Autoxidation of Methyl Linoleate: Identification of the Bis-allylic 11-Hydroperoxide

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ABSTRACT: Based on the understanding of lipid peroxidation as a free radical chain reaction, over 50 yr ago the three primary products of linoleic acid autoxidation were predicted to be the 9-, 11-, and 13-hydroperoxides. The 9- and 13-hydroperoxides were found at the time, but formation of 11-hydroperoxylinoleate or any other bis-allylic fatty acid hydroperoxide has not been reported heretofore as a product of lipid peroxidation reactions. In vitamin E-controlled autoxidation of methyl linoleate, the 11-hydroperoxy derivative was identified as the next most prominent primary peroxidation product after the 9- and 13-hydroperoxides. It was present in approximately 5–10% of the abundance of the 9- or 13-hydroperoxide. The structures of 11-hydroperoxylinoleate and its 11-hydroxy derivative were established by high-pressure liquid chromatography, ultraviolet spectroscopy, gas chromatography–mass spectroscopy, and ^{1}H nuclear magnetic resonance spectroscopy. The 11-hydroperoxide was not detectable in the absence of α-tocopherol, indicating that efficient trapping of the 11-peroxyl radical as the hydroperoxide is critical to permitting its accumulation.

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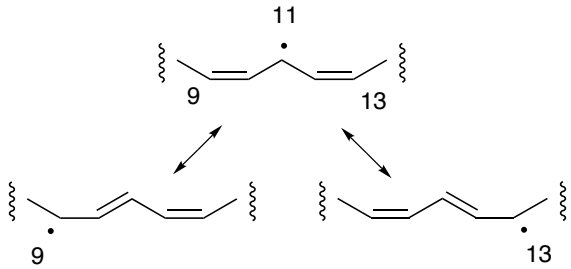
Following development of the concepts of lipid peroxidation as a free radical chain reaction in the 1940s (reviewed in Ref. 1), attempts were made to characterize the nature of the primary peroxidation products of the prototypical polyunsaturated lipid, linoleic acid. It follows from an understanding of tautomerism of the initial free radical that three positions are available for reaction with molecular oxygen, namely, C9, C11, and C13 (Scheme 1).

In studying the products from linoleic acid, Bergstrom and colleagues found the expected 9- and 13-hydroperoxides, whereas the 11-hydroperoxide was not detected (2,3). It was uncertain at the time whether the 11-hydroperoxide was formed in very low yield and/or was highly unstable and thus could not be isolated. In further studies over the years, the allylic nonconjugated 8- and 14-hydroperoxylinoleates were isolated as autoxidation products (4), but detection of the 11-hydroperoxide remained elusive (e.g., Refs. 4–6). The present report describes conditions for the isolation of the bis-allylic hydroperoxide from autoxidation of methyl linoleate.



SCHEME 1

EXPERIMENTAL PROCEDURES

Materials. Methyl linoleate was purchased from Nu-Chek-Prep Inc. (Elysian, MN). Autoxidation products with conjugated diene chromophores were quantified by ultraviolet (UV) spectroscopy (ε = 23,000 M^{-1} cm^{-1} at 235 nm). Vitamin E (α-tocopherol; Sigma Chemical Co., St. Louis, MO) was quantified by using a value of $E_{1\%}$ = 76 at 292 nm in ethanol, (i.e., 100 μg/mL gives an absorbance of 0.76 at 292 nm).

Autoxidation conditions. Reactions were run essentially as described by Peers and Coxon (7) using mixtures of methyl linoleate and 5% by weight of α-tocopherol. The mixture (0.25–0.6 g) was taken to dryness in a 1-L flask, flushed with oxygen, and kept for several days in an oven at 35–37°C. The sample was flushed again with oxygen, usually every day. After 3 d, the sample was examined daily by UV spectroscopy: the whole sample was dissolved in 10 mL dichloromethane, and the UV spectrum of a 5-μL aliquot was recorded in 2 mL of methanol using a Beckman DU-7 (Fullerton, CA) scanning spectrophotometer. Reaction was continued as a dry film under oxygen until ≈20% conversion to conjugated diene was evident from UV spectroscopy. After several days, the distinct 292 nm absorbance of α-tocopherol became less evident and the sample was replenished with an additional 5% α-tocopherol by weight.

