

# 8*R*-Lipoxygenase-catalyzed synthesis of a prominent *cis*-epoxyalcohol from dihomo- $\gamma$ -linolenic acid: a distinctive transformation compared with S-lipoxygenases<sup>S</sup>

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**Abstract** Conversion of fatty acid hydroperoxides to epoxyalcohols is a well known secondary reaction of lipoxygenases, described for S-specific lipoxygenases forming epoxyalcohols with a *trans*-epoxide configuration. Here we report on R-specific lipoxygenase synthesis of a *cis*-epoxyalcohol. Although arachidonic and dihomo- $\gamma$ -linolenic acids are metabolized by extracts of the Caribbean coral *Plexaura homomalla* via 8*R*-lipoxygenase and allene oxide synthase activities, 20:3 $\omega$ 6 forms an additional prominent product, identified using UV, GC-MS, and NMR in comparison to synthetic standards as 8*R*,9*S*-*cis*-epoxy-10*S*-*erythro*-hydroxy-eicosa-11*Z*,14*Z*-dienoic acid. Both oxygens of <sup>18</sup>O-labeled 8*R*-hydroperoxide are retained in the product, indicating a hydroperoxide isomerase activity. Recombinant allene oxide synthase formed only allene epoxide from 8*R*-hydroperoxy-20:3 $\omega$ 6, whereas two different 8*R*-lipoxygenases selectively produced the epoxyalcohol. A biosynthetic scheme is proposed in which a partial rotation of the reacting intermediate is required to give the observed *erythro* epoxyalcohol product. This characteristic and the synthesis of *cis*-epoxy epoxyalcohol may be a feature of R-specific lipoxygenases.—Jin, J., W. E. Boeglin, J. K. Cha, and A. R. Brash. 8*R*-Lipoxygenase-catalyzed synthesis of a prominent *cis*-epoxyalcohol from dihomo- $\gamma$ -linolenic acid: a distinctive transformation compared with S-lipoxygenases. *J. Lipid Res.* 2012. 53: 292–299.

**Supplementary key words** arachidonic acid • total synthesis • Sharpless epoxidation • *trans* epoxide • hepxilin • <sup>18</sup>O<sub>2</sub> incorporation • gas liquid chromatography-mass spectrometry • *Plexaura homomalla*

In the late 1960s and early 1970s, high concentrations of prostaglandin esters were identified in the Caribbean sea whip coral *Plexaura homomalla* (1, 2), and for a few years this abundant octacoral served as a source of prostaglan-

dins for research (3). Due to differences in the prostaglandin profile from that typically seen in mammalian systems, for some time it was suspected that there existed a different prostaglandin biosynthetic pathway in coral (4, 5). Although this putative noncyclooxygenase pathway of prostaglandin synthesis turned out to be a red herring and cyclooxygenase accounts for the biosynthesis (6, 7), research into polyunsaturated fatty acid metabolism in coral extracts uncovered other interesting biochemistry. Bundy and colleagues studied the related coral *Pseudoplexaura porosa* and uncovered 8*R*-lipoxygenase activity, the first known existence of an R-specific lipoxygenase (8). 8*R*-LOX was subsequently found to be widespread in corals including in *P. homomalla* (5, 9) as well as in many marine invertebrates (10), and a 12*R*-LOX is highly conserved and functionally essential in mammals (11, 12). A second novel activity detected in coral extracts was allene oxide synthase (5, 13), which transforms the 8*R*-LOX product, 8*R*-hydroperoxy-eicosatetraenoic acid, to an allene epoxide (5), a proposed intermediate in biosynthesis of cyclopentenones such as the clavulones (14–16), and which hydrolyzes in vitro to an  $\alpha$ -ketol derivative (5).

The work described in the present paper was initiated in the early 1990s, before the cloning of *P. homomalla* cyclooxygenases and lipoxygenases. It concerns an unexpected difference we observed in the metabolism of arachidonic acid (20:4 $\omega$ 6) and dihomo- $\gamma$ -linolenic acid (20:3 $\omega$ 6) in extracts of *P. homomalla*; a prominent, relatively polar, product is formed specifically from 20:3 $\omega$ 6. This difference had been noted before in work from the E. J. Corey laboratory (17).

This work was supported by National Institutes of Health grants GM-15431 and GM-74888 (ARB) and NSF grant CHE0615604 (JKC). Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the National Institutes of Health.

Manuscript received 21 November 2011.

Published, JLR Papers in Press, December 10, 2011  
DOI 10.1194/jlr.M022863

Abbreviations: H(P)ETE, hydro(pero)xyeicosatetraenoic acid; H(P)ETrE, hydro(pero)xyeicosatrienoic acid; LOX, lipoxygenase; RP-HPLC, reverse phase high-pressure liquid chromatography; SP-HPLC, straight phase high-pressure liquid chromatography.

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<sup>S</sup>The online version of this article (available at <http://www.jlr.org>) contains supplementary data in the form of data and references, a scheme, two tables, and two figures.

Although the naturally occurring prostaglandin products in *P. homomalla* are all 2-series derived from arachidonic acid, we included a study of the metabolic fate of 20:3 $\omega$ 6 because it was originally reported as a substrate for the enzymatic activity in the coral (18) and because study of 20:3 $\omega$ 6 metabolism in *P. homomalla* is not complicated by the presence of large amounts of endogenous products. With the availability of cloned recombinant enzymes from *P. homomalla*, we recently returned to the issue of the origin of this extra product from 20:3 $\omega$ 6. The novel product we characterize herein is formed specifically by 8*R*-lipoxygenase metabolism, and its unusual stereochemistry may represent a feature of the secondary reactions of *R*- as opposed to *S*-lipoxygenases.

## MATERIALS AND METHODS

Arachidonic (C20:4 $\omega$ 6) and dihomo- $\gamma$ -linolenic acids (C20:3 $\omega$ 6) were purchased from NuChek Prep Inc. (Elysian, MN). [ $^{14}$ C]20:4 $\omega$ 6 and [ $^{14}$ C]20:3 $\omega$ 6 were purchased from Perkin Elmer Life Sciences (Waltham, MA). *Plexaura homomalla* was collected in the Florida Keys and placed on dry ice until long-term storage in the laboratory at  $-70^{\circ}\text{C}$ .

