solutions was recorded daily. Two methods of extraction were employed: either acidification to pH 4 – 5 and extraction with dichloromethane which was then washed with a small volume of water and taken to dryness under a stream of N_2, or for extraction under neutral conditions with volumes up to 15 ml, direct application without acidification to a 60 mg Oasis cartridge (Waters) equilibrated in methanol then water, with a final water wash to remove salts and elution using 3 ml ethyl acetate.

HPLC-UV analyses

Aliquots of the autoxidation products were analyzed by RP-HPLC using a Waters Symmetry 5 µ C18 column (25 × 0.46 cm), with a solvent of acetonitrile/water/glacial acetic acid (45/55/0.01 by volume), at a flow rate of 0.5 or 1 mL/min, with on-line UV detection at 205 nm, 220 nm, 235 nm, and 270 nm on an Agilent 1100 series diode array detector. Further separation of diastereomers was achieved by normal phase HPLC (NP-HPLC) using an Apollo 5 µ silica column (25 × 0.46 cm) using an isocratic solvent of hexane/isopropanol/glacial acetic acid (100/0.5/0.01, by volume) run at 2 mL/min with on-line UV detection at 235 nm.

LC-MS analyses

HPLC profiles were analyzed using a Thermo TSQ Vantage Triple Quadrapole MS instrument (Thermo Fisher Scientific, Waltham, MA). RP-HPLC-MS analysis was performed with electrospray ionization in the negative ion mode. A Phenomenex Kinetex C18 2.6 µ column (100 X 3 mm) was eluted isocratically with CH_3CN/H_2O/HOAc (40:60:0.01 by volume) at a flow rate of 0.3 mL/min. The electrospray voltage was set at 4.0 k V: vaporizer temperature at 300°C; sheath and auxiliary gas pressure at 50 and 5 'V, respectively; and capillary temperature at 300°C.

GC-MS analyses

Oxidation products were hydrogenated (by Pd/ H_2) and converted to methyl ester and TMS ether derivatives for GC-MS analyses. Methyl ester derivatives were prepared by treatment with diazomethane. TMS ether derivatives were prepared by treatment with 10 µl BSTFA plus 2 µl pyridine for at least 1 h at room temperature. Samples were then evaporated to dryness and dissolved in hexane. Aliquots of ~10 to 100 ng were analyzed by GC-MS utilizing a DB-5 column (30 m × 0.25 mm, Agilent) on a Thermo-Finnigan DSQ mass spectrometer operated in the positive ion electron impact mode (70eV) with temperature programming at 10°C/min from 150 to 300°C.

NMR analyses

^{1}H NMR and ^{1}H, ^{1}H COSY NMR experiments were acquired using a 14.0 T Bruker magnet equipped with a Bruker AV-III console operating at 600.13 MHz. Spectra were acquired in 3 mm NMR tubes using a Bruker 5mm TCI cryogenically cooled NMR probe. Chemical shifts were referenced internally to d_6-benzene (7.16 ppm).

Conclusions

Herein we uncovered a novel mechanism of lipid peroxidation involving intramolecular, macrocyclic oxygen transfer producing a regiospecific oxygenation product, exceptional in the annuls of non-enzymic fatty acid chemistry. As we point out in Discussion, macrocyclic transfer is favored in fourteen to eighteen-membered ring transition states as the effective molarity of reactive centers increases for these ring sizes. Participation of the carboxyl appears to be inherent to the mechanism, leading in this case to the γ-lactone of the trihydroxy-docosapentaenoic acid. On account of the preference for ring-size in the transition, the mechanism is open to other highly polyunsaturated lipids with a donating hydroperoxide and suitably positioned intramolecular recipient.

Data availability

Data are included in the manuscript and Supporting Information. Original data are available upon reasonable request.

Author contributions

MSC. Data acquisition and analysis, manuscript writing and editing; WEB. Data acquisition, manuscript editing; DFS. Data acquisition, manuscript editing; MWC. Data acquisition, manuscript editing; NAP. Experimental design, manuscript writing and editing; ARB.

Experimental design, funding acquisition, data acquisition and analysis, figure generation, manuscript writing and editing.

Conflicts of interest

The authors declare that they have no competing interests.

Author contributions

MSC. Data acquisition and analysis, manuscript writing and editing; WEB. Data acquisition, manuscript editing; DFS. Data acquisition, manuscript editing; MWC. Data acquisition, manuscript editing; NAP. Experimental design, manuscript writing and editing; ARB. Experimental design, funding acquisition, data acquisition and analysis, figure generation, manuscript writing and editing.

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Data availability

Data are included in the manuscript and Supporting Information. Original data is available upon reasonable request.