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High Pressure Liquid Chromatography of Autoxidized Lipids: II. Hydroperoxy-Cyclic Peroxides and Other Secondary Products from Methyl Linolenate

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ABSTRACT

A previous study of autoxidation products by high pressure liquid chromatography (HPLC) of methyl oleate and linoleate was extended to methyl linolenate. Autoxidized methyl linolenate was fractionated by HPLC either after reduction to allylic alcohols on a reverse phase system, or directly on a micro silica column. Isolated oxidation products were characterized by thin layer and gas liquid chromatography and by ultraviolet, infrared, nuclear magnetic resonance and mass spectrometry. Secondary products from the autoxidation mixtures (containing 3.5-8.5% monohydroperoxides) included epoxy unsaturated compounds (0.2-0.3%), hydroxy or hydroperoxy-cyclic peroxides (3.8-7.7%), epoxy-hydroxy dienes (<0.1%), dihydroxy or dihydroperoxides with conjugated diene-triene and conjugated triene systems (0.9-2.9%). Cyclization of the 12- and 13-hydroperoxides of linolenate would account for their lower relative concentration than the 9- and 16-hydroperoxides. Dihydroperoxides may be derived from the 9- and 16-linolenate hydroperoxides. Cyclic peroxides and dihydroperoxides are suggested as important flavor precursors in oxidized fats.

INTRODUCTION

Previous high pressure liquid chromatography (HPLC) studies of oxidized fatty esters were primarily concerned with the separation of hydroperoxide isomers. Chan and Levett (1) separated the eight geometric and positional isomers of autoxidized linolenate hydroperoxides. Funk et al. (2) also resolved by HPLC the hydroperoxides produced by soybean lipoxygenase with α-linolenic acid as substrate. Less attention has been given to HPLC of secondary oxidation products.

Previously, we have used gas chromatography-mass spectrometry (GC-MS) in studies of autoxidized methyl linolenate and obtained indirect evidence of hydroperoxy-cyclic peroxides (3). Haverkamp Begemann et al. (4) obtained linolenate hydroperoxy-cyclic peroxides by partitioning autoxidized linolenate between 80% ethanol and light petroleum ether, followed by liquid-liquid partitioning on Celite coated with carbowax using isooctane-ether as mobile phase. On the basis of hydrogenation experiments, they designated the hydroperoxy-cyclic peroxides as a pair of positional isomers with a six-membered peroxide group, although five-membered cyclic peroxide compounds were not ruled out. Roza and Francke (5) reported positional isomers of five-membered hydroperoxy-cyclic peroxides in enzymatically oxidized linolenate, which was fractionated on a low-pressure silica gel column with a linear solvent gradient of light petroleum and diethyl ether.

A reverse-phase semipreparative HPLC system was previously described which allowed the characterization of secondary autoxidation products from methyl oleate and linoleate (6). This paper reports the extension of these studies to reverse-phase and micro silica HPLC separation of autoxidized methyl linolenate and the identification of secondary oxidation products. Major products identified included several isomeric hydroperoxy-cyclic peroxides. During the course of this study, preliminary reports were published on the preparation of a single 5-membered cyclic peroxide from the enzymatic oxidation of linolenate followed by autoxidation (7), and on the isolation of bicycloendoperoxides from the same oxidation mixtures (8).

EXPERIMENTAL PROCEDURES

Materials

Pure methyl linolenate (100% by GLC and TLC) was prepared by counter double current distribution (9) of linseed methyl esters followed by silicic acid (100 mesh, Mallinckrodt, Paris, KY) chromatography and vacuum distillation. GLC packing and silica gel plates were previously described (6). Reducing agents were triphenylphosphine (Ph_3P) and Ph_3P bonded

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on styrene-divinyl benzene copolymer (2% crosslinked) (Strem Chemicals, Inc., Newburyport, MA). A mixture of trimethylchlorosilane, hexamethyl disilazane, and pyridine (1:2:10) (Regis Chemical Co., Morton Grove, IL) was used as silylating reagent. This reagent was particularly effective for complete silylation of polyhydroxy compounds (10).

