Homolytic Decomposition of Linoleic Acid Hydroperoxide: Identification of Fatty Acid Products[1]

H.W. GARDNER, R. KLEIMAN, and **D. WEISLEDER,** Northern Regional Research Laboratory[2], Peoria, Illinois 61604

ABSTRACT

An isomeric mixture of linoleic acid hydroperoxides, 13-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid (79%) and 9-hydroperoxy-*cis*-12,*trans*-10-octadecadienoic acid (21%), was decomposed homolytically by Fe(II) in an ethanol-water solution. In one series of experiments, the hydroperoxides were decomposed by catalytic concentrations of Fe(II). The 10^{-5} M Fe(III) used to initiate the decomposition was kept reduced as Fe(II) by a high concentration of cysteine added to the reaction in molar excess of the hydroperoxides. Nine different monomeric (no detectable dimeric) fatty acids were identified from the reaction. Analyses of these fatty acids revealed that they were mixtures of positional isomers identified as follows: (I) 13-oxo-*trans,trans*- (and *cis,trans*-) 9,11-octadecadienoic and 9-oxo-*trans,trans*- (and *cis,trans*-) 10,12-octadecadienoic acids; (II) 13-oxo-*trans*-9,10-epoxy-*trans*-11-octadecenoic and 9-oxo-*trans*-12,13-epoxy-*trans*-10-octadecenoic acids; (III) 13-oxo-*cis*-9,10-epoxy-*trans*-11-octadecenoic and 9-oxo-*cis*-12,13-epoxy-*trans*-10-octadecenoic acids; (IV) 13-hydroxy-9,11-octadecadienoic and 9-hydroxy-10,12-octadecadienoic acids; (V) 11-hydroxy-*trans*-12,13-epoxy-*cis*-9-octadecenoic and 11-hydroxy-*trans*-9,10-epoxy-*cis*-12-octadecenoic acids; (VI) 11-hydroxy-*trans*-12,13-epoxy-*trans*-9-octadecenoic and 11-hydroxy-*trans*-9,10-epoxy-*trans*-12-octadecenoic acids; (VII) 13-oxo-9-hydroxy-*trans*-10-octadecenoic and 9-oxo-13-hydroxy-*trans*-11-octadecenoic acids; (VIII) isomeric mixtures of 9,12,13-dihydroxyethoxy-*trans*-10-octadecenoic and 9,10,13-dihydroxyethoxy-*trans*-11-octadecenoic acids; and (IX) 9,12,13-trihydroxy-*trans*-10-octadecenoic and 9,10,13-trihydroxy-*trans*-11-octadecenoic acids. In another experiment, equimolar amounts of Fe(II) and hydroperoxide were reacted in the absence of cysteine. A large proportion of dimeric fatty acids and a smaller amount of monomeric fatty acids resulted. The monomeric fatty acids were examined by gas liquid chromatography-mass spectroscopy. Spectra indicated that the monomers were largely similar to those produced by the Fe(III)-cysteine reaction.

INTRODUCTION

The decomposition of linoleic acid hydroperoxide (LOOH) by enzymes or systems postulated to be enzymes has been studied extensively. Among the products identified were oxooctadecadienoic acid (1); 13-oxotridecadienoic acid, n-pentane (2); dimers (3); isomeric oxohydroxyoctadecenoic acids (4,5); trihydroxyoctadecenoic acid, hydroxyepoxyoctadecenoic acid (6); hydroxyoctadecadienoic acid; (6,7) and an unsaturated ether, 9- (nona-1′,3′-dienoxy)-non-8-enoic acid (8).

In contrast to all this enzymatic research, complete structural determination of products from the decomposition of LOOH by non-enzymatic reactions has been less thoroughly investigated, except for studies concerning the identification of pentane (9), addition products with α-tocopherol (10), and 11-hydroxy-12,13-epoxy-9-octadecenoic acid (11). Most investigators indicate that the overall mechanism of decomposition is probably homolytic. In some studies, fatty ester hydroperoxides were decomposed, neat, both thermally (12) and at low temperatures (13). Products in both instances were primarily dimers, whose structural details were not determined completely.

Transition metal ions are especially effective in catalyzing free radical reactions in the presence of peroxides or hydroperoxides. In their lower valency state, metal ions readily produce alkoxy radicals (RO·) from hydroperoxides (ROOH), whereas higher oxidation states promote peroxy radical (ROO·) formation (14). O'Brien (15) observed that LOOH degraded with Fe(II) yielded numerous products, but he did not identify them. O'Brien also discovered that Fe(III) was much less effective in decomposing LOOH, but, when electron donors like cysteine were present, the reaction resembled the decomposition caused by Fe(II)

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[2]ARS, USDA.

according to his parameters. This similarity can be attributed to the reduction of Fe(III) to Fe(II) by cysteine.

We examined nonvolatile products after homolytic degradation of LOOH in dilute solution. When equimolar Fe(II) was added to the LOOH solutions, we observed facile formation of dimers, which complicated the isolation of the monomeric fatty acids also formed. Catalytic amounts of Fe(III) (10^{-5} M) in the presence of cysteine in molar excess of LOOH moderate decomposition enough that only monomeric fatty acids can be detected. We identified the major fatty acid products of this reaction and compared them with equimolar-Fe(II) products.

METHODS

Hydroperoxides

LOOH was prepared by soybean lipoxygenase ([EC 1.13.1.13] lipoxygenase type 1, Sigma Chemical Co., St. Louis, Mo., 134,000 units/mg) oxidation of linoleic acid. The method of oxidation, including the concentration of enzyme (units/ml), and the isolation of LOOH were the same as used previously (10). By thin layer chromatography (TLC) densitometry (16), LOOH was determined to be an isomeric mixture of 13-hydroperoxy-*trans*-11,*cis*-9-octadecadienoic (79%) and 9-hydroperoxy-*trans*-10,*cis*-12-octadecadienoic (21%) acids.

