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

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We analyzed the autoxidation and non-enzymic reactions of the 17*S*-hydroperoxide of docosahexaenoic acid **1**, a common lipoxygenase product in mammalian cells, which, with its six double bonds, can undergo reactions not accessible to all polyunsaturated fatty acids. Incubation in phosphate-buffered saline for one or more days at 37 °C revealed a dominant product identified by comparison to synthetic standards 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**; however, this epoxide is stable in PBS at pH 7.4, indicating that the γ-lactone is a primary product rather than a secondary product formed from 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 the retention of both hydroperoxy oxygens on the 5- and 17-carbons. Direct involvement of the 17-hydroperoxide group in oxygen transfer with the participation of the C1 carboxyl in a mechanism with high "effective molarity" at the 4,5-double bond can account for the findings. Other fatty acid hydroperoxides with a similar spatial relationship between the peroxide and double bonds could also undergo this intramolecular oxygen transfer, a novel pathway in lipid peroxidation.

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Introduction

The primary reactions during the autoxidation of unsaturated fatty acids were first studied with oleic and linoleic acids, and this was 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 three or four methylene-interrupted double bonds gives further opportunities for complex product outcomes, including secondary transformations giving cyclized carbon rings and endoperoxides (the mechanistic basis of prostaglandin biosynthesis).[6–13]

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 monohydroperoxides.[14,15] In biology, much attention is directed toward the pathways arising from 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 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 successfully investigated the α-tocopherol-controlled formation of dihydro(pero)xides conducted as a neat oil (detailed in the SI). 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.* ref. 20 and 21).

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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 analysis showed two prominent new peaks dominating the chromatogram, with no evidence of the mixture of dihydroperoxides observed in the enzyme reactions and also in 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 17*S*-HPDHA **1** in PBS at 37 °C, over the course of several days, the UV spectrum showed a reduction in the signal of the conjugated diene chromophore with an increase in absorbance around 280 nm, as seen in Fig. 1. RP-HPLC chromatograms of extracts from day 1 and day 6 of autoxidation are illustrated in Fig. 2; they were run at a relatively slow solvent flow rate (0.5 mL min^{-1}) to help optimize resolution. Two single product peaks dominate the day-1 chromatogram, as shown in Fig. 2A. The first, eluting just after 50 min, was recorded mainly in the 270 nm channel and its UV spectrum has λ_{max} of 282 nm in the RP-HPLC column solvent (inset, Fig. 2A). It was readily identified by LC-MS ($[M - H]^-$ *m*/*z*: 273) as a C17 aldehyde fatty acid. Its formation during autoxidation is not unexpected and is well precedented.[5,30]

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

A close inspection of the UV spectra recorded by diode array detection from HPLC reveals important features, as shown in Fig. 3. Notably, the conjugated diene chromophores of the starting material 17-hydroperoxy-DHA and its 17-hydroxy analog clearly differ (Fig. 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 (Fig. 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 lacks a number of non-conjugated double

