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Demonstration of HNE-related aldehyde formation via lipoxygenase-catalyzed synthesis of a *bis*-allylic dihydroperoxide intermediate

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Abstract

One of the proposed pathways to the synthesis of 4-hydroxy-nonenal (HNE) and related aldehydes entails formation of an intermediate bis-allylic fatty acid dihydroperoxide. As a first direct demonstration of such a pathway and proof of principle, herein we show that 8*R*-lipoxygenase (8*R*-LOX) catalyzes the enzymatic production of the HNE-like product (11-oxo-8-hydroperoxy-undeca-5,9-dienoic acid) via synthesis of 8,11-dihydroperoxy-eicosa-5,9,12,14-tetraenoic acid intermediate. Incubation of arachidonic acid with 8*R*-LOX formed initially 8*R*-hydroperoxyeicosatetraenoic acid (8*R*-HPETE) which was further converted to a mixture of products including a prominent HPNE-like enone. A new bis-allylic dihydroperoxide was trapped when the incubation was repeated on ice. Re-incubation of this intermediate with 8*R*-LOX successfully demonstrated its conversion to the enone products, and this reaction was greatly accelerated by co-incubation with NDGA, a reductant of the LOX iron. These findings identify a plausible mechanism that could contribute to the production of 4-hydroxy-alkenals *in vivo*.

Introduction

4-Hydroxy-alkenals are a class of cytotoxic aldehydes that play significant roles in various injurious and disease states involving cellular oxidative stress.^{1–3} Their toxicity resides on the α,β -unsaturated carbonyl moiety which is highly electrophilic and adducts with biological nucleophiles to exert toxic potential.^{4–7} The prototypical 4-hydroxy-alkenal is 4-hydroxy-nonenal (HNE), representing the tail-end nine carbons of the common polyunsaturated lipids, linoleic and arachidonic acids.¹ In the cellular environment this results in the release of a diffusible cytotoxin. As most fatty acids exist in esterified form in cellular membranes, 4-hydroxy-alkenals generated via lipid peroxidation may also remain attached in complex membrane lipids. This has negative consequences through protein or DNA adduction and a resulting cellular toxicity.^{8–10}

One of the intriguing issues in the lipid peroxidation field is the mechanistic origin of HNE and the related 4-hydroxy-alkenals. Multiple mechanistic proposals have appeared over the years, their most common feature being the involvement of a polyunsaturated lipid hydroperoxide. Beyond this step, there are several highly plausible potential pathways, and

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Supporting Information

Figures of identification of other products from the reaction of arachidonic acid with 8*R*-LOX and demonstration of the bis-allylic dihydroperoxide formation using mouse platelet-type 12*S*-LOX and human 15LOX-1. This material is available free of charge via the Internet at <http://pubs.acs.org>.

based on the current evidence there seems little doubt that there are diverse synthetic routes to HNE-related aldehydes. Over two decades ago, hypothetical pathways to HNE were suggested by Pryor and Porter,¹¹ and these remain valid proposals. Indeed, experimental evidence appeared recently supporting one of their schemes involving a pseudo-symmetrical diepoxy carbon radical intermediate.¹² Among other potential routes, we advanced alternate pathways via the intermediacy of fatty acid radical dimers,¹³ and provided experimental support for the concept.¹⁴ Convergence of the activities of 5-lipoxygenase (5-LOX) and cyclooxygenase provides yet another route to these short-chain aldehydes.¹⁵