Reaction Conditions

Fe(III)-cysteine: The final reaction solution was 3.2 mM LOOH, 12.8 mM cysteine (free base, Nutritional Biochemicals Corp., Cleveland, Ohio) and 10^{-5} M $FeCl_3$ in 80% ethanol. The reaction was initiated by the addition of a small volume of $FeCl_3$ solution (10^{-3} M), while stirring vigorously for 1 hr at room temperature in the presence of air. In certain experiments, the reaction proceeded under either pure O_2 or N_2. The reaction mixture was extracted once with a 1.6 times larger volume of chloroform.

Equimolar Fe(II): The reaction solution was 3.2 mM LOOH and 3.2 mM ferrous ammonium sulfate in 80% ethanol. The other conditions were the same as those used with Fe(III)-cysteine, except that the reaction time was 10 min.

Chromatography

Fatty acids initially were isolated with two different chromatographic columns. The first column, serving as a preparative procedure, was packed with silicic acid, as described previously (5). The column was equilibrated with chloroform before the crude product (ca. 0.7 g) was applied. Elution was stepwise with 100 ml chloroform, 150 ml 2% methanol, and 250 ml 10% methanol in chloroform. The free fatty acids were eluted in the first 320 ml, except for trihydroxyoctadecenoic acid which eluted mixed with other minor components between 320-380 ml. The fatty acids (less than 260 mg) collected in the first 320 ml were applied to a second column (inside diameter 2.5 cm) packed with 50 g Mallinckrodt SilicAR CC-4 in isooctane. Stepwise elution was with 40 ml 20% ether, 260 ml 24% ether, 250 ml 30% ether, 250 ml 40% ether, 300 ml 50% ether, 250 ml 70% ether in hexane, 200 ml ether, and 200 ml methanol.

Column fractions found to be mixtures were purified further by TLC (10). Solvent systems were: hexane-ether-acetic acid 50:50:1 and 30:70:1 for most fatty acids and dihydroxyethoxyoctadecenoic acid, respectively; chloroform-methanol-acetic acid 65:10:1 for trihydroxyoctadecenoic acid; hexane-ether 80:20 for methyl oxostearate; hexane-ether 60:40 and 50:50 for most methyl esters; hexane-ether 30:70 for methyl dihydroxyethoxyoctadecenoate; and chloroform:methanol 95:5 for methyl trihydroxyoctadecenoate.

Silyl derivatives of fatty esters were separated by gas liquid chromatography (GLC), as described by Kleiman and Spencer (17). Column temperature was programed from 175-375 C at 4 C/min. Products from oxidations with periodic acid were separated by GLC with a 4 ft x 1/4 in. glass column packed with Silar 5 CP on Gas Chrom Q (Applied Science Laboratories, State College, Pa.). The column temperature was programed from 50-230 C at 4 C/min. Calculated carbon number of the dimer was determined by GLC using a standard wax ester (jojoba oil). The column for this determination was 3 ft x 1/8 in. and packed with 3% OV-1 on Gas Chrom Q. The column was temperature programed from 200-400 C at 2 C/min.

Spectroscopy

Mass spectrometry (MS) was employed in tandem with GLC, as described by Kleiman and Spencer (17). Multiple spectra were recorded throughout the GLC elution of each fatty ester to detect positional isomers. NMR spectra were determined as before (5). IR spectra were recorded by a Perkin-Elmer model 621 with 0.1 mm thick NaCl cells containing 10% solutions in CCl_4 and CS_2.

Derivatives

Diazomethane was used to esterify and H_2-Pd or $NaBH_4$ to reduce (10). Hexamethyldisilazane-trimethylchlorosilane-pyridine 2:1:1

TABLE I

Yield of Fatty Acids Produced by Decomposition of LOOH[a] by Fe(III)-Cysteine as Separated by Column Chromatography[b]

Compound[c]	Wt, mg[d]	Eluant volume, ml
Unknown	4.1	0-210
I	29.4	210-590
II	40.1	590-750
III, IV	12.9	710-810
V	9.4	810-910
VI	16.4	870-1010
Unknown	8.0	980-1080
VII	12.4	1080-1210
VIII[e]	51.5	1150-1260
IX[f]	116	320-380
Other	40.2	Minor components and polar material

[a]LOOH = linoleic acid hydroperoxide.

[b]Crude product (720 mg) was applied to a silicic acid column and eluted with chloroform-methanol. VIII was collected as tabulated. The other components studied (253 mg) were collected as a mixture in the first 320 ml eluted from the column. This mixture was applied to a second silicic acid column and eluted with hexane-ether as tabulated above.

[c]Key to the Roman numerals is given in Figure 1.

[d]Wt determined from actual wt of fractions. Those fractions composed of a mixture of two components were analyzed by thin-layer chromatography densitometry so that individual wt could be calculated.

[e]Two partially separated isomeric forms.

[f]IX plus other minor components as detected by thin layer chromatography.

was selected to silylate fatty esters with more than one hydroxyl group and bis(trimethylsilyl)-trifluoroacetamide (Regis Chemical Co., Chicago, Ill.) for monohydroxylated esters.

Diols and epoxides were oxidized with periodic acid (18), except that epoxides required 1/2 hr at 60 C instead of 15 min at room temperature used for diol oxidation. Quantitation of the molar ratio of the oxidation fragments, hexanal and methyl 9-oxo-nonanoate, was made possible by comparison with fragments from a standard, methyl 9,10,12,13-tetrahydroxystearate, oxidized under the same conditions. Methyl tetrahydroxystearate was prepared by alkaline $KMnO_4$ oxidation of linoleic acid (19) at 0 C reaction temperature followed by esterification and isolation by TLC.

RESULTS

Fe(III)-Cysteine Reaction

Catalytic quantities of Fe(III) ions in the presence of excess cysteine rapidly decompose LOOH to a number of chloroform-ethanol-soluble products. The reaction is complete within 20 min. Fatty acids are 52% of the mixture of lipid products. In addition to fatty acids, a number of ninhydrin-positive lipids are produced and account for the remainder of the total lipid. Other highly polar material was extracted into the chloroform layer, which was not recovered from the silicic acid column, and was subsequently assumed to be nonlipid. Preliminary data indicated that the ninhydrin-positive lipids arose from an additon reaction between cysteine and LOOH. Isolation and characterization of these adducts will be detailed in a subsequent publication. First, we concentrated on identifying the fatty acids. We did not examine the volatiles produced, but surmise their presence from the unusual almost breadlike aroma emanating from the reaction mixture. When Fe(III) was not added to the reaction mixture, LOOH failed to decompose substantially after 1 hr.