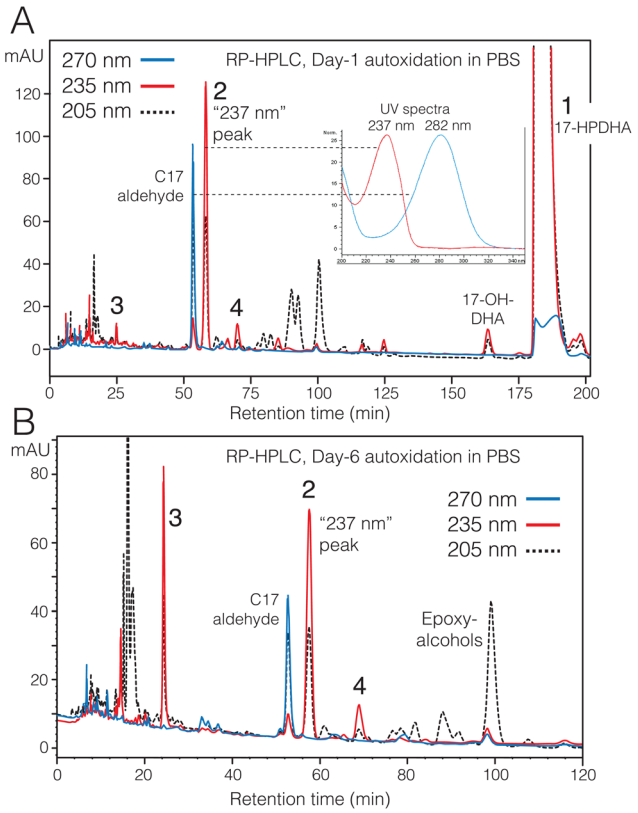


Fig. 2 RP-HPLC analyses of 17*S*-HPDHA autoxidation after 1 day and 6 days in PBS. (A) The RP-HPLC chromatogram of an aliquot after 1 day in PBS at 37 °C. (B) The chromatogram (0–120 min) after 6 days in PBS at 37 °C. The major products are the C17 aldehyde (λ_{max}: 282 nm) and compound **2** (λ_{max}: 237 nm). The small peak **3** at 25 min in panel A becomes prominent in panel B after several days of autoxidation. The small peak at ~70 min in both chromatograms, designated **4**, is of mechanistic significance and is identified later in the Results section. Autoxidation products were analyzed using a Waters Symmetry C18 column (25 × 0.46 cm) and 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^{-1} with diode array detection at 205, 235 and 270 nm.

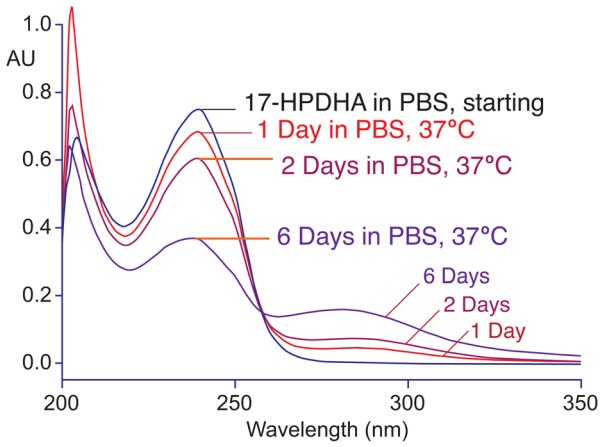


Fig. 1 UV spectra recorded during the course of 17*S*-HPDHA autoxidation in PBS. UV spectra of the autoxidation mixture were recorded in PBS without dilution on days 0, 1, 2 and 6.

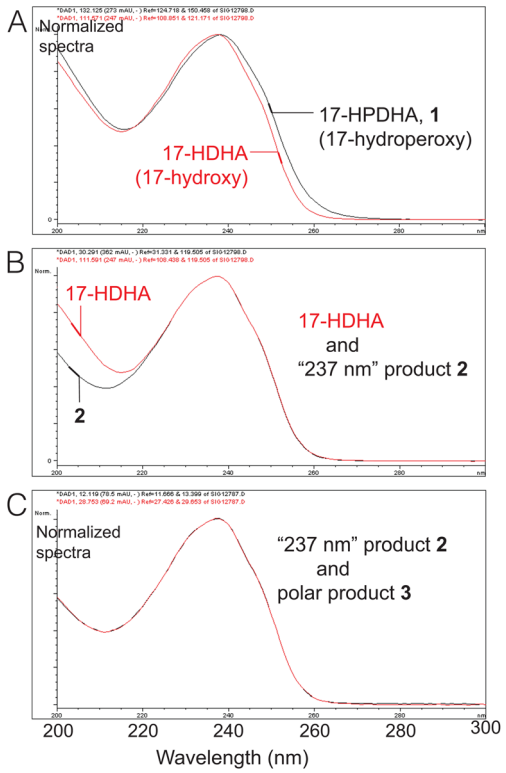


Fig. 3 Overlaid UV spectra from RP-HPLC analysis of 17*S*-HPDHA autoxidation. Spectra of the starting 17-hydroperoxide **1** and autoxidation products were recorded online in RP-HPLC solvent (CH_3CN/H_2O/HAc: 45/55/0.01 by volume, *cf.* Fig. 2) and are normalized to absorbance maxima. (A) 17-HPDHA and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