Among the many possibilities for the synthesis of HNE-related aldehydes during lipid peroxidation, of direct relevance to the present study is the postulated intermediacy of a bis-allylic fatty acid dihydroperoxide (Scheme 1). We had invoked such a pathway to account for retention of the hydroxyl chirality in the transformation of 13*S*-hydro(pero)xy-linoleate (13*S*-hydro(pero)xy-octadecadienoic acid, 13*S*-H(P)ODE) to 4*S*-H(P)NE.^{16,17} It was subsequently proposed also as a potential intermediate in diverse biochemical transformations.^{18–22} In following up on our own observations we had prepared model linoleate dihydroperoxides (diHPODEs) by singlet oxygen treatment of linoleic acid and examined their stability.²³ Disconcertingly, we found that the purified diHPODEs, in the absence of any catalyst, show little propensity to degrade to HNE-related aldehydes when allowed to stand in air at 37°C.²³ By comparison, the mono-hydroperoxides of linoleate do form H(P)NE upon standing in air with no added catalyst. Conversion of the diHPODEs to H(P)NE was induced by treatment with acid, in line with the much-invoked Hock cleavage, an acid-catalyzed reaction (reviewed in ref.²⁴). This, however, does not mimic the neutral environment under which HNE was formed using our autooxidation conditions.²³ If diHPODEs or the corresponding dihydroperoxides of arachidonate (diHPETEs) are involved in formation of HNE, then some other catalyst has to be active. In earlier experiments using the *P. homomalla* 8*R*-LOX²⁵ we noticed the formation of polar products from arachidonic acid, prompting our investigation of their structures and mechanism of formation. This led to the present report in which we identify a bis-allylic dihydroperoxide pathway to an HPNE-related aldehyde with a lipoxygenase (LOX) enzyme promoting the transformations.

Experimental Procedures

Materials

Arachidonic acid was purchased from NuChek Prep Inc. (Elysian, MN). [1-¹⁴C] C20.4ω6 was purchased from Perkin Elmer Life Sciences. 9*E*,11*Z*,14*Z*-20:3ω6 was synthesized by Dr. Jin K. Cha, Wayne State University, as described previously.²⁶ Nordihydroguaiaretic acid (NDGA) was purchased from Cayman Chemical Co. (Ann Arbor, MI). Triphenylphosphine (TPP) was purchased from Sigma.

Expression and purification of 8*R*-lipoxygenase

cDNA of the 8*R*-LOX domain of the *P. homomalla* peroxidase-lipoxygenase fusion protein was subcloned into the pET3a vector (with an N-terminal His₄ tag), and the protein was expressed in *E. coli* BL21 (DE3) cells and purified by nickel affinity chromatography according to a previously published protocol.²⁷ For clarity, this 8*R*-lipoxygenase is referred to here as the recombinant 8*R*-LOX.

Enzymatic incubation

Incubations were performed at room temperature in 1 ml of 50 mM Tris, pH 8.0, 500 mM NaCl, 2 mM CaCl₂ and 0.01% Emulphogene detergent using recombinant 8*R*-LOX (10 μg/ml) and fatty acid substrates, arachidonic acid (25 μg/ml) or purified 8*R*,11*R*-diHPETE (5 μg/ml). Incubations were conducted in a 1 ml quartz cuvette, monitored by repetitive

scanning from 350–200 nm using a Lambda-35 spectrophotometer (Perkin-Elmer) and stopped by acidification to pH ~4.5. After extraction using C18 Oasis cartridges the samples were further analyzed by HPLC.

HPLC analyses

Aliquots of the extracts were analyzed by RP-HPLC using a Waters Symmetry 5 μ column (25 $\times$ 0.46 cm) at a flow rate of 1 ml/min with CH₃CN/H₂O/HAc (45/55/0.01, by volume). Peaks were monitored using on-line UV detection (Agilent 1100 series diode array detector). Further purification was achieved by normal-phase HPLC using a Beckmann Ultrasphere 5 μ silica column (25 $\times$ 0.46 cm) using a solvent of hexane/isopropanol/glacial acetic acid (100/2/0.1, by volume) at 1 ml/min. For chiral analysis, the enantiomers were resolved using a Chiralpak AD column (25 $\times$ 0.46 cm) with a solvent system of hexane/methanol (100/2, by volume) run at 1 ml/min.

Preparation of 8R,11R-diHPETE

Arachidonic acid (100 μ g/ml) in Tris enzyme buffer (pH 8.0, 500 mM NaCl, 2 mM CaCl₂ and containing 0.01% Emulphogene detergent) was reacted with 8R-LOX (10 μ g/ml) for 30 min on ice, with the reaction monitored by scanning UV spectroscopy (200 – 350 nm, as illustrated in Supplementary Figure S1). The reaction was stopped by acidification using 1M KH₂PO₄ and 1N HCl to give pH 4 and the sample applied to an Oasis C18 cartridge, which was washed with water and the products eluted with methanol. The 8R,11R-diHPETE was subsequently purified by RP-HPLC (cf. Figure 3, main text) followed by methylene chloride extraction from the HPLC solvent. The organic phase was washed with water and then blown to dryness under a nitrogen stream. The purified 8R,11R-diHPETE in ethanol was quantified by UV spectroscopy assuming an extinction coefficient of 25,000 at 242 nm, and stored at –30°C prior to use.