High-pressure liquid chromatography (HPLC) analyses. Autoxidized methyl linoleate was analyzed by straight-phase (SP)-HPLC using a Beckman 5 μm silica column and a sol-

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Abbreviations: GC–MS, gas chromatography–mass spectrometry; HETE, hydroxyeicosatetraenoic acid; NMR, nuclear magnetic resonance; RP-HPLC, reversed-phase high-pressure liquid chromatography; SP-HPLC, straight-phase HPLC; TMS, trimethylsilyl; UV, ultraviolet.

vent of hexane/isopropanol 100:1 or 100:0.5 (vol/vol). For analytical work, the products were monitored by on-line detection using a Hewlett-Packard 1040A diode array UV detector (Palo Alto, CA). For collection of products, samples of up to 4 mg total of hydroperoxide products (and including in the sample larger amounts of unchanged methyl linoleate) were injected on an Alltech (Deerfield, IL) 10 µm semipreparative silica column (25 × 1 cm) eluted with hexane/isopropanol (100:0.5, vol/vol) at a flow rate of 5 mL/min. For collection of products, the column effluent was monitored using a Spectrophysics (San Jose, CA) variable wavelength UV detector set at 210 nm. Reversed-phase (RP)-HPLC analysis and purification of 0.5-mg aliquots of the novel product were carried out using a Beckman Ultrasphere 5 µ ODS column (25 × 0.46 cm) with an Upchurch guard column and a solvent system of methanol/water, 85:15 (vol/vol). Final purification by SP-HPLC was carried out on aliquots of up to 0.5 mg per injection using the Beckman analytical silica column and the hexane/isopropanol 100:1 (vol/vol) solvent.

Derivatization. Hydrogenation of 5–10 µg of hydroxy fatty acid methyl ester was carried out by bubbling hydrogen through a suspension of platinum oxide (*ca.* 1 mg) in 100 µL ethanol for 2 min followed by addition of water and extraction with ethyl acetate. Trimethylsilyl (TMS) ether derivatives were prepared by treatment with bis(trimethylsilyl)trifluoracetamide (10 µL) and pyridine (5 µL) for 15 min at room temperature. Subsequently, the reagents were evaporated under a stream of nitrogen and the samples dissolved in hexane for gas chromatography–mass spectrometry (GC–MS) analysis.

GC–MS. Mass spectra were recorded in the electron impact mode using a Hewlett-Packard HP5980 engine mass spectrometer operated at 70 eV and coupled to a Hewlett-Packard 5890 gas chromatograph equipped with an SPB-1 fused-silica capillary column of 5 or 15 m × 0.25 mm inner diameter. Samples were injected on column at an oven temperature of 150°C, and after 1 min the temperature was programmed to 300°C at 10 or 20°C/min.

Nuclear magnetic resonance (NMR) analyses. Spectra were recorded in deuterated benzene using a Bruker 400 MHz instrument (Billerica, MA). Chemical shifts are reported in relation to tetramethylsilane (δ 0.0).

RESULTS

Isolation of a novel product. SP-HPLC analysis of vitamin E-controlled autoxidation reactions of methyl linoleate showed the formation of 13- and 9-hydroperoxy products and one distinct minor product that chromatographed between the two main peaks (Fig. 1). In contrast to the major 13- and 9-hydroperoxides, the minor product did not contain a conjugated diene chromophore and was detectable only at lower wavelengths in the UV. The unreduced product was collected from injections of the autoxidation mixture on a semipreparative SP-HPLC column. Following reduction of the hydroperoxides, the retention times of all the products on SP-HPLC increased, and the minor product shifted its chromatographic mobility and now eluted with the 9-hydroxylinoleate (Fig. 2A), or, on a silica column from a different manufacturer (Alltech), as a peak in the tail of the 9-hydroxylinoleate (not shown).