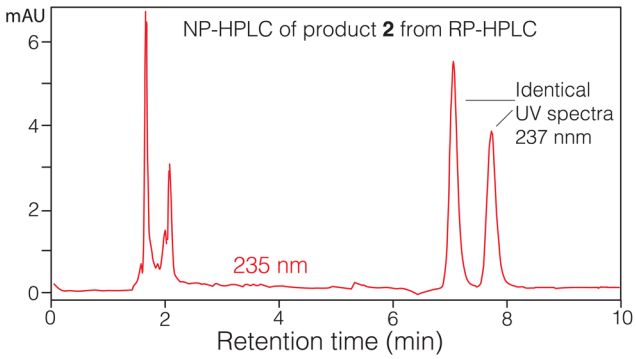


Fig. 4 NP-HPLC analysis of product **2** from 17*S*-HPDHA autoxidation. The 237 nm product **2** from RP-HPLC was analyzed using an Apollo 5 μm silica column (25 × 0.46 cm) with an isocratic solvent of hexane/isopropanol/glacial acetic acid (100 : 5 : 0.02 by volume) at a flow rate of 2 mL min^{-1}, and the UV recording at 235 nm is shown.

bonds of 17-HDHA. Fig. 3C shows that the UV spectra of **2** and the polar product **3** are identical. By a similar line of deduction, this suggests that the two have an identical environment around the conjugated diene and identical non-conjugated double bonds.

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

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 in HPLC. Synthesis of the methyl ester of 4,5-*cis*-epoxy-17-hydroxy-22:5ω3 gave ^{1}H NMR results consistent with a proposed structure (SI Fig. S2) and clearly not matching key chemical shifts in the spectrum of **2** (Fig. 5, discussed below). Significantly, our attempted synthesis of the free acid of the 4,5-epoxy-17-alcohol gave an exact match with the NMR results for **2**. The explanation turned out to be the hydrolysis of the 4,5-epoxy moiety under the mildly acidic conditions used for extraction after soybean lipoxygenase oxygenation at C17. Alkaline hydrolysis of the methyl ester with Oasis cartridge extraction from KOH solution (acidification was not required for efficient extraction) gave the free acid of the authentic 4,5- epoxy-17-alcohol **4**, as confirmed by NMR (SI Fig. S3). This suggested that product **2**, with a 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 discussed below in relation to GC-MS analyses, the polar product **3** is the open form of the γ-lactone and thus 4,5,17-trihydroxy.

The proton NMR spectrum with COSY analysis of **2** shows downfield signals from the *cis–trans* conjugated diene between 5 and 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, as shown in Fig. 5. The existence of the γ-lactone is strongly supported by protons on the lactone ring being split into H2a and H2b, and H3a and 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 the separate signals 9a/9b and 12a/12b; although these do not have chiral neighbors, the environment has chirality due to the folding over of the carbon chain, as originally deduced and as 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