Fatty acids were isolated by column chromatography in quantities sufficient for structural work (Table I). Yields of individual fatty acids fluctuated somewhat from one experiment to another, presumably because the free radical reactions taking place were so complex. Although yields shifted for each product in replicate experiments, the identity of fatty acids predominating remained nearly the same, as far as we could ascertain.

Structures of the fatty acids identified and labeled by Roman numerals are summarized in Figure 1.

In certain experiments, the reactions were made to proceed either under a N_2 atmosphere or under pure O_2, instead of under air. Comparison of products from reactions under the three conditions were strikingly different. Compared to the products from reaction in air, the products from the pure O_2 reaction were observed to have the following changes in product distribution: (A) production of II and III was enhanced and (B) formation of the cysteine-fatty acid addition compounds was inhibited. On the other hand, compared to the air reaction, the N_2 experiment resulted in: (A) no detectable formation of II and III and (B) an enhanced production of cysteine-fatty acid addition compounds.

Structure Determinations

Product I: Its UV spectrum compared favorably with that reported by Binder, et al., (20); λ_{max} (methanol) = 277 nm, ϵ_{max} = 20,300.

An IR spectrum (I methyl ester) also compared with that reported by Binder, et al., (20) for methyl 9-oxo-*trans,trans*-10,12-octadecadienoate, except there was a small absorption at 955 cm^{-1} due to the presence of some *cis, trans*-oxodiene.

TABLE II

Mole Percent of Isomers as Determined by Thin Layer Chromatography or Periodic Acid Oxidation[a]

Compound[b]	Carbons of hydroperoxy-, or oxo-group		Carbons of epoxide- or diol-group	
	9	13	9,10	12,13
LOOH	21	79	—	—
I	26	74	—	—
II	—	—	22	78
III	—	—	51	49
V	—	—	12	88
VI	—	—	29	71
			23	77
IX	—	—	40	60

[a]The appropriate derivatives of linoleic acid hydroperoxide (LOOH) and I were determined by TLC separation of isomers followed by char densitometry. The other compounds were oxidized by periodic acid and their cleavage products quantitated by GLC.

[b]Key to the Roman numerals is given in Figure 1.

Some features in the NMR spectrum were: methylene protons α to the conjugated carbonyl at δ 2.54 (t, 2 H); olefinic proton β to carbonyl centered at δ 7.51 (dd J = 15 Hz, J = 11 Hz, 1 H); olefinic protons centered at δ 6.10 (m, 3 H); methylene protons α to the carboxylic acid at δ 2.34 (t, 2 H); and methylene protons α to the conjugated olefin at δ 2.20 (m, 2 H).

MS confirmed the proposed structure on the basis of a trimethylsilyl oxooctadecadienoate derivative. Ions with normalized intensities greater than 35% were: 67; 73, $(CH_3)_3$ Si+; 75, $(CH_3)_2$SiOH+; 81; 95; 151, cleavage of C-8 and C-9; 166, cleavage of C-7 and C-8 with rearrangement (18); 276, M - 90; 295, fragmentation between C-13 and C-14; 341, M - 15; and 366, M.

Percentages of 13- and 9-oxo isomers, as determined by TLC char-densitometry (21) after hydrogenation and esterification, are reported in Table II. Identities of the corresponding spots scraped from TLC plates were ascertained by MS compared with MS of authentic methyl 13- and 9-oxostearates described by Ryhage and Stenhagen (22).

Product II: The two isomers of II were inseparable by TLC or column chromatography in all solvents tested. On TLC, II reacted readily with a 2,4-dinitrophenylhydrazine spray (1) yielding a yellow-orange spot.

The UV spectrum of II was characteristic of spectra of α,β-unsaturated carbonyls; λ_{max} (ether) = 229 nm, ϵ_{max} = 16,500.

Characteristic IR absorptions (II methyl ester) were 1635 cm^{-1}, olefin α,β to a carbonyl; 1680 and 1700 cm^{-1}, conjugated carbonyl; 1740 cm^{-1}, ester carbonyl; 973 cm^{-1}, *trans*-

