Formation of 12-[^{18}O]Oxo-*cis*-10, *cis*-15-Phytodienoic Acid
from 13-[^{18}O]Hydroperoxylinolenic Acid
by Hydroperoxide Cyclase

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ABSTRACT

13-[^{18}O] Hydroperoxylinolenic acid was permitted to react with an extract of flaxseed acetone powder containing hydroperoxide cyclase activity. The resulting product, 12-oxo-*cis*-10,*cis*-15-phytodienoic acid (12-oxo-PDA), contained ^{18}O in the carbonyl oxygen at carbon 12, suggesting that an epoxide was an intermediate in the hyderoperoxide cyclase reaction. A substrate specificity study showed that a *cis* double bond β,γ to the conjugated hydroperoxide group was essential for the substrate to be converted to a cyclic product by hydroperoxide cyclase.

INTRODUCTION

Polyunsaturated fatty acids with *n*-3,6,9-unsaturation can be converted to cyclic fatty acids containing a cyclopentenone ring (1) by enzymes present in a wide variety of plant tissues (2). We have previously shown that an *n*-6 hydroperoxide, formed by action of lipoxygenase, is an intermediate in the reaction sequence (3). Hydroperoxide cyclase then converts the fatty acid hydroperoxide to a cyclic fatty acid (Fig. 1). The product resulting from (9,12,15)-linolenic acid is 8-[2-(*cis*-2'-pentenyl)-3-oxo-*cis*-4-cyclopentenyl] octanoic acid, for which the common name 12-oxo-*cis*-10,*cis*-15-phytodienoic acid (12-oxo-PDA) has been proposed (1). The purpose of this investigation was to determine the origin of the carbonyl oxygen at carbon 12 of 12-oxo-PDA using ^{18}O-labeled 13-L(*S*)-hydroperoxy-*cis*-9,*cis*-15,*trans*-11-octadecatrienoic acid (13-[^{18}O]-hydroperoxylinolenic acid) as a substrate for the hydroperoxide cyclase enzyme.

EXPERIMENTAL PROCEDURES

Materials

(9,12,15)-Linolenic acid and (6,9,12)-linolenic acid were obtained from Nu-Chek-Prep, Inc., (Elysian, MN), and ^{18}O$_2$ gas (> 99%) was purchased from Stohler Isotope Chemicals (Waltham, MA). Soybean lipoxygenase (21,600 units/mg) was obtained from Sigma Chemical Co. (St. Louis, MO), N,O-*bis*-(trimethylsilyl)-trifluoroacetamide (BSTFA) from Pierce Chemical Co. (Rockford, IL), DC LSX-3-0295 silicone phase for gas chromatography from Applied Science Division (State College, PA) and precoated Anasil HF silica gel thin layer chromatography (TLC) plates from Analabs, Inc. (North Haven, CT).

Mass Spectrometry

Mass spectra were recorded with a Varian/MAT 112S GC-MS system; the glass column was 2 m x 2 mm id containing 3% DC LSX-3-0295 on 100/120 mesh Gas-Chrom Q and was temperature programmed from 165 to 220 C at 2 C/min.

Preparation of 13-[^{18}O]-Hydroperoxylinolenic Acid

Soybean lipoxygenase, which catalyzes the oxygenation of linolenic acid predominantly at carbon 13 and a minor amount at carbon 9, was used to prepare a solution that contained 13-[^{18}O] hydroperoxylinolenic acid. Water and all buffer solutions were degassed under reduced pressure, then kept under a nitrogen atmosphere prior to initiating the reaction.
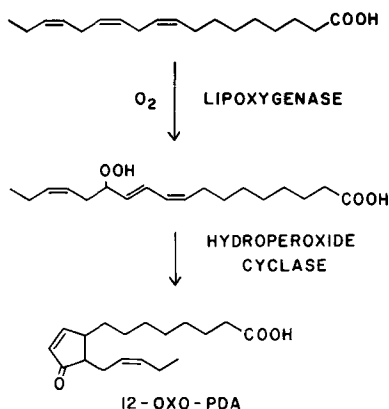

FIG. 1. Reactions catalyzed by lipoxygenase and hydroperoxide cyclase from flaxseed with (9,12,15)-linolenic acid as substrate.