Compound Derivatization

Catalytic hydrogenations were performed in 100 μ L of ethanol using about 1 mg of palladium and bubbling with hydrogen for 2 min at room temperature. The hydrogenated products were recovered by the addition of water and extraction with ethyl acetate. Methyl esters were prepared using ethereal diazomethane in methanol. Methoxime derivatives were prepared by treatment with 5 μ L of methoxylamine hydrochloride in pyridine (10 mg/ml) overnight at room temperature. Trimethylsilyl (TMS) ether derivatives were prepared using bis(trimethylsilyl)-trifluoroacetamide (10 μ L) at room temperature for 2 hour. PFB esters were prepared using 10% diisopropyl ethanolamine in acetonitrile (20 μ L) and 10% pentafluorobenzylbromide (40 μ L) at 37 °C for 15 min.

GC-MS analyses

Analyses of product derivatives were performed in the positive ion electron impact mode (70 eV) using a Thermo Finnigan Trace DSQ ion trap GC-MS. The RTX-1701 fused silica capillary column, 17 m $\times$ 0.25 mm internal diameter, was programmed from 150° to 300° at 20°/min. The samples were subjected to rapid repetitive scanning over the mass range of 50–500 a.m.u. covering the major fragment ions.

NMR analyses

¹H NMR and COSY spectra were recorded on a Bruker 600 MHz spectrometer at 298 K. Chemical shifts are reported relative to tetramethylsilane (0.0 ppm), using the residual non-deuterated NMR solvent as the lock signal (δ = 7.16 ppm for C₆D₆, 7.26 ppm for CDCl₃).

Results

Metabolism of arachidonic acid by 8R-LOX

Recombinant 8R-LOX from the *P. homomalla* AOS-LOX fusion protein oxygenates arachidonic acid specifically to 8R-HPETE, and then on further exposure to the enzyme the 8R-HPETE is slowly transformed to secondary products via multiple oxidative pathways. When the reactions are observed by repetitive scanning in the UV range 200 – 350 nm (Supplementary Figure S1), addition of a high concentration of enzyme (10 µg/ml) to arachidonic acid results in the rapid (< 1 min) conversion to 8R-HPETE, evidenced by appearance of the characteristic conjugated diene chromophore with λ_{max} at 237 nm. Over the next 10 min, the 237 nm chromophore shows a gradual decrease while associated with the appearance of new chromophores characteristic of conjugated trienes with λ_{max} at 270 nm, and a smooth, broad absorbance with λ_{max} at ~320 nm. In fact, subsequent HPLC analysis reveals that additional new products are present, concealed under the residual conjugated diene chromophore in the UV scanning analysis.

