Discovery of an Arachidonic Acid C-8 Lipoxygenase in the Gorgonian Coral *Pseudoplexaura porosa*

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The gorgonian coral *Pseudoplexaura porosa* contains a lipoxygenase capable of converting exogenous arachidonic acid into (8R)-8-hydroperoxy-5,9,11,14-eicosatetraenoic acid. The (8R)- (or 8-L-) configuration in this product, opposite to that observed in previously reported 8-lipoxygenase products, was determined unambiguously by comparison of oxidative ozonolysis fragments with authentic malic acid-derived standards. Extracts from the coral contained no detectable prostaglandins (PGs, PGBs, PGEs, or PGFs). Although arachidonic acid represents one of the most abundant of the common fatty acids found in the phospholipid and total lipid fractions of *P. porosa*, products ascribable to the arachidonic acid 8-lipoxygenase pathway ((8R)-8-hydroperoxy-5,9,11,14-eicosatetraenoic acid, the corresponding alcohol 8-hydroxyeicosatetraenoic acid, further transformation products) have not yet been identified in the coral extracts. The physiological significance of the 8-lipoxygenase in this species remains a matter for speculation.

In 1982, Doerge and Corbett (1) reported the partial purification of a novel lipoxygenase from the gorgonian coral *Pseudoplexaura porosa*. This lipoxygenase exhibited a specificity for arachidonic acid and catalyzed the introduction of oxygen at C-15 forming (15(S))-15-hydroperoxy-5,8,11,13-eicosatetraenoic acid (15-HPETE), identical to material synthesized by soybean (2, 3) and mammalian (4) lipoxygenases. Since *P. porosa* is relatively abundant and readily available, we set out to determine whether it might be competitive with commercially available soybean lipoxygenase, in terms of yield or experimental simplicity, as a source of research quantities (mg to g) of 15-HPETE.

We found that when no attempt was made to purify the coral lipoxygenase, the predominant oxidation product after incubation with arachidonic acid and oxygen was not 15-HPETE, but rather (8R)-8-hydroperoxy-5,9,11,14-eicosatetraenoic acid (8-HPETE). The corresponding alcohol, 8-HETE, has been obtained previously by a variety of procedures: enzymatically (from arachidonic acid) using human neutrophils (5) or rat hepatic microsomal cytochrome P-450 (6); chemically from arachidonic acid via regio-random oxidation by Cu2+/H2O2 (7), air (8), or singlet oxygen (9, 10); and via total chemical synthesis (11). However, due primarily to its formation in only low yield and the difficulty of obtaining pure samples, 8-HETE remains one of the least well-studied of the monohydroxyeicosatetraenoic acids. Only its chemotactic activity toward human neutrophils has been reported (6, 12).

In this report, we document the rather surprising ability of an unstable lipoxygenase in the gorgonian *P. porosa* to convert exogenous arachidonic acid into 8-HPETE and assign unambiguously the (8R)-stereochemistry to the product.

**EXPERIMENTAL PROCEDURES**

**Preparation of 8-HPETE**

A. Using Acetone Powder—A 35-g sample (small branches) of the gorgonian coral *P. porosa* (collected in the Florida area by Dr. David G. Anderson, University of Miami) was extracted with 250 ml of acetone in a Waring blender at high speed for 5 min. Dry ice chips were added intermittently to keep the temperature at 10–15 °C. Following filtration through a medium-porosity sintered glass funnel, the solids were extracted with three additional 250-ml portions of acetone. The resulting tan sandy-looking solids were superficially dried by pulling nitrogen through the funnel for 30 min; the solids then weighed 10.6 g.

Oxygen was introduced via a gas dispersion tube below the surface of a stirred solution of 220 mg (0.72 mmol) of arachidonic acid (NucChek) in 500 ml of Tris pH 8.0 buffer (50 mM Tris in distilled water, adjusted to pH 8.0 with 1 M hydrochloric acid) containing 1.7 g of the acetone-insoluble coral solids from above. A vigorous stream of oxygen through a second tube held above the reaction mixture served to minimize foaming problems. After 45 min, the mixture was filtered through Celite, and the solids were washed with two 100-ml portions of the pH 8 buffer, then with 250 ml of 25% ethyl acetate/hexane, and finally 200 ml of ethyl acetate. The combined aqueous filtrate was saturated with salt, adjusted to pH 3.0–3.5, and extracted with the organic washes from the preceding operation. The extracts were washed with water (containing a trace of sodium bisulfate) and brine, dried over anhydrous sodium sulfate, and concentrated in vacuo.