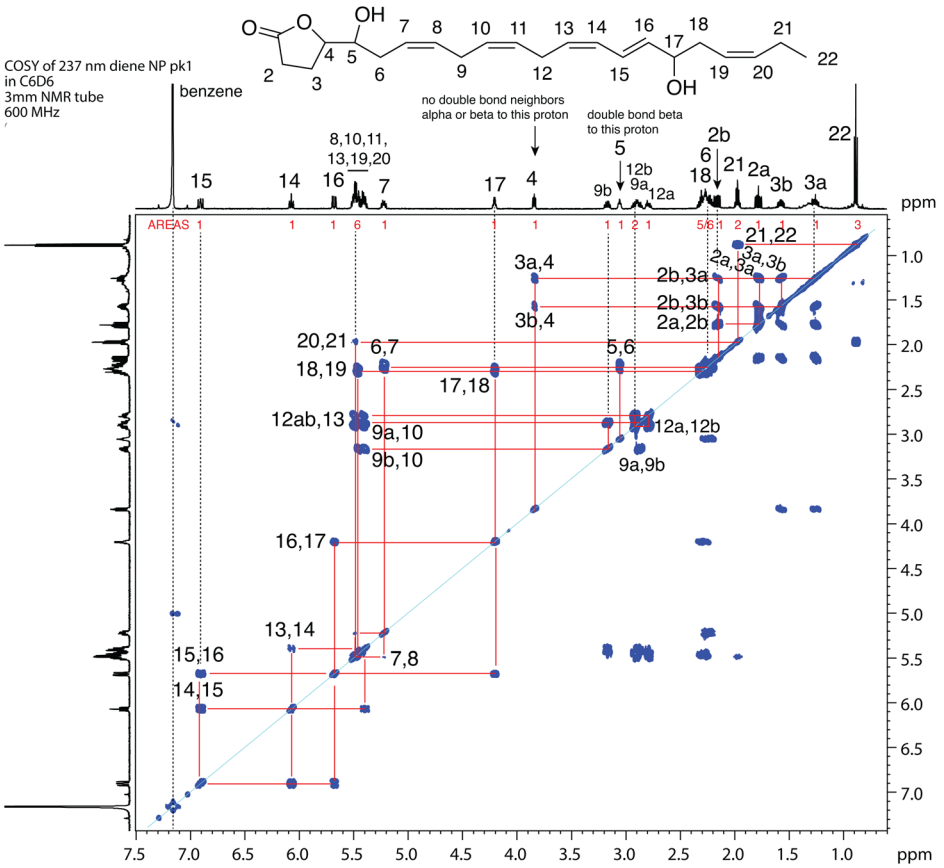


Fig. 5 The proton NMR spectrum and COSY analysis of the 237 nm product **2** in d_6-benzene. The spectrum and COSY analysis were recorded from the first eluting diastereomer from NP-HPLC (*cf.* Fig. 4).

(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 the *intra*molecular transfer of hydroperoxy oxygen 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 oxygen from outside sources would give ^{18}O^{16}O mixtures, readily discernible by mass spectrometry. GC-MS analysis 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, as shown in Fig. 6A. The mass spectrum of the γ-lactone product **2** shows *m*/*z* 173/175 ions with a ratio suggesting the slightly less than complete retention of ^{18}O, although the higher mass ions (*m*/*z* 443/447) show that two ^{18}O are mainly retained (Fig. 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 that these are at C5 and C17 (Fig. 6C).

The percent retention of ^{16}O/^{18}O labels in the products was quantified by LC-MS, as this gave a superior signal-to-noise ratio, which was helpful for the M + 2 signals representing mixed ^{16}O–^{18}O compositions. The results for product **2** measured for [M − H + acetate]$^-$ at *m*/*z* 419/423 showed 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 the 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 the slow hydrolysis of the γ-lactone product. This was confirmed by the incubation of the purified γ-lactone product for 5 days in PBS, resulting in 65% hydrolysis to its open form, the trihydroxy derivative **3** (SI Fig. S4).