### Incubation with coral extracts

Frozen *P. homomalla* was cut into small pieces with scissors and placed in 10 vols of 50 mM Tris, pH 8, containing 1 M NaCl on ice and homogenized using a Polytron blender (Brinkmann) in 10-s bursts. The homogenate was allowed to settle under gravity for up to 30 min; aliquots of the supernatant were diluted 10-fold into fresh buffer for incubations with fatty acid substrates (100  $\mu\text{M}$ ), typically for 5 min at room temperature. Products were extracted by the addition of 1 M  $\text{KH}_2\text{PO}_4$  plus sufficient 1 N HCl to give pH 4, followed by extraction with 2 vols of ethyl acetate. The organic phase was collected, washed with water to remove traces of acid, and taken to dryness under nitrogen. The extracts were redissolved in a small volume of MeOH before HPLC analysis.

Acetone powders of *P. homomalla* were prepared as described (5) and stored at  $-70^{\circ}\text{C}$  until use. Typically, a 3 mg/ml suspension/solution in 50 mM Tris (pH 8) containing 1 M NaCl was prepared for incubations with substrates (5 min at room temperature). For recovery of 8-hydroperoxides from these incubations, the 3 mg/ml suspension was diluted 10-fold, and the incubation time was extended to 20 min; a few milligrams of 8*R*-HPETE or 8*R*-HPETrE could be prepared and purified from 0.5 l of the dilute acetone powder incubations. Products were extracted as described above. If required, before HPLC, hydroperoxides were reduced using a molar excess of triphenylphosphine in MeOH (5 min at room temperature).

### HPLC analyses

Typically, aliquots of the extracts were analyzed initially by RP-HPLC using an ODS Ultrasphere 5 $\mu$  column (Beckman) (25  $\times$  0.46 cm) or Waters Symmetry column (25  $\times$  0.46 cm) using a solvent of MeOH/ $\text{H}_2\text{O}$ /HAc (80/20/0.01 or 75/25/0.01 by volume) at a flow rate of 1 ml/min with on-line UV detection (1100 series diode array detector; Agilent, Santa Clara, CA) and radioactive monitoring (Radiomatic Flo-One). Larger amounts (0.5–1 mg of total fatty acids) were injected for collection of products, or a semi-preparative column (Ultrasphere ODS, 25  $\times$  1 cm; Beckmann) was used for larger quantities. Further analysis and purification was achieved by SP-HPLC using a 5- $\mu$  silica column (Alltech) or a Beckmann Ultrasphere 5  $\mu$  silica column using a

solvent of hexane/isopropanol/glacial acetic acid (100/2/0.1 for H(P)ETE free acids and 100/1 for methyl esters; 100/5/0.1 and 100/3 for more polar derivatives and their methyl esters) run at 1 or 2 ml/min.

### Synthesis of the *threo*-epoxy (8*R*, 9*S*, 10*R*)- and *erythro*-epoxy (8*R*, 9*S*, 10*S*)-eicosanoates

The synthetic approach is outlined in Supporting Data and supplementary Scheme 1.

### Expression and purification of 8*R*-lipoxygenase

cDNA of the 8*R*-LOX domain of the *P. homomalla* peroxidase-lipoxygenase fusion protein (19) was subcloned into the pET3a vector (with an N-terminal His4 tag), and the protein was expressed in *Escherichia coli* BL21 (DE3) cells and purified by nickel affinity chromatography according to a previously published protocol (20). For clarity, this 8*R*-lipoxygenase is referred to herein as the recombinant 8*R*-LOX.

The second *P. homomalla* 8*R*-lipoxygenase tested here was the soluble enzyme purified in 1996 (21); aliquots from the original purification were stored at  $-70^{\circ}\text{C}$  and these retained sufficient activity for use 15 years later. This enzyme is referred to here as the soluble 8*R*-LOX.

### Incubation with enzymes

Side-by-side incubations were performed at room temperature in 1 ml of 50 mM Tris pH 8.0 containing 500 mM NaCl, 2 mM  $\text{CaCl}_2$  and 0.01% Emulphogene detergent using [ $^{14}$ C]20:3 $\omega$ 6 or [ $^{14}$ C]20:4 $\omega$ 6 fatty acids (each 25  $\mu\text{g}/\text{ml}$  and 300,000 CPM) and recombinant 8*R*-LOX (10  $\mu\text{g}$  in 1 ml) or soluble 8*R*-LOX. Under these conditions the recombinant enzyme completely metabolized 50  $\mu\text{M}$  20:4 $\omega$ 6 or 20:3 $\omega$ 6 substrate within 1 min, while 50  $\mu\text{l}$  ( $\sim$ 50  $\mu\text{g}$ ) of the soluble 8*R*-LOX converted 50  $\mu\text{M}$  20:4 $\omega$ 6 or 20:3 $\omega$ 6 to the corresponding 8*R*-hydroperoxide in 5 min; an additional 20  $\mu\text{l}$  enzyme was added to promote further metabolism of the 8*R*-hydroperoxide. Incubations were conducted in a 1 ml quartz cuvette and the rate of reaction monitored by repetitive scanning from 350–200 nm using a Lambda-35 spectrophotometer (Perkin-Elmer). Reactions were stopped by addition of 500  $\mu\text{l}$  MeOH and the solution placed on ice. After addition of 3 ml of water, 100  $\mu\text{l}$  1M  $\text{KH}_2\text{PO}_4$ , and 40  $\mu\text{l}$  1N HCl to give pH  $\sim$ 4.5, the samples were extracted using C18 Oasis cartridge and eluted with MeOH and analyzed by HPLC as described above.