Oxidations

Methyl linolenate, 5 g, was stirred at room temperature in an oxygen atmosphere for 88 hr to a peroxide value (PV) of 1113 (sample I). This sample was reduced with Ph_3P (11) at 0 C in water-saturated diethyl ether with stirring for 1 hr, and then fractionated by reverse-phase HPLC. A second linolenate sample (6 g) was autoxidized at 40 C in an oxygen atmosphere for 21 hr to a PV of 904 and was not reduced (sample II) prior to silicic acid fractionation and HPLC on micro silica columns. Before GC-MS, hydroperoxy cyclic peroxides were reduced with Ph_3P bonded to styrene-divinylbenzene copolymer (100% molar excess Ph_3P/mol hydroperoxide) in diethyl ether at room temperature with stirring for 2-3 hr. The polymeric reducing agent was removed by filtration in ether through a Pasteur pipette packed with Celite under nitrogen pressure.

HPLC

Reverse-phase HPLC of the methyl linolenate sample autoxidized at room temperature (I) was done on a 122 x 0.78 cm column packed with C-18 hydrocarbon bonded to Porasil B (Waters Associates, Milford, MA) at room temperature and 5 mL/min flow with a Waters Model 6000A pumping system. The column eluant was monitored with a variable wavelength ultraviolet detector (Schoeffel Instruments, Westwood, NJ) set at 212 nm for ester functionality (12). A stepgradient of H_2O and CH_3CN mixtures was used for elution. The column was cleaned between runs with $CHCl_3$. Samples (100 μL neat) were introduced with the Waters U-6K injector.

The reverse-phase HPLC fractions were concentrated by partial removal of CH_3CN on a rotating evaporator at 40 C, and they were extracted with diethyl ether after addition of brine. The fractions were then combined on the basis of functional group purity as determined by TLC and stored in diethyl ether or CH_3CN at -20 C.

The linolenate sample autoxidized at 40 C (II) was fractionated first on silicic acid column with diethyl ether/hexane eluants by a procedure similar to that of Gardner (13). Most of the unoxidized linolenate was eluted with 200 mL 1:9 ether/hexane. The following oxidation products were then eluted with 100-mL portions of ether/hexane mixtures of the volume proportions indicated: (2:8) epoxy compounds; (3:7) hydroperoxide mixture; (4:6) mixture of hydroperoxides and hydroperoxy-cyclic peroxides; (1:1) hydroperoxy-cyclic peroxide mixture; (6:4) dihydroperoxides; and (7:3) unidentified polar compounds. The remaining oxidation products were eluted with 100% diethyl ether and methanol.

The mixture of hydroperoxy-cyclic peroxide was separated with a 50 x 0.94 cm column at room temperature packed with 10 μ silica (Magnum 9, Partisil 10, Whatman, Inc., Clifton, NJ), 5.0 mL/min (1000 psi, and 0.3% absolute ethanol in hexane. Typical sample sizes were 16-20 mg dissolved in mobile phase.

Methods

Infrared (IR), gas chromatography (GC), and gas chromatography-mass spectrometry (GC-MS) methods used were as previously described (6). Except as noted, TLC was conducted with diethyl ether/hexane/acetic acid (50:50:1, v/v/v) on silica gel "60" plates (with fluorescent 254 nm indicator, E. Merck, Darmstadt, Germany). ^{1}H-NMR, except as noted, and ^{13}C-NMR spectra were obtained on a Bruker WH-90 Fourier transform spectrometer. The ^{13}C-NMR spectra were obtained at 22.63 MHz with proton noise decoupling. All spectra were taken in solutions of deuterochloroform, which also served as an internal deuterium lock. Chemical shifts are given as δ-values in ppm downfield from the internal tetramethylsilane signal.

RESULTS

Chromatographic Fractionation of Autoxidized Linolenate after Reduction (Fig. 1)

Reverse-phase HPLC separation of sample I (Fig. 1) yielded fractions containing dihydroxy, epoxy-hydroxy, hydroxy-cyclic peroxy, monohydroxy, epoxy esters and unoxidized starting material. The reverse phase HPLC system separated autoxidized linolenate as previously reported for the autoxidized oleate and linoleate (6) according to functional group with partial separation of positional and geometric isomers. The dihydroxy, hydroxy-cyclic peroxides and hydroxy trienes are apparently derived from the corresponding hydroperoxides. The hydroxy trienes are partially separated into *trans,cis* and *trans,trans* conjugated diene-triene isomers.