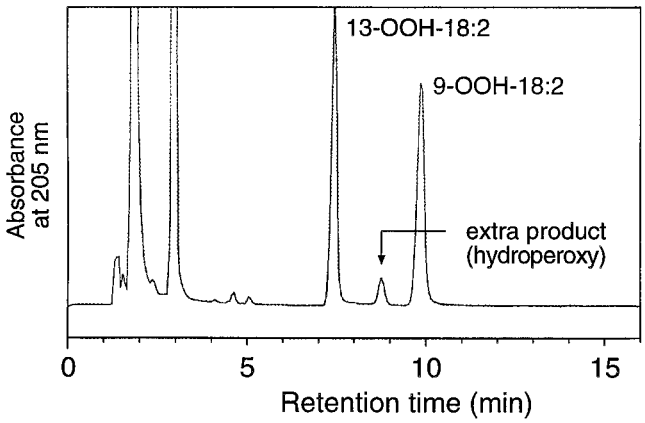

FIG. 1. Straight-phase high-pressure liquid chromatographic (SP-HPLC) analysis of autoxidized methyl linoleate. Vitamin E-controlled autoxidation of methyl linoleate was analyzed on an Alltech (Deerfield, IL) silica column (25 × 0.46 cm) using a solvent of hexane/isopropanol (100:0.5, vol/vol) eluted at 2 mL/min with ultraviolet detection at 205 nm. The large peak near the solvent front is methyl linoleate and the second large peak at 3 min is α-tocopherol. The novel product is marked as "extra product, (hydroperoxy)."

On the RP-HPLC system used for further purification (Fig. 2B), the novel product (as the hydroperoxide or the hydroxy derivative) eluted at ≈10 mL, substantially earlier than the combined peak of 9- and 13-hydro(per)oxides (retention volume ≈13 mL), allowing complete removal of these contaminants from the semipreparative SP-HPLC. The hydroperoxy and hydroxy derivatives of the new product were not resolved from each other on RP-HPLC; they formed shoulders of the same chromatographic peak. Prior to GC–MS and NMR analyses, the new product was finally re-purified as the hydroperoxide or hydroxy derivative by SP-HPLC. It was recovered in a yield of approximately 5–10% relative to one of two main products, or up to a 5% yield of the combined products. About 0.5–0.75 mg of 11-hydroperoxide was recovered pure from autoxidations that gave 7.5 mg each of purified 9- and 13-hydroperoxides.

Spectral analyses. When analyzed as the methyl ester TMS ether of the triphenylphosphine-reduced (hydroxy) derivative, the novel product had a similar mass spectrum to 9- and 13-hydroxylinoleates. The most prominent ions were present at *m/z* 382 (M^+, 35% relative abundance), *m/z* 311 ($M - C_5H_{11}$, 48%), *m/z* 225 (C9–C18, base peak), and *m/z* 130 (55% relative abundance). The differences from the spectra of the 9- and 13-hydroxylinoleates were in ion abundances, making it difficult to distinguish the different compounds with the double bonds still present. The position of the hydroxyl group in the new product was established unambiguously from the mass spectrum of the methyl ester TMS ether derivative of the hydrogenated product (Fig. 3). The

