ARTICLE

Linoleic Acid Metabolism in the Red Alga *Lithothamnion corallioides*: Biosynthesis of 11*(R)*-Hydroxy-9*(Z)*,12*(Z)*-octadecadienoic Acid

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Incubation of [1-^{14}C]linoleic acid with an enzyme preparation obtained from the red alga *Lithothamnion corallioides* CROUAN resulted in the formation of 11-hydroxy-9*(Z)*,12*(Z)*-octadecadienoic acid as well as smaller amounts of 9-hydroxy-10*(E)*,12*(Z)*-octadecadienoic acid, 13-hydroxy-9*(Z)*,11*(E)*-octadecadienoic acid and 11-keto-9*(Z)*,12*(Z)*-octadecadienoic acid. Steric analysis showed that the 11-hydroxyoctadecadienoic acid had the *(R)* configuration. The 9- and 13-hydroxyoctadecadienoic acids were not optically pure, but were due to mixtures of 75% *(R)* and 25% *(S)* enantiomers (9-hydroxyoctadecadienoate), and 24% *(R)* and 76% *(S)* enantiomers (13-hydroxyoctadecadienoate). 11-Hydroxyoctadecadienoic acid was unstable at acidic pH. In acidified water, equal parts of 9*(R,S)*-hydroxy-10*(E)*,12*(Z)*-octadecadienoate and 13*(R,S)*-hydroxy-9*(Z)*,11*(E)*-octadecadienoate, plus smaller amounts of the corresponding *(E),(E)* isomers were produced. In aprotic solvents, acid treatment resulted in dehydration and in the formation of equal amounts of 8,10,12- and 9,11,13-octadecatrienoates. The enzymatic conversion of linoleic acid into the hydroxyoctadecadienoic acids and the ketooctadecadienoic acid was oxygen-dependent; however, inhibitor experiments indicated that neither lipoxygenase nor cytochrome P-450 were involved in the conversion. This conclusion was supported by experiments with $^{18}O_2$ and $H_2{}^{18}O$, which demonstrated that the hydroxyl oxygen of the hydroxyoctadecadienoic acids and the keto oxygen of the 11-ketooctadecadienoic acid were derived from water and not from molecular oxygen.

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Studies carried out during the last years have documented the importance of lipoxygenases in the metabolism of polyunsaturated fatty acids in marine algae (for a review, see ref. 2). The majority of oxylipins thus produced are formed by sequences that involve arachidonic acid 12-lipoxygenase in the initial step. An example of this is the conversion of arachidonic acid into 12*(R)*,13*(S)*-dihydroxy-5*(Z)*,8*(Z)*,10*(E)*,14*(Z)*-eicosatetraenoic acid (12*(R)*,13*(S)*-diHETE) (3), a transformation that occurs by initial lipoxygenase-catalyzed oxygenation of arachidonic acid into 12*(S)*-hydroperoxy-5*(Z)*,8*(Z)*,10*(E)*,14*(Z)*-eicosatetraenoic acid (12*(S)*-HPETE), followed by hydroperoxide isomerase-catalyzed conversion of the hydroperoxide into the diol fatty acid (1).

Guerriero *et al.* (4) recently isolated the ethyl esters of an array of oxylipins from the calcareous red algae *Lithothamnion corallioides* and *Lithothamnion calcareum.* Apart from the previously known esters of 5-, 11-, 12- and 15-hydroxyeicosatetraenoic acids (5-, 11-, 12- and 15-HETE), three new esters oxygenated at C-13 were obtained, *i.e.,* ethyl 13-hydroxy-5*(Z)*,8*(Z)*,11*(Z)*,14*(Z)*-eicosatetraenoate, ethyl 13-hydroxy-5*(Z)*,8*(Z)*,11*(Z)*,14*(Z)*,17*(Z)*-eicosapentaenoate and ethyl 8-hydroxy-13-keto-5*(Z)*,9*(E)*,11*(E)*,14*(E)*-eicosatetraenoate. The unusual structures of the three latter compounds prompted an investigation of the metabolism of ^{14}C-labeled polyunsaturated fatty acids in *Lithothamnion.* The present work is concerned with transformations of [^{14}C]linoleic acid in the presence of an enzyme preparation of *Lithothamnion corallioides* and with the reaction mechanism.

EXPERIMENTAL PROCEDURES

Linoleic acid was purchased from NuChek Prep, Inc. (Elysian, MN). [1-^{14}C]Linoleic acid was obtained from Amersham Laboratories (Amersham, U.K.) and added to the unlabeled acid to provide a specimen having a specific radioactivity of 5.3 kBq/μmol. 9*(R,S)*-HOD, 9*(R,S)*-hydroxy-10*(E)*,12*(E)*-octadecadienoic acid, 13*(R,S)*-HOD and 13*(R,S)*-hydroxy-9*(E)*,11*(E)*-octadecadienoic acid were prepared by treatment of the corresponding hydroperoxides (5) with sodium borohydride. Methyl 11-hydroxy-12-octadecenoate was obtained by deoxygenation of methyl 11-hydroxy-12,13-epoxyoctadecanoate (Hamberg, M., unpublished data). [9,10,12,13-$^2H_4$]Linoleic acid was prepared by partial deuteriation of 9,12-octadecadiynoic acid as described previously (6). The isotopic composition of the sample was 93% tetradeuteriated and 7% trideuteriated molecules. $^{18}O_2$ (97–98%) was obtained from Cambridge Isotope Laboratories (Woburn, MA) and $H_2{}^{18}O$ (98.7 atom%) was purchased from Isotec, Inc. (Miamisburg, OH).

Enzyme preparation. The red alga *Lithothamnion corallioides* CROUAN was collected at 10–15 m depth in a semi-exposed sound on the Southern coast of Norway in June, 1991, and kept at −77°C. Batches of about 90 g of frozen algae were crushed and ground in a mortar with liquid nitrogen. The coarse powder (85 g) was added to 0.09 M potassium phosphate buffer, pH 7.4 (85 mL), and homogenized at 0°C with an Ultra-Turrax (Janke and Kunkel, Staufen, Germany). The homogenate was centrifuged at 9,300 × *g* for 15 min and the resulting supernatant was further centrifuged at 105,000 × *g* for 60 min.