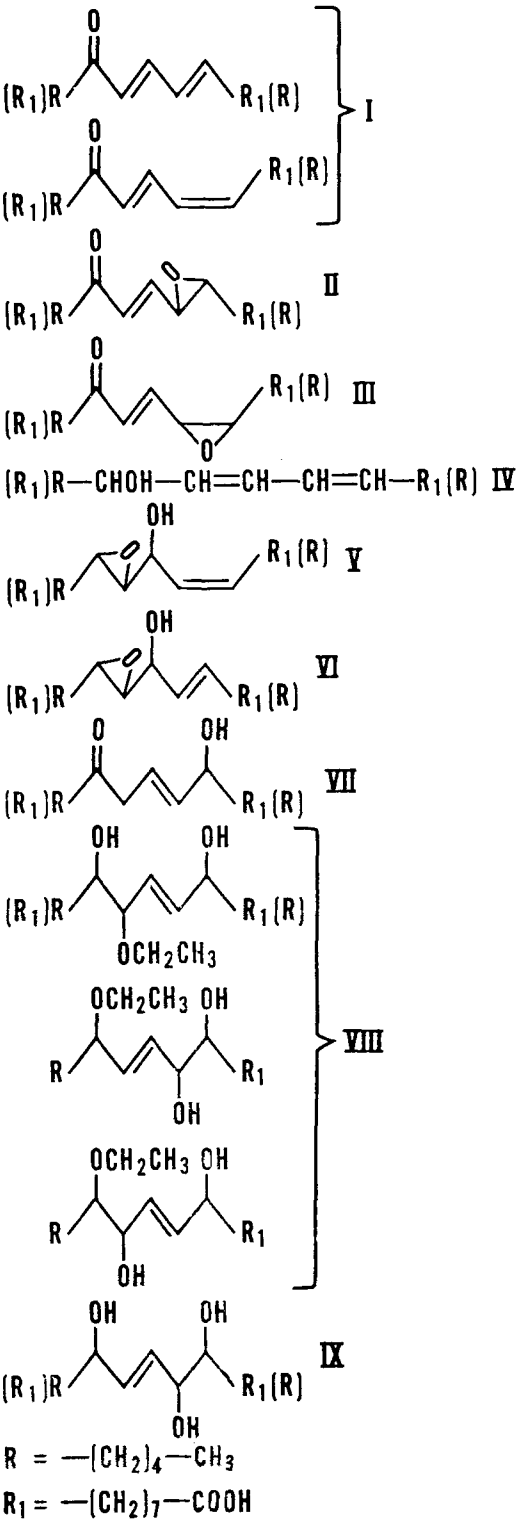

FIG. 1. Numerical key and structures of fatty acids isolated as products of the Fe(III)-cysteine reaction.

monoene; and 885 cm^{-1}, *trans*-epoxide (23).

NMR delineated the sequence of the substituents as follows: epoxide proton α to methylene at δ 2.90 (m, 1 H); epoxide proton α to unsaturation at δ 3.20 (dd, J = 6 Hz, J = 2 Hz, 1 H); olefinic proton α to the epoxide centered at δ 6.57 (dd, J = 16 Hz, J = 6 Hz, 1 H); olefinic proton α to carbonyl centered at δ 6.36 (d, J = 16 Hz, 1 H); methylene α to conjugated carbonyl at δ 2.52 (t, 2 H); and methylene α to carboxylic acid carbonyl at δ 2.34 (t, 2 H). Double irradiation of the epoxide proton at 3.20 δ confirmed its position α to the double bond. The epoxide protons were assigned a *trans*-configuration because of their upfield absorption compared to the corresponding *cis*-epoxide, product III. The difference in chemical shift between the *cis*- and *trans*-epoxide protons α to methylene and α to olefin were 0.24 and 0.27 δ, respectively, which correspond closely to 0.22 δ difference reported for *cis*- and *trans*-epoxides of saturated fatty esters (24). Reduction of II with $NaBH_4$ yielded the corresponding hydroxyepoxyoctadecenoic acid, which was examined by NMR. The epoxide protons α to methylene and α to olefin absorbed at 2.78 and 3.05 δ, respectively, upfield from the corresponding *cis*-epoxide reported by Graveland (6).

MS of the trimethylsilyl ester of II resulted in poor spectra which could not be interpreted readily. However, $NaBH_4$ reduction of II, esterification and preparation of the trimethylsiloxy derivative yielded a compound that fragmented into intense ions. MS compared favorably with the reported by Graveland (6) who also examined a mixture of methyl 13-trimethylsiloxy-9,10-epoxy-11-octadecenoate and methyl 9-trimethylsiloxy-12,13-epoxy-10-octadecenoate. Our spectra differed from Graveland's in that the cleavage between the epoxide and the double bond was reduced greatly, which resulted in small intensitites of m/e 199 and 285.

Periodic acid oxidation showed that the epoxide was mostly at C-12,13 (Table II).

Product III: III migrated in TLC with a slightly lower R_f compared with II, as expected for *cis*- vs *trans*-epoxides (25). III eluted from column chromatography mixed with IV but was isolated almost completely from IV by esterification followed by TLC.

The UV spectrum of III was similar to the one reported for II, λ_{max} (ether) = 229 nm.

An IR spectrum (III methyl ester) had features similar to the spectrum of II. The notable difference was a shift in epoxide absorption from 885 cm^{-1} in II to 825 cm^{-1} in III, indicating that III was a *cis*-epoxide (23).

Assignments of an NMR spectrum of III (methyl ester) are as follows: epoxide proton α to methylene at δ 3.14 (m, 1 H); epoxide proton α to unsaturation at δ 3.47 (dd, J = 6 Hz, J = 4 Hz, 1 H); olefinic proton α to epoxide at δ 6.63 (dd, J = 16 Hz, J = 6 Hz, 1 H); the other olefinic proton at δ 6.34 (d, J = 16 Hz, 1 H); carbomethoxy methyl at δ 3.62 (S, 3 H); methylene α to ester carbonyl at δ 2.27 (t, 2 H); and other assignments identical to those observed for II. Double irradiation experiments confirmed the structure.

MS (III methyl ester) was less than satisfactory; however, a small molecular ion (m/e 324), M - 18, and M - 31 were observed. After $NaBH_4$ reduction and trimethylsilylation, the MS was similar to the comparable derivative of II.

III was shown to be a nearly equal mixture of positional isomers by periodic acid oxidation (Table II).

Product IV: IV was present in trace quantities compared to the other constituents and thus was difficult to isolate. After preparative TLC of the column fraction containing a mixture of III and IV, a partially purified sample of IV was obtained. The TLC fraction was separated further by GLC after trimethylsiloxy derivatization. Replicate MS sampled over the appropriate GLC peak gave spectra comparable to trimethylsiloxy derivatives of methyl 9-hydroxy-*trans,trans*-10,12-octadecadienoate and others more comparable to methyl 13-hydroxy-*cis*-9,*trans*-11-octadecadienoate, as indicated by a shift in ratios of m/e 225 to 311 (17). The geometric configuration of the diene was not determined.

Product V: Product V separated from the analogous product VI both by column chromatography and TLC. Although the difference in structures reported here is proposed to be *cis*- vs *trans*-unsaturation, this difference would not be expected to result in chromatographic separation with the techniques used in this study. We did not examine these products for either erythro or threo isomerism. This type of isomerism may be the additional criterium that caused the separation of V from VI.

Characteristic IR absorptions (V methyl ester) were 890 cm^{-1}, *trans*-epoxide, and 3480 cm^{-1}, hydroxyl. Absorptions at 970 cm^{-1}, indicative of a *trans*-double bond, and at 1660 or 710 cm^{-1}, indicative of *cis*-unsaturation, were weak and not definitive enough to assign either geometry to the unsaturation.