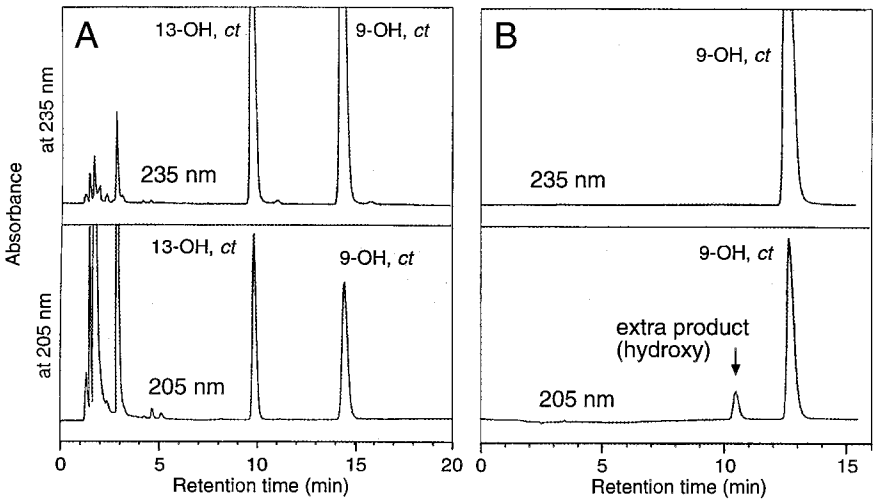

FIG. 2. SP-HPLC and reversed-phase (RP)-HPLC analyses of hydroxylinoleate methyl esters. (A) Following reduction with triphenylphosphine to the corresponding hydroxy products, the sample shown in Figure 1 was analyzed by SP-HPLC using the identical chromatographic conditions. The absorbance scales at 205 and 235 nm are set at identical values [0.35 absorbance units (AU) full scale]. (B) The peak of 9-hydroxylinoleate methyl ester collected from SP-HPLC was chromatographed on RP-HPLC using a Beckman 5 μm ODS Ultrasphere column (25 × 0.46 cm; Fullerton, CA) and a solvent of methanol/water (85:15, vol/vol) with a flow rate of 1 mL/min. The absorbance scales at 205 and 235 nm are set at identical values (0.5 AU full scale). For abbreviation see Figure 1.

base peak was recorded at *m/z* 73. The two most prominent ions above *m/z* 100 were the two α-cleavage ions at *m/z* 287 and 201 (26 and 62% relative abundance, respectively). This establishes the position of the hydroxyl group at C-11. Other structurally significant ions were recorded at *m/z* 371 (M – 15, <0.5% relative abundance), *m/z* 355 (M – 31, <0.5%), and *m/z* 339 (M – 47, 1%). Additional prominent ions above *m/z* 100 were *m/z* 258 [M – 128, 7%, an ion that