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Abbreviations: ETYA, 5,8,11,14-eicosatetraynoic acid; GC/MS, gas chromatography/mass spectrometry; GLC, gas-liquid chromatography; 12*(R)*,13*(S)*-diHETE, 12*(R)*,13*(S)*-dihydroxy-5*(Z)*,8*(Z)*,10*(E)*,14*(Z)*-eicosatetraenoic acid; 5-HETE, 5-hydroxy-6*(E)*,8*(Z)*,11*(Z)*,14*(Z)*-eicosatetraenoic acid; 11-HETE, 11-hydroxy-5*(Z)*,8*(Z)*,12*(E)*,14*(Z)*-eicosatetraenoic acid; 12-HETE, 12-hydroxy-5*(Z)*,8*(Z)*,10*(E)*,14*(Z)*-eicosatetraenoic acid; 15-HETE, 15-hydroxy-5*(Z)*,8*(Z)*,11*(Z)*,13*(E)*-eicosatetraenoic acid; 9-HOD, 9-hydroxy-10*(E)*,12*(Z)*-octadecadienoic acid; 13-HOD, 13-hydroxy-9*(Z)*,11*(E)*-octadecadienoic acid; 12*(S)*-HPETE, 12*(S)*-hydroperoxy-5*(Z)*,8*(Z)*,10*(E)*,14*(Z)*-eicosatetraenoic acid; MC, (−)-menthoxycarbonyl; Me_3Si, trimethylsilyl; NDGA, nordihydroguaiaretic acid; TLC, thin-layer chromatography. The term "oxylipin" was introduced recently (ref. 1) as an encompassing term for oxygenated compounds which are formed from fatty acids by reaction(s) involving at least one step of mono- or dioxygenase-catalyzed oxygenation.

Ammonium sulfate fractionation carried out with the high speed supernatant provided precipitates obtained at 0-30%, 30-55% and 55-80% saturation. The 30-55% precipitate, which contained more than 90% of the linoleic acid oxidizing activity, was dissolved in potassium phosphate buffer and used as the enzyme source. The protein concentration (7) was 2 mg/mL. If desired, the 30-55% ammonium sulfate precipitate could be stored at −30°C for several weeks without loss of activity.

Incubations and treatments. [1-^{14}C]Linoleic acid (300 μM) was stirred with enzyme preparation at 22°C for the times indicated. Five volumes of methanol were added and the mixture diluted with water, acidified to pH 4, and rapidly extracted with two portions of diethyl ether. The combined ether phases were washed until neutral reaction and dried over magnesium sulfate. The product obtained after evaporation of the ether was esterified by treatment with diazomethane and subjected to thin-layer radiochromatography. Recovery of radioactivity from added labeled substrate was 90-95%.

Chemical methods. Hydrogenation was carried out with palladium on calcium carbonate as the catalyst (8). Preparation of (−)-menthoxycarbonyl (MC) derivatives and procedures for oxidative ozonolysis and steric analysis of MC derivatives by gas-liquid chromatography (GLC) were performed as described (9,10).

Chromatographic and instrumental methods. Thin-layer chromatography (TLC) was carried out with precoated plates (Kieselgel 60, 0.25 mm) from E. Merck (Darmstadt, Germany). The solvent systems consisted of mixtures of ethyl acetate/hexane in the proportions indicated. Thin-layer argentation chromatography was performed with plates coated with Silica gel G/$AgNO_3$ (9:1, w/w) and a solvent system consisting of ethyl acetate/hexane (25:75, vol/vol). Radioactivity on TLC plates was determined with a Berthold Dünnschichtscanner II (Wildbad, Germany) interfaced with a Macintosh SE/30 PC. GLC was carried out with a Hewlett-Packard (Avondale, PA) model 5890 gas chromatograph equipped with a methyl silicone capillary column (length, 25 m; film thickness, 0.33 μm). Gas chromatography/mass spectrometry (GC/MS) was performed with a Hewlett-Packard Model 5970B mass selective detector connected to a Hewlett-Packard Model 5890 gas chromatograph. Ultraviolet spectroscopy was carried out with a Hewlett-Packard Model 8450A diode array spectrophotometer, and infrared spectra were obtained with a Perkin-Elmer (Überlingen, Germany) model 257 infrared spectrrophotometer. Radioactivity was measured with a Packard (Downers Grove, IL) Tri-Carb Model 4450 liquid scintillation counter.

RESULTS

Isolation of products formed from linoleic acid. Figure 1A shows a thin-layer radiochromatogram of the esterified product obtained following incubation of 300 μM [1-^{14}C]-linoleic acid with the enzyme preparation at 22°C for 20 min. The reaction product consisted of three compounds (Compounds A_1, A_2 and A_3) having a polarity typical for hydroxyoctadecadienoates, as well as a fourth compound (Compound B; $R_f = 0.52$), which migrated like a ketooctadecadienoate. Compound B was directly obtained in a purity which was satisfactory for structural analysis. In order to obtain Compounds A_1-A_3 in pure form, a broad

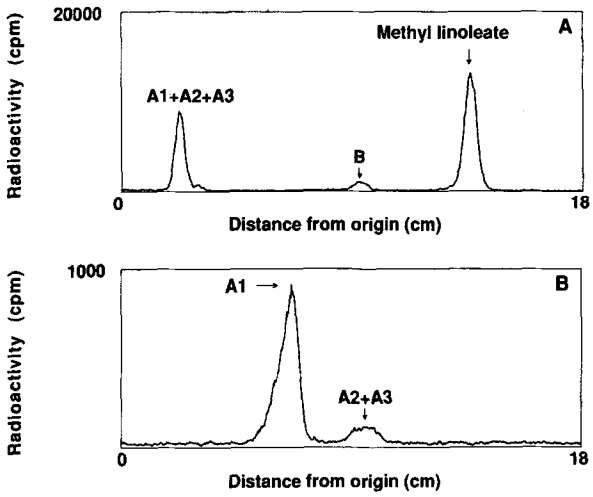

FIG. 1. A: Thin-layer radiochromatogram of esterified product obtained following incubation of [1-^{14}C]linoleic acid (300 μM) with enzyme preparation (2 mL) at 22°C for 20 min. Solvent system, ethyl acetate/hexane (1:9, vol/vol). B: Thin-layer argentation radiochromatogram of material present in zone of compounds $A_1 + A_2 + A_3$ in A. Solvent system, ethyl acetate/hexane (25:75, vol/vol).

zone of silica gel containing these compounds was scraped off and the material subjected to thin-layer argentation chromatography. As seen in Figure 1B, this resulted in separation of a major hydroxyoctadecadienoate (Compound A_1; $R_f = 0.37$) from two minor hydroxyoctadecadienoates (Compounds A_2 and A_3). The two latter compounds were separated on plain silica gel using a solvent system of ethyl acetate/hexane (2:8, vol/vol; Compound A_2, $R_f = 0.39$; Compound A_3, $R_f = 0.44$).

