Biosynthesis of a linoleic acid allylic epoxide: mechanistic comparison with its chemical synthesis and leukotriene A biosynthesis[S]

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Abstract **Biosynthesis of the leukotriene A (LTA) class of epoxide is a lipoxygenase-catalyzed transformation requiring a fatty acid hydroperoxide substrate containing at least three double bonds. Here, we report on biosynthesis of a dienoic analog of LTA epoxides via a different enzymatic mechanism. Beginning with homolytic cleavage of the hydroperoxide moiety, a catalase/peroxidase-related hemoprotein from *Anabaena* PCC 7120, which occurs in a fusion protein with a linoleic acid 9*R*-lipoxygenase, dehydrates 9*R*-hydroperoxylinoleate to a highly unstable epoxide. Using methods we developed for isolating extremely labile compounds, we prepared and purified the epoxide and characterized its structure as 9*R*,10*R*-epoxy-octadeca-11*E*,13*E*-dienoate. This epoxide hydrolyzes to stable 9,14-diols that were reported before in linoleate autoxidation (Hamberg, M. 1983. Autoxidation of linoleic acid: Isolation and structure of four dihydroxy octadecadienoic acids. *Biochim. Biophys. Acta* 752: 353–356) and in incubations with the *Anabaena* enzyme (Lang, I., C. Göbel, A. Porzel, I. Heilmann, and I. Feussner. 2008. A lipoxygenase with linoleate diol synthase activity from Nostoc sp. PCC 7120. *Biochem. J.* 410: 347–357). We also prepared an equivalent epoxide from 13*S*-hydroperoxylinoleate using a "biomimetic" chemical method originally described for LTA_4 synthesis and showed that like LTA_4, the C18.2 epoxide conjugates readily with glutathione, a potential metabolic fate in vivo. We compare and contrast the mechanisms of LTA-type allylic epoxide synthesis by lipoxygenase, catalase/peroxidase, and chemical transformations. These findings provide new insights into the reactions of linoleic acid hydroperoxides and extend the known range of catalytic activities of catalase-related hemoproteins.**—Niisuke, K., W. E. Boeglin, J. J. Murray, C. Schneider, and A. R. Brash. **Biosynthesis of a linoleic acid allylic epoxide: mechanistic comparison with its chemical synthesis and leukotriene A biosynthesis. *J. Lipid Res.* 2009.** 50: **1448–1455.**

Supplementary key words lipoxygenase • biomimetic synthesis • *Anabaena* • cyanobacterium • catalase • peroxidase • hemoprotein • ^{1}H-nuclear magnetic resonance • gas liquid chromatography-mass spectrometry

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The plethora of products formed through oxygenation of polyunsaturated fatty acids was extended recently through characterization of the activities of an enzyme from *Anabaena* PCC 7120 (1), a photosynthetic cyanobacterium which is also termed *Nostoc* sp. PCC 7120 (2). The enzyme in question is a plasmid-encoded fusion protein with a catalase-related hemoprotein on the N-terminal side and a lipoxygenase at the C-terminal end. The catalase-related domain is 39 kDa in size, directly connected to the lipoxygenase (49 kDa), giving a molecular mass of 88 kDa for the full-length fusion protein. The full-length fusion protein was successfully expressed in *Escherichia coli*, and although the cDNA encoding only the catalase-related domain did not express as active protein, the cDNA of the lipoxygenase (LOX) domain expressed very well by itself, allowing the dual catalytic activities of the fusion protein to be clearly distinguished. The LOX domain converts C18 fatty acids to the corresponding 9*R*-hydroperoxide (3, 4). This is an unusual LOX activity, forming products enantiomeric to the 9*S*-LOX of plants (5), and characterized earlier in *Hydra vulgaris* (6), in the marine alga *Ulva conglobata* (7), and as an activity of *Gersemia fruticosa* arachidonate 11*R*-LOX (8). We showed that the catalase-related domain converts the 9*R*-hydroperoxide of α-linolenic acid to two unstable epoxides that we isolated and characterized (1). One is an allylic 9,10-epoxide with C13-C16 of the carbon chain cyclized into a [1.1.0]bicyclobutane, a unique structural motif in biology. A second product, an allylic epoxide of the leukotriene A (LTA) type, 9*R*,10*R*-octadeca-11*E*,13*E*,15*Z*-trienoic acid, is also formed from the same 9*R*-hydroperoxylinolenic acid substrate (1).

Abbreviations: H(P)ETE, hydro(pero)xyeicosatetraenoic acid; H(P)ODE, hydro(pero)xyoctadecadienoic acid; LT, leukotriene; LOX, lipoxygenase; RP-HPLC, reversed-phase high-pressure liquid chromatography; SP-HPLC, straight-phase high-pressure liquid chromatography.

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The metabolism of linoleic acid by this *Anabaena* fusion protein was studied by Feussner and colleagues and reported to form 9*R*-HPODE via the LOX domain and three more polar dihydroxy products (2). The major diol was identified as 9,14-dihydroxyoctadeca-10*E*,12*E*-dienoic acid. Formation of a 9,10-dihydroxyoctadeca-11*E*,13*E*-dienoic acid and an 8,11-dihydroxyoctadeca-9*E*,12*E*-dienoic acid was also described. Additional experiments using incubations conducted using $H_2{}^{18}O$ or $^{18}O_2$ indicated that the hydroxyl at C-9 in the main diol arises from molecular oxygen and the hydroxyl at C14 from water. The fusion protein was termed a linoleate diol synthase (2).

Based on the structures of the linoleic acid-derived 9,14- and 9,10-diols formed by the *Anabaena* fusion protein and the incorporation of water at C14 in the main diol, they are likely to be the stable end products of unstable epoxide intermediate(s) that hydrolyze under the typical conditions of incubation at room temperature. If this were the case, based on analogy to LTA_4 and other allylic epoxides (e.g., Refs. 9–11), hydrolysis should produce two diastereomeric 9,14-diols as the main products together with two minor 9,10-diols. In the first part of this article, we clarify this issue. We then report the isolation and characterization of the unstable epoxide precursor. Third, we compare the stereochemical structure of this product to the isomeric epoxides formed through a chemical transformation. The chemical method was developed originally as a "biomimetic" synthesis in starting from a fatty acid hydroperoxide and giving the natural epoxide as one of the products, although the steps in between are completely different from the enzymology (12–14). Originally developed for preparation of the conjugated triene-containing allylic epoxide LTA_4, here, we report its application to preparation of conjugated diene-containing epoxides.