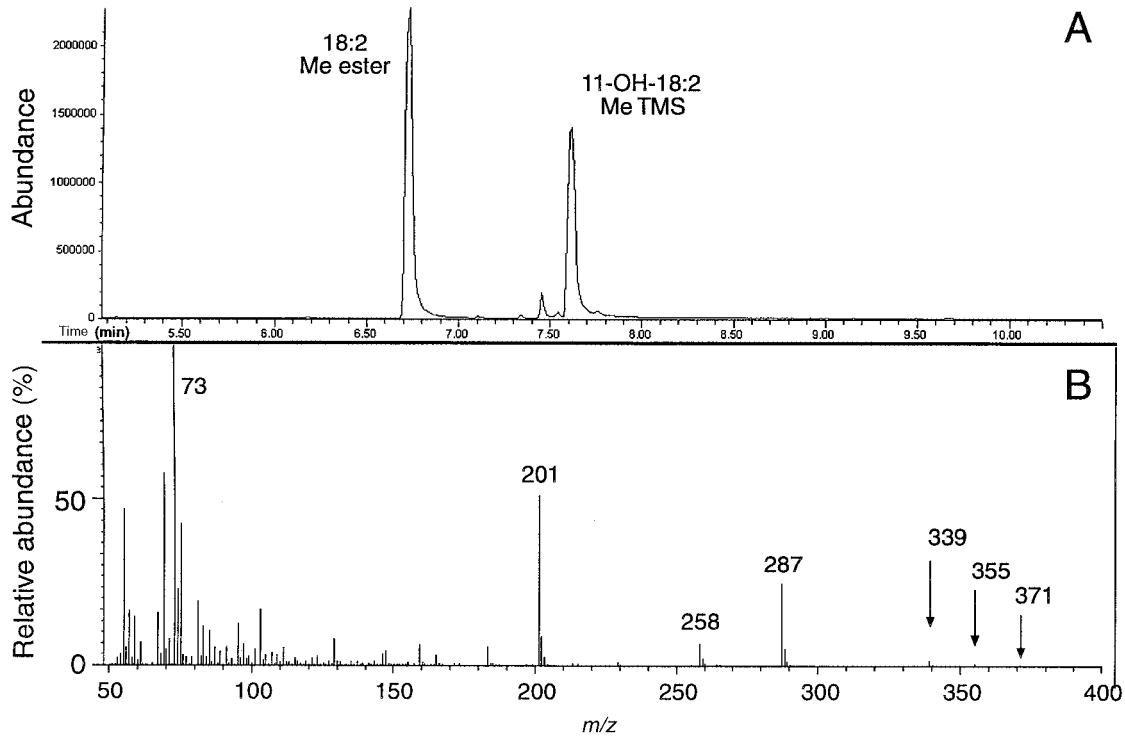

FIG. 3. Gas chromatography–mass spectrometry analysis of hydrogenated 11-hydroxylinoleate, methyl ester trimethylsilyl (TMS) ether derivative. Panel A shows the total ion chromatogram. The largest peak is methyl stearate (18:2 Me ester) formed by loss of the C-11 hydroxyl during hydrogenation. (B) The mass spectrum of the methyl ester TMS ether derivative of 11-hydroxy-stearate (11-OH-18:2 MeTMS) is shown.

had the same chromatographic profile as the major diagnostic ions in the GC peak; it may represent migration of the TMS group to the ester carbonyl followed by cleavage at C-10/C-11 and elimination of the fragment OHC–$(CH_2)_6$–CH_3], *m/z* 183 (6%), *m/z* 159 (7%), *m/z* 129 (8%), and *m/z* 103 (16%).

The ^{1}H NMR spectra of the 11-hydroperoxide and the 11-hydroxylinoleate are in accord with the spectral characteristics reported for the bis-allylic arachidonate products, 13-hydroxyeicosatetraenoic acid (13-HETE) (8) and 10-HETE (9). Most notably, the geminal hydro(pero)xy proton at C-11 has a chemical shift unusually far downfield and it appears among the olefinic protons (Fig. 4). In the hydroxy derivative (Fig. 4B), H11 occurs at 5.37 ppm as a double triplet. The triplet is accounted for by the equivalent coupling to H10 and H12 (J = 8.5 Hz), while a 3.5 Hz coupling that splits the triplet occurs between the geminal H11 proton and the proton on the hydroxyl group itself; the latter is a doublet (J = 3.5 Hz) at 1.00 ppm. The C-11 geminal proton reverted to a simple triplet upon exchange of the hydroxyl proton with D_2O, with concomitant elimination of the hydroxyl signal at 1.00 ppm. In the hydroperoxide, H11 appears as a clean triplet at 5.75 ppm (Fig. 4A) accounted for by the equal couplings to H10 and H12. An additional 3.5 Hz coupling can occur through the oxygen atom in the hydroxy derivative, but no significant long-range coupling is possible through both oxygens of the hydroperoxide.