The reduced linolenate sample was analyzed by GLC after silylation. The GC chromatogram

showed the same products as obtained by HPLC (Fig. 2). However, the hydroxy-cyclic peroxides (TMS ethers) were not stable under our GC conditions, and only minor amounts were detected. Therefore, GC was not suitable for quantitation. However, approximate quantitative analyses of autoxidized linolenate were obtained below in the nonreduced sample by silicic acid column chromatography.

Epoxy unsaturated esters. GC of this fraction gave two partially resolved peaks with retentions 1.32 and 1.39 relative to linolenate. TLC showed one spot with R_f 0.89 relative to linolenate. IR (CS_2) (1734 cm^{-1}, ester carbonyl), (3002 cm^{-1}, *cis* unsaturation). ^{1}H-NMR supported *cis* unsaturation at 5.43 ppm (4H) and indicated the presence of a *cis* epoxide ring with absorptions at 2.79 and 2.98 ppm (2H) (14). These data support the presence of *cis* olefinic *cis* epoxy esters.

Hydroxy octadecatrienoates. GC of the silyl derivative of this fraction showed two major peaks (50.6 and 37.3%) followed in elution by two minor peaks (8.1 and 3.9%) with retentions 1.67, 1.77, 1.79 and 1.87, respectively, relative to linolenate. GC of this HPLC fraction after hydrogenation and silylation showed two peaks with retentions 1.51 and 1.72 relative to methyl stearate. TLC had two UV active spots of R_f 0.56 and 0.48 relative to linolenate. UV showed conjugated diene with a maximum at 232 nm. GC-MS m/e (rel intensity) of this fraction after silylation: 380 (M+,0.11); 365 (M-15,0.15) and 349 (M-31,0.15) and characteristic mass fragments for the 9-, 12-, 13- and 16-OTMS stearates (3). These data confirm GC-MS studies for autoxidized linolenate hydroperoxides (3).

Hydroxy-cyclic peroxy octadecadienoates. GC of this fraction after silylation gave two small peaks of retention 2.11 and 2.19 relative to methyl linolenate. After hydrogenation and silylation, GC showed two peaks of approximately equal area with the same retentions (2.46 and 2.88) relative to methyl stearate as methyl 9,10,12- and 13,15,16-trihydroxyoctadecanoate respectively (TMS ethers) (3). TLC gave one UV active spot of R_f 0.40 relative to linolenate. UV showed a maximum at 233 nm for conjugated diene. IR (CS_2) hydroxy absorption (3575 cm^{-1}, free C-OH), (3700-3220 cm^{-1}, H-bonded C-OH), and (3005 cm^{-1}, olefinic-H), (988 and 950 cm^{-1}, conjugated *cis,trans* unsaturation). ^{1}H-NMR supported the IR analysis with signals for the methine proton of the carbinol carbon 3.84 ppm (m) (1H), and the conjugated olefinic system with absorption centered at 6.49 and 5.39 ppm (4H). Additional absorptions were observed for cyclic peroxide methine protons at 4.79 and 4.29 ppm (2H) and for cyclic peroxide methylene protons at 2.50 to 2.93 ppm (2H). A five-membered peroxide ring was assigned on the basis of the 360 MHz ^{1}H-NMR data of Chan et al. (7) for 16-hydroperoxy-13,15-peroxy-9,11-octadecadienoate and the ^{1}H-NMR data of Porter et al. (15) for methyl 6-hydroxy-7,9-peroxy-10,12-octadecadienoate. GC-MS m/e (rel intensity) after silylation: 396 (M-16,23) ion. After hydrogenation and silylation, GC-MS

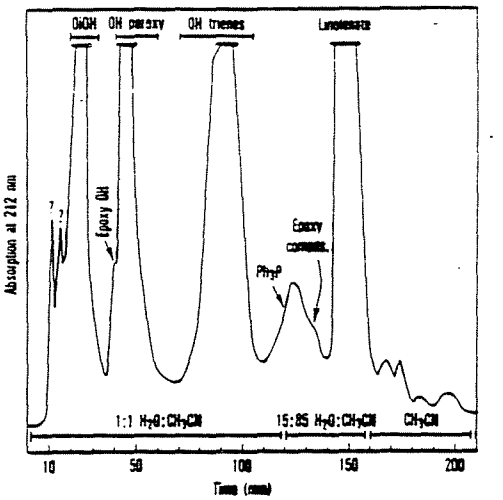


FIG. 1. Reverse phase (C-18 on Porasil B) HPLC chromatogram of Ph_3P-reduced linolenate, autoxidized at 27 C, PV 1113, (I) (flow 5 mL/min, detector set at 212 nm at 2 ABS units).