After extraction using a C18 cartridge, RP-HPLC analysis with diode-array detection at multiple wavelengths shows a complex mixture of products (Figure 1). The peaks labeled 1–4 each exhibit a smooth UV chromophore typical of a conjugated enone, while the later-eluting groups of products (mainly 8,15-diH(P)ETEs) exhibit the characteristic UV chromophore of a conjugated triene. After further chromatography, the main components of peak 1 were separated and identified by NMR as a mixture of a C₇ keto acid, 5-oxo-hept-6-enoic acid, and C₇ aldehyde acid, 7-oxo-hept-5E-enoic acid (Supplementary data, Figures S2, S3, S4). It turns out that the peak 2 in Figure 1 is directly relevant to the *bis*-allylic dihydroperoxide pathway to HNE-related aldehydes. 2 exhibits a UV chromophore (λ_{max} 218 nm) similar in character to the spectrum of HPNE.²⁸ Treatment of the extracts with triphenylphosphine converted 2 to a slightly more polar derivative (λ_{max} 221 nm) consistent with reduction of the hydroperoxy analogue to its hydroxy counterpart. The hydroxy analogue of 2 was analyzed by GC-MS as the methyl ester TMS ether methoxime derivative, Figure 2A. Structurally diagnostic ions were observed at *m/z* values of 327 (*M*⁺, 1% abundance), 312 (*M*-15, 2%), 296 (*M*-31, 2%), 186 (C₈-C₁₁, 100%) and 155 (16%), compatible with the methyl ester of a C₁₁ fatty acid containing an 8-hydroxyl and C₁₁ aldehyde. The proton NMR spectrum of the parent 2 defined the structure, Figure 2B (with COSY analysis, Figure S5). Thus, the doublet at 9.6 ppm in Figure 2B represents the aldehyde proton (*J*_{10,11} = 7.7 Hz), with coupling to the doublet of doublets at 6.3 ppm (H10, *J* = 7.7 and 16.0 Hz), the 16.0 Hz *J* value indicating a *trans* double bond with H9 (dd at 6.8 ppm, *J* = 16.0 and 6.0 Hz), next to the C8 proton geminal with the hydroperoxide (C8, q, *J* = 6 Hz), with further clear couplings through to H2. Consistent with the conjugated enone chromophore and GC-MS analysis, and taking into account its formation from 8R-HPETE, the 2 was identified as 8R-hydroperoxy-11-oxo-undeca-5Z,9E-dienoic acid. This HPNE-like aldehyde is formed by cleavage of the C₂₀ precursor between C11 and C12. The yield of 2, estimated from an experiment using [1-¹⁴C]8R-HPETE as substrate, was approximately 8% of the recovered radioactive products. This result is compatible with the intensity of the UV absorbance traces, given that the molar extinction coefficient of a conjugated enone chromophore is in the range 10,000 – 15,000, only one third of the values for the conjugated trienes (8,15-diH(P)ETEs, ϵ 40,000 – 55,000^{29,30}) that appear prominent in Figure 1.

Before describing experiments on the mechanism of formation of 2, we will briefly outline the identification of other 8R-LOX products in Figure 1. The several compounds with UV spectra characteristic of all-*trans* or *trans-cis-trans* conjugated trienes^{30,31} were readily identified as 8,15-diHETEs or 8,15-diHPETEs based on the availability of synthetic standards, and the change in chromatographic mobility of the hydroperoxides on reduction

with triphenylphosphine; their proposed synthesis is shown in Supplementary Scheme S1. Peak **3** eluting at 15 min with λ_{max} at 272 nm exhibits a conjugated dienone chromophore, and was identified by GC-MS and ^1H -NMR as the C_{11} aldehyde 6-hydroperoxy-(2*E*,4*E*)-undecenal (Supplementary Figures S6, S7). Peak **4** at 24 min retention time has the UV absorbance characteristic of a conjugated trienone (λ_{max} 316 nm), and was identified as the C_{13} aldehyde, 8-hydroperoxy-(2,4,6)-tridecenal (Supplementary Figure S8). Experiments using [$1\text{-}^{14}\text{C}$]arachidonic acid as 8*R*-LOX substrate confirmed that the C_7 keto and aldehyde acids in peak **1** and the HPNE-related **2** retain the $1\text{-}^{14}\text{C}$ label, and for example, that the C_{11} and C_{13} aldehydes from the methyl end of the fatty acid were unlabeled. The radioactivity also facilitated the detection of two additional polar products that are resolved using RP-HPLC solvent containing 90% water, Supplementary Figures S9. LC-MS analysis indicated that these extra products, which absorb very weakly near 200 nm in the UV are C_7 hydroxy acids (M-H ion at m/z 143), likely 5-hydroxy-hept-6-enoic acid and 7-hydroxy-hept-5-enoic acid, expected as α -cleavage products from 8-HPETE or 8,15-diHPETE (cf.^{32–34}), and one of which was identified in an earlier 8-LOX metabolism study by NMR.³⁵