### GC-MS analysis of $^{18}\text{O}_2$ incorporation in product from coral

Incubation of 20:3 $\omega$ 6 (100  $\mu\text{M}$ ) with an extract of *P. homomalla* acetone powder (3 mg powder/ml pH 8 buffer) was conducted under an atmosphere of  $^{18}\text{O}_2$ . The products were purified initially by RP-HPLC (MeOH/ $\text{H}_2\text{O}$ /HAc, 80/20/0.01 by volume), and then further purified by SP-HPLC (Hex/IPA/HAc, 100/5/0.1 by volume for the epoxyalcohol). Aliquots of HETrE (prepared by TPP reduction) and epoxyalcohol from the  $^{18}\text{O}_2$  incubation, together with unlabeled samples, were hydrogenated ( $\text{H}_2$ , palladium on carbon in ethanol for 2 min) and after addition of water and extraction with ethyl acetate, they were converted to the pentafluorobenzyl (PFB) ester TMS ether derivative. The  $^{18}\text{O}$  content was determined by GC-MS analysis in the negative ion/chemical ionization mode using a Nermag R10-10B instrument with a 5 m SPB-1 capillary column programmed from 150 $^{\circ}$  to 300 $^{\circ}$  at 20 $^{\circ}/\text{min}$ . The samples were subjected to rapid repetitive scanning over a 10 a.m.u. mass range (0.2 s per scan) covering the prominent M-181 ion (loss of PFB, resulting in the  $\text{RCOO}^-$  ion of product); approximately 30 scans were collected during elution of the GC peak, and these were averaged for calculation of the relative ion abundances. For analysis of hydrogenated

HETrE as the PFB-TMS derivative, the range scanned was  $m/z$  397-406 (M-181 ion for unlabeled molecules at  $m/z$  399,  $^{18}\text{O}_1$ -labeled at  $m/z$  401, and  $^{18}\text{O}_2$ -labeled at  $m/z$  403). For the hydrogenated epoxyalcohol product as the PFB-TMS derivative, the scanning range was  $m/z$  411-420 with M-181 for the unlabeled molecules at  $m/z$  413, and the  $^{18}\text{O}$ -labeled molecules at  $m/z$  415 or 417.

### LC-MS analysis of $^{18}\text{O}$ incorporation in product from 8R-LOX

$[^{18}\text{O}_2]$ 8R-HPETrE was prepared using recombinant 8R-LOX (from the *P. homomalla* fusion protein) reacted with C20:3 $\omega$ 6 (20  $\mu\text{g}/\text{ml}$ ) in pH 8.0 Tris buffer (5 ml) under an atmosphere of  $^{18}\text{O}_2$ ; (the 100 ml bulb of oxygen gas contained about  $\sim 35\%$  of normal air ( $^{16}\text{O}_2$ ), because it had been used three times previously for  $^{18}\text{O}$  syntheses). The  $[^{18}\text{O}_2]$ 8R-HPETrE labeled in the hydroperoxy group was purified by SP-HPLC and reacted with recombinant 8R-LOX under a normal atmosphere to produce the corresponding epoxyalcohol. The  $^{18}\text{O}$  contents of the 8R-HPETrE and its corresponding epoxyalcohol product (which share the same molecular weight, 338 for the unlabeled species) were measured by negative ion electrospray LC-MS using a ThermoFinnigan TSQ Quantum instrument by rapid repetitive scanning over the mass range encompassing the M-H anions ( $m/z$  330-350, 5 scans/sec). A total of 20-30 scans over the HPLC peaks were averaged to obtain the partial mass spectra of labeled and unlabeled epoxyalcohol and 8R-HPETrE.

### NMR analysis

$^1\text{H}$  NMR and  $^1\text{H}, ^1\text{H}$  COSY NMR spectra were recorded on a Bruker 400 MHz or Bruker DRX 500 MHz spectrometer at 298 K. The parts/million values are reported relative to residual nondeuterated solvent ( $\delta = 7.16$  ppm for  $\text{C}_6\text{H}_6$ , 7.26 ppm for  $\text{CDCl}_3$ ).

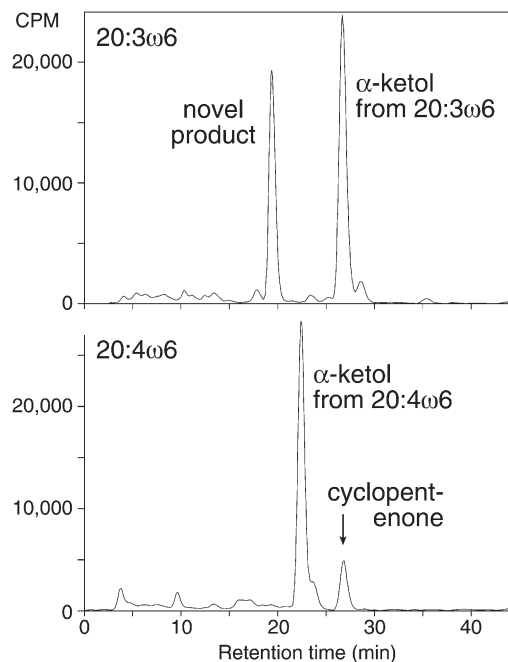
## RESULTS

### Metabolism in extracts of *P. homomalla*

As originally reported (5), when arachidonic acid (20:4 $\omega$ 6) is incubated with extracts of *P. homomalla*, the fatty acid is rapidly metabolized by 8R-LOX, and the resulting 8R-HPETE is further transformed by allene oxide synthase, leading to the appearance of  $\alpha$ -ketol and cyclopentenone end products (Fig. 1, lower panel). Metabolism of dihomo- $\gamma$ -linolenic acid (20:3 $\omega$ 6) is similar, except for the appearance of a prominent, more polar product that is absent (or present in insignificant amounts) in the arachidonic acid incubations (Fig. 1).