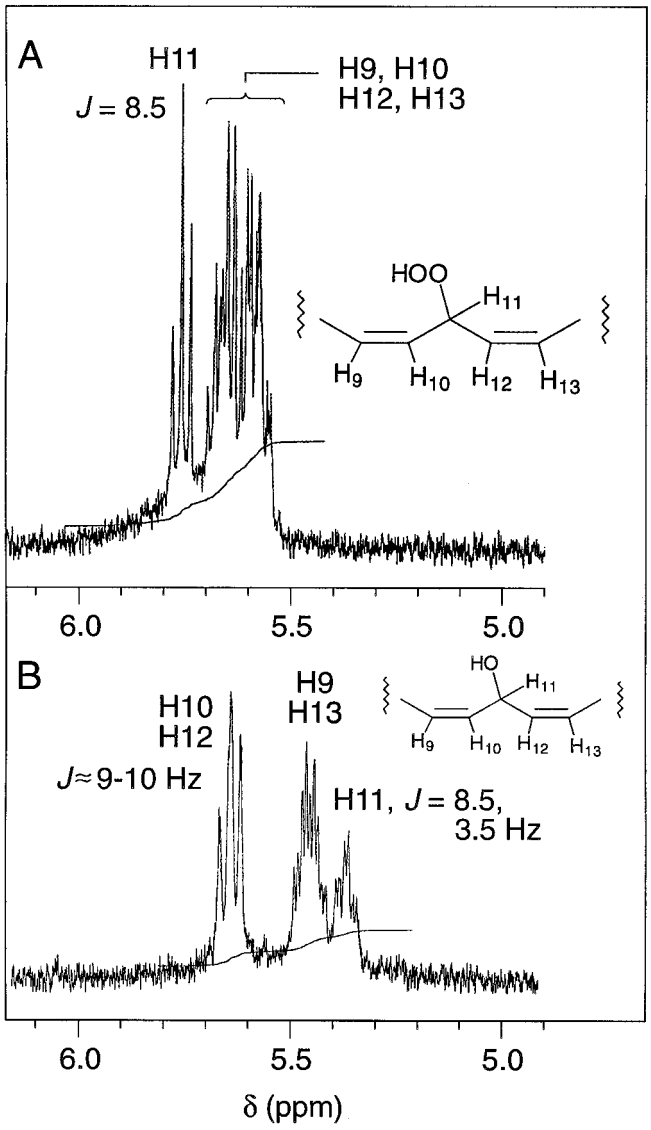

FIG. 4. Olefinic and H11 protons in the ^{1}H nuclear magnetic resonance spectra (400 MHz) of 11-hydroperoxy- and 11-hydroxylinoleate methyl esters in d_6-benzene. The two panels show the signals from H9, H10, H11, H12, and H13 of (A) 11-hydroperoxylinoleate, and (B) 11-hydroxylinoleate.

The spectrum of 11-hydroxylinoleate has the four olefinic protons resolved into two pairs, permitting assessment of the double-bond configurations. As the signals from H10 and H12 at 5.65 ppm are superimposed, the olefinic region centered on C11 must be symmetrical. The slightly broad triplet of H10/H12 (almost a doublet of doublets) is caused by the partially superimposed couplings of 8.5 Hz to the geminal proton H11 ($J_{10,11} = J_{11,12}$) and the ≈10 Hz coupling across the double bonds ($J_{9,10} = J_{12,13}$). The latter defines the two double bonds as *cis*.

^{1}H NMR (400 MHz, in deuterated benzene, using 7.24 ppm for the residual protons in the solvent) gave for 11-hydroperoxylinoleate methyl ester (ppm): δ 0.95 (*t*, 3 protons, H18), 1.2–1.5 (*m*, 14 protons, H4, H5, H6, H7, H15, H16, H17), 1.65 (*p*, 2 protons, H3), 2.2 (*m*, 6 protons, H2, H8, H14), 3.45 (*s*, 3 protons, C$\underline{H}_3$O), 5.55–5.7 (*m*, 4 protons, H9, H10, H12, H13), 5.75 (*t*, 1 proton, H11, $J_{9,10} = J_{11,12}$ = 8.5 Hz), 7.55 (*s*, 1 proton, –OO$\underline{H}$). For 11-hydroxylinoleate methyl ester (ppm): δ 0.95 (*t*, 3 protons, H18), 1.0 (*d*, 1 proton, <1 in area, –OH, $J_{11,-OH}$ = 3.5 Hz), 1.2–1.4 (*m*, 14 protons, H4, H5, H6, H7, H15, H16, H17), 1.6 (*p*, 2 protons, H3), 2.2 (*m*, 6 protons, H2, H8, H14), 3.45 (*s*, 3 protons, C$\underline{H}_3$O), 5.37 (*dt*, 1 proton, H11, $J_{10,11} = J_{11,12}$ = 8.5 Hz, $J_{11,-OH}$ = 3.5 Hz), 5.45 (*m*, 2 protons, H9, H13), 5.65 (*t/dd*, 2 protons, H10, H12, J ≈ 8.5–10 Hz).