EXPERIMENTAL PROCEDURES

Materials

Fatty acids were purchased from NuChek Prep (Elysian, MN). [1-^{14}C]Linoleic acid was purchased from Perkin-Elmer Life Sciences. 9*R*-Hydroperoxy-C18.2ω6 (9*R*-HPODE) was prepared using the LOX domain of the *Anabaena* enzyme in pH 7.5 Tris buffer using methods described previously for the linolenic acid analog (1); the reaction of linoleic acid with the LOX domain in buffer alone exhibits strong substrate inhibition at fatty acid concentrations over 30 μM, necessitating large volumes for preparation of milligram quantities (e.g., 4 mg in 200 ml), but this can be largely overcome by including 0.1% CHAPS detergent in the solution (e.g., allowing reaction of 5 mg fatty acid in 50 ml buffer). After reaction was complete as judged by UV analysis, the 9*R*-HPODE product was extracted with dichloromethane at pH 4 and purified by straight-phase high-pressure liquid chromatography (SP-HPLC) (1).

Expression and purification of *Anabaena* enzyme constructs

The cDNA for the full-length peroxidase-LOX fusion protein from *Anabaena* sp. strain PCC 7120 was cloned into pET17b for expression in *E. coli* as described previously (1). The full-length construct included the native cDNA sequence with a $(His)_6$ tag on the N terminus (MHHHHHHDLNTY——LMMSINI.). The protein was expressed in *E. coli* BL21 (DE3) cells (Novagen) using methodology described previously (15) and was purified on nickel-nitrilotriacetic acid agarose (Qiagen, Valencia, CA) according to the manufacturer's instructions and quantified from the main Soret band at 406 nm assuming $\varepsilon = 100{,}000$. The LOX-only domain (starting at amino acid 344 with the amino acid sequence KDDLPGK…, and comprising the last 430 amino acids of the full-length construct) was expressed with an N-terminal His_6 tag and purified by nickel affinity chromatography (3).

Enzyme incubations and extraction of stable end products

Incubations with purified enzyme were conducted in 50 mM Tris and 150 mM NaCl, pH 7.5, at room temperature. Small-scale reactions (0.5 ml and 1 ml) were monitored by UV spectroscopy by repetitive scanning in the range 200–300 nm or by monitoring the decrease in absorbance over time at 235 nm. For preparation of products for NMR analysis, reactions were conducted in 50 ml buffer with up to 200 μM 9*R*-HPODE as substrate, added in a small volume of ethanol (≤1% by volume). After 20 min, samples were acidified by addition of a previously titrated amount of 1 M KH_2PO_4 plus sufficient 1 N HCl to give pH 6 and applied to a preequilibrated Bond-Elut C18 cartridge (1 g cartridge per 25 ml original buffer), and after washing with water the products were eluted with methanol.

HPLC analysis of stable end products

Samples were run on an Agilent (Palo Alto, CA) 1100 HPLC equipped with a diode array detector (and for radiolabeled samples, connected online to a Flo-One A-100 radioactive detector; Radiomatic Instruments and Chemical, Meridian, CT). Samples were analyzed initially using a Waters C18 Symmetry column (25 × 0.46 cm) with a solvent system of methanol/water/glacial acetic acid (75/25/0.01, by volume), a flow rate of 1 ml/min, and with UV detection at 205, 220, 235, and 270 nm; for improved resolution, in some runs, the solvent proportions were changed to 65/35/0.01 (methanol/water/glacial acetic acid, by volume). For methyl esters, a solvent system of methanol/water 75/25 (v/v) was employed. Subsequent SP-HPLC of individual products used a Beckman Ultrasphere 5 μ silica column (25 × 0.46 cm), a solvent system of hexane/isopropanol/acetic acid (100:5:0.1, by volume), a flow rate of 1 ml/min for free acids, and a solvent of hexane/isopropanol (100:3, v/v) for methyl esters. For chiral phase HPLC, we employed a Daicel Chiralpak AD column (0.46 × 25 cm), hexane/methanol (100:2 or 100:5, by volume), and 1 ml/min (16).

Preparation and purification of the unstable epoxide

Enzyme reactions were conducted at 0°C essentially as previously described (1). Briefly, reaction was initiated by vigorous vortex mixing of a hexane solution of 9*R*-HPODE (100–400 μM in 20 ml) with the *Anabaena* enzyme (0.8 nmole) in 200 μl of phosphate buffer, pH 8. After 1 min of mixing, the hexane phase was collected, evaporated to ~2 ml under nitrogen, and the fatty acids methylated using ethereal diazomethane in the presence of 1% ethanol. The sample was evaporated to dryness, redissolved in hexane, and stored at −80°C until further analyzed. Initially, we experimented with HPLC under cold conditions (silica column, hexane/diethyl ether solvent, 100:5, v/v, run at −10–15°C) as used previously for isolation of allene epoxides (17). Later, the use of HPLC conditions previously developed for chromatography of LTA_4 proved more satisfactory, either SP-HPLC of methyl esters using triethylamine in the solvent (14, 18) or our modification of a reversed-phase HPLC (RP-HPLC) procedure, suitable for methyl esters or acids (1, 19). For SP-HPLC, an Altex silica guard column (5 μm, 45 × 4.6 mm) was run with a solvent of hexane/triethylamine

(100:0.5, by volume) eluted at 3 ml/min. For RP-HPLC, the hexane extracts were analyzed and purified using a Waters Symmetry C18 5 μm column (0.46 × 25 cm) eluted at a flow rate of 1 ml/min with methanol/20 mM potassium phosphate pH 8 (replaced with triethylamine for LC-MS analysis, see below) in the proportions 80:20 (by volume), with UV detection at 205, 220, 235, and 270 nm using an Agilent 1100 series diode array detector. The main products from RP-HPLC were recovered by extraction with cold hexane followed by evaporation to dryness under a strong stream of nitrogen; products were promptly redissolved in hexane/triethylamine (100:0.1, by volume) and stored at −80°C prior to further analysis.

Incubation in $H_2{}^{18}O$ and LC-MS analysis of the products

LC-MS of the stable products from reaction of 9*R*-HPODE with the *Anabaena* enzyme in $H_2{}^{18}O$ buffer (^{18}O:^{16}O, 3:1) was performed using a Thermo Finnigan LC Quantum instrument. A Waters Symmetry C18 column (15 × 0.2 cm) was eluted with methanol/10 mM ammonium acetate (80:20, by volume) at 0.2 ml/min. The heated capillary ion lens was operated at 220°C. Nitrogen was used as a nebulization and desolvation gas. The electrospray potential was held at 4 kV. Source-induced dissociation was set at −10 eV. Negative ion mass spectra were acquired over the mass range *m/z* 100 to 500 at 2 s/scan. Collision-induced dissociation was performed at −15 eV.