NMR assignments are as follows: carbinol methine proton at C-11, δ 4.63 (dd, J = 4 [epoxide] and 8 [olefin] Hz, 1 H); epoxide proton α to carbinol methine, δ 2.77 (dd, J = 2 [epoxide] and 4 [carbinol methine] Hz, 1 H); epoxide proton α to methylene, δ 2.98 (m, 1

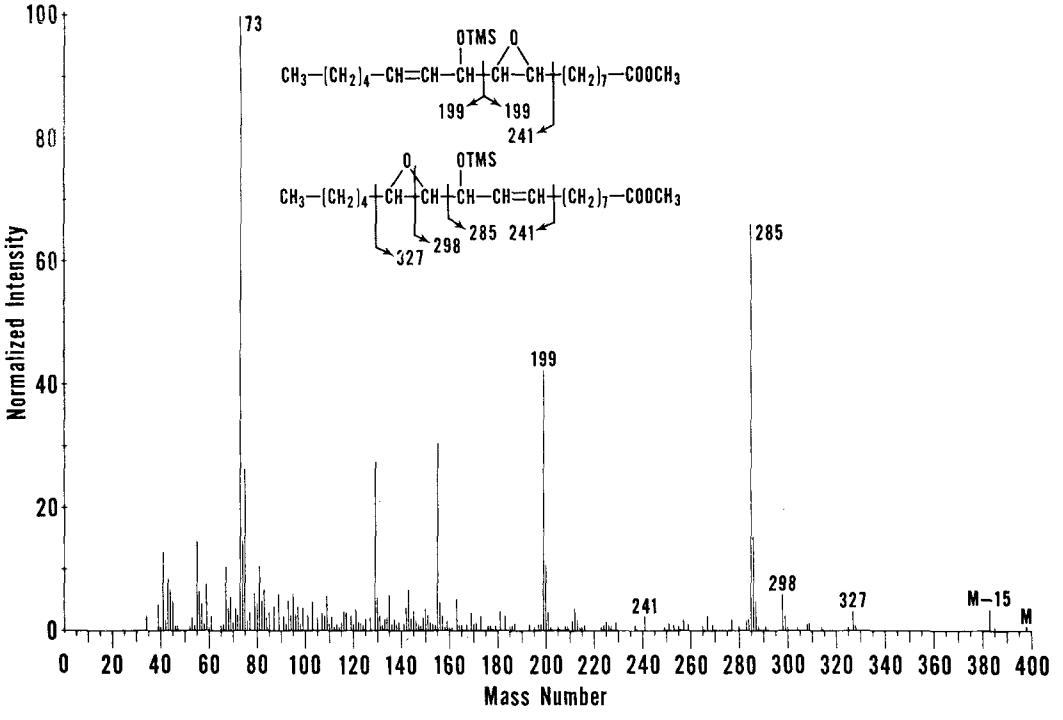

FIG. 2. Mass spectrum of VI; methyl ester-trimethylsiloxy derivative.

H); olefinic proton α to carbinol methine, δ 5.32 (dd, J = 11 [olefin] Hz, 1 H); olefinic proton α to methylene, centered at δ 5.60 (m, J = 11 [olefin], 1 H); methylene α to olefin, δ 2.06 (m, 2 H). Double resonance experiments determined the sequence of substituents, as well as the J values. The coupling between epoxide protons of 2 Hz indicates that the epoxide is *trans-* which agrees with the coupling constant of similar epoxides (26). The coupling observed between the olefinic protons of 11 Hz suggests that the double bond is probably *cis-*.

MS of V clearly supported the proposed structure and were identical to the MS obtained with VI (Fig. 2). Replicate MS taken over the GLC peak showed first a spectrum of the one isomer, the derivative of 11-hydroxy-12,13-epoxy-9-octadecenoic acid, characterized by m/e 285 ion. At later elution times, the former isomer mixed with the derivative of 11-hydroxy-9,10-epoxy-12-octadecenoic acid was observed as characterized by both m/e 285 and 199. The m/e 285 ion predominated in intensity relative to the 199 ion in all the spectra taken pointing to a preponderance of the one positional isomer. The observation on positional isomerism was confirmed by periodic acid oxidation (Table II), i.e. a preponderance of 12,13-epoxide.

Product VI: IR absorptions were largely similar to those observed with V. Relative to V, VI had a more intense absorption of a *trans-* epoxide at 890 cm^{-1}; however, a weak absorption at 840 cm^{-1} indicated the possibility of a minor amount of *cis-*epoxide. Like V, the absorptions characteristic of unsaturation were not definitive for VI.

NMR was most helpful in determining the difference between V and VI. The olefinic coupling constant could not be determined, but the narrow absorption at δ 5.54 (m, 2 H) indicates that the double bond is *trans-* rather than the *cis-* in V. Absorption of the C-11 carbinol methine proton was at δ 4.25 (dd, J = 6 [epoxide] and 8 [olefin] Hz, 1 H). The difference in chemical shifts of the C-11 methine in products V and VI is 0.38 ppm. This difference in shift is of the magnitude and direction expected for a difference in geometry (*cis-* vs *trans-*) of the α-unsaturation (27). Other absorptions are similar to those observed in the spectrum of V, except that the proton of the epoxide α to methylene was slightly upfield at δ 2.93 (m, 1 H). Coupling of the epoxide protons is the same as in V, i.e. J = 2 Hz, and, therefore, the epoxide is predominantly *trans-*.

