8R-Lipoxygenase-catalyzed synthesis of a prominent cis-epoxyalcohol from dihomo-γ-linolenic acid: a distinctive transformation compared with S-lipoxygenases.

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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 epoxyalcohol configuration. Here we report on R-specific lipoxygenase synthesis of a cis-epoxyalcohol. Although arachidonic and dihomo-γ-linolenic acids are metabolized by extracts of the Caribbean coral Plexaura homomalla via 8R-lipoxygenase and allene oxide synthase activities, 20:3ω6 forms an additional prominent product, identified using UV, GC-MS, and NMR in comparison to synthetic standards as 8R,9S-cis-epoxy-10S-erythro-hydroxy-eicos-11Z,14Z-dienoic acid. Both oxygens of 18O-labeled 8R-hydroperoxide are retained in the product, indicating a hydroperoxide isomerization activity. Recombinant allene oxide synthase formed only allene epoxide from 8R-hydroperoxy-20:3ω6, whereas two different 8R-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 epoxalcohol may be a feature of R-specific lipoxygenases.—Jin, J., W. E. Boeglin, J. K. Cha, and A. R. Brash. 8R-Lipoxygenase-catalyzed synthesis of a prominent cis-epoxyalcohol from dihomo-γ-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 • hepoxilin • 18O 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 octocoral served as a source of prostaglandins 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 8R-lipoxygenase activity, the first known existence of an R-specific lipoxygenase (8). 8R-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 12R-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 8RLOX product, 8&hydroperoxyl-icosatetraenoic acid, to an allene epoxide (5), a proposed intermediate in biosynthesis of cycloartenones such as the clavulones (14–16), and which hydrolyzes in vitro to an α-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 (20:4ω6) and dihomo-γ-linolenic acid (20:3ω6) in extracts of P. homomalla; a prominent, relatively polar, product is formed specifically from 20:3ω6. This difference had been noted before in work from the E. J. Corey laboratory (17).

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

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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 20:3ω6 because it was originally reported as a substrate for the enzymatic activity in the coral (18) and because study of 20:3ω6 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 20:3ω6. The novel product we characterize herein is formed specifically by 8R-lipoxygenase metabolism, and its unusual stereochemistry may represent a feature of the secondary reactions of 8R as opposed to 4lipoxygenases.

**MATERIALS AND METHODS**

Arachidonic (C20:4ω6) and dihomo-γ-linolenic acids (C20:3ω6) were purchased from NuChek Prep Inc. (Elysian, MN). [1-14C]20:4ω6 and [1-13C]20:3ω6 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°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 in ice and homogenized using a Polytron blender (Brinkmann) in 50°C and stored at −70°C.

**Acetone powders** of *P. homomalla* were prepared as described (5) and stored at −70°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 into fresh buffer for incubations with fatty acid substrates (100 μM), typically for 5 min at room temperature. Products were extracted by the addition of 1 M KH2PO4 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°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 8R-HETE or 8R-HPETE 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 μ column (Beckman) (25 x 0.46 cm) or Waters Symmetry column (25 x 0.46 cm) using a solvent of MeOH/H2O/HAc (80/20/0.01 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 x 1 cm; Beckman) was used for larger quantities. Further analysis and purification was achieved by SP-HPLC using a 5-μ silica column (Alltech) or a Beckmann Ultrasphere 5 μ 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 three-epoxy (8R, 9S, 10R)- and erythro-epoxy (8R, 9S, 10S)-eicosaenoates**

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

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

cDNA of the 8R-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 clarification, this 8R-lipoxygenase is referred to herein as the recombinant 8R-LOX.