Biomimetic synthesis of C18.2 epoxides

The synthesis followed the method described for the conversion of 5- hydroperoxyeicosatetraenoic acid (5-HPETE) to LTA_4 as the methyl ester (14) and the corresponding reaction of 15-HPETE methyl ester to the 14,15-epoxide analog of LTA_4 (13), as used previously in our laboratory (18). For synthesis of the C18.2 allylic epoxides, 3 mg (10 μmol) 13*S*-HPODE methyl ester was evaporated to an oil under N_2 in a dry 300 μl Reactivial (Pierce) to which a small triangular Teflon stirrer was added. An argon atmosphere, instilled via a syringe port cap, and 80 μl freshly distilled THF:CH_2Cl_2 (1:1) was added. To this was added 6.0 μl 1,2,2,6,6-pentamethylpiperidene (35 μmol), freshly distilled under vacuum from CaH_2; the resulting solution was cooled to −70°C by means of a dry ice-acetone bath. Five minutes later, 3.0 μl trifluoromethane sulfonic anhydride (Alfa or Aldrich) was added, which gave a pale-yellow color. The reaction was maintained at −70°C and was complete by 45 min. Triethylamine (10 μl) was added, and the reaction warmed to room temperature and transferred to 500 μl hexane:Et_2O:triethylamine (50:50:1). This was washed twice with equal volumes of brine and the organic phase evaporated under N_2 to give a yellow oil that was promptly reconstituted with 800 μl hexane/triethylamine (100:1, v/v), layered with argon, and stored at −70°C for analysis. The crude mixture was purified in aliquots by SP-HPLC at room temperature using a Waters μPorasil column (10 μ, 3.9 × 250 mm) and a solvent system of hexane:triethylamine (100:1 v/v/) at 1.0 ml/min with UV detection at 250 nm. This synthesis was completed in our laboratory in 1984, and 1 mg samples of the two HPLC-purified epoxide methyl esters were stored at −70°C in HPLC column solvent since that time. Aliquots of the two epoxides were repurified by RP-HPLC in conjunction with this study using the HPLC conditions described above for the *Anabaena* C18.2 epoxide methyl ester. Using a Waters Symmetry C18 column (25 × 0.46 cm), a solvent of methanol/water/triethylamine (85/15/0.1) at a flow rate of 1 ml/min with UV detection at 250 nm, the epoxide methyl esters eluted in the same order as on SP-HPLC at retention times of 14 and 15.5 min, respectively. Their UV spectra were acquired with the online diode array detector, and ^{1}H-NMR spectra were recorded in d_6-benzene.

Derivatization and GC-MS analysis

Methyl esters were prepared using ethereal diazomethane/methanol (5:1). Catalytic hydrogenations were performed in 100 μL of ethanol using about 1 mg of palladium on alumina 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. Trimethylsilyl ester and trimethylsilyl ether derivatives were prepared by treatment with *bis*(trimethylsilyl)-trifluoracetamide (10 μL) and pyridine (5 μL) at room temperature for 2 h. Subsequently, the reagents were evaporated under a stream of nitrogen, and the samples were dissolved in hexane for GC-MS analysis. GC-MS was carried out in the positive ion electron impact mode (70 eV) using a Thermo Finnigan Trace DSQ ion trap GC-MS with the Xcalibur data system, version 1.3. Samples were injected at 150°C, and after 1 min, the temperature was programmed to 300°C at 12 or 20°C/min.

NMR analyses

^{1}H-NMR spectra were recorded on a Bruker 600 MHz instrument at 283K. Samples were dissolved in d_6-benzene or $CDCl_3$, and the chemical shifts are reported relative to the benzene (δ 7.16 ppm for hydrogen) or $CHCl_3$ (δ 7.24 ppm) signals. The chemically synthesized epoxide methyl esters were analyzed originally on a Bruker 400 MHz instrument using carbon tetrachloride as solvent. (Although the epoxides were stable in this solvent and satisfactory spectra were obtained, the use of CCl_4 for proton NMR necessitates the addition of tetramethylsilane or other reference compound to provide a lock signal for our current instruments.)

RESULTS

Metabolism of linoleic acid by the *Anabaena* fusion protein

When linoleic acid is metabolized by the recombinant *Anabaena* fusion protein, it is rapidly oxygenated to 9*R*-HPODE by the LOX domain (2–4) and then further transformed to polar products. Analysis of the polar products was carried out using purified 9*R*-HPODE as substrate, and a typical RP-HPLC chromatogram is shown in **Fig. 1**. The two most prominent peaks, which exhibit identical conjugated diene chromophores with λmax at 230.5 nm (Fig. 1, peaks 1 and 2, inset) were identified as 9,14-dihydroxyoctadeca-10*E*,12*E*-dienoic acids by GC-MS and ^{1}H-NMR (see supplementary data). Two smaller and later eluting peaks exhibited conjugated dienes with a very slight shift to higher wavelength (peaks 3 and 4, λmax 231.5 and 231.8 nm, respectively; Fig. 1, inset) and were identified as 9,10-dihydroxy-octadeca-11*E*,13*E*-dienoic acids (see supplementary data). Based on the stereochemistry of 9,14-diols deduced by Hamberg (20), and assuming that the original *R* chirality of the hydroperoxide is retained in the C9 hydroxyl, the two 9,14-diols are the 9*R*,14*S* (peak 1) and 9*R*,14*R* (peak 2) diastereomers. These are predicted to be the major products of hydrolysis of an unstable allylic 9,10-epoxide, with the 9,10-diols being the minor hydrolysis products. This was supported by LC-MS analysis of an incubation conducted in $H_2{}^{18}O$, in which the incorporation of one atom of ^{18}O from water was confirmed in both the 9,14- and 9,10-diols (data not shown).