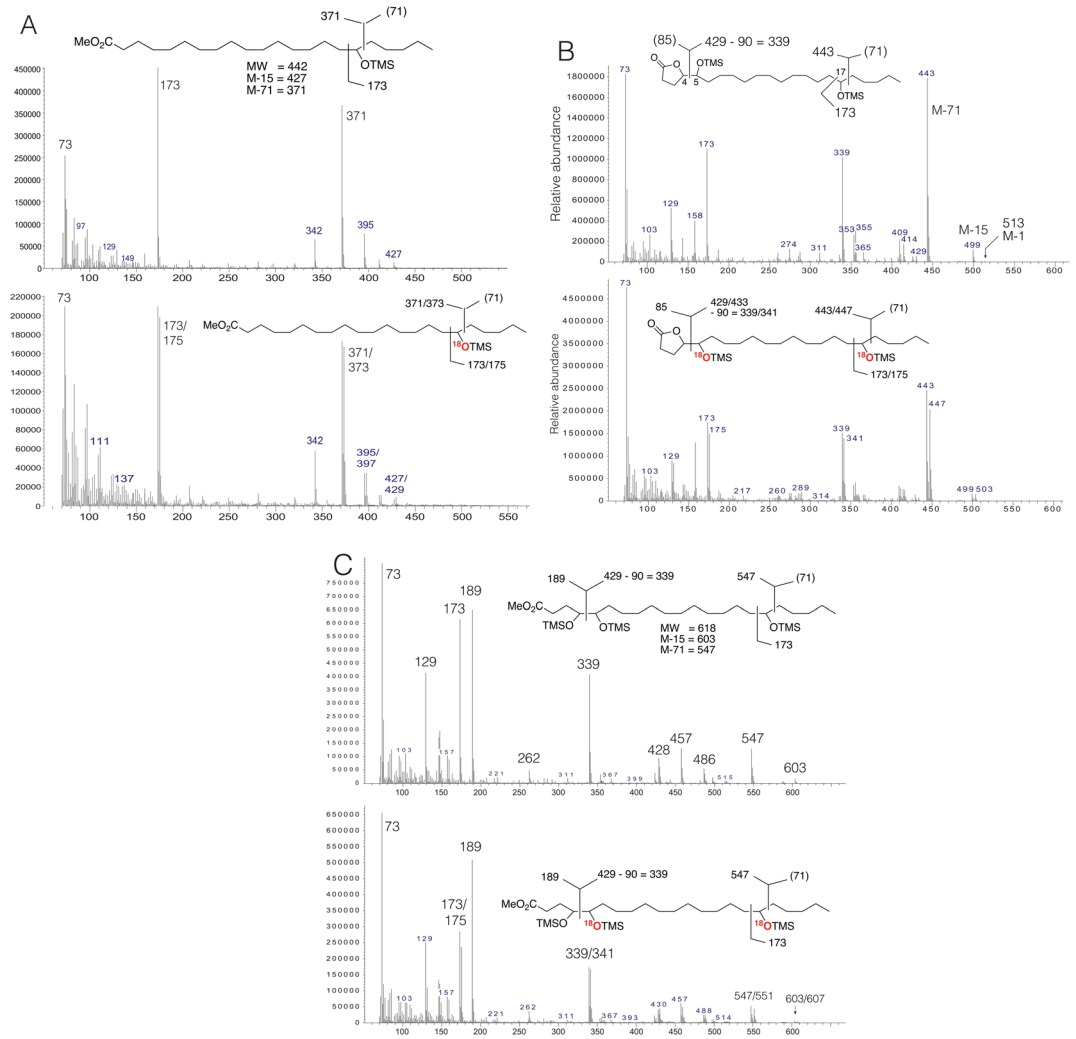


Fig. 6 Mass spectra from GC-MS analysis of unlabeled and $^{16}O/^{18}O$-labeled 17*S*-HDHA **1**, γ-lactone **2**, and trihydroxy **3**. The compounds were hydrogenated and derivatized and were then analyzed by GC-MS in electron impact mode. (A) Unlabeled and $^{16}O/^{18}O$-labeled 17-HDHA prepared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

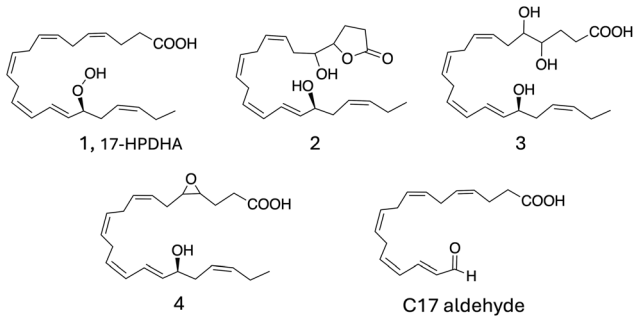
Identification of 4,5-epoxy-17*S*-hydroxy-22:5ω3 in the autoxidation

In Fig. 2, the small 70 min peak eluting after the γ-lactone, designated as **4**, was found to match with synthetic 4,5-*cis*-epoxy-17*S*-hydroxy-22:5ω3 in terms of the UV spectrum and retention time from both RP-HPLC and NP-HPLC, and the mass spectrum from LC-MS. 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.* ref. 33 and 34), but there are no nearby peaks with the required 235 nm absorbance and with an appropriate UV spectrum, strongly suggesting that the product is exclusively the *cis*-epoxide. The synthetic product (prepared from racemic 4,5-*cis*-epoxide) gave two equal-sized peaks in NP-HPLC, whereas the proportions of the diastereomers from 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 incubation in PBS, or is the 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 the definitive answer of no detectable lactonization (SI Fig. S5), suggesting that the γ-lactone is a primary product of the oxygen transfer reaction.

Discussion

Herein, we found an unusual, unexpected and unique major product from the incubation of 17*S*-HPDHA **1** in PBS at 37 °C



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

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, as shown in Scheme 1 with the associated products.

As established by the oxygen-18 experiments, the two diastereomers of the γ-lactone **2** are formed *via* internal oxygen transfer from the 17-hydroperoxide. Almost certainly, this oxygen transfer is facile on account of the 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, and it is 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 of transfer from 17-HPDHA, there is no apparent propensity for the involvement of the fatty acid carboxyl. For 18-hydroperoxy-EPA reacting at the 5,6-double bond, there is the potential for the formation of a δ-lactone with the C1 carboxyl, and the direct involvement of the carboxyl group may also facilitate this overall transformation.

The 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 an 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 naturally occur (*e.g.* ref. 5 and 35–38).