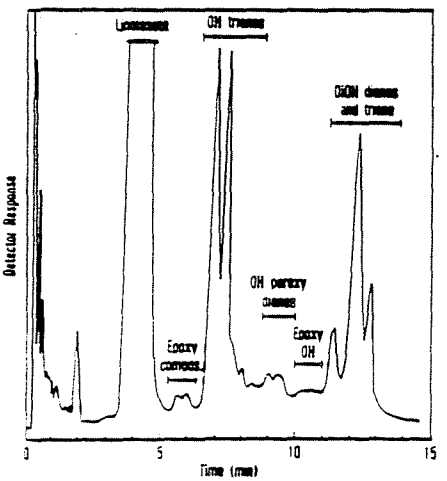


FIG. 2. Gas chromatogram of Ph_3P-reduced linolenate, autoxidized at 27 C, PV 1113, (I) (TMS ethers) (3% JXR packing in a 6 ft x 1/8 in. column, 180-250 C at 4 C/min temperature program).

indicated a mixture of 9,10,12- and 13,15,16-trihydroxystearates expected from methyl 9-hydroxy-10,12- and 16-hydroxy-13,15-cyclic peroxy octadecadienoates (3).

Epoxy-hydroxy octadecadienoates. GC showed three partially resolved peaks with retentions 2.41, 2.49 and 2.56 relative to linolenate. IR (CS_2) (3600 cm^{-1}, free C-OH), (3640-3140 cm^{-1}, H-bonded C-OH), and (3005 cm^{-1}, olefinic-H), (900 cm^{-1}, isolated *trans* unsaturation). ^{1}H-NMR supported the IR analysis with signals for the methine proton 4.18 ppm (1H), of the carbinol carbon and for isolated *trans* unsaturation 5.62 ppm. NMR also indicated the presence of a *cis* epoxide ring (14) with absorptions at 3.15 and 2.85 ppm (2H). GC-MS m/e (rel intensity) after Ph_3P reduction and silylation: 381 (M-15,11) ion corresponding to epoxy-hydroxy octadecadienoates and fragment ions, which indicated epoxy groups on carbon positions 9 and 10: 199 (5), 15 and 16: 71 (36), and 12 and 13: 111 (10). These data support the presence of epoxy-hydroxy or epoxy-hydroperoxy dienes in autoxidized linolenate. The epoxy-hydroxy compounds are apparently not artifacts of the Ph_3P reduction because MS of the silylated Ph_3P reduced hydroxy or hydroperoxy cyclic peroxides gave no evidence for these compounds (see below).

Dihydroxy octadecatrienoates. GC after silylation showed three peaks with retentions 2.69, 2.90 and 3.01 relative to linolenate. TLC showed two UV active spots of R_f 0.10 and 0.12 relative to linolenate. UV showed maxima at 229 and 267 nm for conjugated diene and triene, respectively. IR (CS_2) (3650-3120 cm^{-1}, bonded C-OH), (3005 cm^{-1}, olefinic) (998 and 950 cm^{-1}, conjugated *cis,trans*), (976 cm^{-1}, isolated *trans* unsaturation). ^{1}H-NMR supported the IR analysis with signals for the methine proton on the carbinol carbon 4.17 ppm and for the olefinic protons 5.61 ppm (center HC=CH). GC-MS m/e (rel. intensity) after silylation: 468 (M+,2) and 437 (M-31,5), for the TMS ethers of the dihydroxy triene. After hydrogenation and silylation, GC-MS m/e (rel intensity) showed evidence for dihydroxy stearate (OTMS ethers); 443 (M-31,18), with hydroxy on carbon-9:259 (96) and carbon-13:315 (39) on one end and on carbon-12:187 (36) and carbon-16:131 (63) on the other end. The spectral evidence supports a mixture of dihydroxy conjugated diene-triene and conjugated triene structures.