Structure of Compound A_1. Compound A_1 accounted for 71% of the reaction product formed from linoleic acid. The UV spectrum of Compound A_1 did not show any specific absorption band in the region 200-320 nm, demonstrating the absence of a conjugated diene structure. Infrared spectroscopy showed absorption bands at *inter alia* 3350-3620 cm^{-1} (hydroxyl) and 1735 cm^{-1} (ester carbonyl). No band was observed in the region 950-1000 cm^{-1}, thus excluding the presence of *(E)* double bond(s). Analysis of the Me_3Si derivative of Compound A_1 by GC/MS showed a single peak having a C-value of 19.41. The mass spectrum (Fig. 2) showed prominent ions at *inter alia m/e* 382 (M, 38%), 311 [M − 71; loss of $\cdot(CH_2)_4$-CH_3, 32], and 225 ([CH=CH-CH($OSiMe_3$)-CH=CH-$(CH_2)_4$-CH_3]$^+$, 44). The spectrum was similar to those of the Me_3Si derivatives of the methyl esters of 9- and 13-hydroxyoctadecadienoic acids (9- and 13-HOD). Catalytic hydrogenation performed on Compound A_1 provided methyl 11-hydroxy-stearate as judged by GC/MS analysis of the Me_3Si derivative. Prominent ions appeared at *m/e* 371 (M − 15; loss of $\cdot CH_3$, 4%), 339 [M − (15 + 32), 19], 287 [Me_3SiO^+=CH-$(CH_2)_9$-$COOCH_3$, 100], and 201 [Me_3SiO^+=CH-$(CH_2)_6$-CH_3, 71].

The data mentioned indicated that Compound A_1 was a methyl octadecadienoate having a hydroxyl group at C-11. In order to determine the absolute configuration of C-11 and the positions of the double bonds, a sample of Compound A_1 (250 μg) was stirred with palladium on calcium carbonate (5 mg) in ethyl acetate (3 mL) under

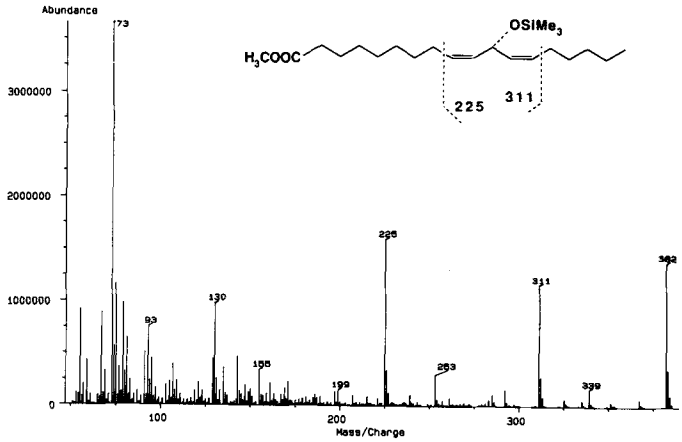

FIG. 2. Mass spectrum of the Me_3Si derivative of methyl 11*(R)*-hydroxy-9*(Z)*,12*(Z)*-octadecadienoate (Compound A_1).

hydrogen gas for 3 min. The partially hydrogenated product obtained was a mixture of 11-hydroxystearate, two isomeric 11-hydroxyoctadecenoates, and unreacted methyl 11-hydroxyoctadecadienoate as judged by GC/MS analysis. Oxidative ozonolysis performed on the MC derivatives yielded methyl hydrogen azelate (from methyl 11-hydroxy-9,12-octadecadienoate and methyl 11-hydroxy-9-octadecenoate), the MC derivative of 2*(R)*-hydroxynonanoic acid (from methyl 11-hydroxy-9-octadecenoate), as well as the MC derivative of methyl hydrogen 2*(S)*-hydroxy-1,12-dodecanedioate (from methyl 11-hydroxy-12-octadecenoate). On the basis of the results obtained, Compound A_1 was assigned the structure of methyl 11*(R)*-hydroxy-9*(Z)*,12*(Z)*-octadecadienoate.

Structures of Compounds A_2 and A_3. Compounds A_2 and A_3 each accounted for 5% of the product formed from linoleic acid. The UV spectra of the two compounds showed a strong absorption band at 234 nm (solvent, methanol; $\varepsilon = 26{,}000$), indicating the presence of a conjugated diene structure. The C-value of the Me_3Si derivatives found on GLC analysis of Compounds A_2 and A_3 was 19.81 [references, Me_3Si derivatives of the methyl esters of 9-HOD (C-19.81), 13-HOD (C-19.81), 9-hydroxy-10*(E)*,12*(E)*-octadecadienoic acid (C-20.20), and of 13-hydroxy-9*(E)*,11*(E)*-octadecadienoic acid (C-20.36)]. The mass spectra of the Me_3Si derivative of Compounds A_2 and A_3 were identical with those of the corresponding derivatives of the methyl esters of 9- and 13-HOD, respectively. Prominent ions were observed at *m/e* 382 (M), 311 [M − 71; loss of $\cdot(CH_2)_4$-CH_3] and 225 {due to the ion Me_3SiO^+=CH-$(CH{=}CH)_2$-$(CH_2)_4$-CH_3 in the spectrum of the derivative of 9-HOD, due to the ion [$(CH{=}CH)_2$-$CH(OSiMe_3)$-$(CH_2)_4$-CH_3]$^+$ in the spectrum of the derivative of 13-HOD}. The fragmentation patterns of the derivatives of 9- and 13-HOD were similar; however, the intensity of the *m/e* 225 ion was greater than that of the *m/e* 311 ion in the spectrum of the derivative of 9-HOD, whereas the reverse was true in the spectrum of the derivative of 13-HOD. Catalytic hydrogenation of Compounds A_2 and A_3 produced methyl 9- and 13-hydroxystearates, respectively, as demonstrated by GC/MS. Oxidative ozonolysis performed on the MC derivative of Compound A_2 yielded the MC derivative of methyl hydrogen 2-hydroxysebacate [*(R)/(S)*, 75:25], while the same treatment of the MC derivative of Compound A_3 afforded methyl hydrogen azelate plus the MC derivative of 2-hydroxyheptanoic acid [*(R),(S)*, 24:76]. Thus, Compound A_2 was methyl 9-hydroxy-10*(E)*,12*(Z)*-octadecadienoate [75% of the *(R)* enantiomer], whereas Compound A_3 was identical to methyl 13-hydroxy-9*(Z)*,11*(E)*-octadecadienoate [76% of the *(S)* enantiomer].