Trapping the intermediate to the HNE-like aldehyde

To aid in the isolation of unstable or rapidly metabolized intermediates, the incubation of arachidonic acid with 8*R*-LOX was repeated at ice-cold temperature. RP-HPLC of the on-ice incubation showed a relative decrease in formation of all cleavage products, including those in peaks **1** – **4**, with increased recovery of the 8,15-diH(P)ETEs and, eluting just after the 8,15-diHPETEs, a prominent new peak that was hardly detected in the incubation at room temperature (Figure 3A). The new peak showed an unfamiliar UV chromophore with a smooth profile (as opposed to the angular shoulders on a typical conjugated diene) and a relatively high λ_{max} at 242 nm (Figure 3B). After hydrogenation to remove the double bonds, GC-MS analysis of the TMS ester TMS ether derivative showed diagnostic ions at m/z values of 545 (M-15, 1% abundance), 455 (M-90, 1%), 359 ($\text{C}_8\text{--C}_{20}$, 7%), 343 ($[\text{C}_1\text{--C}_{11}]\text{--}90$, 92%), 303 ($\text{C}_1\text{--C}_8$, 97%), 269 ($[\text{C}_8\text{--C}_{20}]\text{--}90$, 89%) and 229 ($\text{C}_{11}\text{--C}_{20}$, 73%) (Figure 3C). The major α -cleavage fragment ions define the position of the two hydroxyl groups at C_8 and C_{11} . The parent 8,11-diHPETE (5*Z*,9*E*,12*E*,14*Z*) exhibits a distinctive conjugated diene chromophore (12*E*,14*Z*) modulated mainly by the presence of the nearby 9*E* double bond.

Conversion of purified 8,11-diHPETE to the aldehydes in peaks **1** and **2**

To examine the relationship between 8,11-diHPETE and the secondary cleavage products, purified 8,11-diHPETE was incubated with 8*R*-LOX and formation of the cleavage products was checked by RP-HPLC (Figure 4). The conversion was minimal using 8*R*-LOX alone, but upon co-incubation with NDGA, a reductant of the LOX iron, 8,11-diHPETE was completely consumed, and accompanied by production of the C_7 aldehyde acid of peak **1** and the HPNE-related C_{11} acid **2**. Control experiments examining the conversions of 8,15-diHPETEs by 8*R*-LOX with or without added NDGA established that neither condition produced peaks **1** and **2** (data not shown).

Determination of the C_{11} -hydroperoxyl configuration

Formation of 8,11-diHPETE by 8*R*-LOX appears to involve a secondary oxygenation via a “frame-shift” along the carbon chain of the 8*R*-HPETE substrate, in which case formation of an 11*R* hydroperoxy moiety is predicted. To provide support for this proposed biosynthetic mechanism, we examined the 8*R*-LOX metabolism of 9*E*,11*Z*,14*Z*-eicosatrienoic acid, a synthetic C_{20} fatty acid containing a conjugated diene in the same position as in 8-HPETE, but lacking the 8-hydroperoxyl (Figure 5A). RP-HPLC analysis of the reaction products demonstrated, in addition to the formation of two conjugated triene-containing products

(presumably oxygenated at C9, thus extending the conjugation to 10,12,14), a more prominent product exhibiting a UV chromophore similar in profile to that of 8,11-diHPETE, except with the λ_{max} 4 nm lower, at 239 nm. GC-MS analysis of the TPP-reduced and hydrogenated product as the TMS ester TMS ether derivative unequivocally established the position of the hydroxy at C11 (Figure 5B). The parent is therefore 11-hydroperoxy-eicosa-9,12,14-trienoic acid. To determine the stereochemistry at C11, the hydrogenated and TPP-reduced derivative was converted to the PFB ester and compared by chiral column HPLC with the PFB esters of hydrogenated 11*R*-HETE and 11*RS*-HETE. The chiral phase-HPLC analysis showed mainly the 11*R* enantiomer (Figure 5C), and therefore by direct analogy, the structure of the 8,11-diHPETE generated from arachidonic acid is expected to be 8*R*,11*R*-diHPETE.

Discussion

Herein we establish that 8*R*-LOX forms 8*R*,11*R*-diHPETE as one of the transformation products of 8*R*-HPETE, and that this bis-allylic dihydroperoxide is further converted in the course of the ongoing enzymatic reactions, forming the 4-hydroperoxy-alkenal **2** as a product of this pathway. (Formation of other products is covered in the Supplement). Considering initially the generation of 8,11-diHPETE, it is notable that detection of such bis-allylic fatty acid hydroperoxides is uncommon, for several reasons. At the top of the list is that, until the year 2000, bis-allylic hydroperoxides remained undiscovered as products of lipid peroxidation³⁶ and therefore they were simply not looked for in lipoxygenase reactions. Secondly, the instability of bis-allylic hydroperoxides during typical acid extraction conditions, (most extensively studied with the corresponding bis-allylic hydroxy derivatives e.g. refs.^{37,38}), limits the prospects of their detection by chance. Thirdly, aside from the fungal enzyme manganese-lipoxygenase that forms 11-hydroperoxy-linoleate as a major product,³⁹ the initial reaction of lipoxygenases with polyunsaturated fatty acids forms the bis-allylic hydroperoxides as very minor products (1% abundance).^{27,40} By comparison, bis-allylic dihydroperoxides are relatively prominent among the products of lipoxygenase secondary reactions, as evidenced here by their detection using the *P. homomalla* 8*R*-LOX (Figure 3), the mouse platelet-type 12*S*-LOX, and human 15-LOX-1 (Supplementary Figures S10, S11).