### Identification of the novel 20:3 $\omega$ 6 product

The structure was established based on UV, NMR, and GC-MS data. The purified polar product displayed only end absorbance in the UV data, indicating no conjugated double bonds. A quantity of  $\sim 100$   $\mu\text{g}$  was prepared, and the proton NMR and COSY spectra were recorded in  $\text{CDCl}_3$ . These results (see supplementary Table I) indicated the presence of an 8,9-*cis* epoxide [H8, dd, 3.03 ppm; H9, dt, 2.92 ppm;  $J_{8,9} = 4$  Hz; cf. *cis* epoxides 4-5 Hz, *trans* epoxides  $\sim 2$  Hz (22)], with  $\alpha$ -hydroxyl at C-10 and two *cis* double bonds at 11,12 and 14,15. So far this established the covalent structure as 8,9-*cis*-epoxy-10-hydroxy-eicosa-11Z, 14Z-dienoic acid, an epoxyalcohol of the hepoxilin B-type that is distinctive in being a *cis*-epoxide (23).



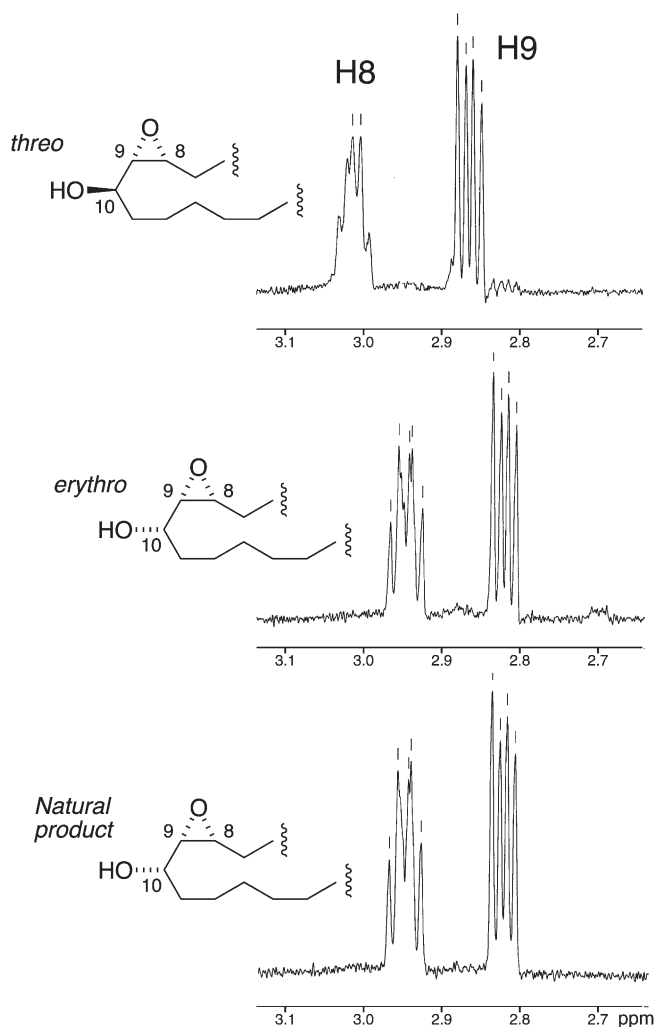
**Fig. 1.** RP-HPLC analysis of products formed from 20:3 $\omega$ 6 and 20:4 $\omega$ 6 in homogenates of *Plexaura homomalla*. The fatty acids [ $^{14}\text{C}$ ]20:3 $\omega$ 6 or [ $^{14}\text{C}$ ]20:4 $\omega$ 6 (100  $\mu\text{M}$ , 500,000 CPM) were incubated side-by-side with a homogenate of *P. homomalla* for 5 min at room temperature in 50 mM Tris buffer (pH 8) containing 500 mM NaCl. The samples were extracted using a C18 cartridge and analyzed by RP-HPLC using a Beckman ODS Ultrasphere column (5 $\mu$ , 25  $\times$  0.46 cm) with a solvent of MeOH/ $\text{H}_2\text{O}$ /HAc (75/25/0.01 by volume) and a flow rate of 1 ml/min with on-line UV detection (Hewlett-Packard 1040A diode array detector) and radioactive monitoring (Radiomatic Flo-One detector).

### Determination of the C-10 hydroxyl configuration

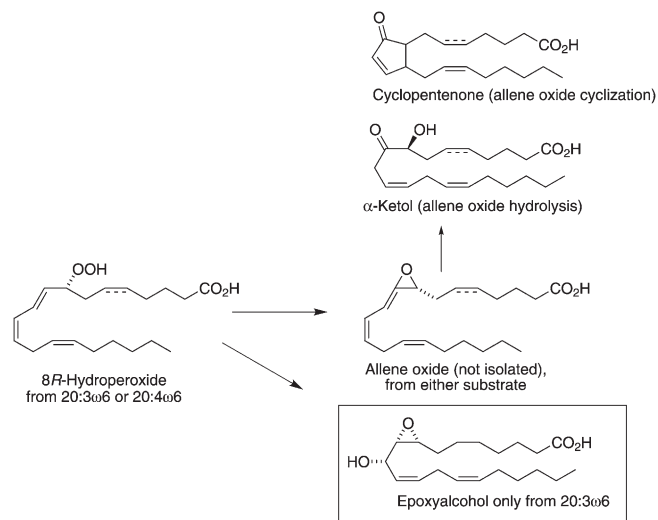
To establish the relative stereochemistry of the epoxide to the C-10 hydroxyl, two saturated analogs of the natural product were prepared by total chemical synthesis as outlined in the supplementary data and in supplementary Scheme I.