Structure of Compound B. Compound B accounted for 13% of the reaction product formed from linoleic acid. The UV spectrum showed a strong absorption band at 255 nm (solvent, methanol; $\varepsilon = 17{,}000$). Mass spectrometric analysis of Compound B showed a molecular ion at *m/e* 308 (6%), as well as high intensity ions at *m/e* 277 (M − 31; loss of $\cdot OCH_3$, 6), 211 [M − 97; loss of $\cdot$CH=CH-$(CH_2)_4$-CH_3, 16], 183 [M − 125; loss of $\cdot$CO-CH=CH-$(CH_2)_4$-CH_3, 16], 151 {[CH=CH-CO-CH=CH-$(CH_2)_4$-CH_3]$^+$, 42}, and 125 [O^+≡C-CH=CH-$(CH_2)_4$-CH_3, 36]. Catalytic hydrogenation of Compound B yielded methyl 11-ketostearate as judged by GC/MS. The data mentioned indicated that Compound B was a methyl 11-ketooctadecadienoate. The ultraviolet spectroscopic data further suggested that the two double bonds were not present as a conjugated diene, but were part of a 3-keto-1,4-pentadiene structure.

Proof for the location and geometry of the double bonds was obtained by an experiment in which the products formed from Compound B upon sodium borohydride reduction were analyzed. Thus, Compound B (240 μg) was treated with $NaBH_4$ (30 mg) in methanol (3 mL) at 22°C for 30 min. Analysis by TLC showed two bands of similar intensity. The more polar material was identified as methyl 11-hydroxy-9*(Z)*,12*(Z)*-octadecadienoate by GC/MS using the authentic compound as reference. The less polar material was due to a 1:1 mixture of methyl 11-hydroxy-9-octadecenoate (C-value of Me_3Si derivative, 19.54; prominent ions at *m/e* 369 (M − 15; loss of $\cdot CH_3$, 1%), 337 [M − (15 + 32), 4] and 285 [Me_3SiO^+=CH-CH=CH-$(CH_2)_7$-$COOCH_3$), 100]) and methyl 11-hydroxy-12-octadecenoate (C-value of Me_3Si derivative, 19.65; prominent ions at *m/e* 369 (M − 15; loss of $\cdot CH_3$, 2%), 337 [M − (15 + 32), 5], and 199 [Me_3SiO^+=CH-CH=CH-$(CH_2)_4$-CH_3, 100; mass spectrum identical with that of the Me_3Si derivative of the authentic compound]. The positions of the hydroxyl group and of the double bond in the two hydroxyoctadecenoates were established by oxidative ozonolysis performed on the MC derivatives. This treatment afforded methyl hydrogen azelate and the MC derivative of 2*(R,S)*-hydroxynonanoic acid (from the MC derivative of methyl 11-hydroxy-9-octadecenoate) and the MC derivative of methyl hydrogen 2*(R,S)*-hydroxy-1,12-dodecanedioate (from the MC derivative of methyl 11-hydroxy-12-octadecenoate). On the basis of the data presented, Compound B was assigned the structure methyl 11-keto-9*(Z)*,12*(Z)*-octadecadienoate.

Acid-induced conversions of methyl 11(R)*-hydroxy-9*(Z)*,12*(Z)*-octadecadienoate.* A solution of methyl 11*(R)*-hydroxy-9*(Z)*,12*(Z)*-octadecadienoate (250 μg) in dimethoxyethane (5 mL) was treated with water (10 mL) and 2 M HCl (0.1 mL) at 22°C for 15 min. Analysis of the product by TLC and GC/MS demonstrated complete conversion of the methyl 11-hydroxyoctadecadienoate into four isomeric hydroxyoctadecadienoates. These compounds were identified, using the authentic materials as references, as the methyl esters of 9-HOD (43%), 13-HOD (43%),

9-hydroxy-10*(E)*,12*(E)*-octadecadienoic acid (7%) and 13-hydroxy-9*(E)*,11*(E)*-octadecadienoic acid (7%). The four hydroxyoctadecadienoates were racemic as shown by oxidative ozonolysis performed on the MC derivatives, which produced the MC derivatives of methyl hydrogen 2*(R,S)*-hydroxysebacate and 2*(R,S)*-hydroxyheptanoic acid.

In another experiment, methyl 11*(R)*-hydroxy-9*(Z)*,12*(Z)*-octadecadienoate (27 μg) in methanol (3 mL) was treated with 2 M HCl (2 μL) at 23°C. This treatment was accompanied by the appearance of a UV absorption band with λ_{max} = 234 nm (ε = 27,000). As shown in Figure 3, appearance of the absorption band was rapid. Disappearance of the 11-hydroxyoctadecadienoate in the acidified methanol solution followed first order kinetics with a half-life time of 32 s. The products formed were analyzed by GC/MS and found to be a 1:1 mixture of 9-methoxy-10,12-octadecadienoate [base peak at *m/e* 167, due to CH_3O^+=CH-(CH=CH)$_2$-(CH$_2$)$_4$-CH$_3$] and 13-methoxy-9,11-octadecadienoate [base peak at *m/e* 253, due to CH_3O^+=CH-(CH=CH)$_2$-(CH$_2$)$_7$-COOCH$_3$]. GC/MS analysis of the hydrogenated derivatives was in full accordance with these structures.