(9,12,15)-Linolenic acid substrate solution (8 mM) was prepared according to the Surrey method (4) and the soybean lipoxygenase solution was prepared at a concentration of 1 mg/ml in 10 mM borate buffer (pH 9). A 16-ml test tube filled with water was placed in an inverted position in a chamber filled with water. Water was displaced from the tube with 8 ml of $^{18}O_2$; the tube was sealed with a teflon-coated septum and removed from the chamber. The buffered soybean lipoxygenase solution (1.6 ml) was added with a syringe to the remaining 8 ml of water in the tube. The oxygenation reaction was then initiated by the introduction of 0.8 ml of (9,12,15)-linolenic acid substrate solution. After 20 min, the septum was removed and the solution was adjusted from pH 9 to pH 7 with 0.2 M K-phosphate buffer (pH 6.5).

For determination of the percentage of $^{18}O_2$ incorporated into 13-hydroperoxylinolenic acid, a portion of this solution was adjusted to pH 4 and extracted with chloroform/methanol (2:1, v/v); the chloroform phase was removed, the solvent evaporated and the sample esterified with diazomethane. Hydroperoxide groups were reduced to hydroxyl groups concurrently with the saturation of double bonds by passing hydrogen through a solution of the sample dissolved in methanol with platinum oxide catalyst. The trimethylsilyloxy (OTMS) derivative of the hydroxyl group was prepared with BSTFA. Selected ion monitoring by GC-MS of the mass fragments m/e 175 vs m/e 173 $[CH_3(CH_2)_4CH(OTMS)]^+$ indicated that 94% of the hydroperoxide formed contained ^{18}O. Summation of the gas chromatographic peak areas generated by monitoring for mass fragments m/e 175 and 317 (13 isomer) and comparison with the sum of the areas generated by fragments m/e 231 and 261 (9 isomer) showed that 96% of the product was 13-hydroperoxylinolenic acid.

Formation of 12-oxo-cis-10, cis-15-Phytodienoic Acid

An enzyme solution containing hydroperoxide cyclase activity was prepared by extracting a flaxseed acetone powder (1 g) with 10 ml of 50 mM K-phosphate buffer (pH 7.0) for 30 min, then centrifuging the extract at 12,000 x g for 10 min. The enzyme solution (0.3 ml) was added to 13-[^{18}O]hydroperoxy-linolenic acid solution (8.3 ml) prepared as already described. After 90 min, the pH was adjusted to 4 and the products were extracted into 10 ml of chloroform/methanol solvent (2:1, v/v). Separation of the products by TLC was accomplished with a chloroform/acetic acid

solvent system (100:1, v/v) with 4 developments. The 12-oxo-PDA, which migrated just ahead of the 12,13-ketol (formed from hydroperoxide isomerase) and just behind unreacted (9,12,15)-linolenic acid, was eluted from the gel with ethyl ether, esterified with diazomethane and analyzed by gas chromatography mass spectrometry (GC-MS).

Reaction of 9-Hydroperoxy-cis-6, cis-12,trans-10-Octadecatrienoic Acid with Hydroperoxide Cyclase

Tomato lipoxygenase was used to prepare the 9-hydroperoxide of (6,9,12)-linolenic acid by the Matthew et al. method (5). The hydroperoxide product was purified by TLC (hexane/ethyl ether/acetic acid, 65:35:1, v/v), then eluted from the gel with ethyl ether. The solvent was evaporated and the sample was redissolved in 95% ethanol (0.2 ml). A small portion of this preparation (ca. 0.2 μmol) was esterified and analyzed as the reduced, saturated, trimethylsilyloxy derivative by summing ions m/e 173 plus 315 (13 isomer) and m/e 229 plus 259 (9 isomer) by GC-MS as already described. The results indicated that 81% of the product was the 9-hydroperoxy isomer, thus assuring that the desired product, 9-hydroperoxy-cis-6,cis-12,trans-10-octadecatrienoic acid [9-hydroperoxy-(6,10,12)-linolenic acid], had been obtained.