The transformation from arachidonic acid to the HPNE-related aldehyde fragment involves a dual catalytic activity of the lipoxygenase active site iron. The dioxygenase activity by the ferric iron oxygenates C8 and C11 in a sequential manner and produces 8*R*,11*R*-diHPETE. If the oxygenation proceeded efficiently, then ferric iron would be regenerated in each catalytic cycle and free ferrous iron would not become available. Although the first oxygenation to 8*R*-HPETE is fast and efficient, the secondary reactions by 8*R*-LOX are slow, and they produce a complex mixture of products. Therefore, the primary product, 8*R*-HPETE, can be considered as a poor substrate of the 8*R*-LOX, and the weak secondary reactions may not be well controlled. For instance, with 8*R*-HPETE in the enzyme active site there may be, in effect, a low concentration of suitably-positioned molecular oxygen available to intercept the LOX-generated radicals (cf. refs.^{41–43}), and/or radical intermediates may escape the active site.^{42,43} Either possibility will leave the non-heme iron in the ferrous state, now available to catalyze reductive transformations. These reductive reactions were inhibited significantly by the use of ice-cold temperatures during the enzyme incubation, likely by improving the efficiency of the oxygenation reactions, allowing recovery of the bis-allylic dihydroperoxide.

There is ample evidence from our product analyses to establish that reduction to alkoxyl radicals is catalyzed by the 8*R*-LOX enzyme. This reaction is involved in synthesis of each of the aldehydic products detected (cf.⁴⁴). This reduction leaves the LOX iron in the

oxidized state, and addition of NDGA helps promote ferric-to-ferrous recycling and is required for 8R-LOX transformation of 8,11-diHPETE. By contrast, as noted above, the cycling occurs fairly efficiently in the complex mixture of reactions starting from arachidonic acid. As a proposed sequence of reactions using 8*R*,11*R*-diHPETE as substrate, the ferrous iron catalyzes a homolytic cleavage of the 11-hydroperoxide and generates an alkoxyl radical at C11. The subsequent carbon chain cleavage between C11 and C12 could then be promoted by β -cleavage of the alkoxyl radical and give the HPNE-like aldehyde, **2** (Scheme 2). Alternative mechanisms might be considered including the possibility of an alkoxy radical cyclization followed by epoxycarbonyl radical cleavage²¹. The C11–C12 carbon chain cleavage should also produce 3*Z*-nonenal (Scheme 2), although this fragment was not detected in our experiments. Our prior experience with 3*Z*-nonenal is that it chromatographs very poorly on RP-HPLC, and with its weak UV absorbance (end absorbance only) such a product is difficult to detect. Although it might isomerize to 2*E*-nonenal or oxidize to HPNE, which chromatograph nicely and are more readily detectable, we checked using authentic standards for comparison and neither was present on the chromatograms.

Carbon chain cleavage leading to aldehydes was recognized four decades ago as a secondary lipoxygenase activity (promoted under anaerobic conditions).⁴⁵ This well-recognized reaction occurs via alkoxyl radical formation then carbon chain cleavage at the C(O•)-CH₂ bond distal to the conjugated diene. The resulting products are akin to the C11 and C13 aldehydes from the methyl end of the molecule identified in the present study. A similar cleavage activity by lipoxygenase that is not reliant on anaerobic conditions was established more recently,⁴⁶ although mechanistic studies postulate a lack of oxygen availability in the LOX active site as a predisposing factor.⁴² The formation of a bis-allylic dihydroperoxide as observed here appears to offer an alternative option for carbon chain cleavage. Formation of **2** occurs with fragmentation at the C(O•)-conjugated diene bond, thus creating the 4-hydroperoxy-alkenal. These results establish the precedent for involvement of lipoxygenase in synthesis of fatty acid dihydroperoxide and its transformation to H(P)NE-related aldehydes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