Some very minor products (≤5% abundance of the main 9,14-diols) were structurally characterized. These

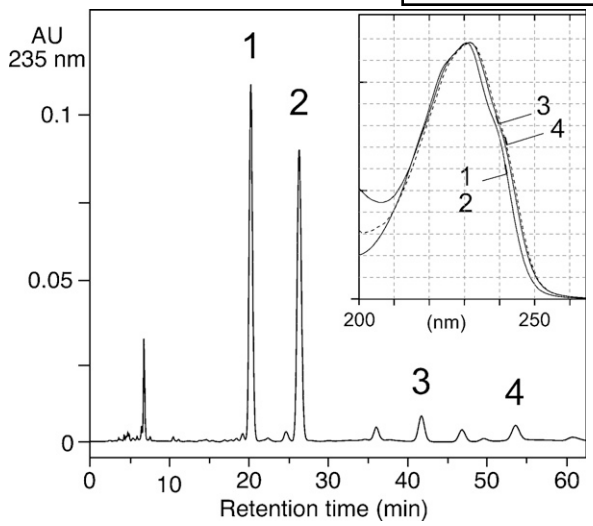

Fig. 1. RP-HPLC analysis of the stable end products of linoleic acid metabolism by the *Anabaena* peroxidase-LOX fusion protein. Column: Waters Symmetry C18, 25 × 0.46 cm; solvent, methanol/water/glacial acetic acid (65:35:0.01, by volume); flow rate, 1 ml/min; UV detection at 235 nm. Inset: the UV spectra of peaks 1–4; the spectra of peaks 1 and 2 are identical to each other (λmax 230.5 nm), while 3 is shifted to 231.5 nm and 4 (dashed line) to 231.8 nm.

included an unusual 9,12-dihydroxy-octadeca-10,13-dienoic acid hydrolysis product that chromatographs immediately after the second 9,14-diol peak on RP-HPLC (see supplementary data). We also found a minor epoxyalcohol, 9,10-epoxy-11-hydroxy-octadeca-12-enoic acid that chromatographed between the two 9,10-diols and gave a mass spectrum as the methyl ester TMS ether derivative similar to that attributed by Lang et al. to an 8,11-diol (2). Its relatively featureless and not very informative mass spectrum, dominated by the cleavage ion between the epoxide and hydroxyl that retains the OTMS moiety, is typical of fatty acid epoxides with an α-hydroxyl (hepoxilin B-type epoxyalcohols) (e.g., Refs. 21–23) (see supplementary data).

Isolation and characterization of an unstable epoxide intermediate

Using a protocol we developed for the synthesis and instantaneous extraction of unstable intermediates, we isolated a nonpolar conjugated diene-containing product from reaction of 9*R*-HPODE with the *Anabaena* fusion protein. After conversion to the methyl ester derivative, the product was purified by SP-HPLC using a solvent containing triethylamine to help stabilize acid-sensitive molecules or the use of cold conditions as in **Fig. 2A**. The UV spectrum of the product showed a conjugated diene chromophore with an unusually broad top and λmax at 241 nm (Fig. 2B). The complete covalent structure was elucidated by ^{1}H-NMR and correlation spectroscopy analysis (**Fig. 3**). The *trans* epoxide configuration follows from the coupling constant between the two epoxide protons (J = 2 Hz) and the coupling constants across the two double bonds ($J_{11,12}$ = 15.6 Hz; $J_{13,14}$ = 15.0 Hz) establish the *trans-trans* configuration of the conjugated diene. Assuming that the chirality at C9 is retained as 9*R*, then the complete structure of the epoxide product is 9*R*,10*R*-epoxyoctadeca-11*E*,13*E*-dienoic acid.

Using the fusion protein, an equivalent product was also prepared from 11*R*-HPETE (the main product of the *Anabaena* LOX domain from arachidonic acid) and identified as 11*R*,12*R*-epoxyeicosa-5*Z*,8*Z*,13*E*,15*E*-tetraenoate by ^{1}H-NMR (see supplementary data).

Biomimetic chemical synthesis of linoleate allylic epoxide

For the chemical reaction, we used 13*S*-HPODE as starting material as it is readily available in multimilligram quantities using soybean LOX-1, and one of the resulting 12,13-epoxyoctadeca-8,10-dienoate products has a symmetrical epoxydiene core structure to the corresponding epoxide formed by the *Anabaena* enzyme from 9-HPODE. In common with the chemical reaction using HPETE to produce LTA-type epoxides (14), the chemical transformation of methyl 13-HPODE gave three products. These eluted on SP-HPLC at retention volumes of 12.6, 13.8,

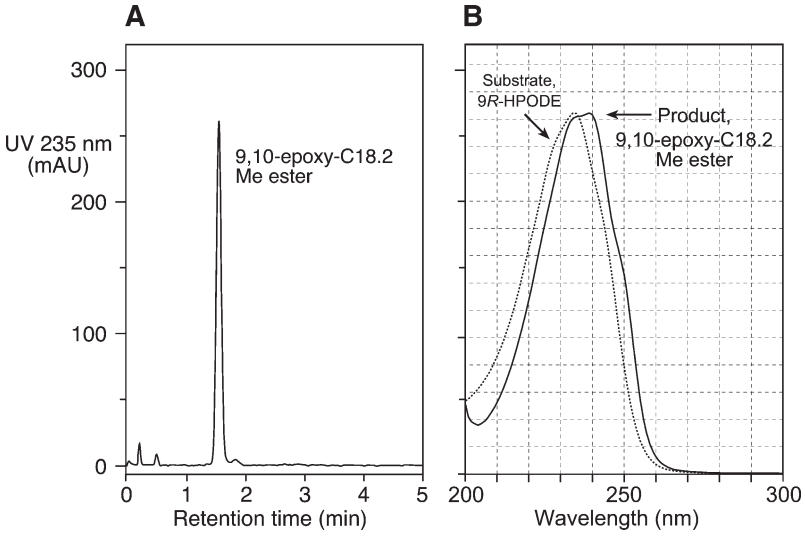

Fig. 2. Chromatography of the linoleate allylic epoxide. A: The sample was run on an Altex/Beckman silica column (5 μ, 4.5 × 0.46 cm) immersed in an ethanol/ice bath at approximately −10 to −15°C with a solvent of hexane/diethyl ether (100:5, v/v) at a flow rate of 3 ml/min with UV detection at 235 nm. B: UV spectrum of the linoleate allylic 9,10-epoxide prepared using the *Anabaena* enzyme. For comparison, the spectrum of 9-HPODE is shown as the dotted line. Solvent: hexane.