The following experiment was carried out in order to investigate the fate of methyl 11*(R)*-hydroxy-9*(Z)*,12*(Z)*-octadecadienoate in an acidified aprotic solvent. Methyl 11-hydroxyoctadecadienoate (18 μg) in acetonitrile (3 mL) was treated with 70% (w/w) perchloric acid (5 μL). The UV spectrum recorded after 1 min at 23°C showed absorption bands indicative of a conjugated triene, *i.e.*, at 259 nm, 269 nm (ε = 50,000), and 280 nm [Fig. 4; reported for 9*(Z)*,11*(E)*,13*(E)*-octadecatrienoic acid: bands at 261, 270 and 281 nm; and for 9*(E)*,11*(E)*,13*(E)*-octadecatrienoic acid: bands at 258, 268 and 279 nm (11)]. GC/MS analysis of the material showed two pairs of peaks, *i.e.*, at C-18.99 (21%) and 19.11 (21%), and at C-19.41 (29%) and 19.45 (29%). The mass spectra recorded on these peaks showed an intense molecular ion at *m/e* 292, but were otherwise relatively non-informative. Catalytic hydrogenation performed on the product yielded a single compound, *i.e.*, methyl stearate (*m/e* 298). It was thus clear that the product formed upon acid treatment of methyl 11-hydroxy-

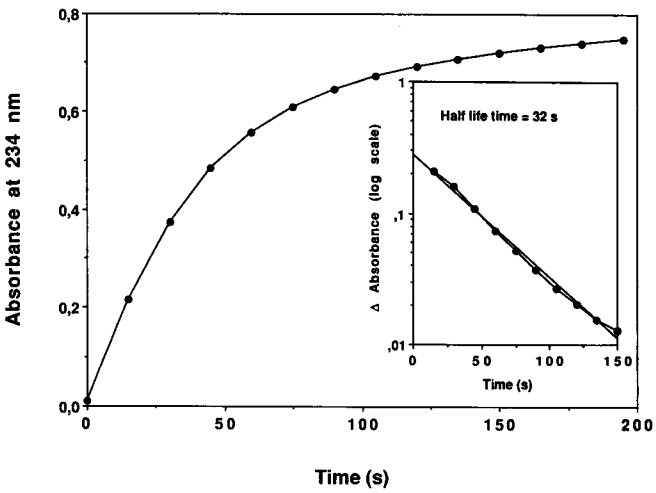

FIG. 3. Appearance of absorbance at 234 nm upon treatment of methyl 11*(R)*-hydroxy-9*(Z)*,12*(Z)*-octadecadienoate (27 μg) in methanol (3 mL) with 2 μL of 2 M HCl. Inset: Plot of change in absorbance at 234 nm (log scale) *vs.* time.

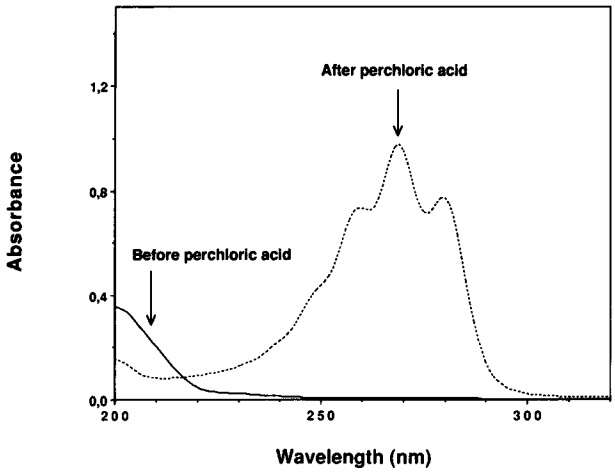

FIG. 4. UV spectra recorded on a solution of 18 μg of methyl 11*(R)*-hydroxy-9*(Z)*,12*(Z)*-octadecadienoate in 3 mL of acetonitrile before (solid line) and 1 min after (dashed line) addition of 5 μL of 70% (w/w) perchloric acid.

octadecadienoate in acetonitrile was due to four isomeric methyl octadecatrienoates. It appeared that the two factors causing separation of the octadecatrienoates upon GLC were different positions of the triene moiety, and geometrical isomerism of the triene. The geometrical configurations of the conjugated trienes were not determined; however, the fact that oxidative ozonolysis performed on the octadecatrienoate mixture yielded equal amounts of methyl hydrogen suberate and methyl hydrogen azelate localized the conjugated triene structures in the carbon chain, and demonstrated that the octadecatrienoates were due to equal amounts of methyl 8,10,12- and 9,11,13-octadecatrienoates.

Incubation of 11-hydroxy- and 11-ketooctadecadienoates. Figure 5A shows the time course of the formation of oxidized products (9-, 11- and 13-hydroxyoctadecadienoic acids plus 11-ketooctadecadienoic acid) from linoleic acid when incubated with the enzyme preparation. As seen, oxidation proceeded linearly in the time interval 0–30 min and then leveled off because of depletion of substrate. No oxidation was observed when heat-inactivated enzyme was used. In order to obtain information of possible interconversions between the 11-oxygenated products, samples of [1-^{14}C]11*(R)*-hydroxy-9*(Z)*,12*(Z)*-octadecadienoic acid and [1-^{14}C]11-keto-9*(Z)*,12*(Z)*-octadecadienoic acid were prepared by incubation of [1-^{14}C]linoleic acid and isolated as the free acids by silicic acid open column chromatography. The 11-ketooctadecadienoic acid thus obtained was radiochemically pure, whereas the 11-hydroxyoctadecadienoic acid was contaminated with 14% of a mixture of equal parts of 9- and 13-HOD. Re-incubation of [1-^{14}C]11-ketooctadecadienoic acid (100 μM) with the enzyme preparation at 22°C for 20 min did not result in conversion into hydroxyoctadecadienoate(s) or other products. Similar re-incubation of [1-^{14}C]11-hydroxyoctadecadienoic acid resulted in a low yield (5%) of 11-ketooctadecadienoic acid. Slow conversion of 11-hydroxyoctadecadienoic acid into 11-ketooctadecadienoic acid was confirmed by a time-course study (Fig. 5B). Significant formation of 9- and 13-HOD from 11-hydroxyoctadecadienoic acid was not observed.