The crude product (310 mg) was chromatographed on a 20-g column of Mallinckrodt CC-4 acid-washed silica gel. The column was packed and eluted (1-mL fractions) with 25% ethyl acetate/hexane. Fractions 32–49 contained 140 mg (64%) of recovered arachidonic acid (NuChek) in 500 ml of Tris pH 8.0 buffer (50 mM Tris in distilled water, adjusted to pH 8.0 with 1 M hydrochloric acid) containing 1.7 g of the acetone-insoluble coral solids from above. A vigorous stream of oxygen through a second tube held above the reaction mixture served to minimize foaming problems. After 45 min, the mixture was filtered through Celite, and the solids were washed with two 100-ml portions of the pH 8 buffer, then with 250 ml of 25% ethyl acetate/hexane, and finally 200 ml of ethyl acetate. The combined aqueous filtrate was saturated with salt, adjusted to pH 3.0–3.5, and extracted with the organic washes from the preceding operation. The extracts were washed with water (containing a trace of sodium bisulfate) and brine, dried over anhydrous sodium sulfate, and concentrated in vacuo.

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When identical oxygenations were carried out in the absence of coral enzyme, no detectable hydroperoxides were formed (<0.1%). Thus, under the conditions of these experiments, nonenzymatic oxidation is insignificant.

The acetone extracts from the coral extraction were evaporated to dryness, and the residue was diluted with 250 ml of brine (pH 4.55) and extracted with two 250-ml portions of ethyl acetate. The extracts were washed with brine, dried over magnesium sulfate, and evaporated, thereby affording 1.13 g of dark brown-green oil. This oil was analyzed by TLC in several solvent systems, using assorted prostaglandins as markers.
glANDs (PGAs, PGEs, PGFs) and 8-HETE as comparison standards.

**B. Using Buffer Extracts**—A 200-ml portion of pH 8 Tris buffer (described in part A above) was cooled to 0 °C and then used to extract 21.7 g of P. porosa branches in a Waring blender (2 min). The cold aqueous solution was then filtered through a pad of Celite on a medium-porosity sintered glass funnel. A 10-mL aliquot of the clear aqueous filtrate was acidified to pH 3 and extracted with ethyl acetate, and the extracts were analyzed by TLC. Arachidonic acid (110 mg) was added to the remaining aqueous filtrate, and oxygenation was conducted exactly as described in part A above. Workup was exactly as described in part A (except the filtration was omitted) and afforded 120 mg of crude product which was almost identical to that obtained in part A as judged by TLC and reverse-phase HPLC analysis. (HPLC analysis was performed on a 10 × 250-mm Altex Ultrasphere ODS 5-μm particle size column; 79:21:0.1 methanol/water/acetic acid; flow rate 3.5 ml/min; detector at 235 nm.)

Both the part A and part B oxygenations were repeated without adding the arachidonic acid to demonstrate the absence of coral-derived artifacts and the nonparticipation of endogenous arachidonic acid.

**Conversion of 8-HETE to 8-HETE Methyl Ester**

Sodium borohydride reduction of 8-HETE (42 mg; from part A above) followed standard procedures (10 ml of methanol, 100 mg of sodium borohydride, 25 min, pH 3 extractive workup with ethyl acetate). The crude 8-HETE in 1 ml of methanol was esterified with excess ethereal diazomethane (0 °C, 5 min), and the product was purified on 20 g of 40-60-μm silica gel (elution with argon-purged 25% ethyl acetate in hexane). This procedure afforded 15-HETE methyl ester (2 mg) and pure 8-HETE methyl ester (29 mg). Silylation of the material prior to mass spectral analysis was done with Regisil (99% bis(trimethylsilyl)-trifluoroacetamide 1% trimethylsilyl chloride) in pyridine at 25 °C for 90 min. The high-resolution mass spectrum in Fig. 2 was obtained on a CEC 21-110 spectrometer (direct probe), with source temperature 230 °C, ionizing current 100 pA, electron energy 70 eV. HPLC analysis of the 8-HETE prior to silylation was performed on a 4.6 × 250-mm Whatman Partisil 10 column, 2:0:97.9:2-propanol/HOAc/hexane, 2 ml/min, detector at 235 nm (9). 8-HETE exhibited 440033, m/z 453.2; 8-HETE methyl ester 29.0 min.