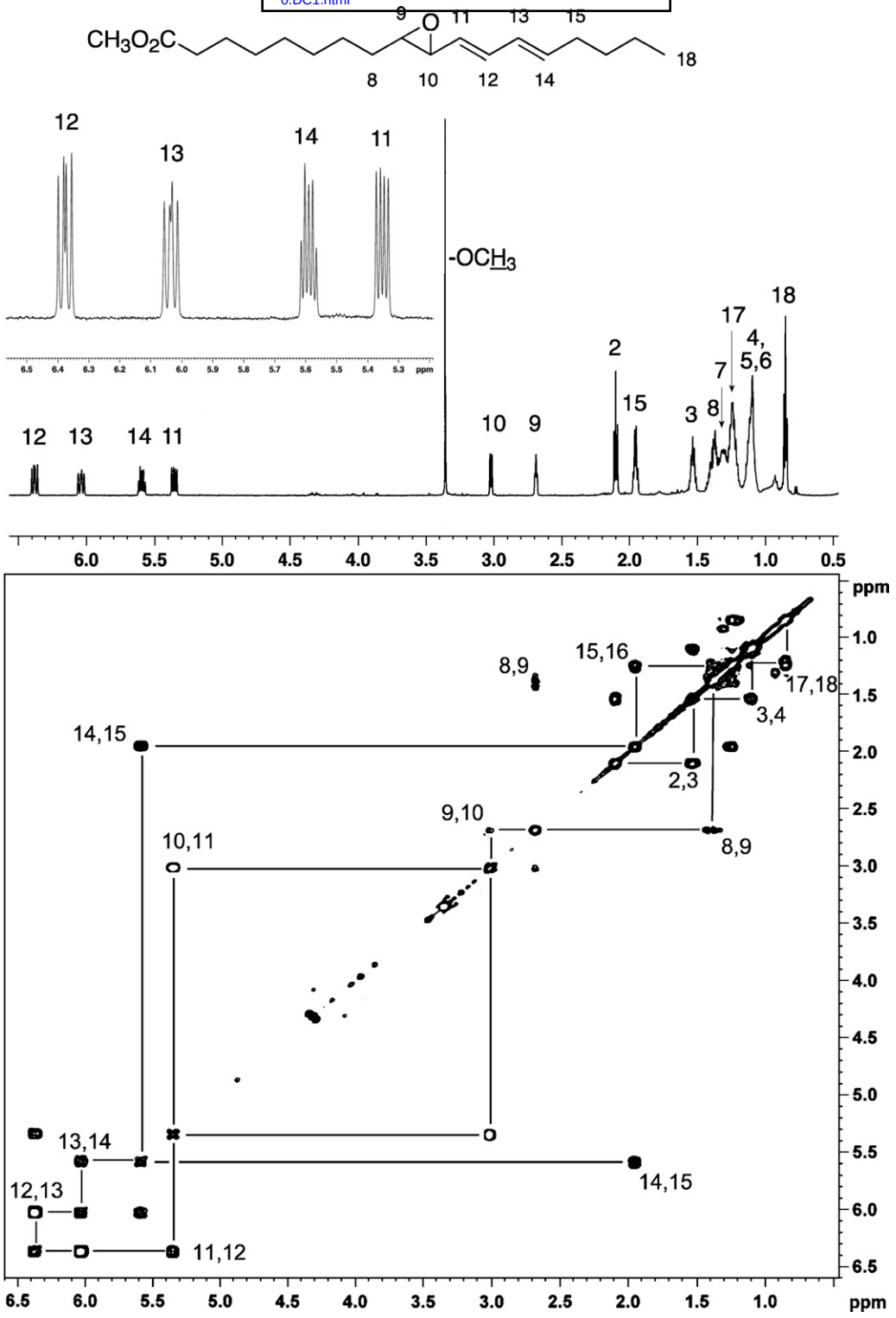

Fig. 3. ^{1}H-NMR spectrum of the methyl ester of the unstable epoxide formed from linoleic acid by the *Anabaena* peroxidase-LOX fusion protein. The inset displays the olefinic protons in more detail. The product is identified as 9*R*,10*R*-epoxyoctadeca-11*E*,13*E*-dienoate methyl ester. Solvent: d6-benzene. Chemical shift δ, multiplicity, number of protons, proton number, J: 6.38, dd, 1, H12, $J_{11,12}$ = 15.6, $J_{12,13}$ = 10.6; 6.04, dd, 1, H13, $J_{12,13}$ = 10.7, $J_{13,14}$ = 15.0; 5.59, td, 1, H14, $J_{13,14}$ = 15.1, $J_{14,15}$ = 7.1; 5.35, dd, 1, H11, $J_{10,11}$ = 8.0, $J_{11,12}$ = 15.4; 3.36, s, 3, CH_3O; 3.02, dd, 1, H10, $J_{9,10}$ = 1.3, $J_{10,11}$ = 8.0; 2.69, dt, 1, H9, $J_{8,9}$ = 6.3, $J_{9,10}$ = 1.7; 2.10, t, 2, H2; 1.95, q, 2, H15; 1.53, p, 2, H3; 1.45–1.33, m, 2, H8; 1.28–1.26, m, 4, H16,17; 1.15–1.06, m, 8, H4,5,6,7; 0.86, t, 3, H18.

and 19.2 ml, respectively, in relative proportions of approximately 8:7:5. The first two peaks were identified as 12*S*,13*S*-epoxyoctadeca-8,10-dienoates with 8*Z*,10*E* and 8*E*,10*E* configurations of the conjugated diene, respectively, and the third eluting product was 13-keto-octadeca-9,11-dienoate. The UV spectra of the two epoxides differed from each other and were quite distinctive (**Fig. 4A**). The NMR spectra showed that both are *trans* epoxides ($J_{12,13}$ = 2 Hz) and allowed designation of the 8*Z*,10*E* conjugated diene of the first eluting epoxide and the 8*E*,10*E* conjugation in the second (see supplementary data). The UV spectrum and NMR spectrum of the second 12,13-epoxide were superimposable with those of the 9,10-epoxide prepared using the *Anabaena* enzyme. Although arising from 13-HPODE and 9-HPODE, respectively, the two epoxides are symmetrical in the epoxy-diene moiety.

Hydrolysis of the *Anabaena* 9,10-epoxide

The purified linoleate 9,10-epoxide of the *Anabaena* enzyme was hydrolyzed as the methyl ester derivative, yielding two major 9,14-diols and two minor 9,10-diols that matched the corresponding methyl ester derivatives of the products of the enzyme incubations. The minor 9,12-diol that chromatographs immediately after the second 9,14-diol on RP-HPLC was also found at a similar (minor) level (data not shown).