MS of VI is shown in Figure 2. A shift in ion intensities during the GLC elution of VI suggested a separation of the two positional

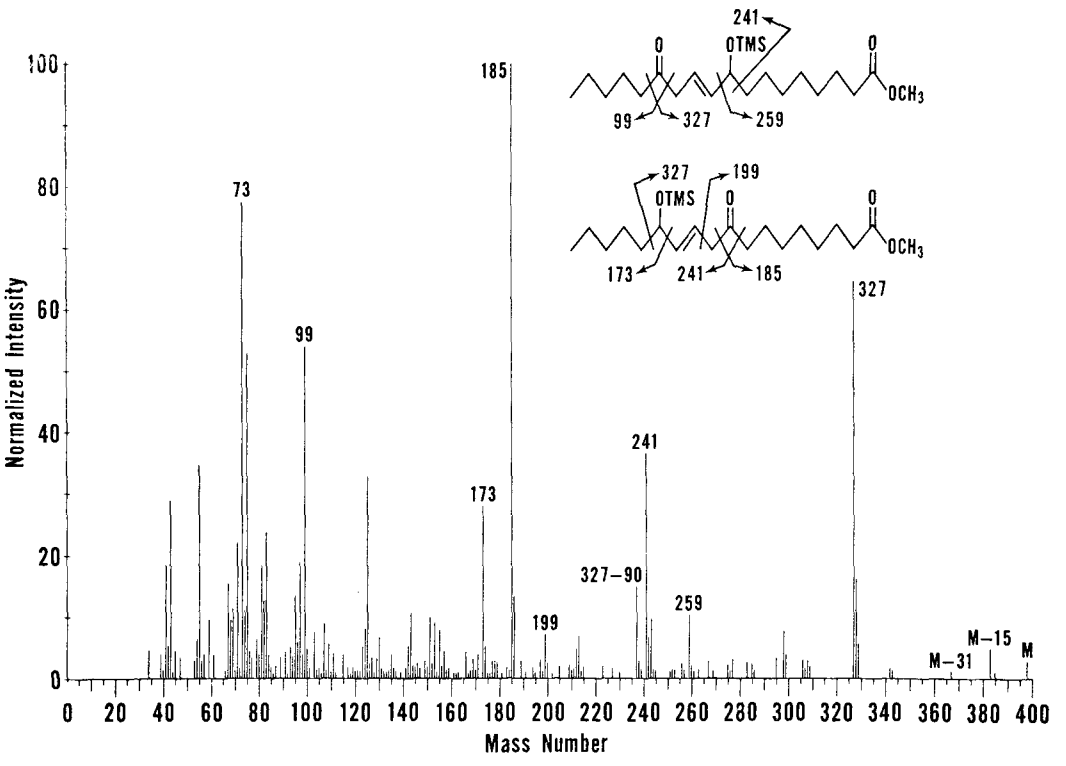

FIG. 3. Mass spectrum of VII; methyl ester-trimethylsiloxy derivative.

isomers as described for V. The 12,13-epoxide predominated over the 9,10-epoxide, Table II.

Product VII: The positional isomers of VII were not chromatographically separable by our methods. On TLC, VII reacted with 2,4-dinitrophenylhydrazine spray forming orange-yellow spots.

Features of the IR spectrum (methyl ester) are 970 cm^{-1}, isolated *trans*-olefin; 1717 cm^{-1}, oxo-carbonyl; 1740 cm^{-1}, ester carbonyl; and 3460 cm^{-1}, hydroxyl.

The NMR spectrum is interpreted as follows: olefinic proton α to carbinol C-H at δ 5.56 (m, J = 16 Hz, 1 H); olefinic proton α to methylene centered at δ 5.7 (m, 1 H); carbinol proton at δ 4.08 (m, 1 H); methylene groups α to ester and ketone carbonyl at δ 2.33 (t, 4 H); and methylene protons between the carbonyl and oiefin at δ 3.11 (d, J = 6 Hz). Integration of the absorption at δ 3.11 yielded 1.1 H, instead of the 2 expected. Another NMR study, (H.W. Gardner and D. Weisleder, unpublished results) of 12-oxo-13-hydroxy-*cis*-9-octadecenoic acid showed that similar protons at C-11 usually integrated considerably less than two. Double irradiation experiments completed the NMR studies and confirmed the positions of the functional groups.

MS (Fig. 3) demonstrated fragment ions characteristic of two positional isomers. MS scans of the GLC peak showed a shift in ion intensities indicating a partial separation of one isomer from the other.

Product VIII: There was a partial separation in two isomers of VIII that could be discerned both in column chromatography and TLC. We do not have sufficient information, especially of the minor component, to understand the reason for the separation. Identification of VIII was based upon the major component; however, spectral data for the minor component were similar to the major one. There is the possibility of geometric isomerism (erythro-threo), as well as three possible positional isomers each of 9,10,13-dihydroxyethoxy-*trans*-11-octadecenoic and 9,12,13-dihydroxy-ethoxy-*trans*-10-octadecenoic acids that depend upon the positions of the hydroxyls relative to the ethoxy. Certain isomers were considered to be less probable on the basis of some of the NMR and MS evidence.

Characteristic absorptions of the IR spectrum are: 978 cm^{-1}, isolated *trans*-olefin; 1090 cm^{-1}, ether-alcohol C-O stretch; and 3420 cm^{-1}, hydroxyl.