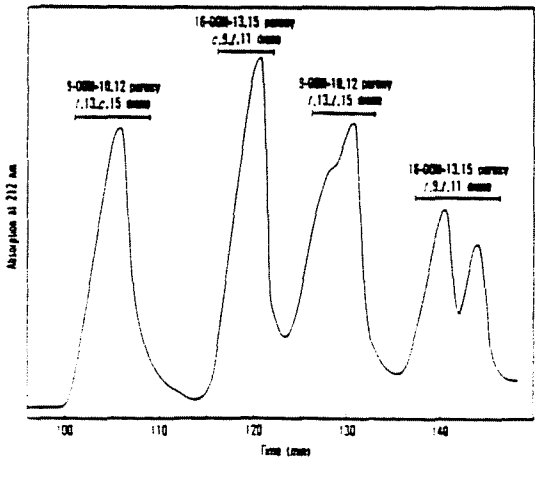


FIG. 3. 10-μ Silica HPLC chromatogram of hydroperoxy-cyclic peroxide mixture from linolenate, autoxidized at 40 C, PV 904, (II) (flow 5.0 mL/min, mobile 0.3% ethanol/hexane, detector set at 212 nm at 2 ABS units).

Chromatographic Fractionation of Nonreduced Autoxidized Linolenate (Fig. 3)

A hydroperoxy-cyclic peroxide mixture was first obtained by ordinary silicic acid column chromatography of linolenate autoxidized at 40 C (sample II). This mixture was then separated by HPLC on microsilica into positional and geometric isomers of the 9- and 16-hydroperoxy-cyclic peroxide dienes (Fig. 3). The *cis,trans* isomers of the hydroperoxy-cyclic peroxides were eluted before the *trans,trans* isomers in the same order as previously observed for the dienol isomers of linolenate hydroperoxides (1). The *trans,trans* isomers were also partially resolved apparently into their epimeric forms with respect to the hydroperoxy-bearing carbon, since the left- and right-side components of the partially resolved peaks in Figure 3 had similar ^{1}H-NMR characteristics, except for slightly different shifts for the proton at the hydroperoxy-bearing carbon.

Weights of oxidation products were estimated from fractions with the same functional group eluted by silicic acid column chromatography of autoxidized linolenate (Table I). Total recovery after silicic acid chromatography was ca. 98%. From peak areas in Figure 3, the relative composition is 27.8 and 24.5% for the respective 9-hydroperoxy *cis,trans* and *trans, trans* isomers, and 27.0 and 20.7% for the 16-hydroperoxy *trans,cis* and *trans,trans* isomers.

9-Hydroperoxy-10,12-peroxy-trans-13,cis-15-octadecadienoate. TLC (1:1 diethyl ether/hexane) showed one UV active spot of R_f 0.53 relative to linolenate. GC after reduction with Ph_3P and silylation gave a small peak of retention 2.11 relative to linolenate. GC of the silylated hydrogenated derivative gave one peak

TABLE I

Weight-Percent Composition of Silicic Acid Fractions from Two Samples of Linolenate Autoxidized at 40 C (II)

Fraction identification	PV 904	PV 1286
Linolenate	87.9	74.8
Epoxy unsaturated esters	0.2	0.3
Monohydroperoxides	3.5	8.4
Hydroperoxy-cyclic peroxides	3.8	7.7
Epoxy-hydroxy dienes	<0.1	<0.1
Dihydroperoxy compounds	0.9	2.9
Unidentified polar materials	3.7	5.9

with same retention relative to methyl stearate as methyl 9,10,12-triOTMS stearate. UV showed a maximum at 231 nm (E_m 24,200) for conjugated diene. IR (neat) (3700-3100 cm^{-1}, H-bonded OH or OOH), (3005 cm^{-1}, olefinic), (990-950 cm^{-1}, conjugated *cis,trans* unsaturation) and (900 cm^{-1}, peroxide) (16).

^{1}H-NMR data (Table II) are consistent with those of Chan (7) in establishing the cyclic 5-membered ring. All other assignments were corroborated with decoupling experiments. ^{13}C-NMR assignments (Table III) also confirm the identity of the title compound.