The 11-hydroperoxy and 11-hydroxy products do not contain a conjugate diene chromophore yet their UV spectra can be distinguished from the spectrum of linoleate and from each other (Fig. 5). A similar extension to the end absorbance of the nonconjugated chromophore was reported for bis-allylic 7-, 10-, and 13-HETE (10).

Autoxidation in the absence of vitamin E. In autoxidation of a dry film of methyl linoleate in the absence of α-tocopherol (analyzed after ≈15% conversion to conjugated diene), the SP-HPLC profile showed prominent peaks for the 9- and 13-hydroperoxides with both *cis-trans* and *trans-trans* conjugated dienes (Fig. 6). The 11-hydroperoxide was not visible, even though, based on retention time, it should have appeared as a peak in the tail of the 13-hydroperoxy-9-*trans*,11-*trans*-linoleate. When the peak and tail of the *trans-trans* 13-hydroperoxide were collected from SP-HPLC and examined on RP-HPLC at 205 and 235 nm, there was no trace of a product with the chromatographic and spectral characteristics of 11-hydroperoxylinoleate methyl ester.

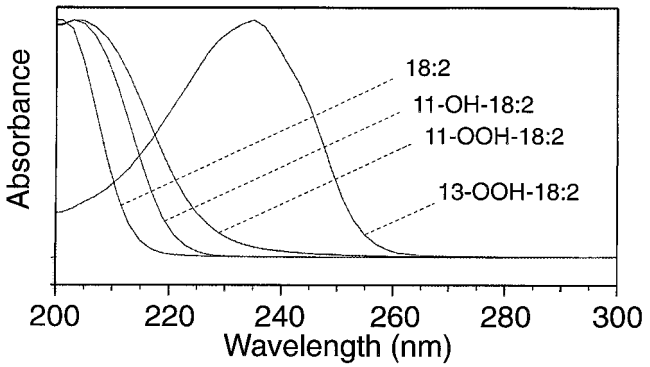

FIG. 5. Ultraviolet spectra of methyl linoleate and its 11-hydroperoxy and 11-hydroxy derivatives. Spectra were recorded using a Hewlett-Packard 1040A diode array detector (Palo Alto, CA) during an RP-HPLC run in methanol/water (85:15 vol/vol) solvent (or 100% methanol for methyl linoleate, 18:2). To avoid saturation of the signal at the low wavelengths, the maximal absorbance at 205 nm was kept below 0.5 AU; spectra at the apex and on the upslope of chromatographic peaks were indistinguishable, confirming that there was no significant saturation of the signal in these spectra. The spectrum of 13-hydroperoxylinoleate is included for comparison. The spectra are normalized to λ_{max} and do not convey relative molar extinction values. Previous studies suggest that at 205 nm the bis-allylic products and the nonconjugated fatty acid derivatives have similar molar absorptions (Ref. 10), estimated here as around 10,000 for the linoleic acid derivatives. For abbreviations see Figure 2.

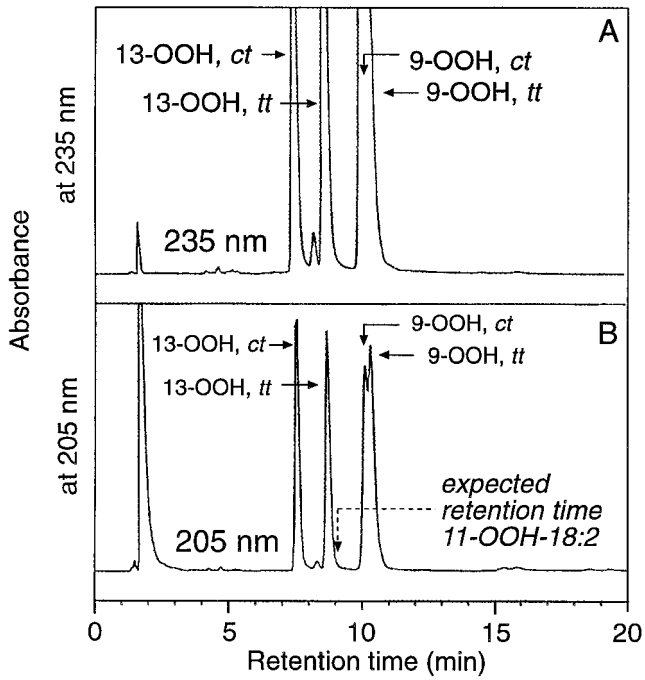

FIG. 6. SP-HPLC analysis of methyl linoleate autoxidized in the absence of vitamin E. Chromatographic conditions were identical to those given in the legend of Figure 1. The absorbance scales at 205 and 235 nm are set at identical values (0.35 AU full scale). See Figures 1 and 2 for abbreviations.