The second *P. homomalla* 8R-lipoxygenase tested here was the soluble enzyme purified in 1996 (21); aliquots from the original purification were stored at −70°C and these retained sufficient activity for use 15 years later. This enzyme is referred to here as the soluble 8R-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 CaCl2 and 0.01% Emulphogene detergent using [14C]20:3ω6 or [15C]20:4ω6 fatty acids (each 25 μg/mL and 300,000 CPM) and recombinant 8R-LOX (10 μg in 1 ml) or soluble 8R-LOX. Under these conditions the recombinant enzyme completely metabolized 50 μM 20:4ω6 or 20:3ω6 substrate within 1 min, while 50 μL (~50 μg) of the soluble 8R-LOX converted 50 μM 20:4ω6 or 20:3ω6 to the corresponding 8R-hydroperoxide in 5 min; an additional 20 μL enzyme was added to promote further metabolism of the 8R-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 μL MeOH and the solution placed on ice. After addition of 3 ml of water, 100 μL 1M KH2PO4, and 40 μL 1N HCl to give pH ~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 18O2 incorporation in product from coral**

Incubation of 20:3ω6 (100 μM) with an extract of *P. homomalla* acetone powder (3 mg powder/ml pH 8 buffer) was conducted under an atmosphere of 18O2. The products were purified initially by RP-HPLC (MeOH/H2O/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 epoxylcohol). Aliquots of HETE (prepared by TPP reduction) and epoxylcohol from the 18O2 incorporation, together with unlabeled samples, were hydrogenated (H2, 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 18O 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° to 300° at 20°/min. The samples were subjected to rapid repetitive scanning over a 10 a.m.u. mass range (0.2 a per scan) covering the prominent M-181 ion (loss of PFB, resulting in the 173 ion) of this extra product from 20:3ω6. We characterize herein is formed specifically by 8R-lipoxygenase metabolism, and its unusual stereochemistry may represent a feature of the secondary reactions of 8R as opposed to 4lipoxygenases.
present in insignificant amounts) in the arachidonic acid end products (leading to the appearance of $8^R$-linolenic acid is rapidly metabolized by $8^R$-LOX under a normal atmosphere to produce the corresponding epoxyalcohol. The $^{18}$O contents of the $8^R$-HPETE 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 $8^R$-HPETE.

**NMR analysis**

$^1$H NMR and $^1$H,$^1$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 CD$_2$D$_2$, 7.26 ppm for CDCl$_3$).

**RESULTS**

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

As originally reported (5), when arachidonic acid (20:4$\alpha$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 dihomoy-linolenic acid (20:3$\alpha$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$\alpha$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 $\mu$g was prepared, and the proton NMR and COSY spectra were recorded in CDCl$_3$. These results (see supplementary Table I) indicated the presence of an $8,9$-cis epoxide [H$8$, dd, 3.03 ppm; H$9$, dt, 2.92 ppm; $J_{8,9} = 4$ Hz; cf. cis epoxides 4-5 Hz, trans epoxides $\sim2$ 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).

**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, $8^R$9-cis-epoxy-10-hydroxy-eicosanotes 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° to 300° at 20°/min) each as well resolved peaks with retention times of 4 min 52 s and 5 min 13 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-5, 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 8R-LOX activity, the *cis* epoxide moiety can be assigned as the 8R,9S enantiomer. Thus, the complete structure of the novel product from 20:3o6 is established as 8R,9S-cis-epoxy-10-hydroxy-eicosa-11Z,14Z-dienoic acid. Metabolism in the coral extracts is summarized in Scheme 1.

![Scheme 1. Biosynthesis from 20:3o6 and 20:3o6 in *P. homomalla*.](image)

### Origin of the epoxyalcohol oxygens in the novel 20:3o6 product

To investigate the mechanism of formation of the epoxyalcohol, 20:3o6 substrate (100 μM) was incubated with extracts of *P. homomalla* acetone powder (10 ml) under an atmosphere of 18O2 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 18O 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 216O:18O-16O:218O of 160:1:900, indicating that most of the molecules of epoxyalcohol contain two oxygen atoms from 18O2 (see supplementary Fig. II). Because the precursor of the epoxyalcohol is 8R-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 8R-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 20:3o6-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 α-ketol hydrolysis product and cyclopentenone (5)] from 8R-HPETrE or 8R-HPETrE (data not shown).