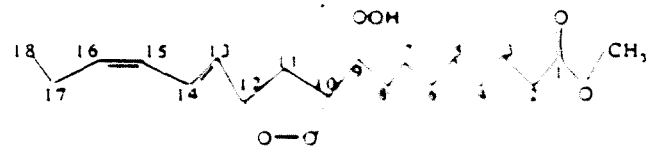
MS m/e (rel intensity) after reduction with Ph_3P and silylation: 396 (M-16,41) and 397 (M-15,10), for the hydroxy-cyclic peroxide as TMS ether, with OTMS on carbon-9: 259 (54). As suggested by GC retention data, MS after hydrogenation and silylation gave the expected methyl 9,10,12-triOTMS stearate derivative (3).

16-Hydroperoxy-13,15-peroxy-cis-9,trans-11-octadecadienoate. TLC (1:1 diethyl ether/hexane) showed one UV active spot of R_f 0.50 relative to linolenate. GC of the silylated Ph_3P-reduced derivative showed a peak of retention 2.19 relative to linolenate. GC of the silylated-hydrogenated derivative showed only one peak with the same retention as methyl 13,15,16-triOTMS stearate relative to methyl stearate. UV (methanol) showed a maximum at 234 nm (E_m 28,700) for conjugated diene. IR (CS_2) (3530 cm^{-1}, free OH or OOH), (3720-3200 cm^{-1}, bonded C-OH or C-OOH), and (3005 cm^{-1}, olefinic-H), and (982 and 950 cm^{-1}, conjugated *cis,trans* unsaturation). The ^{1}H-NMR (Table IV) and ^{13}C-NMR (Table V) assignments confirm the structure of the title compound. MS m/e (rel intensity) after reduction with Ph_3P and silylation: 396 (M-16,10) and 397 (M-15,3), for the hydroxy-cyclic peroxide as OTMS ether, with OTMS on carbon-16: 131 (76). MS after hydrogenation and silylation showed the expected derivative methyl 13,15,16-triOTMS stearate (3).

Mixture of 9-hydroperoxy-10,12-peroxy and 16-hydroperoxy-13,15-peroxy octadecadieno-

TABLE II

^{1}H-NMR of 9-Hydroperoxy-10,12-peroxy-*trans*-13,*cis*-15-octadecadienoate



δ ppm	Multiplicity[a]	J/Hz	Number of protons	Assignment
9.43	br. s			OOH
6.67	dd	15,11		H-14
6.01	br. t	11,10	4	H-15
5.62	dd	15,8		H-13
5.50	m			H-16
4.80	ddd	8,8,5		H-12
4.45	ddd	8,5,4		H-10
4.15	m	-		H-9
3.66	s	-		CH_3O
2.84	ddd	12,8,5	-	H-11β
2.47	ddd	12,8,8	-	H-11α
2.30	t	-	-	H-2
2.07	d	-	-	H-17
1.35-1.15	m	-	-	H-3-8
1.00	t		-	H-18

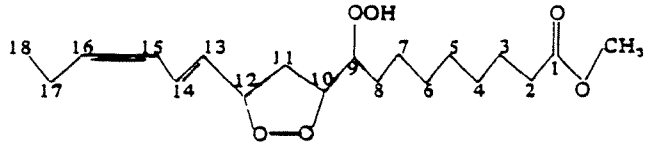
[a]Multiplicity: br = broad, s = singlet, d = doublet, m = multiplet, t = triplet.

ates. TLC (1:1 diethyl ether/hexane) showed a UV active major spot of R_f 0.46 relative to linolenate. GC of the silylated hydrogenated derivative showed two peaks with retentions 2.46 (70%) and 2.88 (30%) relative to methyl stearate for methyl 9,10,12- and 13,15,16-triOTMS stearates, respectively. Apparently, the second peak tailed into the third (Fig. 3). UV showed a maximum at 235 nm (E_m 27,900) for conjugated diene.