Reaction of the synthetic 12,13-epoxyoctadecadienoate with glutathione

The original description of the biomimetic synthesis of LTA_4-related epoxides included the preparation of the LTC_4 analogs, the glutathione adducts (13). We used the reported methods to react the 12,13-epoxyoctadeca-8*E*,10*E*-dienoate with GSH and obtained the predicted 13-hydroxy-12-glutathionyl adduct, purified the adduct by RP-HPLC, and recorded its distinctive UV spectrum (Fig. 4B). The UV spectrum of authentic LTC_4 shows a bathochromic shift compared with plain fatty acid conjugated trienes, whereas only the longer wavelength shoulder in the spectrum of the conjugated diene-containing glutathione adduct of the linoleate epoxide is extended to higher wavelength.

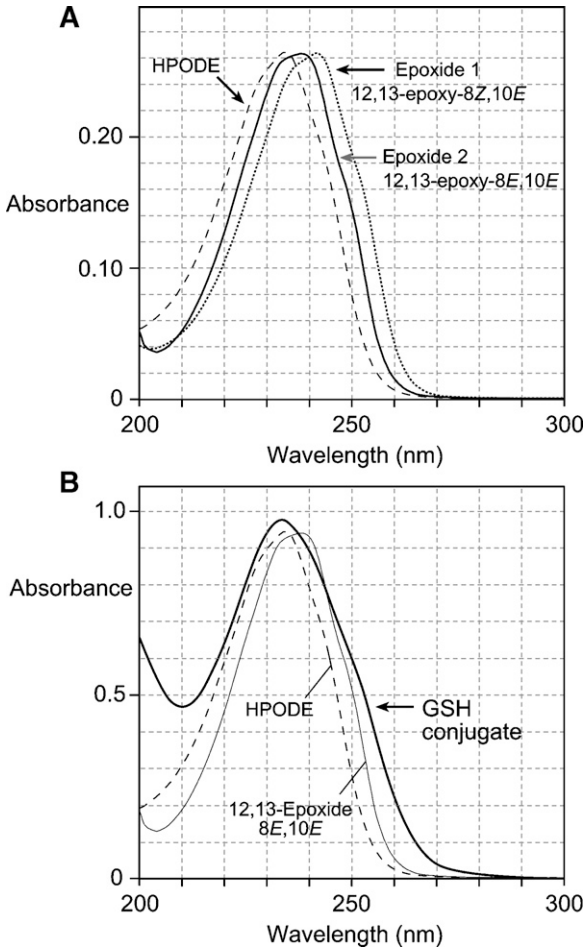

Fig. 4. UV spectra of the 12,13-epoxides and a glutathione conjugate. A: The two epoxides formed by chemical synthesis from 13-HPODE methyl ester, recorded in hexane. B: The GSH conjugate of epoxide 2, the 12,13-epoxy-octadeca-8*E*,10*E*-dienoate recorded in methanol. The spectrum of 13-HPODE is included as the dotted line in the figures for comparison.

DISCUSSION

Here, we show that the product from reaction of linoleic acid or 9*R*-HPODE with the *Anabaena* peroxidase-LOX fusion protein is an unstable allylic epoxide, a novel oxylipin of enzymatic origin. The existence of this type of epoxide was invoked previously to account for the formation of 9,14-diols during autoxidation of linoleic acid and linoleate hydroperoxides (10, 20), and an epoxide was speculated to be an intermediate of the linoleate diol synthase activity of this *Anabaena* enzyme (2). Actually, the latter name for the enzyme is not very appropriate, although we are not offering a more correct and succinct alternative. With all the polyunsaturated fatty acids tested, the heme domain acts as an allylic epoxide synthase, although with distinctive product characteristics from the individual substrates. There are true linoleate diol synthases in which a specific diol stereoisomer is formed under enzymatic control (24, 25) but that is not the case here. Hydrolysis of the linoleate epoxide in vitro is nonenzymatic, giving a 1:1 mixture of the two major 9,14-diol diastereomers. The enzymatic end product from linoleic acid is the allylic 9,10-epoxide.

Chemical synthesis of an analog of the *Anabaena* allylic epoxide was achieved using 13-HPODE methyl ester as starting material (**Fig. 5C**). Using a method originally developed for synthesis of LTA_4 and related conjugated trienoic epoxides (13, 14), we obtained the equivalent results with C18.2 starting material. In parallel to the more detailed original report (14), the chemical transformation of linoleate hydroperoxide gave two allylic epoxides plus a keto-octadecadienoate as products. The 12,13-*trans*-epoxide with *trans-trans* conjugated diene is analogous in structure to the *Anabaena* 9,10-epoxide. Although both the chemical reaction and the *Anabaena* epoxide synthesis are initiated