These synthetic standards, 8R,9S-*cis*-epoxy-10-hydroxy-eicosanoates with the 9,10 *erythro* and *threo* relative configurations, were first analyzed by GC-MS (EI mode) in comparison to the hydrogenated natural product as the methyl ester TMS ether derivatives. The *threo* 8,9-*cis*-epoxy-10-hydroxy-eicosanoate standard eluted before the *erythro* diastereomer (5 m SPB-1 capillary column, 150 $^\circ$  to 300 $^\circ$  at 20 $^\circ$ /min) each as well resolved peaks with retention times of 4 min 52 s and 5 min 1 s, respectively. Their mass spectra had a noticeably different pattern of ion fragments, especially at the lower  $m/z$  values (see supplementary Fig. I). Significant ions in the *threo* methyl ester TMS derivative were observed at  $m/z$  values of 413 (6%), 397 (1%), 321 (3%), 257 (35%), 243 (base peak), and 211, 183, and 165 (all  $\sim 15$ -20%), 143 (29%), and  $m/z$  129 (60%). The later eluting *erythro* standard had structurally diagnostic ions at  $m/z$  values of 413 (M-15, 2% relative abundance), 397 (M-31, 1%), 287 (C1-C10, 18%), 271 (287-16, 4%), 257 (8%), 243 (C10-C20, base peak), with other prominent ions at  $m/z$  values of 211 (8%), 197 (16%), 165 (90%), and 129 (41%). The ion assignments were confirmed by analysis of the mass spectra of the corresponding TMS ester TMS ether

derivatives (data not shown). The *erythro* standard had an indistinguishable mass spectrum and retention time to the hydrogenated epoxyalcohol product of *P. homomalla*. Their structural identity was confirmed by comparison of the NMR spectra of the saturated natural product with the synthetic standards (Fig. 2). These data confirmed the *erythro* relative configuration at 9,10 in the natural product. Because *P. homomalla* exhibits only 8*R*LOX activity, the *cis* epoxide moiety can be assigned as the 8*R*,9*S* enantiomer. Thus, the complete structure of the novel product from 20:3 $\omega$ 6 is established as 8*R*,9*S*-*cis*-epoxy-10*S*-hydroxy-eicosa-11*Z*,14*Z*-dienoic acid. Metabolism in the coral extracts is summarized in Scheme 1.



**Fig. 2.** Partial  $^1\text{H}$ -NMR spectra of the hydrogenated *P. homomalla* product from 20:3 $\omega$ 6 with *erythro* and *threo* epoxyalcohol fatty acid standards. The natural product was converted to the methyl ester, hydrogenated, and reperfired for comparison with the corresponding synthetic 8*R*,9*S*-*cis*-epoxy-10-hydroxy-eicosanoates. The region encompassing the epoxide protons is illustrated. In the *threo* isomer the epoxide protons are located at  $\delta$  3.02 (1H, H8) and 2.87 (1H, dd,  $J_{8,9} = 4.35$  Hz,  $J_{9,10} = 7.94$  Hz), and in the *erythro* diastereomer at  $\delta$  2.95 (1H, ddd, H8) and 2.82 (1H, dd,  $J_{8,9} = 4.2$  Hz,  $J_{9,10} = 7.69$  Hz). Other signals in each diastereomer were observed at  $\delta$  3.65 (3H, s, -OCH<sub>3</sub>), 3.5 (1H, m, H10), 2.3 (2H, t, H2), 1.7–1.45 (m), 1.45–1.2 (m), and 0.88 (3H, t, H20). The spectra were recorded in CDCl<sub>3</sub> using a Bruker 400 MHz instrument.



**Scheme 1.** Biosynthesis from 20:4 $\omega$ 6 and 20:3 $\omega$ 6 in *P. homomalla*.

### Origin of the epoxyalcohol oxygens in the novel 20:3 $\omega$ 6 product

To investigate the mechanism of formation of the epoxyalcohol, 20:3 $\omega$ 6 substrate (100  $\mu\text{M}$ ) was incubated with extracts of *P. homomalla* acetone powder (10 ml) under an atmosphere of  $^{18}\text{O}_2$  at room temperature for 10 min. The products were extracted and purified by reverse-phase (RP)-HPLC and straight-phase (SP)-HPLC; aliquots were hydrogenated and then analyzed for  $^{18}\text{O}$  content by GC-MS of the PFB ester TMS ether derivatives. The ion profile in the hydrogenated epoxyalcohol (see supplementary Fig. I) gave a ratio of  $2^{16}\text{O}:^{18}\text{O}:^{16}\text{O}:2^{18}\text{O}$  of  $\sim 6:4:90$ , indicating that most of the molecules of epoxyalcohol contain two oxygen atoms from  $^{18}\text{O}_2$  (see supplementary Fig. II). Because the precursor of the epoxyalcohol is 8*R*-HPETrE (an assumption proved formally using purified enzymes, *vide infra*), these results are compatible with essentially complete retention of the hydroperoxy oxygens from the precursor 8*R*-HPETrE.

### Lack of product using allene oxide synthase

There are several precedents for the transformation of fatty acid hydroperoxides to epoxyalcohols catalyzed by allene oxide synthase (AOS) and related enzymes (24–26), and it seemed possible that this might account for the formation of the 20:3 $\omega$ 6-derived epoxyalcohol. However, experiments with the expressed AOS domain of the *P. homomalla* AOS-LOX fusion protein (19) produced only allene oxide as product [detected as the major  $\alpha$ -ketol hydrolysis product and cyclopentenone (5)] from 8*R*-HPETE or 8*R*-HPETrE (data not shown).