16-Hydroperoxy-13,15-peroxy-trans-9,trans-11-octadecadienoate. TLC (1:1 diethyl ether/hexane) gave one UV active spot of R_f 0.44 relative to linolenate. GC of the silylated hydro-

TABLE III

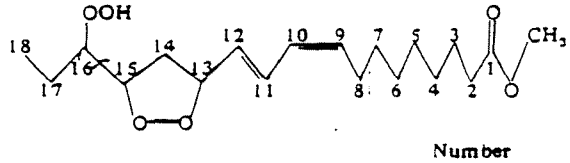
^{13}C-NMR 9-Hydroperoxy-10,12-Peroxy-*trans*-13,*cis*-15-octadecadienoate



δ ppm	Carbon assignment (17)
174.3	1
136.8,131.8,126.6,126.2	13-16
86.0	9
83.8	10,12
83.0	
51.3	OCH_3
41.3	11
34.1	2
29.6	4-8
29.0	
25.6	17
24.9	3
14.0	18

TABLE IV

^{1}H-NMR of 16-Hydroperoxy-13,15-Peroxy-*cis*-9,*trans*-11-octadecadienoate



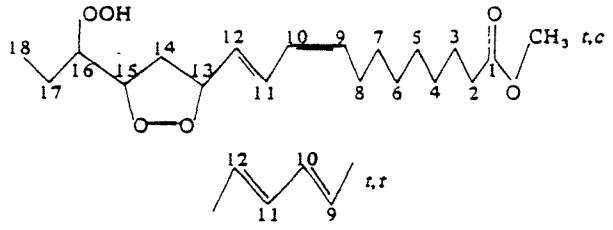
δ ppm	Multiplicity[a]	J/Hz	Number of protons	Assignment
9.38	br, s		1	OOH
6.67	dd	15,11		H-11
6.01	br, t	11,10	4	H-10
5.62	dd	15,8		H-12
5.55	m	10		H-9
4.80	ddd	8,8,8	1	H-13
4.49	ddd	9,7,5	1	H-15
4.15[b]	ddd	9,7,3	1	H-16
3.66	s		3	CH_3O
2.84	ddd	12,8,9	–	H-14β
2.47	ddd	12,8,5	–	H-14α
2.30	t	–	–	H-2
2.08	d	–	–	H-8
1.89-1.20	m	–	–	H-7-3
1.03	t		–	H-18

[a]See footnote a, Table II.

[b]The shift difference between protons for C-16 (Table VI) is apparently due to different epimeric forms.

TABLE V

^{13}C-NMR of 16-Hydroperoxy-13,15-peroxy-9,11-octadecadienoate and Isomers



δ *t,c* ppm	δ *t,t*.ppm	C assignments (17)
174.3		1
135.2,131.8,127.3,126.3	127.3,126.6	9-12
87.4	87.2	16
83.5,82.9	83.8,82.6	13,15
51.4	51.4	OCH_3
41.3	43.7	14
34.1	34.2	2
29.4,29.1	29.1	4-7
27.8	–	8
25.0	25.0	3
22.8	22.1	17
10.2	10.2	18

genated derivative showed only one peak with the same retention relative to methyl stearate as methyl 13,15,16-triOTMS stearate. UV (methanol) showed the maximum at 233 nm (E_m 28,800) for conjugated diene. IR (CS_2) indicated hydroxy or hydroperoxy absorption (3530 cm^{-1}, free OH or C-OOH), (3700-3140 cm^{-1}, bonded C-OH or C-OOH) and (3005 cm^{-1}, olefinic-H), and (984 cm^{-1} conjugated *trans,trans* unsaturation).

^{1}H-NMR data (Table VI) confirm the identity of the title structure.

MS m/e (rel intensity) after reduction with Ph_3P and silylation showed OTMS on carbon-16: 131 (100); MS after hydrogenation and silylation showed the expected derivative for methyl 13,15,16-triOTMS stearate (3).