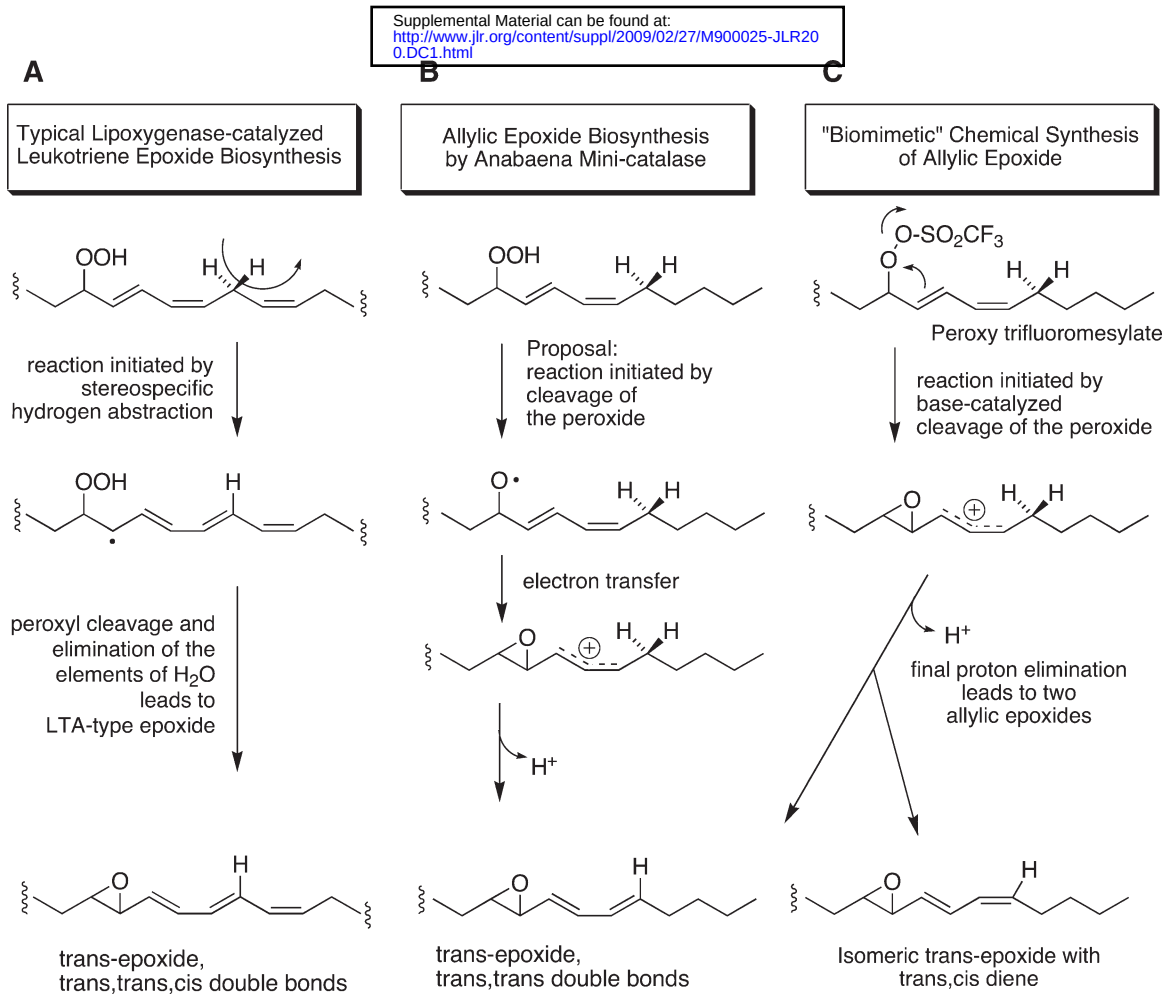

Fig. 5. Mechanisms of transformation to allylic epoxides. A: LOX-catalyzed LTA-type epoxide biosynthesis. B: Transformation of 9*R*-HPODE by the *Anabaena* enzyme. C: Chemical biomimetic synthesis of allylic epoxide.

by activation of the hydroperoxide moiety, the enzyme maintains control of the transformation and makes a single epoxide product.

Allylic epoxides with conjugated trienes are well known in mammalian systems, particularly the arachidonic acid-derived 5-LOX product LTA_4, the immediate precursor of the pro-inflammatory dihydroxy LTB_4, and the bronchoconstrictor glutathionyl adduct LTC_4 (26). Transformation of the fatty acid hydroperoxide to the allylic epoxide is mechanistically quite distinct in the 5-LOX and *Anabaena* reactions (Fig. 5A, B). Conventional LTA_4 synthesis by 5-LOX is initiated by abstraction of a bis-allylic hydrogen from the fatty acid hydroperoxide (Fig. 5A). The hydrogen abstraction is stereospecific and associated with a primary isotope effect, both typical features of a LOX-catalyzed reaction (27–29). The presence of the third double bond in the substrate is required to facilitate reaction; this reduces the bond dissociation energy of the bis-allylic hydrogen targeted for abstraction, thus permitting its LOX-catalyzed removal. Reaction then continues with cleavage of the hydroperoxide and formation of the LTA_4 epoxide with conjugated triene. The corresponding reaction from a dienoic hydroperoxide substrate is beyond the capabilities of a LOX enzyme because the corresponding hydrogens on the carbon chain (at C14 of 9*R*-HPODE) have only one allylic double bond and thus are more tightly bound. We checked using the isolated LOX domain of the *Anabaena* fusion protein and confirmed that the polar epoxide-derived products were completely absent. We propose that transformation of 9*R*-HPODE by the hemoprotein domain of the *Anabaena* enzyme is catalyzed via homolytic cleavage of the hydroperoxide moiety, formation of a carbocation, and subsequent elimination of a proton to give the allylic epoxide as product (Fig. 5, top of middle column). This is compatible with the mechanism of conversion of 9*R*-hydroperoxy-linolenic acid shown earlier, which produces an allylic epoxide with bicyclobutane and an LTA-type epoxide (1).

In a cellular environment, allylic epoxides are easily hydrolyzed, but they may also react with nucleophiles, such as glutathione, a reaction that would be promoted by glutathione transferases. Such a possibility might help explain the apparent absence of detectable linoleate diols in *Anabaena* strains expressing the hemoprotein-LOX enzyme. The presence of 9*R*-HODE and 9*R*-HOTrE is established (2, 30), but no further derivatives of the hydroperoxides have been reported. Analysis of the metabolic fate of the epoxides described in this and our earlier report (1) might help clarify the pathways of linole(n)ic acid metabolism in *Anabaena* and point toward their biological role.