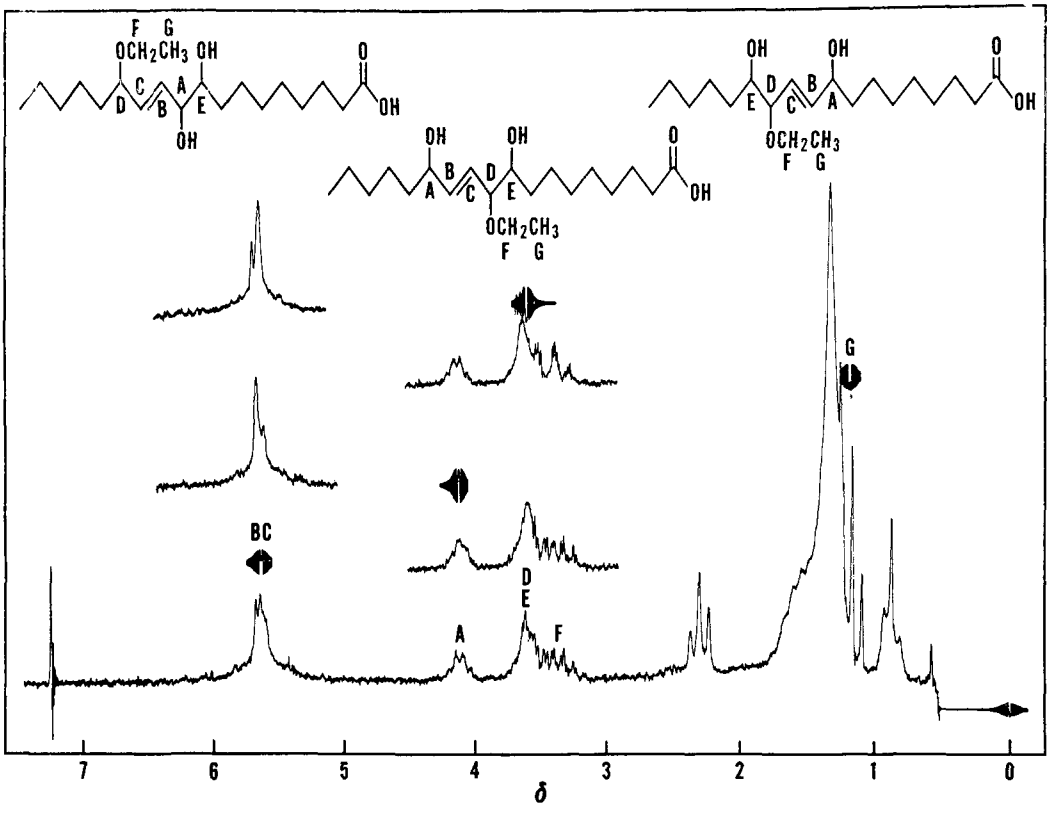

FIG. 4. NMR spectrum of VIII.

As shown in Figure 4, absorptions in the NMR are: methyl of ethoxy group at δ 1.16 (t, 3 H); methylene of ethoxy group centered at δ 3.44 (m, showing splitting due to two or more isomeric forms, 2 H); carbinol C-H proton α to olefin at δ 4.12 (m, 1 H); ether C-H α to olefin and carbinol C-H at δ 3.62; carbinol OH at δ 5.60 (2 H); and olefinic protons at δ 5.63 (m, 2 H). Double irradiation (Fig. 4) indicated that the double bond is situated between a secondary alcohol and an ether C-H, at least in a significant proportion of the total isomeric mixture. Thus, 9,12-dihydroxy-13-ethoxy-*trans*-10-octadecenoic and 10,13-dihydroxy-9-ethoxy-*trans*-11-octadecenoic acids were probably not the prevalent isomers.

Replicate GLC-MS were made on both chromatographically separable components of VIII. MS of both yielded similar fragment ions. One of these spectra is (Fig. 5) sampled from the center of the GLC peak. The fragment ions observed would be characteristic of four different isomers (Fig. 1), including 9,12-dihydroxy-13-ethoxy-*trans*-10-octadecenoic acid, although NMR data are not in accord with this structure. The two isomers with a 9-ethoxy group were eliminated from consideration because the characteristic expected ions, m/e 215, 215-46, or 215+73, were absent in all the MS. Because two pairs of the remaining four isomers have fragmentation ions that are identical, one cannot select which are prevalent. However, the varying intensities of m/e 343 or 416 vs 257 from one spectrum to another suggest that both 9,13-dihydroxy-12-ethoxy-*trans*-10-octadecenoic and 9,13-dihydroxy-10-ethoxy-*trans*-11-octadecenoic acids are present. The existence of 9,10-dihydroxy-13-ethoxy-*trans*-11-octadecenoic acid was considered to be unlikely, since the rearrangement ion, m/e 332 (TMSO-CH-$(CH_2)_7$-COTMS-OCH_3^+), was not present in the MS (17). On the basis of NMR, one might assume that 9,12-dihydroxy-13-ethoxy-*trans*-10-octadecenoic also was not present; however, decoupling experiments by NMR would probably not be sensitive to minor components. Even though the four isomers shown in Figure 1 must be considered as possible, we conclude that 9,13-dihydroxy-12-ethoxy-*trans*-10-octadecenoic and 9,13-dihydroxy-10-ethoxy-*trans*-11-octadecenoic acids are the major components of product VIII on the basis of all the

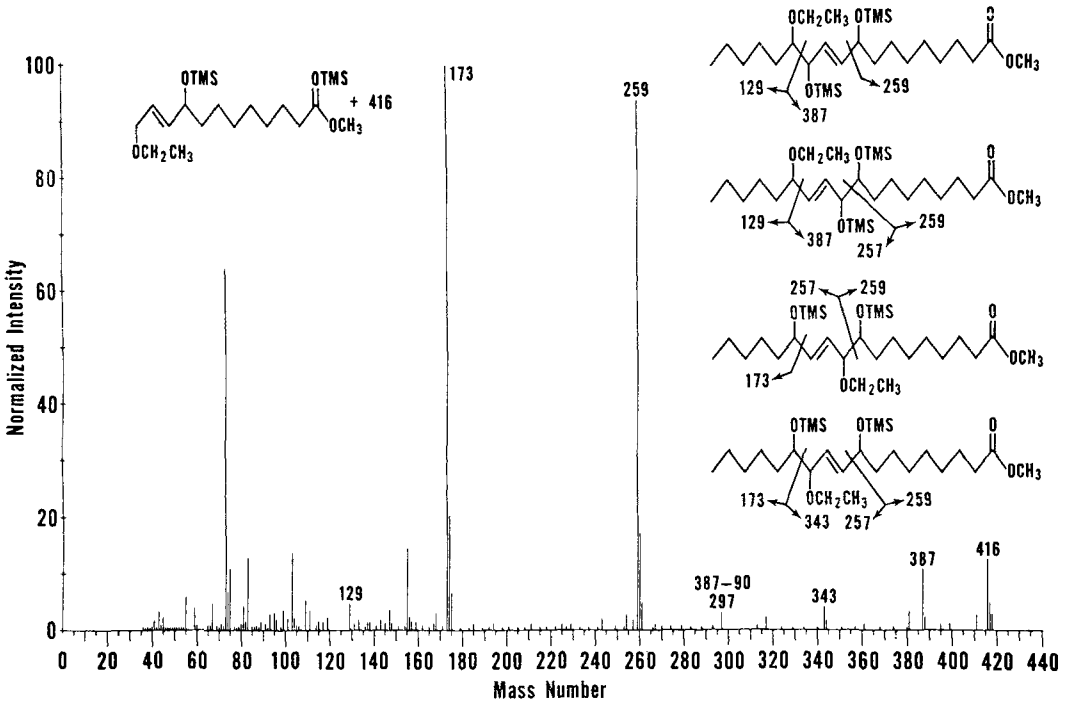

FIG. 5. Mass spectrum of VIII; methyl ester-trimethylsiloxy derivative.

available information.