**Oxidative Degradation of 8-HETE Methyl Ester to Urethane 6**

A solution of 7 mg of 8-HETE methyl ester 4 in 0.5 ml of toluene was treated with 25 μl of (S)-α-methylbenzyl isocyanate and then heated at 90 °C for 20 h. Evaporation of the toluene and chromatographic purification of the residue (silical gel, 20% ethyl acetate/hexane) afforded pure urethane 5 (65% of theory). A 4-mg portion of the urethane 5 in 2 ml of methyl chloride was cooled to −78 °C and treated with excess ozone (10 min). Nitrogen was then bubbled through the cold solution to remove the ozone, and then the residue was removed in a stream of nitrogen at 25 °C. The residue was oxidized (400 μl of 30% hydrogen peroxide, 800 μl of acetic acid, 50 °C, 16 h) and esterified (50 μl of methanol, excess ethereal diazomethane, 0 °C, 10 min), and the crude product was purified by preparative TLC (silica gel, 2% acetone/methylene chloride). HPLC analysis of the derivative 6 was performed on a 4.6 × 250-mm Baker (R)-N-3,5-dinitrobenzoyl phenylglycine chiral covalent column, elution (1 ml/min) with 13% isopropanol/hexane, detector at 258 nm.

**Oxidative Degradation of 8-HETE Methyl Ester to 1-Methyl Carbonate Derivative 8**

A solution of 7 mg of 8-HETE methyl ester 4 and 0.5 ml of 0.1 M (−)-menthyl chloroformate in toluene (Regis Chemical Co. code no. 440005) was treated with 6.3 μl of pyridine and allowed to stand at 25 °C for 1 h in a tightly stoppered vial under an argon atmosphere. Water (3 ml) was added, and the reaction vial was shaken vigorously for 5 min. The organic layer was separated by pipette, dried over anhydrous magnesium sulfate, and concentrated in a stream of nitrogen. Chromatographic purification of the residue (10 g of silica gel, 8% acetone/hexane) afforded 9 mg of 8-HETE methyl ester, 8-menthyl carbonate (83% of theory). Ozonolysis, oxidation, and diazomethane esterification exactly as described in the preceding experiment then yielded product 7, which was analyzed by gas chromatography (Hewlett-Packard 5892A gas chromatograph/mass spectrometer, 6 foot 1% QF-1 on Supelcoport 100/120 column, 150 °C, 20 ml/min flow rate).

**Derivatization of Malic Acid Standards**

In separate experiments, racemic malic acid and (S)-(−)-malic acid were each esterified (excess diazomethane, 0 °C, 5 min), then converted into urethanes 10 and 13, and into methyl carbonate derivatives 11 and 14 using procedures analogous to those in the two preceding experiments.

**Analysis of Coral Lipid Fatty Acids**

The total lipids were extracted from branches of P. porosa by homogenization in 2:1 chloroform/methanol using a Sorvall Omni-mixer and partitioned according to the procedure of Folch et al. (13). The organic phase was evaporated to dryness under an atmosphere of nitrogen and then reconstituted in a known volume of chloroform. Aliquots of this solution (in duplicate) were spotted on 250-μm Silica Gel H thin layer plates, and the plates were developed with 70:30:1 benzene/ether/acetic acid. The area at the origin of the plates, containing the phospholipid fraction, was scraped from the plates into a screw-cap tube containing 0.5 ml of chloroform and 0.5 ml of 14% boron trifluoride in methanol. Aliquots from the total coral lipid extract were treated in the same fashion. The tubes were boiled for 45 min, and 1 ml of distilled water added to each tube to halt the reaction. Hexane (2.0 ml) was added to each tube with vortexing. The hexane layers were removed and evaporated under a stream of nitrogen, and the residue was redissolved in a small volume of hexane. The hexane extracts were chromatographed on a Silica Gel H thin layer plate developed in benzene. Fatty acid methyl esters were located by spraying a single outside lane of the plate, containing a standard fatty acid ester mixture, with a solution of 0.2% 2,7-dichlorofluorescein in ethanol, followed by UV visualization. The areas corresponding to fatty acid methyl esters were scraped into tubes containing 2 ml of 1:2 ether/hexane and 1.0 ml of water and mixed thoroughly. The organic layer was then analyzed by gas chromatography.