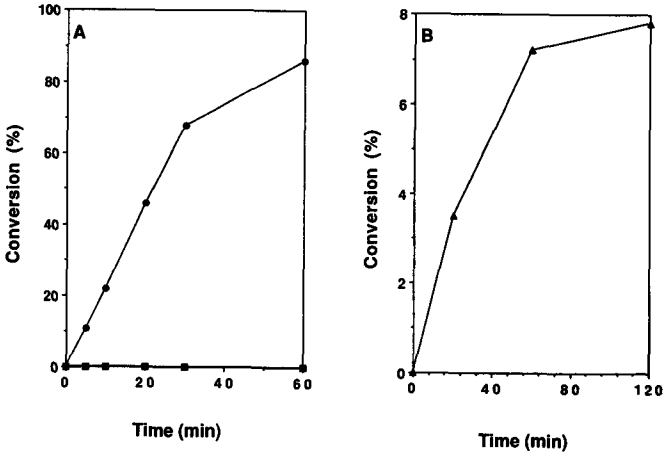

FIG. 5. A: Time course of the oxidation of linoleic acid into 9-, 11- and 13-hydroxyoctadecadienoic acids and 11-ketooctadecadienoic acid. Enzyme preparation (10 mL; -●-●-●-) or heat-inactivated (95°C, 10 min) enzyme preparation (10 mL; -■-■-■-) were stirred with [1-^{14}C]linoleic acid (300 μM) at 22°C and aliquots of 2 mL were removed at times indicated. The percentage conversion of linoleic acid into the three hydroxyoctadecadienoic acids and the 11-keto-octadecadienoic acid was determined by thin-layer radiochromatography. B: Time course of the conversion of 11-hydroxyoctadecadienoic acid into 11-ketooctadecadienoic acid. Enzyme preparation (8 mL) was stirred with [1-^{14}C]11-hydroxyoctadecadienoic acid (100 μM) at 22°C and aliquots of 2 mL were removed at times indicated. The percentage conversion into 11-ketooctadecadienoic acid was determined by thin-layer radiochromatography.

In order to facilitate interpretation of results obtained with linoleic acid incubated in the presence of $^{18}O_2$ and $H_2{}^{18}O$ (see below), re-incubations of 11-hydroxy- and 11-ketooctadecadienoic acids (100 μM) were also carried out with enzyme preparation dissolved in potassium phosphate buffer (1.6 mL) and $H_2{}^{18}O$ (0.4 mL). As seen in Table 1, 11-ketooctadecadienoic acid, either produced from 11-hydroxyoctadecadienoic acid or recovered following incubation of 11-ketooctadecadienoic acid, contained a small fraction of solvent ^{18}O (incorporation, 7-8% of the theoretical). As expected, 11-hydroxyoctadecadienoic acid recovered following incubation of this hydroxy acid did not contain any ^{18}O in the C-11 hydroxyl group.

^{18}O experiments. Linoleic acid (300 μM) was incubated at 22°C for 20 min with the enzyme preparation under an atmosphere of $^{18}O_2$. As seen in Table 2, the 11-hydroxy- and 11-ketooctadecadienoic acids produced were completely devoid of ^{18}O. A small (2.5%) incorporation was observed in 9- and 13-HOD. As a check, following aspiration of the reaction mixture at the end of the incubation period, the incubation vessel was injected with linoleic acid and soybean lipoxygenase. The linoleic acid 13-hydroperoxide thus formed was reduced into 13-HOD. Mass spectrometric analysis of the Me_3Si derivative of the methyl ester of this compound showed satisfactory incorporation of ^{18}O.

In another experiment, linoleic acid was stirred with the enzyme preparation in the presence of $H_2{}^{18}O$. As seen in Table 2, the resulting 11-hydroxy- and 11-ketooctadecadienoates showed virtually complete incorporation of ^{18}O in the hydroxyl and keto groups, respectively. Incorporation of ^{18}O in 9- and 13-HOD was extensive but not complete, *i.e.*, 79-86% of the theoretical.

Incubation of [9,10,12,13-$^2H_4$]linoleic acid. Enzyme preparation (2 mL) was stirred at 22°C for 20 min with 300 μM [9,10,12,13-$^2H_4$]linoleic acid. The hydroxyoctadecadienoates were isolated and converted into the Me_3Si derivatives. Selected monitoring of the ions *m/e* 225, 226, 227, 228 and 229 showed that 11-hydroxyoctadecadienoic acid, as well as 9- and 13-HOD, were due to 93% of tetradeuteriated and 7% trideuteriated molecules. This isotopic composition was the same as that of the incubated deuteriated linoleic acid, thus showing that the hydrogens at carbons 9, 10, 12 and 13 were retained in the conversion of linoleic acid into the three hydroxyoctadecadienoic acids.

Effect of anaerobiosis and enzyme inhibitors. Conversion of linoleic acid into oxidized products was strictly dependent on the presence of oxygen (Table 3). Furthermore, sodium azide in 5 mM concentration inhibited the reaction. The lipoxygenase inhibitor nordihydroguaiaretic acid gave only weak inhibition, and two other well-established lipoxygenase inhibitors, 5,8,11,14-eicosatetraynoic acid and esculetin, were without effect. One cytochrome P-450 inhibitor, metyrapone, lacked inhibitory effect, whereas another one, SKF-525A, gave moderate inhibition when tested in 1 mM concentration. Furthermore,

TABLE 1

Isotope Composition of Compounds Isolated Following Incubation of 11-Hydroxy- and 11-Ketooctadecadienoic Acids with Enzyme Preparation in the Presence of $H_2{}^{18}O$

	Isotope composition (%)[b]			
	11-Hydroxyoctadecadienoic acid		11-Ketooctadecadienoic acid	
Compound incubated[a]	$^{18}O_0$	$^{18}O_1$	$^{18}O_0$	$^{18}O_1$
11-Hydroxyoctadecadienoic acid	100	0 (0)	98.5	1.5 (7)
11-Ketooctadecadienoic acid	—	—	98.4	1.6 (8)

[a] Enzyme preparation (1.6 mL) and $H_2{}^{18}O$ (98.7 atom%, 0.4 mL) were stirred at 22°C for 20 min with 11*(R)*-hydroxy-9*(Z)*,12*(Z)*-octadecadienoic acid (100 μM) or 11-keto-9*(Z)*,12*(Z)*-octadecadienoic acid (100 μM). Five volumes of methanol were added and the products isolated and derivatized as described in the text.

[b] The isotopic compositions were determined by selected monitoring of the following ions: *m/e* 225 and 227 ($[CH=CH-CH(OSiMe_3)-CH=CH-(CH_2)_4-CH_3]^+$, Me_3Si derivative of the methyl ester of 11-hydroxyoctadecadienoic acid, and *m/e* 151 and 153 ($[CH=CH-CO-CH=CH-(CH_2)_4-CH_3]^+$; methyl ester of 11-ketooctadecadienoic acid. The dwell time used for data acquisition was 50 ms. Numbers in parentheses indicate content of ^{18}O relative to the content of ^{18}O in the water of the incubation mixture.