### Formation of epoxyalcohol by 8*R*-LOX enzymes

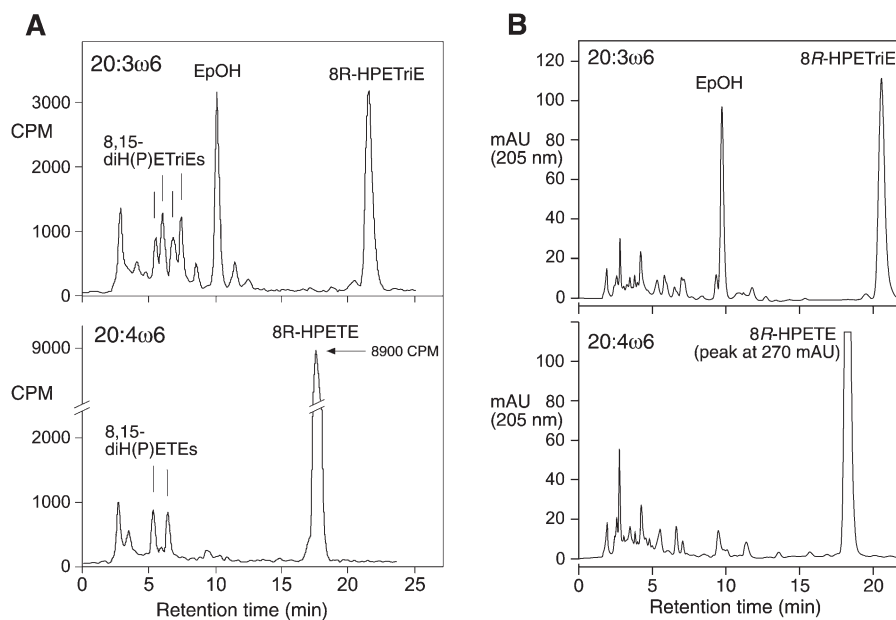
By contrast, use of the recombinant LOX domain of the AOS-LOX fusion protein gave positive results. When sufficient enzyme was used to quickly transform (<1 min) all the fatty acid to the corresponding 8*R*-hydroperoxide, further reaction generated secondary products. When observed by repetitive scanning in the UV, the rapid appearance of the

conjugated diene at 237 nm was followed by the gradual decrease in intensity at this wavelength, with the appearance of a new chromophore characteristic of a conjugated triene(s) centered on  $\sim 270$  nm and a weaker broad absorbance in the area of 300–350 nm. The main product of the 20:3 $\omega$ 6 reaction absorbs relatively weakly, at 205 nm, and is not detected by UV scanning (see below). In side-by-side incubations monitored in the UV, it was apparent that the 20:3 $\omega$ 6-derived 8*R*-HPETriE disappeared more quickly than the corresponding arachidonic acid-derived 8*R*-HPETE. These side-by-side reactions were also conducted using  $^{14}$ C-labeled fatty acid substrate, and, after extraction of these samples using C18 cartridges, RP-HPLC analysis showed distinctly different profiles of products (Fig. 3A). The results confirmed the more extensive metabolism of the 20:3 $\omega$ 6-derived 8*R*-HPETriE (less remaining compared with 8*R*-HPETE) and, more significantly, the prominent appearance of a polar product unique to 20:3 $\omega$ 6 metabolism. This distinctive peak at  $\sim 10$  min is the most abundant secondary product from 20:3 $\omega$ 6, detected at 205 nm in the UV. In larger-scale incubations, this polar product from 20:3 $\omega$ 6 was prepared in sufficient amounts for structural analysis by  $^1$ H-NMR (see supplemental Tables I and II). On the basis of these data, the 8*R*-LOX product was shown to be identical to the coral epoxyalcohol 8*R*,9*S*-*cis*-epoxy-10*S*-hydroxy-eicosa-11*Z*,14*Z*-dienoic acid.

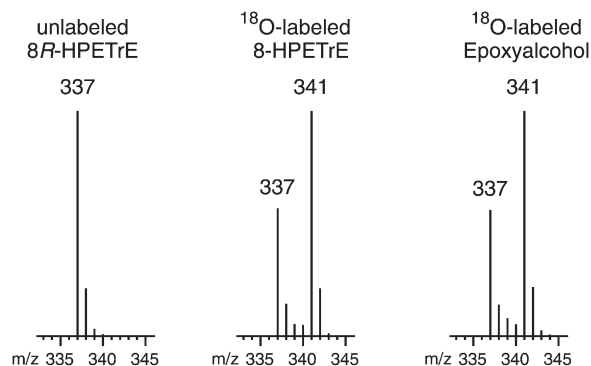
We also tested the soluble 76-kDa 8*R*-LOX from *P. homomalla*, which was available in limited quantities from the original purification (21). It reacted very similarly to the recombinant 8*R*-LOX from the AOS-LOX fusion protein. The substrates 20:4 $\omega$ 6 and 20:3 $\omega$ 6 were comparable for oxygenation to the corresponding 8*R*-hydroperoxide; however, 8*R*-HPETriE was converted to further products at over twice the rate of 8*R*-HPETE. When reactions with identical amounts of enzyme were analyzed and stopped at the same time (with half of the 20:3 $\omega$ 6 hydroperoxide consumed), subsequent RP-HPLC analysis confirmed the more extensive metabolism of 8*R*-HPETriE and the appearance of a single prominent, more polar product detected at 205 nm, with no comparable prominent product from 8*R*-HPETE (Fig. 3B). This polar product from 20:3 $\omega$ 6 was identified as the same epoxyalcohol identified earlier by its identical UV profile and cochromatography on both RP-HPLC and SP-HPLC with the epoxyalcohol formed by the recombinant 8*R*-LOX.

### Retention of hydroperoxy oxygens in the epoxyalcohol

When 8*R*-HPETriE containing an  $\sim 1:2$  mixture of  $^{216}$ O and  $^{218}$ O in the hydroperoxide group was reacted with the recombinant 8*R*-LOX, the  $^{18}$ O contents of the substrate and epoxyalcohol product were almost indistinguishable (Fig. 4). Close inspection indicated 98% retention of both hydroperoxy oxygens in the epoxyalcohol, pointing to a



**Fig. 3.** RP-HPLC analysis of products formed from 20:3 $\omega$ 6 and 20:4 $\omega$ 6 by two purified 8*R*-lipoxygenases. A: Recombinant 8*R*-LOX (20) (10  $\mu$ g in 1 ml) was reacted with [ $^{14}$ C]20:3 $\omega$ 6 or [ $^{14}$ C]20:4 $\omega$ 6 fatty acids (each 25  $\mu$ g/ml and 300,000 CPM) in 50 mM Tris pH 8 containing 500 mM NaCl, 2 mM CaCl<sub>2</sub>, and 0.01% Emulphogene detergent for 10 min at room temperature. An aliquot of the extract was analyzed by RP-HPLC using a Waters Symmetry column (25  $\times$  0.46 cm), a solvent of MeOH/H<sub>2</sub>O/HAc (80/20/0.01 by volume), at a flow rate of 1 ml/min, with on-line UV detection (Agilent 1100 series diode array detector) and radioactive monitoring (Radiomatic Flo-One). B: Reactions of soluble 8*R*-LOX (21) ( $\sim 50$   $\mu$ g/ml) with unlabeled 20:3 $\omega$ 6 and 20:4 $\omega$ 6 (50  $\mu$ M) were conducted in 1 ml UV cuvettes in 50 mM Tris pH 8 containing 500 mM NaCl, 2 mM CaCl<sub>2</sub>, and 0.01% Emulphogene detergent at room temperature. The transformations were observed in the UV by repetitive scanning from 350 to 200 nm. When about half of the initially formed 8*R*-HPETriE was consumed (at 30 min), the reactions were stopped, extracted, and analyzed by RP-HPLC with UV detection as outlined above. The UV profiles at 205 nm are illustrated.