Gas chromatographic analysis was performed on a Hewlett-Packard 5790A instrument, utilizing a 15-m DB5 normal capillary column (0.245 mm, inner diameter, 0.1-μm film thickness) and flame ionization detection. Column pressure was 8 p.s.i., and instrument conditions were as follows: injector temperature 300 °C, detector temperature 350 °C, splitter ratio 40:1, hydrogen carrier gas flow 1.40 ml/min, auxiliary gas nitrogen flow 30 ml/min, air flow rate 40 ml/min, column volume 2.83 ml. The column was programmed from 140–250 °C at 4 °C/min after an initial 2 min at 140 °C. Fatty acids in the unknown samples were identified by comparison of their retention times with those of known fatty acid ester standards (Nu-Chek). The data were collected and integrated on a Vista 01 data system. Results are expressed as percentage of the total fatty acid methyl ester areas and are a very close approximation of molar quantities.

**RESULTS**

As described in detail under "Experimental Procedures," incubation of arachidonic acid in an oxygen atmosphere with the solids remaining after thorough acetone extraction of P. porosa led to the formation of two new more polar products in a ratio of approximately 10:1. The minor product (the faster moving of the two on TLC) was the expected (15) HPEPE, identified by direct TLC, HPLC, and spectral comparison with an authentic sample prepared by soybean lipoxynegenation of arachidonic acid (2, 3). Like 15-HPEPE, the major product isolated in 18% yield (50% if account was taken of recovered arachidonic acid) was strongly positive to a peroxide-specific TLC spray reagent (14). The fact that sodium borohydride reduction of the new lipoxynegenase product afforded a mono-alcohol, not a diol, showed that the positive peroxide test was due to a hydroperoxide, not a peroxide.

The position of the oxygen functionality in the new lipoxynegenase product was readily determined by mass spectral analysis of the trimethyisilyl derivative of the corresponding 8-HETE ester 4 from sodium borohydride reduction and diazomethane esterification (Fig. 1). The base peak at m/z 448.
Arachidonic Acid C-8 Lipooxygenase in Coral

FIG. 1. Formation and characterization of 8-HPETE from arachidonic acid by coral lipooxygenase.

FIG. 2. Mass spectrum of trimethylsilyl ether of (8R)-8-HETE methyl ester 4. Ion at m/z 406 corresponds to M′ for trimethylsilyl ether of 4. Ion at m/z 391.2643 represents M′ – CH₃ (calculated 391.2668); ion at m/z 265.1968 results from C-7/C-8 cleavage (calculated for C₁₁H₁₂SiO₂, 265.1988).

FIG. 3. NMR of vinyl region of (8R)-8-HETE methyl ester. Top, normal spectrum at 200 MHz; bottom, decoupled by irradiation of CHO signal at δ 4.2 ppm.

FIG. 4. Synthesis of oxidative ozonolysis standards from malic acid. Top, shows the vinyl region of 8-HETE ester 4. The C-9 proton at approximately δ 5.7 is split by the C-10 proton with J = 6.41 Hz and by the C-8 proton with J = 6.41 Hz. In Fig. 3, bottom, irradiation of the proton on C-8 (δ 4.20) simplified the C-9 proton signal to a doublet, retaining the trans -CH=CH- coupling with the proton at C-10.

The configuration at C-8 in 3 and 4 was determined by ozonolysis of urethane 5 and menthy carbonate 7 and comparison of the fragments with authentic malic acid-derived standards (Fig. 4). Racemic malic acid was converted to diastereomeric urethane mixture 10 with (S)-α-methylbenzyl isocyanate (16, 17) to allow optimization of the analytical technique used to separate diastereomers. Although it was diastereomers of 10 and not enantiomers that were being separated, a chiral HPLC column support was required to effect the separation (Fig. 5). The urethane derivative of the pure (S)-isomer of malic acid, 13, constituted the slower moving (14.2 min) component of the mixture (Fig. 5, B and
analysis indicated that the crude product was almost identical to that obtained with the enzyme powder preparation above (the only difference being the virtual absence of 15-HPETE in this product). Acidification and ethyl acetate extraction of a small portion of the lipoxigenase-containing aqueous extracts again showed no peroxide-positive material and no prostaglandins. The yield and positional specificity of the 8-HETE prepared by either of these procedures is quite remarkable, as no attempt was made to optimize pH, concentration, enzyme/substrate ratio, etc.