Product IX: IR, NMR, and MS were essentially as described by Graveland (6), who also examined a mixture of 9,12,13-trihydroxy-10-octadecenoic and 9,10,13-trihydroxy-11-octadecenoic acids. We found a few minor differences in MS data; for example, the intensity of the rearrangement ion, m/e 460 (TMSO-CH-CH=CH-CHOTMS-$[CH_2]_7$-COTMS-OCH_3), was much reduced. Also, the MS we obtained of hydrogenated IX presistently had more fragment ions, especially at m/e less than 173 and a persistent ion of 213 (303-90) in all spectra taken. We found m/e 389 to be virtually absent, but m/e 389-90 was intense. Three oxidation products of IX (methyl ester) were identified by GLC-MS after periodic acid treatment: hexanal, methyl 9-oxononanoate, and 4-hydroxy-2-nonenal. The molar quantities of hexanal and methyl 9-oxononanoate are reported in Table II.

Equimolar Fe(II) Reaction

LOOH was decomposed with an equimolar quantity of ferrous ammonium sulfate. The reaction occurred immediately as expected and could be assessed visually by the formation of brown Fe(III). Products were separated by column chromatography, and fractions from it were separated further by GLC. Among the products, we detected substances giving MS and chromatographic properties identical to I, IV, V (and VI), VIII, and IX, as well as many compounds other than those resulting from the LOOH-cysteine-Fe(III) reaction. Because of the complexity of the Fe(II) system, the presence of trace quantities of II, III, and VII could easily have been missed. A determination of the geometry of the epoxides or unsaturation was not possible, except for oxooctadeca-(*trans, trans*)-dienoic acid which was isolated relatively pure in ca. 15% yield.

Other products detected were compounds yielding MS and chromatographic data that indicated they were ethoxyoctadecadienoic acid and diethoxyhydroxyoctadecenoic acid. Additional IR data confirmed the identity of ethoxyoctadecadienoic acid. Other components, including a large amount of compounds tentatively identified as dimer, were not researched in detail. Typically, the silylated methyl ester of the dimer eluted from GLC as one peak centered at calculated carbon number of 41.4 calculated from the standard wax esters. The 41.4 value is consistent with a structure of dimeric C-18 esters containing some oxygenated functional groups. Detailed structure determinations of the dimer were not made, except that a few MS recorded had fragments up to m/e 706. In the more polar

fractions eluting from a hexane-ether silicic acid column (over 800 ml), dimers accounted for 69-92% of the total as determined by GLC. The percentage dimer decreased with decreasing elution volumes.

DISCUSSION

The homolytic decomposition of LOOH by a catalytic concentration of Fe(III), 10^{-5} M, was made possible by keeping Fe(III) reduced as Fe(II) by cysteine in molar excess of LOOH. According to present theories, the homolytic breakdown of LOOH by Fe(II) proceeds through the alkoxydiene radical (14). Supposedly, the variety of products formed results from further reactions of the alkoxydiene free radical. More evidence is needed to determine whether the final products are formed entirely by a free radical mechanism or by participation in part or total by ionic mechanisms. Cysteine in the absence of 10^{-5} M Fe(III) was relatively ineffective in decomposing LOOH, an observation that indicates the reaction was at least initiated homolytically.

Many of the same products were produced with Fe(II) equimolar to LOOH without cysteine. The major difference between equimolar Fe(II) and the Fe(III)-cysteine system was formation of dimer by the former. Apparently, the rapid reaction with equimolar Fe(II) caused a high concentration of radicals that promoted termination reactions.

Information provided by quantitative analyses of positional isomers gave clues as to the origin of the products. When the 9- to 13-oxo ratio of I is compared to the 9- to 13-hydroperoxy ratio of LOOH (Table II), it can be concluded that I was derived from LOOH by a net loss of one molecule of water. The *cis, trans*-diene of LOOH was only partially retained in the formation of I as the *trans,trans*-diene was a significant portion of the mixture of isomers found. In a previous study (10) of the decomposition of LOOH in the presence of α-tocopherol, I was observed as one of the major products, except that only the *trans, trans*-diene was detected.

Because IV was only a trace product, the 9- to 13-hydroxy ratio of isomers could not be determined readily, but IV is assumed to be formed from the alkoxydiene radical, possibly through gain of a hydrogen radical from sulfhydryl. It was surprising that the yield of IV was so low, leading us to believe IV may have been utilized in secondary reactions.

Epoxides were located predominantly at carbons-12,13 in a molar percentage corresponding to the percentage of 13-hydroperoxide used as a reactant (Table II). The exception was III, which was a minor product compared to II, V, and VI. Apparently, the predominant reaction is cyclization of the hydroperoxy group to the α-unsaturation. These epoxides may have formed either through a free radical mechanism similar to that proposed by Gardner, et al., (10) or through an ionic mechanism as outlined by Hamberg and Gotthammar (11). Even though epoxide formation by the addition of peroxide oxygen across a double bond is a well documented reaction, the epoxides found by us do not appear to be produced in this manner, because the positional isomers largely do not correlate.

At this time little can be said of the formation of products VII, VIII, and IX, as well as the mechanistic details of the formation of the other compounds. We currently are attempting to sort out the various pathways and plan to report further on the details of these reactions in the future.

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