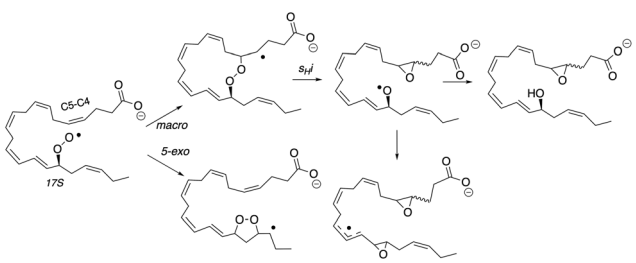
The evidence points to the γ-lactone being a primary product of the 17-HPDHA reaction and not a secondary product from a 4,5-epoxide. There is a small peak from 4,5-*cis*-epoxy-17-alcohol **4** in the HPLC chromatograms but a control experiment established that it is stable at pH 7.4, and it does not lactonize under conditions of incubation in PBS. Nonetheless, as expected, the synthetic 4,5-*cis*-epoxy-17-hydroxy docosanoid is acid labile, as we discovered when using acidic conditions for its extraction (as described in the Results section). 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 the formation of the γ-lactone moiety. A peroxyl radical mechanism would involve 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]

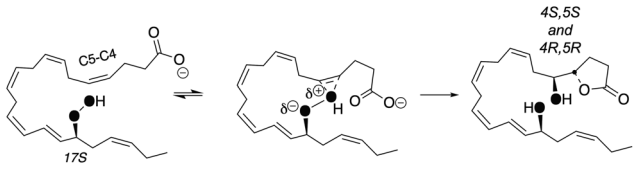
16-*endo* macrocyclization of the peroxyl would provide a parallel mechanistic pathway to the product epoxides. In support of this mechanism, free radical macrocyclizations are well-known[40] and this mechanism would account for the ^{18}O labelling results as well, with one oxygen from the hydroperoxide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

There are, however, reasons to doubt 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 in 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 the formation of *trans*-substituted epoxides, but the two diastereomeric lactone products are selectively formed from independently prepared *cis* epoxides. Fourth, there is ample precedent for the formation of the intermediate alkoxyl radical in 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, which has, on balance, more points in its favor, is presented in Scheme 3.



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



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

This suggests the 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 and 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 the early results above) by acid hydrolysis of 4,5-*cis*-epoxy-17-hydroxy-22:5ω3.

There are other examples of oxygen transfer 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 occurs 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, as the effective molarity of one reactive center with its partner reactant is high. Seven to twelve-membered ring formation is not favored as these ring sizes have destabilizing cross-ring steric interactions, and the effective molarity of reactant centers forming mid-size rings is lower than 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.

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 oxygen transfer 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 the 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 hydroperoxide transfer.

A final caveat for any discussion of mechanisms relating to the conversion of 17*S* HPDHA to the γ-lactone products is the fact that the reaction is carried out in a pH 7.4 buffer, while the precedents for free radical reactions previously 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 the selectivity and reactivity of peroxyl radical species.

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× PBS (P7059) were obtained 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) was obtained from Icon stable isotopes (Summit, NJ).