TABLE 2

Isotope Composition of Compounds Isolated Following Incubation of Linoleic Acid with Enzyme Preparation in the Presence of $^{18}O_2$ and $H_2{}^{18}O$

Compound analyzed[a]	Isotope composition (%)			
	Incubation with $^{18}O_2$[b]		Incubation with $H_2{}^{18}O$[c]	
	$^{18}O_0$	$^{18}O_1$	$^{18}O_0$	$^{18}O_1$
11-Hydroxyoctadecadienoic acid	100.0	0	80.1	19.9 (101)
9-HOD	97.5	2.5	83.0	17.0 (86)
13-HOD	97.5	2.5	84.5	15.5 (79)
11-Ketooctadecadienoic acid	100.0	0	80.6	19.4 (98)

[a]The isotopic composition was determined by selected monitoring of the ions *m/e* 225 and 227 (hydroxyoctadecadienoates), and 151 and 153 (11-ketooctadecadienoate).

[b]Enzyme preparation (10 mL) in a sealed vessel was purged with argon and evacuated in five cycles. $^{18}O_2$ (97–98 atom%, 0.1 L) was introduced, and the reaction was started by injection of linoleic acid (300 μM. The mixture was stirred at 22°C for 20 min and then added to five volumes of methanol. Products were isolated and derivatized as described in the text.

[c]Enzyme preparation (4 mL) and $H_2{}^{18}O$ (98.7 atom%, 1 mL) was stirred at 22°C for 20 min with 300 μM linoleic acid. Five volumes of methanol were added and the products isolated and derivatized as described in the text. Numbers in parentheses indicate percentage content of ^{18}O relative to the content of ^{18}O in the water of the incubation mixture.

TABLE 3

Effect of Anaerobiosis and Enzyme Inhibitors on the Oxidation of Linoleic Acid

Condition[a]	Conversion (% of control)[b]
Control	100
Heat-treated enzyme (95°C, 10 min)	0
Anaerobiosis	< 1
Sodium azide (5 mM)	22
SKF-525A (1 mM)	50
Diethyldithiocarbamate (1 mM)	63
NDGA (0.1 mM)	75
2,2′-Dipyridyl (1 mM)	100
Esculetin (0.1 mM)	101
Metyrapone (1 mM)	101
ETYA (0.1 mM)	101

[a]The enzyme preparation was stirred at 22°C for 5 min with inhibitor and then stirred for additional 20 min in the presence of [1-^{14}C]-linoleic acid. Anaerobiosis was accomplished by repeated argon-purging and evacuation of the enzyme preparation contained in a sealed vessel.

[b]The control incubation showed 49% conversion into the three hydroxyoctadecadienoic acids and the ketooctadecadienoic acid.

slight inhibition was noted in the presence of the copper-chelating agent, diethyldithiocarbamate.

DISCUSSION

Several mechanisms exist for introduction of molecular oxygen in the formation of oxylipins. A quantitatively large part of oxylipins is formed by sequences that are initiated by lipoxygenase-catalyzed oxygenation. Compounds formed in this way include leukotrienes and lipoxins formed by initial 5-lipoxygenation of arachidonic acid in animal tissue (12), jasmonic acid and a host of other biologically active compounds formed by initial 9- and 13-lipoxygenation of linoleic and α-linolenic acids in plants (13), and a variety of products formed in algae by initial 12-lipoxygenation of arachidonic and eicosapentaenoic acids (2). Prostaglandins and thromboxanes constitute a group of oxylipins which is formed by initial dioxygenation catalyzed by prostaglandin endoperoxide synthase (14). Oxylipins also may be biosynthesized by cytochrome P-450-catalyzed monooxygenation, in animal (15) as well as in plant tissue (16,17). An additional, recently discovered mechanism of biosynthesis of oxylipins consists of hydroperoxide-dependent epoxidation of fatty acids and hydroxy acids in higher plants (18,19). The present study is concerned with transformation of linoleic acid into hydroxy and ketooctadecadienoic acids in the red alga *Lithothamnion corallioides* by a mechanism which appears to be distinct from previously recognized mechanisms of oxylipin biosynthesis.

The major compound formed from linoleic acid upon incubation with the enzyme preparation of *Lithothamnion* was found to have the structure 11*(R)*-hydroxy-9*(Z)*,12*(Z)*-octadecadienoic acid (Fig. 6). This novel hydroxy acid was related to 13-hydroxyarachidonate and 13-hydroxyeicosapentaenoate recently isolated as natural products from *Lithothamnion* (4), and also to the methyl ester of 11-hydroperoxylinoleic acid, which had been obtained earlier by chemical synthesis (20). The transformation of linoleic acid into 11*(R)*-hydroxy-9*(Z)*,12*(Z)*-octadecadienoic acid demonstrated in the present paper constitutes the first example of an enzymatic reaction in which oxygen is introduced into a *bis*-allylic methylene group of a polyunsaturated fatty acid. Interestingly, the oxygen incorporated originated in water rather than dioxygen. This was an unexpected result, and a number of control experiments were performed in order to verify it. For example, that the lack of incorporation of ^{18}O in 11-hydroxyoctadecadienoic acid biosynthesized from linoleic acid under $^{18}O_2$ was not due to technical problems with the $^{18}O_2$ incubations or with the ^{18}O-gas itself was shown by a control experiment in which linoleic acid was incubated with soybean lipoxygenase using the same atmosphere of $^{18}O_2$

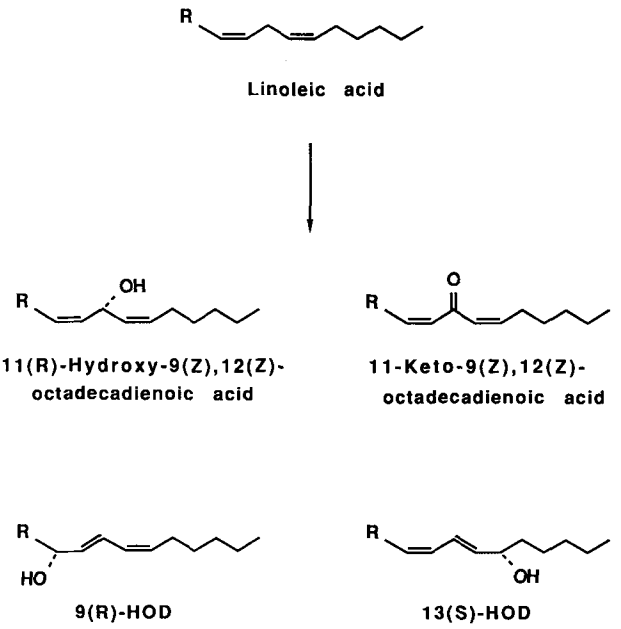

FIG. 6. Transformations of linoleic acid in *Lithothamnion corallioides*. The major enantiomers of the 9- and 13-HOD isolated are shown. R = $(CH_2)_7$-COOH.

as that used for the *Lithothamnion* incubation. The linoleic acid 13-hydroperoxide thus produced showed the expected incorporation of ^{18}O. Furthermore, that the incorporation of ^{18}O into 11-hydroxyoctadecadienoic acid formed from linoleic acid in the presence of $H_2{}^{18}O$ was not due to secondary chemical or enzymatical exchange of ^{18}O into 11-hydroxyoctadecadienoic acid was apparent from an experiment in which 11-hydroxyoctadecadienoic acid was separately incubated with the enzyme preparation in the presence of $H_2{}^{18}O$. No incorporation of ^{18}O in the re-isolated 11-hydroxyoctadecadienoic acid could be detected (Table 1).