HPODE	hydroperoxy-octadecenoic acid
diHPODE	dihydroperoxy-octadecenoic acid
HPETE	hydroperoxyeicosatetraenoic acid
diHPETE	dihydroperoxyeicosatetraenoic acid
4-HNE	4-hydroxy-2 <i>E</i> -nonenal

4-HPNE	4-hydroperoxy-2 <i>E</i> -nonenal
LOX	lipoxygenase
PFB	pentafluorobenzyl
RP-HPLC	reversed-phase high-pressure liquid chromatography

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Figure 1. RP-HPLC analysis of reaction of arachidonic acid with 8R-LOX

Recombinant 8R-LOX (10 $\mu\text{g/ml}$) was reacted with arachidonic acid (25 $\mu\text{g/ml}$) in 50 mM Tris pH 8 containing 500 mM NaCl, 2 mM CaCl_2 plus 0.01% Emulphogene detergent for 10 min at room temperature. An aliquot of the reaction was analyzed by RP-HPLC using a Waters Symmetry column (25 $\times$ 0.46 cm), a solvent of $\text{CH}_3\text{CN}/\text{H}_2\text{O}/\text{HAc}$ (45/55/0.01, by volume), at a flow rate of 1 ml/min, with diode array detection. The different wavelengths are on the same scale and are offset for clarity.

Figure 2. GC-MS and NMR analyses of 2

A. Mass spectrum of the methyl ester TMS ether methoxime derivative of TPP-reduced **2**.

B. ¹H-NMR spectrum of **2**; the COSY spectrum is shown as Supplementary Figure S5.

Figure 3. Identification of the bis-allylic 8,11-diHPETE as a product of the on-ice incubation of arachidonic acid with 8R-LOX

A. Recombinant 8R-LOX (10 $\mu\text{g}/\text{ml}$) was reacted with arachidonic acid (50 μM) in 50 mM Tris pH 8 containing 500 mM NaCl, 2 mM CaCl_2 plus 0.01% Emulphogene detergent for 15 min at the ice-cold temperature. An aliquot of the reaction was analyzed by RP-HPLC using a Waters Symmetry column (25 $\times$ 0.46 cm), a solvent of $\text{CH}_3\text{CN}/\text{H}_2\text{O}/\text{HAc}$ (45/55/0.01, by volume), at a flow rate of 1 ml/min, with diode array detection. The peaks labeled 1–4 correspond to those in Figure 1. The different wavelengths are on the same scale and are offset for clarity. **B.** Overlay of the UV spectra of 8R-HPETE and 8,11-diHPETE. **C.** GC-MS analysis of the TMS ester TMS ether derivative of the TPP-reduced hydrogenated 8,11-diHPETE.

Figure 4. Conversion of purified 8,11-diHPETE to peaks 1 and 2

Reactions of recombinant 8R-LOX (10 $\mu\text{g/ml}$) with purified 8,11-diHPETE (5 $\mu\text{g/ml}$) were conducted in 50 mM Tris pH 8 containing 500 mM NaCl, 2 mM CaCl_2 plus 0.01% Emulphogene detergent for 15 min at room temperature without (top) or with (below) adding 5 μM NDGA. Aliquots of the reaction were analyzed by RP-HPLC with diode array detection as outlined above. The UV profiles at 220 nm are illustrated.

Figure 5. Determination of C-11 hydroperoxyl configuration of 8,11-diHPETE

A. Scheme of 8*R*-LOX reactions with 9*E*,11*Z*,14*Z*-C20:3. **B.** GC-MS analysis of TMS ether TMS ester of TPP-reduced hydrogenated 11-hydroperoxy-C20:3. **C.** Chiral phase-HPLC analysis of PFB ester of TPP-reduced hydrogenated 11-hydroperoxyl C20:3 using a Chiralpak AD column with a solvent system of hexane/methanol (100/2, by volume) at a flow rate of 1 ml/min. The UV profile at 205 nm is illustrated.

Scheme 1. Bis-allylic fatty acid hydroperoxide and its cleavage to a 4-hydro(pero)xy-alkenal

Scheme 2. Proposed routes of formation of compound 2