Synthesis and purification of 17*S*-hydroperoxydocosahexaenoic acid, 1

The stereospecific 17*S*-hydroperoxydocosahexaenoic acid **1** was synthesized by soybean lipoxygenase-catalyzed reactions following the methods described for linolenate with minor modifications.[48] The DHA substrate (50 mg, redissolved in 200 μL of ethanol) was prepared in dipotassium phosphate buffer (100 mL, 0.1 M, pH 8.7) from a stock solution (20 mg mL^{-1}). 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 absorption of conjugated dienes) with 10 μL aliquots in 490 μL of buffer. After the activity was observed to have stalled after ~30 minutes, while avoiding the formation of undesired products observable at ~270 nm (conjugated trienes), the reaction was terminated by adding hydrochloric acid dropwise to adjust the pH to <5. Products were extracted with dichloromethane, and the extract was washed with a small volume of water, evaporated under a stream of N_2, and purified by preparative SP-HPLC using a TLC Advantage semi-preparative silica column (25 × 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^{-1}. Injections of 3–5 mg were chromatographed and purified 17*S*-HPDHA was quantified by UV in ethanol (λ_{max}: 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.* ref. 50 and 51, possibly also due to 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 was then washed twice with a half volume of cold 0.1 M phosphate at pH 8, followed by washing with cold water. The resulting epoxides were separated on a semi-preparative Waters Symmetry 7 μm C18 column (15 × 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^{-1}. The last-eluting epoxide, separated by 5 min from the others at a retention time of 29 min, was collected and a small aliquot was used for quantification 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 of 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 the reaction was completed, as confirmed by UV scanning, the 17-hydroperoxy group was reduced with $NaBH_4$ *in situ*, and alkaline solution was 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 of water until the eluate was neutral, and the product was collected by elution with 3 mL of ethyl acetate. The 4*RS*-epoxy-17*S*-hydroxy diastereomers were resolved by NP-HPLC using a Grace Apollo 5 μm silica column (25 × 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^{-1} with retention times of 8.34 and 8.73 min, and they were then subject to NMR analysis in d_6-benzene (illustrated for the first-eluting diastereomer in SI Fig. 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 was purified by RP-HPLC (Beckman semi-preparative C18 column, 25 × 1 cm; solvent: CH_3CN/H_2O/HAc, 80 : 20 : 0.01 by volume; retention time: 8.7 min at 5 mL min^{-1}). To form the 4-hydroxy-17-hydroperoxy derivative, the γ-lactone (200 μg in a 5 mL React-ivial) was opened in 20 μl of 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^{-1} soybean LOX (Sigma, type V); UV scanning (200–350 nm) showed within 30 s that 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 was washed with water, and the product was eluted with ethyl acetate. Treatment with molar excess of triphenylphosphine in 50 μl of MeOH for 15 min at RT gave the 4,17-dihydroxy derivative that was purified by RP-HPLC (Waters Symmetry C18 column, 25 × 0.46 cm; solvent: CH_3CN/H_2O/HAc, 55 : 45 : 0.01 by volume; retention time: 9.8 min at 1 mL min^{-1}) and the proton NMR spectrum was acquired in d_6-benzene of the two diastereomers together. ^{1}H NMR (600 MHz, C_6D_6); chemical shift, number of protons, multiplicity, *J*, 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× PBS at pH 7.4 in 0.1% volume of ethanol to give a final concentration of approximately 30–40 μM (giving ∼1.0 AU absorbance at 237 nm) and this was incubated at 37 °C for up to 6 days. UV spectra of the PBS solutions were recorded daily. Two methods of extraction were employed: either acidification to pH 4–5 and extraction with dichloromethane, which was then followed by washing with a small volume of water and taking to dryness under a stream of N_2; or extraction under neutral conditions with volumes up to 15 mL and direct application without acidification to a 60 mg Oasis cartridge (Waters) equilibrated in methanol then water, followed by a final water wash to remove salts and elution using 3 mL of ethyl acetate.

HPLC-UV analyses

Aliquots of the autoxidation products were analyzed by RP-HPLC using a Waters Symmetry 5 μm 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^{-1}, 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 μm 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^{-1} 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 negative ion mode. A Phenomenex Kinetex C18 2.6 μm column (100 × 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^{-1}. The electrospray voltage was set at 4.0 kV; the vaporizer temperature at 300 °C; the sheath and auxiliary gas pressure at 50 and 5 Arb, respectively; and the 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 of BSTFA plus 2 μL of 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 positive ion electron impact mode (70 eV) with temperature programming at 10 °C min^{-1} from 150 to 300 °C.

NMR analyses

^{1}H NMR and ^{1}H, ^{1}H COSY NMR experiments were performed 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 5 mm 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 annals of non-enzymic fatty acid chemistry. As described in the Discussion section, macrocyclic transfer is favored in fourteen- to eighteen-membered-ring transition states as the effective molarity of reactive centers increases for these ring sizes. The 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 ring-size preference in the transition, the mechanism is open to other highly polyunsaturated lipids with a donating hydroperoxide and suitably positioned intramolecular recipient.

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.

Data availability

Data are included in the manuscript and supplementary information (SI). Original data is available upon reasonable request. The SI contains analysis of vitamin E-controlled 17-HPDHA autoxidation, indicating no detectable formation of Protectin D1 (text, Scheme S1 and Fig. S1); proton NMR spectra and COSY analyses of the methyl ester and free acid of synthetic 4,5-*cis*-epoxy-17-hydroxy-22:5ω3 (Fig. S2 and S3); RP-HPLC showing hydrolysis of the γ-lactone after 5 days in PBS (Fig. S4); and RP-HPLC analysis showing 4,5-*cis*-epoxy-17-hydroxy-22:5ω3 unchanged after incubation overnight in PBS (Fig. S5).

Supplementary information is available. See DOI: https://doi.org/10.1039/d6ob00371k.

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