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REFERENCES

1. Schneider, C., K. Niisuke, W. E. Boeglin, M. Voehler, D. F. Stec, N. A. Porter, and A. R. Brash. 2007. Enzymatic synthesis of a bicyclobutane fatty acid by a hemoprotein-lipoxygenase fusion protein from the cyanobacterium *Anabaena* PCC 7120. *Proc. Natl. Acad. Sci. USA.* **104:** 18941–18945.
2. Lang, I., C. Göbel, A. Porzel, I. Heilmann, and I. Feussner. 2008. A lipoxygenase with linoleate diol synthase activity from Nostoc sp. PCC 7120. *Biochem. J.* **410:** 347–357.
3. Zheng, Y., W. E. Boeglin, C. Schneider, and A. R. Brash. 2008. A 49 KD mini-lipoxygenase from *Anabaena* sp. PCC 7120 retains catalytically complete functionality. *J. Biol. Chem.* **283:** 5138–5147.
4. Andreou, A. Z., M. Vanko, L. Bezakova, and I. Feussner. 2008. Properties of a mini 9R-lipoxygenase from Nostoc sp. PCC 7120 and its mutant forms. *Phytochemistry.* **69:** 1832–1837.
5. Liavonchanka, A., and I. Feussner. 2006. Lipoxygenases: occurrence, functions and catalysis. *J. Plant Physiol.* **163:** 348–357.
6. Di Marzo, V., C. Gianfrani, L. De Petrocellis, A. Milone, and G. Cimino. 1994. Polyunsaturated-fatty-acid oxidation in *Hydra*: regioselectivity, substrate-dependent enantioselectivity and possible biological role. *Biochem. J.* **300:** 501–507.
7. Akakabe, Y., K. Matsui, and T. Kajiwara. 2002. Enantioselective formation of (R)-9-HPODE and (R)-9-HPOTrE in the marine green alga *Ulva conglobata*. *Bioorg. Med. Chem.* **10:** 3171–3173.
8. Mortimer, M., R. Järving, A. R. Brash, N. Samel, and I. Järving. 2006. Identification and characterization of an arachidonate 11*R*-lipoxygenase. *Arch. Biochem. Biophys.* **445:** 147–155.
9. Borgeat, P., and B. Samuelsson. 1979. Arachidonic acid metabolism in polymorphonuclear leukocytes: unstable intermediate in formation of dihydroxy acids. *Proc. Natl. Acad. Sci. USA.* **76:** 3213–3217.
10. Hamberg, M. 1983. A novel transformation of 13-L_S-hydroperoxy-9,11-octadecadienoic acid. *Biochim. Biophys. Acta.* **752:** 191–197.
11. Hamberg, M. 1999. An epoxy alcohol synthase pathway in higher plants: biosynthesis of antifungal trihydroxy oxylipins in leaves of potato. *Lipids.* **34:** 1131–1142.
12. Corey, E. J., A. E. Barton, and D. A. Clark. 1980. Synthesis of the slow reacting substance of anaphylaxis leukotriene C-1 from arachidonic acid. *J. Am. Chem. Soc.* **102:** 4278–4279.
13. Corey, E. J., A. Marfat, and G. Giichi. 1980. Simple synthesis of the 11,12-oxido and 14,15-oxido analogs of leukotriene A and the corresponding conjugates with glutathione and cysteinylglycine, analogs of leukotrienes C and D. *J. Am. Chem. Soc.* **102:** 6607–6608.
14. Atrache, V., J. K. Pai, D. E. Sok, and C. J. Sih. 1981. Biomimetic synthesis of leukotriene-A. *Tetrahedron Lett.* **22:** 3443–3446.
15. Boutaud, O., and A. R. Brash. 1999. Purification and catalytic activities of the two domains of the allene oxide synthase-lipoxygenase fusion protein of the coral *Plexaura homomalla*. *J. Biol. Chem.* **274:** 33764–33770.
16. Schneider, C., Z. Yu, W. E. Boeglin, Y. Zheng, and A. R. Brash. 2007. Enantiomeric separation of hydroxy and hydroperoxy eicosanoids by chiral column chromatography. *Methods Enzymol.* **433:** 145–157.
17. Brash, A. R., S. W. Baertschi, C. D. Ingram, and T. M. Harris. 1988. Isolation and chacterization of natural allene oxides: unstable intermediates in the metabolism of lipid hydroperoxides. *Proc. Natl. Acad. Sci. USA.* **85:** 3382–3386.
18. Maas, R. L., C. D. Ingram, A. T. Porter, J. A. Oates, D. F. Taber, and A. R. Brash. 1985. Investigation of the chemical conversion of hydroperoxy-eicosatetraenoate to leukotriene epoxide using stereospecifically labeled arachidonic acid. *J. Biol. Chem.* **260:** 4217–4228.
19. Fitzpatrick, F. A., D. R. Morton, and M. A. Wynalda. 1982. Albumin stabilizes leukotriene A_4. *J. Biol. Chem.* **257:** 4680–4683.
20. Hamberg, M. 1983. Autoxidation of linoleic acid: isolation and structure of four dihydroxy octadecadienoic acids. *Biochim. Biophys. Acta.* **752:** 353–356.
21. Hamberg, M., and B. Gotthammar. 1973. A new reaction of unsaturated fatty acid hydroperoxides: formation of 11-hydroxy-12,13-epoxy-9-octadecenoic acid from 13-hydroperoxy-9,11-octadecadieneoic acid. *Lipids.* **8:** 737–744.
22. Funk, C. D., and W. S. Powell. 1983. Conversion of 8,11,14-eicosatrienoic acid to 11,12-epoxy-10-hydroxy-8-heptadecenoic acid by aorta. *Prostaglandins.* **25:** 299–309.
23. Pace-Asciak, C. R., E. Granstrom, and B. Samuelsson. 1983. Arachidonic acid epoxides. Isolation and structure of two hydroxy epoxide intermediates in the formation of 8,11,12- and 10,11,12- trihydroxy-eicosatrienoic acids. *J. Biol. Chem.* **258:** 6835–6840.
24. Gerwick, W. H., M. Moghaddam, and M. Hamberg. 1991. Oxylipin metabolism in the red alga *Gracilariopsis lemaneiformis*: mechanism of formation of vicinal dihydroxy fatty acids. *Arch. Biochem. Biophys.* **290:** 436–444.
25. Hamberg, M., L. Y. Zhang, I. D. Brodowsky, and E. H. Oliw. 1994. Sequential oxygenation of linoleic acid in the fungus *Gaeumannomyces graminis*: stereochemistry of dioxygenase and hydroperoxide isomerase reactions. *Arch. Biochem. Biophys.* **309:** 77–80.
26. Samuelsson, B., S. E. Dahlen, J. A. Lindgren, C. A. Rouzer, and C. N. Serhan. 1987. Leukotrienes and lipoxins: structures, biosynthesis, and biological effects. *Science.* **237:** 1171–1176.
27. Maas, R. L., C. D. Ingram, D. F. Taber, J. A. Oates, and A. R. Brash. 1982. Stereospecific removal of the D_R hydrogen atom at carbon 10 of arachidonic acid in the biosynthesis of leukotriene A_4 by human leukocytes. *J. Biol. Chem.* **257:** 13515–13519.
28. Panossian, A., M. Hamberg, and B. Samuelsson. 1982. On the mechanism of biosynthesis of leukotrienes and related compounds. *FEBS Lett.* **150:** 511–513.
29. Maas, R. L., and A. R. Brash. 1983. Evidence for a lipoxygenase mechanism in the biosynthesis of epoxide and dihydroxy leukotrienes from 15(S)-hydroperoxyicosatetranoic acid by human platelets and porcine leukocytes. *Proc. Natl. Acad. Sci. USA.* **80:** 2884–2888.
30. Murakami, N., H. Shirahashi, A. Nagatsu, and J. Sakakibara. 1992. Two unsaturated 9R-hydroxy fatty acids from the cyanobacterium *Anabaena* flos-aquae f. flos-aquae. *Lipids.* **27:** 776–778.