When either the coral enzyme powder or the aqueous buffer oxygencations were carried out in the absence of added arachidonic acid, no peroxide-positive products were obtained.

An analysis of the fatty acid composition of P. porosa was performed using standard techniques (see "Experimental Procedures"), and the results are summarized in Table I. The presence of an arachidonic acid 8-lipoxigenase in a coral would generate only marginal interest if the coral had no access to arachidonic acid. On the contrary (Table I), arachidonic acid comprised the most abundant single component of the total coral lipids (20%) and was second only to palmitic acid in the phospholipid fraction. Thus, although no 8-lipoxigenase products of arachidonic acid have yet been identified from P. porosa, both the enzyme and the substrate are present.

**DISCUSSION**

This report documents the first biochemical preparation of optically active 8-HPETE with known C-8 stereochemistry. The C-8 configuration of the 8-HETE prepared from arachidonic acid with either human neutrophils (5) or rat hepatic microsomes (6) was not determined, while the 8-HETE obtained via regio-random chemical (i.e., nonenzymatic) oxidation of arachidonic acid (7-10), as well as that from total chemical synthesis (11), was racemic.

Several examples of the lipoxigenase-mediated introduction of oxygen into C-8 of C-20 fatty acids have been reported, and in each case it was the (8S)-isomer which was formed, opposite to that produced by the P. porosa lipoxigenase. Bishomo-γ-linolenic acid was transformed by lipoxigenases

**TABLE I**

*Analysis of fatty acid content of P. porosa*

The acids categorized as "others" are probably isomers of unsaturated fatty acids or branched chain acids. Since the purpose of this experiment was primarily to show the presence of arachidonic acid in reasonable amounts, only the common fatty acids were identified.

<table>
<thead>
<tr>
<th>Fatty Acid Type</th>
<th>Relative abundance (area %) in P. porosa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phospholipids</td>
<td>Total Lipids</td>
</tr>
<tr>
<td>14:0</td>
<td>0.75</td>
</tr>
<tr>
<td>16:1</td>
<td>0.335</td>
</tr>
<tr>
<td>16:0</td>
<td>13.43</td>
</tr>
<tr>
<td>18:2</td>
<td>1.7</td>
</tr>
<tr>
<td>18:1 and 18:3</td>
<td>1.35</td>
</tr>
<tr>
<td>18:0</td>
<td>7.2</td>
</tr>
<tr>
<td>20:4</td>
<td>11.77</td>
</tr>
<tr>
<td>20:2</td>
<td>0.3</td>
</tr>
<tr>
<td>20:3 and 20:1</td>
<td>0.22</td>
</tr>
<tr>
<td>22:6</td>
<td>5.3</td>
</tr>
<tr>
<td>22:1</td>
<td>0.25</td>
</tr>
<tr>
<td>22:0</td>
<td>0.99</td>
</tr>
<tr>
<td>24:1 and 24:0</td>
<td>0.233</td>
</tr>
<tr>
<td>Others (unidentified)</td>
<td>56.17</td>
</tr>
</tbody>
</table>

C). The (S)-isomer of malic acid was chosen because it was already on hand and because we anticipated the (8S)-stereochemistry in the 8-HETE based on other lipoxigenase precedents (see "Discussion"). As shown in Fig. 5, D and E, the urethane 8 derived from ozonolysis of 8-HETE ester 5 exhibited HPLC retention time identical to the faster moving (13.5 min) component of the 10 mixture and hence possessed the (8R) configuration. In a separate experiment, recovery of isomerically pure urethane 13 following exposure to the same oxidative ozonolysis conditions used with 5 ensured that the C-2 asymmetric center in 8 was stable to the degradation sequence. Thus the (8R)-configuration in 3 and 4 may be assigned with confidence.