The *bis*-allylic alcohol group present in 11-hydroxyoctadecadienoic acid made the compound sensitive to acid. In acidified protic solvents, 11-hydroxyoctadecadienoic acid underwent rapid solvolysis to produce 9- and 13-substituted derivatives having one pair of conjugated double bonds. In aprotic solvents elimination of water took place with the formation of derivatives having a conjugated triene structure. Fatty acids containing conjugated triene and tetraene structures, *e.g.*, elaeostearic and parinaric acids, occur naturally in plants (21). Possibly the facile dehydration of 11-hydroxyoctadecadienoate into conjugated triene acids may have relevance for the biosynthesis of such compounds.

Two other hydroyoctadecadienoates, *i.e.*, 9- and 13-HOD, were obtained as minor compounds following incubation of linoleic acid with *Lithothamnion*. Undoubtedly, part of these compounds was formed non-enzymatically from 11-hydroxyoctadecadienoic acid during the incubation period and the isolation procedure. However, steric analysis showed that the 9- and 13-HOD isolated were not racemates, but due to *ca.* 75% of the *(R)* and *(S)* enantiomers, respectively. It thus seemed likely that *ca.* 50% of the 9- and 13-HOD were formed as the racemic compounds by chemical solvolysis of 11-hydroxyoctadecadienoic acid, while the remaining 50% were formed as the pure *(R)* and *(S)* compounds, respectively, by enzymatic oxidation of linoleic acid. Interestingly, this oxidation occurred by a non-lipoxygenase mechanism as judged by the extensive incorporation of ^{18}O from $H_2{}^{18}O$, but not from $^{18}O_2$, in the hydroxyl groups of 9- and 13-HOD (Table 2). A fourth compound isolated following incubation of linoleic acid, 11-keto-9*(Z)*,12*(Z)*-octadecadienoic acid, was apparently formed by enzymatic oxidation of 11*(R)*-hydroxy-9*(Z)*,12*(Z)*-octadecadienoic acid (Fig. 5B). As expected from its formation from 11-hydroxyoctadecadienoic acid, the oxygen of the C-11 keto group of 11-ketooctadecadienoic acid was derived from water, and not from dioxygen (Table 2).

The transformation of linoleic acid into 11*(R)*-hydroxy-9*(Z)*,12*(Z)*-octadecadienoic acid was oxygen-dependent (Table 3), involved incorporation of 1 atom of oxygen from water (Table 2), and was not inhibitable, or only moderately so, by lipoxygenase and cytochrome P-450 inhibitors (Table 3). If the transformation occurred by a single enzymatic step it followed, per definition, that the enzyme involved was not an oxygenase. It may be speculated that an oxidase was responsible for the conversion; however, it is apparent that further studies on the enzyme(s) involved in the transformation are necessary before any conclusions as to the mechanism may be drawn. Such work is in progress in our laboratory.

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REFERENCES

1. Gerwick, W.H., Moghaddam, M.F., and Hamberg, M. (1991) *Arch. Biochem. Biophys. 290,* 436-444.
2. Gerwick, W.H., and Bernart, M.W. (1992) in *Advances in Marine Biotechnology: Pharmaceutical and Bioactive Natural Products* (Zaborsky, O.R., and Attaway, D.H., eds.) Vol. 1, Plenum Press, New York, in press.
3. Moghaddam, M.F., and Gerwick, W.H. (1991) *J. Nat. Prod. 54,* 1619-1624.
4. Guerriero, A., D'Ambrosio, M., and Pietra, F. (1990) *Helv. Chim. Acta 73,* 2183-2189.
5. Hamberg, M. (1989) *Lipids 24,* 249-255.
6. Hamberg, M. (1987) *Biochim. Biophys. Acta 920,* 76-84.
7. Bradford, M.M. (1976) *Anal. Biochem. 72,* 248-254.
8. Hamberg, M., and Fahlstadius, P. (1992) *Plant Physiol.*, in press.
9. Hamberg, M. (1971) *Anal. Biochem. 43,* 515-526.
10. Hamberg, M. (1991) *Lipids 26,* 407-415.
11. Crombie, L., and Jacklin, A.G. (1957) *J. Chem. Soc.*, 1632-1646.
12. Samuelsson, B., Dahlén, S.-E., Lindgren, J.-Å., Rouzer, C.A., and Serhan, C.N. (1987) *Science 237,* 1171-1176.
13. Gardner, H.W. (1991) *Biochim. Biophys. Acta 1084,* 221-239.
14. Smith, W.L., and Marnett, L.J. (1991) *Biochim. Biophys. Acta 1083,* 1-17.
15. Capdevila, J., Chacos, N., Werringloer, J., Prough, R.A., and Estabrook, R.W. (1981) *Proc. Natl. Acad. Sci. USA 78,* 5362-5366.
16. Croteau, R., and Kolattukudy, P.E. (1975) *Arch. Biochem. Biophys. 170,* 61-72.
17. Salaün, J.-P., Benveniste, I., Reichhart, D., and Durst, F. (1978) *Eur. J. Biochem. 90,* 155-159.
18. Hamberg, M., and Hamberg, G. (1990) *Arch. Biochem. Biophys. 283,* 409-416.
19. Blée, E., and Schuber, F. (1990) *J. Biol. Chem. 265,* 12887-12894.
20. Porter, N.A., Ziegler, C.B., Khouri, F.F., and Roberts, D.H. (1985) *J. Org. Chem. 50,* 2252-2258.
21. Badami, R.C., and Patil, K.B. (1981) *Prog. Lipid Res. 19,* 119-153.

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