DISCUSSION

The presence of α-tocopherol in the autoxidation of methyl linoleate allowed detection of a previously unrecognized primary product of lipid peroxidation, the bis-allylic 11-hydroperoxide. Efficient trapping of peroxyl radicals by α-tocopherol and other hydrogen atom donors is known to suppress the isomerization of the *cis-trans* conjugated 9- and 13-hydroperoxyl radicals and thus reduce the formation of *trans-trans* isomers and other secondary products (cf. Figs. 1 and 6) (7,11). This activity also appears to be the basis of the trapping and preservation of the bis-allylic product. The critical competition is at the peroxyl radical stage. The relatively unstable bis-allylic peroxyl radical is formed as originally predicted (1,2,12). If vitamin E is present, the peroxyl radical is trapped; the bis-allylic hydroperoxide product is stable under the conditions of its formation, and it does not convert to the 9- and 13-hydroperoxides. If vitamin E is not present, the 11-peroxyl radical fragments and re-adds molecular oxygen to give the thermodynamically more stable conjugated diene peroxyls, which then go on to the 9- and 13-hydroperoxide products.

In the course of relatively recent experiments on the oxygenation of polyunsaturated fatty acids, bis-allylic hydroxy or hydroperoxy products have been found as enzymatic products. The first report was of the occurrence of the 13-hydroxyeicosapentaenoic acid in the marine red algae *Lithothamnion corallioides* and *L. calcareum* (8). As a product of linoleic acid metabolism in *L. corallioides* (13), 11*R*-hydroxylinoleate was subsequently found. Later in the 1990s, bis-allylic product(s) were detected as hydroxy derivatives in cytochrome P450 reactions (10,14,15) and in the myoglobin-catalyzed monooxygenation of linoleic acid (16), and as hydroperoxide products of the manganese-containing lipoxygenase of the fungus *Gäumannomyces graminis* (17) and of the 8*R*-lipoxygenase domain of the peroxidase-lipoxygenase fusion protein from the coral *Plexaura homomalla* (18).

The bis-allylic 11-hydroperoxylinoleate derivative is fairly stable to chromatographic and other analytical manipulations, provided the sample is not subjected to low pH (10,14). For methyl esters, as in the present study, there is no need to acidify during chromatography or HPLC. For long-chain free fatty acids the pK_a values are approximately pH 7–8 for the parent fatty acids (19,20) and probably in the region of pH 6–7 for the monohydro(pero)xy products, so it is not necessary to acidify to pH 3–4 to achieve an efficient extraction. The long-term stability of these products is similar to other fatty acid hydroperoxides. A sample of oxidized linoleate methyl ester kept in the −20°C freezer for 4 yr (in ethanol in the presence of α-tocopherol from the original autoxidation) contained a similar proportion of the 11-hydroperoxide as freshly autoxidized methyl linoleate.

Under the appropriate conditions, formation and accumulation of bis-allylic products can be expected for other polyunsaturated lipids. As evidence of this, several years ago we detected the 10,15-dihydro(pero)xyeicosatrienaoate among the primary oxygenation products in the vitamin E-controlled autoxidation of 15-hydroxyeicosa-8,11,13-trienoate methyl ester (Brash, A.R., and Boeglin, W.E., unpub-

lished observations). At the time there was some doubt as to the origin of this bis-allylic hydroperoxy derivative, but it is now evident that its formation as a primary oxygenation product is no longer in question.

ACKNOWLEDGMENTS

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