To further confirm the (8R)-configurational assignment, a similar series of experiments was carried out on menthyl malic acid 13; C incorporation of 10 and 13; D, urethane from (S)-(+)malic acid 13; C, coinjection of 10 and 13; D, urethane from ozonolysis of 8-HETE; E, coinjection of 6 and racemic standards 10.

DISCUSSION

This report documents the first biochemical preparation of optically active 8-HPETE with known C-8 stereochemistry. The C-8 configuration of the 8-HETE prepared from arachidonic acid with either human neutrophils (5) or rat hepatic microsomes (6) was not determined, while the 8-HETE obtained via regio-random chemical (i.e., nonenzymatic) oxidation of arachidonic acid (7-10), as well as that from total chemical synthesis (11), was racemic.

Several examples of the lipoxigenase-mediated introduction of oxygen into C-8 of C-20 fatty acids have been reported, and in each case it was the (8S)-isomer which was formed, opposite to that produced by the P. porosa lipoxigenase. Bishomo-γ-linolenic acid was transformed by lipoxigenases.
in rabbit peritoneal neutrophils (20) and potato tuber homogenates (22) into \((\text{S},\text{S})\)-8-hydroxy-9,11,14-eicosatrienoic acid I (Fig. 6). The same potato enzyme also catalyzes the conversion of arachidonic acid into 5-HPETE, as well as the latter’s further transformation into leukotriene \(A_4\) (22). \((\text{S},\text{S},\text{S})\)-8,15-diHETE (Fig. 6, II) has been isolated following incubation of arachidonic acid with soybean lipoxygenase (23,24) or with eosinophil-rich human leukocyte preparations (25). This potent chemotactic factor (26) has also been obtained via total chemical synthesis (27).

Considerable information is available regarding the relationship (antarafacial) between the stereochemistry of the reaction of arachidonic acid with soybean lipoxygenase and the stereochemistry of the products. Evidently (and less likely), the coral 8-lipoxygenase could convert arachidonic acid into 8,15-diHETE (Fig. 6), but this is not a product of the coral lipoxygenase pathway except under specific circumstances. The stereochemical consequences of this point can be determined via the same mechanism as the earlier studied lipoxygenases, which is removed during the platelet lipoxygenase transformation of arachidonic acid into (12S)-HETE (29). Alternately (and less likely), the coral 8-lipoxygenase could conceivably function via a different mechanism with different stereochemical consequences. This point can be determined in straightforward fashion by performing the coral lipoxygenase reaction on stereospecifically 10-monotriitated arachidonic acid (29, 30). Mechanistic details not withstanding, it is noteworthy that the 8-HPETE produced by the \(P.\ porosa\) lipoxygenase possesses cleanly the (8R)-configuration, opposite to that found in previously reported 8-lipoxygenase products.

Although lipoxygenases have long been known in the plant kingdom (31) and their importance in mammalian fatty acid metabolism is becoming increasingly apparent (32), the current work constitutes only the second report of a lipoxygenase in a relatively primitive invertebrate species (1). The presence of the 8-lipoxygenase in \(P.\ porosa\) was not observed by Doerge and Corbett (1) presumably because of their efforts to purify the 15-lipoxygenase. They indicated that the 15-lipoxygenase was unstable and lost 50% of its activity in 25 h even at 0 °C (1). Although we have not tried to reproduce their purification scheme, it is possible that the 8-lipoxygenase may have been even less stable and was thus preferentially destroyed during their isolation process.

The biological role of this rather efficient arachidonic acid 8-lipoxygenase in \(P.\ porosa\) is not yet clear. Our inability so far to isolate products from the coral attributable to the 8-lipoxygenase pathway may simply be a matter of not knowing exactly what to look for (e.g. more complex rearrangement or further oxidation products of 5-HPETE). Although fatty acid analysis of the coral indicates that arachidonic acid is present in abundance, it is likely tied up in various esterified forms (especially phospholipids) and thus may be unavailable for lipoxygenase oxidation except under specific circumstances.

Further study of the lipoxygenase capabilities of marine organisms is warranted in several areas to determine (a) the biological significance of the lipoxygenase in these species, (b) the distribution of lipoxygenases in other forms of marine life, and (c) whether the coral 8-lipoxygenase, like the potato enzyme (22), is capable of effecting the 5-HPETE-leukotriene \(A_4\) conjugate dehydration, even though it removes the “wrong” hydrogen at C-10 (33).

REFERENCES