### Formation of epoxyalcohol by 8R-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 8R-hydroperoxide, further reaction generated secondary products. When observed by repetitive scanning in the UV, the rapid appearance of the...
We also tested the soluble 76-kDa 8\(\text{R}\)-LOX from \textit{P. homomalla}, which was available in limited quantities from the original purification (21). It reacted very similarly to the recombinant 8\(\text{R}\)-LOX from the AOS-LOX fusion protein. The substrates 20:3\(\omega\)6 and 20:3\(\omega\)6 were comparable for oxygenation to the corresponding 8\(\text{R}\)-hydroperoxide; however, 8\(\text{R}\)-HPETrE was converted to further products at over twice the rate of 8\(\text{R}\)-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\(\text{R}\)-HPETrE and the appearance of a single prominent, more polar peak detected at 205 nm, with no comparable prominent product from 8\(\text{R}\)-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\(\text{R}\)-LOX.

Retention of hydroperoxy oxygens in the epoxyalcohol

When 8\(\text{R}\)-HPETrE containing an \(\sim 1:2\) mixture of \(\text{\textsuperscript{16}}\)O and \(\text{\textsuperscript{18}}\)O in the hydroperoxide group was reacted with the recombinant 8\(\text{R}\)-LOX, the \(\text{\textsuperscript{18}}\)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\(\text{R}\)-lipooxygenases. A: Recombinant 8\(\text{R}\)-LOX (20) (10 \(\mu\)g in 1 ml) was reacted with [\(\text{\textsuperscript{14}}\text{C}\)]20:3\(\omega\)6 or [\(\text{\textsuperscript{14}}\text{C}\)]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\(_2\), 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 x 0.46 cm), a solvent of MeOH/H\(_2\)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\(\text{R}\)-LOX (21) (\~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\(_2\), and 0.01% Emulphogene detergent at room temperature. The transformations were observed in the UV by repetitive scanning from 350 to 290 nm. When about half of the initially formed 8\(\text{R}\)-HPETE 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.
mechanism involving close control of the transformation by the 8R-LOX enzyme.

DISCUSSION

Hydroperoxide isomerase activity

The typical dioxygenase activity of lipoygenase 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 O2 (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 O2 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 O2 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-

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 5aHPETE to cis-epoxy LTA4, not to the well known trans-epoxy leukotriene A4 (38). Although the mechanisms of epoxyalcohol and LTA4 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.
lipooxygenases can diffuse out of the active site or be subject to interception by molecular oxygen, an event that promotes lipooxygenase 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 epoxycalcohol product (Fig. 5).

Wrap-up of a historical issue

The striking and unexpected difference between 20:4ω6 and 20:3ω6 metabolism in *P. homomalla* was detected in the original investigations of prostaglandin biosynthesis by Corey and Ensley, and the prominent extra product from 20:3ω6 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_{20}H_{30}O_{4}, all a perfect match for the epoxycalcohol 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 epoxycalcohol 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).

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**Fig. 5.** Mechanism of 8R-LOX-catalyzed epoxycalcohol synthesis from 8R-HPETE. The scheme accounts for the stereochemistry of the epoxycalcohol (8R-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 epoxyaliphatic radical intermediate must either rotate at the 9,10 bond (left) or flip over (right) to produce the epoxycalcohol product. In the box: reaction of $S$-configuration fatty acid hydroperoxide forms a $trans$-epoxy $threo$-hydroxy epoxycalcohol.

Other biosyntheses of cis-epoxyalcohols

Although heretofore only *trans*-epoxyalcohols have been reported from lipooxygenase catalysis (e.g., 23, 28, 39–41), other enzymes can make the *cis*-epoxyalcohols. The majority of these are mechanistically quite distinct, however, because the epoxide is formed via oxygen transfer. The lipooxygenase 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 hydroperoxy 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 peroxynases, 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 13S-hydroperoxylinoleic acid to the 11S-*threo*-hydroxy-12R,13S-*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* epoxycalcohol. By comparison, the equivalent intermediate in the hydroperoxy isomerase activity of
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