**Fig. 4.** Mass spectrometric analysis of 8R-LOX-catalyzed transformation of [ $^{18}\text{O}$ ]8R-HPETrE to epoxyalcohol. Epoxyalcohol formed by recombinant 8R-LOX from  $^{18}\text{O}$ -labeled 8R-HPETrE (comprised of a  $\sim 1:2$  ratio of  $2^{16}\text{O}$  to  $2^{18}\text{O}$ ). The ion abundances were measured by LC-MS (details in Methods) for the unlabeled species (left), the hydroperoxy substrate (middle), and epoxyalcohol product (side). A  $\sim 2\%$  increase in relative abundance in the channel representing  $^{18}\text{O}$ - $^{16}\text{O}$  ions ( $m/z$  339) corresponds to a very minor loss of one of the oxygens of  $^{18}\text{O}$ - $^{18}\text{O}$  labeled substrate. Overall, the results show  $\sim 98\%$  retention of both hydroperoxy oxygens in the epoxyalcohol product.

mechanism involving close control of the transformation by the 8R-LOX enzyme.

## DISCUSSION

### Hydroperoxide isomerase activity

The typical dioxygenase activity of lipoxygenase enzymes involves activation of the resting ferrous enzyme to the ferric form, then cycling of the ferric enzyme as it catalyzes reaction with polyunsaturated fatty acid and  $\text{O}_2$  (27). By contrast, the epoxyalcohol biosynthesis we characterize here fits the criteria for a LOX enzyme acting as a hydroperoxide isomerase (28, 29). In this case, the reaction cycle is initiated by the ferrous enzyme. Several lines of evidence suggest that a lack of access of molecular oxygen within the active site promotes hydroperoxide isomerase activity (30). If present, molecular oxygen reacts readily with radical intermediates, thus intercepting and blocking hydroperoxide isomerase cycling. Furthermore, molecular oxygen promotes enzyme activation to the ferric form, also inhibiting isomerase activity (29, 31). Therefore, one can deduce that the 8R-HPETrE is an acceptable substrate for interaction with the ferrous iron and that  $\text{O}_2$  is excluded from intercepting the radical intermediates. With the arachidonic acid-derived 8R-hydroperoxide, the overall rate of reaction is comparatively sluggish, and very little epoxyalcohol product is formed. The main products are dihydroperoxides or leukotriene A-related diols, both of which are products of the ferric enzyme. This suggests that the selective reaction with the 20:3 8R-hydroperoxide is facilitated by exclusion of  $\text{O}_2$  within a critical part of the active site and that this does not occur with binding of the arachidonate analog.

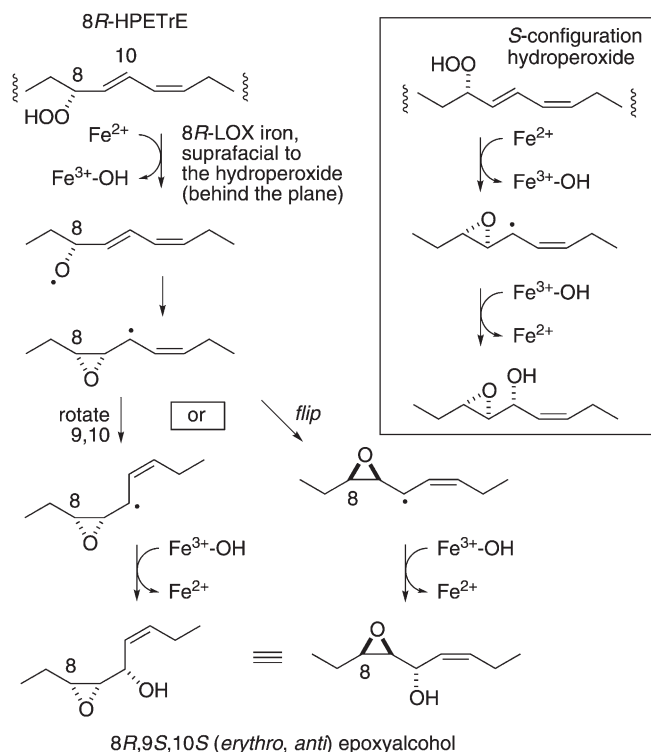
### Assignment of the 10S (erythro, anti) configuration

In postulating a mechanism for the hydroperoxide cycling with 8R-HPETrE, there is some difficulty in account-

ing for the *erythro* configuration of the epoxyalcohol product (discussed in the following subsection), and therefore it is imperative that the structural assignment is secure. For *trans*-epoxy epoxyalcohols, there are empirical rules that reliably allow assignment of the *erythro* or *threo* configuration. These rules relate to their relative polarity on TLC, relative retention time on GC, and both the relative chemical shifts and coupling constants on NMR (22, 32, 33). However, for *cis*-epoxy products there are fewer closely analogous examples in the literature (e.g., all fatty acid-related epoxyalcohols with data available are *trans* epoxides), and the differences for *erythro* and *threo* on NMR are small or nonexistent (34). Our assignment is founded on the well preceded *threo* product in Sharpless' hydroxyl-directed epoxidation of Z-allylic alcohols with  $\text{Ti}(\text{OiPr})_4$  (see supplementary Scheme I, epoxidation of 10R-3) (34–37). This allowed assignment of the two epoxide diastereomers (*erythro* and *threo*) obtained via Sharpless asymmetric epoxidation (see supplementary Scheme I). Indeed, the latter assignment shows good agreement with precedent using closely related model compounds (34, 35). For example, the asymmetric epoxidation (using L-(+)-diisopropyl tartrate) of 3-hydroxy-4Z-undecenol yields an unreactive 3R enantiomer with 2:3 ratio of *erythro:threo* products (35); our results concur exactly with this precedent and others (34, 36, 37).