The 9-hydroperoxy-(6,10,12)-linolenic acid was reacted with an extract of flaxseed acetone powder containing hydroperoxide cyclase activity. The ethanolic solution of the compound (ca. 4 μmol) was added to 20 ml of 50 mM K-phosphate buffer (pH 7.0) and 1 ml of flaxseed acetone powder extract (1 g in 10 ml of 50 mM K-phosphate buffer, pH 7) was added. After 1 hr, the reaction mixture was adjusted to pH 4 and the products were extracted with 35 ml of chloroform/methanol (2:1, v/v). Separation of the products was done using TLC with chloroform/acetic acid solvent (100:1, v/v) with 3 developments. Products were visualized by exposing a portion of the plate to iodine vapor, then eluted from the gel, esterified with diazomethane and analyzed by GC-MS.

RESULTS AND DISCUSSION

Figure 2 shows the mass spectrum of 12-[^{18}O]oxo-PDA formed enzymically from 13-[^{18}O]hydroperoxylinolenic acid. The molecular ion at m/e 308 and the mass fragments at m/e 277 [M-OCH$_3$]$^+$, m/e 240 [M-(C$_5$H$_9$) + H]$^+$, m/e 179 [M-(CH$_2$)$_5$COOCH$_3$]$^+$ and m/e 165 [M-(CH$_2$)$_6$COOCH$_3$]$^+$ were 2 daltons

higher than the corresponding masses obtained when 13-[^{16}O] hydroperoxylinolenic acid was the substrate (m/e 306, 275, 238, 177 and 163). Comparison of these ^{18}O fragments in Figure 2 with the intensities of the corresponding ^{16}O fragments indicated that the compound contained 90% ^{18}O in the oxo group compared to 94% in the hydroperoxide. This small apparent decrease in ^{18}O enrichment was not regarded as experimentally significant. Thus, the mass spectra indicated that 13-hydroperoxy-linolenic acid was converted to 12-oxo-PDA with nearly complete retention of ^{18}O in the carbonyl oxygen at carbon 12. This result is similar to that reported for the synthesis of 12-oxo-13-hydroxy-cis-9-octadecenoic acid (α-ketol) from 13-hydroperoxylinoleic acid, catalyzed by hydroperoxide isomerase. This enzyme from flaxseed (6) and corn germ (7) has been shown to catalyze the formation of the α-ketol with retention of ^{18}O in the 12-oxo group, but with ^{16}O in the 13-hydroxy group, presumably from water.

Gardner has recently suggested a mechanism for hydroperoxide isomerase action based on the incorporation of ^{18}O into the 12-oxo group and on work in his own laboratory, which showed that substitution by nucleophiles other than water could occur at the hydroperoxide carbon atom with inversion of stereoconfiguration from S to R (8). He proposed the formation of an epoxy-cation intermediate by loss of OH$^-$ from the hydroperoxide group. The intermediate could react with a nucleophile (OH$^-$) in a bimolecular nucleophilic substitution (S_N2) reaction at the carbon originally bearing the hydroperoxide group. This mechanism accounted for the transfer of a hydroperoxide oxygen to a vicinal carbon and the inversion of configuration at the hydroperoxide carbon.