### Proposed catalytic cycle

The reaction is catalyzed and controlled by the active site iron, which must first cleave the hydroperoxide and subsequently catalyze an oxygen rebound and hydroxylate the intermediate epoxyallylic radical while both hydroperoxy oxygens are retained in the epoxyalcohol product (Fig. 5). This is easy to conceptualize for the reactions of S-configuration fatty acid hydroperoxides because all steps occur on the same face of the reacting molecule, allowing formation of a *trans* epoxide and *threo* alcohol (Fig. 5, box). Our results with the R-configuration hydroperoxide indicate not only formation of a *cis*-epoxide, which itself presents no conceptual problem, but also the *erythro* configuration of the alcohol. Assuming the iron is in control, this necessitates either a 9,10 bond rotation before hydroxylation or flipping over of the reacting epoxyallylic radical intermediate (Fig. 5, right and left options). Perhaps the 8R-hydroperoxide sits partly turned away from square so that the epoxyallylic intermediate, when formed, further rotates to expose the opposite face of the intermediate for hydroxylation. We note too that the formation of *cis*-epoxides may be a characteristic of 8R-LOX because the activity in *P. homomalla* extracts was shown to convert 5S-HPETE to *cis*-epoxy LTA<sub>4</sub>, not to the well known *trans*-epoxy leukotriene A<sub>4</sub> (38). Although the mechanisms of epoxyalcohol and LTA<sub>4</sub> synthesis differ, the reactions being initiated by the ferrous and ferric enzymes, respectively, the substrate conformation that predisposes to *cis*-epoxide formation is dictated by binding in the active site and thus could be dictated in similar fashion by an enzyme that favors R versus S oxygenation.



**Fig. 5.** Mechanism of 8R-LOX-catalyzed epoxyalcohol synthesis from 8R-HPETrE. The scheme accounts for the stereochemistry of the epoxyalcohol (8,9-*cis*-epoxy, 9,10 *erythro*) and the complete retention of the hydroperoxy oxygens. Assuming that the active site iron cleaves the hydroperoxide and momentarily binds the distal hydroperoxy oxygen, the epoxyallylic radical intermediate must either rotate at the 9,10 bond (left) or flip over (right) to produce the epoxyalcohol product. In the box: reaction of *S*-configuration fatty acid hydroperoxide forms a *trans*-epoxy *threo*-hydroxy epoxyalcohol.

### Other biosyntheses of *cis*-epoxyalcohols

Although heretofore only *trans*-epoxyalcohols have been reported from lipoxygenase catalysis (e.g., 23, 28, 39–41), other enzymes can make the *cis*-epoxides. The majority of these are mechanistically quite distinct, however, because the epoxide is formed via oxygen transfer. The epoxyalcohol synthase activities in the fish parasitic fungus *Saprolegnia parasitica* (42) and in potato leaves and beetroot (43, 44) catalyze oxygen transfer from the hydroperoxy fatty acid to the adjacent conjugated diene; the original hydroperoxide moiety is reduced to an alcohol, while the transferred oxygen produces *trans*- or *cis*-epoxidation of the *trans* and *cis* double bonds, respectively. In the case of plant peroxygenases, epoxidation may occur via intermolecular or intramolecular oxygen transfer from a fatty acid hydroperoxide to a *cis* double bond (45, 46). More similar to our reaction, but forming the *threo* product, is the conversion of 13*S*-hydroperoxylinoleic acid to the 11*S*-*threo*-hydroxy-12*R*,13*S*-*cis*-epoxide by a cytochrome P450 in the amphioxus *Branchiostoma floridae* (26). Notably, the oxygen rebound step in P450 catalysis is very fast ( $\sim 10^{-9}$  s), tending to favor suprafacial hydroxylation of the intermediate, forming the *threo* epoxyalcohol. By comparison, the equivalent intermediate in the hydroperoxide isomerase activity of

lipoxygenases can diffuse out of the active site or be subject to interception by molecular oxygen, an event that promotes lipoxygenase activation to the ferric form (30). Accordingly, one might expect there is more time in the 8R-LOX reaction for the rotation required to form the observed *erythro* epoxyalcohol product (Fig. 5).

### Wrap-up of a historical issue

The striking and unexpected difference between 20:4 $\omega$ 6 and 20:3 $\omega$ 6 metabolism in *P. homomalla* was detected in the original investigations of prostaglandin biosynthesis by Corey and Ensley, and the prominent extra product from 20:3 $\omega$ 6 was partially characterized (17). For example, it was shown to exhibit only weak end absorbance in the UV, to not react with sodium borohydride, to contain two double bonds and an alcohol and a possible epoxy functionality, and to have a molecular formula as the methyl ester of C<sub>21</sub>H<sub>36</sub>O<sub>4</sub>, all a perfect match for the epoxyalcohol we identify. Furthermore, the reported mass spectrum of the hydrogenated product as the methyl ester TMS derivative [listed in tabular form in the thesis (17)] contains all the major ions and similar ion abundances as reported in our Results section. There is little doubt that this product and our epoxyalcohol are the same compound. The existence of 8R-LOX metabolism in *P. homomalla* was not uncovered until the mid-1980s, a decade after these early biosynthetic studies (8), and it was only around the years 1995–2000 that the origin of the coral prostaglandins via cyclooxygenase was firmly established (6, 7, 47–49).<sup>10</sup>

The authors thank Prof. Thomas M. Harris and Donald F. Stec, Ph.D., for help with the NMR analyses.

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