A similar mechanism involving an epoxy-cation intermediate appears likely for the hydroperoxide cyclase reaction (Fig. 3). Abstraction of a proton from carbon 12 by the enzyme could lead to an enolate anion at carbons 12 and 13; rearrangement of this intermediate would give cyclization between carbons 9 and 13. 13-Hydroperoxylinoleic acid, which differs from 13-hydroperoxy-linolenic acid only by the absence of unsaturation at carbon 15, is unreactive with hydroperoxide cyclase (3) (Fig. 4A). However, when 9-hydroperoxy-(6,10,12)-linolenic acid was allowed to react with a flaxseed extract contain-
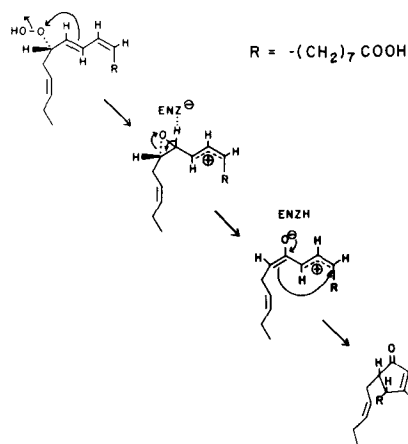
FIG. 3. Proposed mechanism for the cyclization of 13-hydroperoxylinolenic acid by the hydroperoxide cyclase enzyme.
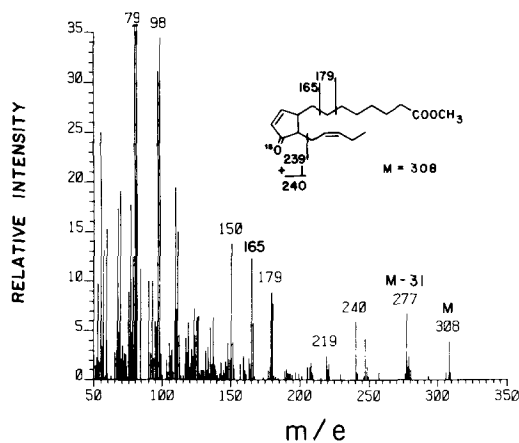
FIG. 2. Mass spectrum of 12-[^{18}O] oxo-cis-10,cis-15-phytodienoic acid (12-oxo-PDA) resulting from the reaction of 13-[^{18}O] hydroperoxylinolenic acid with an extract of flaxseed acetone powder containing hydroperoxide cyclase activity.
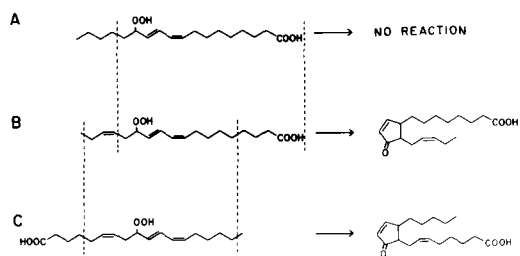
FIG. 4. Reactions showing the products of hydroperoxide cyclase activity from (A) 13-hydroperoxy-linoleic acid, (B) 13-hydroperoxylinolenic acid, and (C) 9-hydroperoxy-(6,10,12)-linolenic acid. Dashed lines indicate portions of molecules with identical structure. Experiments showed that only 13-hydroperoxylinolenic acid (B) and 9-hydroperoxy-(6,10,12)-linolenic acid (C) were reactive with hydroperoxide cyclase.

ing hydroperoxide cyclase activity, a cyclic compound was identified as a product. Over a range of 13 carbons, this substrate had the same chemical structure as 13-hydroperoxy-linolenic acid. The cyclic product of this hydroperoxide cyclase reaction was proposed to be 8-(2-oxo-5-pentyl-*cis*-3-cyclopentenyl)-*cis*-6-octanoic acid on the basis of its mass spectrum, which showed ions at m/e 306 [M]$^+$, m/e 275 [M-OCH$_3$]$^+$, and m/e 152 [C$_5$H$_4$O(CH$_2$)$_4$CH$_3$+H]$^+$. Figure 4C shows the structure of the hydroperoxide substrate and the proposed structure of the cyclic product.

In a previous paper, we reported that *n*-3 unsaturation in the fatty acid was necessary for recognition by the hydroperoxide cyclase enzyme (3). However, the results reported here demonstrated that a *cis* double bond β,γ to the conjugated hydroperoxide group was the essential feature. Apparently, the carboxyl group was not a factor in the attachment of the substrate hydroperoxide to the enzyme. It is likely that the substrate can attach to the active site with the carboxyl group in either direction. The important factor is that the substrate molecule for hydroperoxide cyclase must have a 4(S)-hydroperoperoxy-*cis*-1,*cis*-7,*trans*-5-octa-triene group.

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