Dihydroperoxy octadecatrienoates. Silicic acid chromatography gave a dihydroperoxide fraction, more polar than the hydroperoxy-cyclic peroxide mixture, upon elution with 60:40 diethyl ether/hexane. TLC (1:1 diethyl ether/hexane) showed two UV active spots of R_f 0.31 and 0.27 relative to linolenate, that gave a strongly KI positive peroxide test (18). UV (methanol) showed maxima at 235 nm and 267 nm (triplet) for conjugated diene and triene, respectively. IR (CS_2): (3530 cm^{-1}, free OH or OOH), (3712-3210 cm^{-1}, bonded C-OH or C-OOH), (3005 cm^{-1}, olefinic-H), 988 and 950 cm^{-1}, conjugated *cis,trans*) and (968 cm^{-1}, isolated *trans*). ^{1}H-NMR ($CDCl_3$, 100 MHz) supported the IR analysis with signals for the methine protons on the hydroperoxy-containing carbons 4.40 ppm (2H), and for olefinic system 6.28-5.26 ppm. There was no indication of hydroxy-containing carbons (3.8-4.2 ppm). GC-MS after hydrogenation and silylation showed evidence for dihydroxystearate (TMS ethers): 443 (M-31,10) ion, with the hydroxy on carbon-9: 259 (29) and carbon-13: 315 (15) on one end of the molecule and on carbon-12: 187 (15) and carbon-16 131 (48) on the other end. The UV, IR, NMR and MS data support dihydroperoxy conjugated diene-triene and conjugated triene structures.

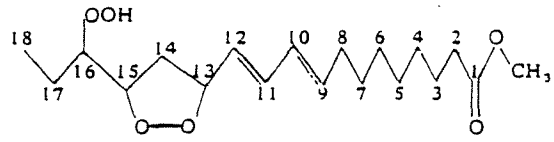
DISCUSSION

Secondary oxidation of hydroperoxides produces a complex mixture of volatile and nonvolatile compounds that may contribute either directly or as precursors to flavor deterioration in unsaturated fats (19-21). In this work, hydroperoxy-cyclic peroxides have been identified as major secondary products in autoxidized linolenate and dihydroperoxides as minor products. Other minor products identified after reduction (Ph_3P) include epoxy and epoxy-hydroxy esters.

Our previous studies (3,22,23) of autoxidized methyl linolenate showed the formation of significantly more 9- and 16-hydroperoxides (~5-8%) than of 12- and 13-hydroperoxides (18-25%). The evidence of significant amounts of 9,10,12- and 13,15,16-trihydroxystearates in

TABLE VI

^{1}H-NMR of 16-Hydroperoxy-13,15-peroxy-*trans*-9,*trans*-11-octadecadienoate



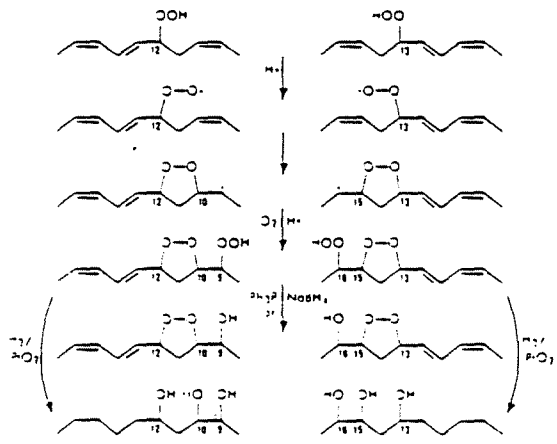
δ ppm	Multiplicity[a]	J/Hz	Number of protons	Assignment
8.98	br, s		1	OOH
6.65	dd	15,11		H-11
5.99	m	15,11	4	H-10
5.60	m	15,8		H-12
5.46	m	15,-		H-9
4.77	ddd	8,8,8	1	H-13
4.47	m	8,8	1	H-15
3.86[b]	m	8,5	1	H-16
3.66	s		3	CH_3O-
2.75	m	12,8,5	–	H-14β H-14α
2.30	t	–	–	H-2
2.09	d	–	–	H-8
1.84-1.17	m	–	–	H-7-4
0.87	t		–	H-18

[a]See footnote a, Table II.

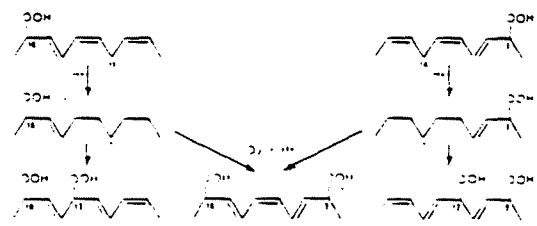
[b]The shift difference between protons for C-16 (Table VI) is apparently due to different epimeric forms.

the hydrogenated derivatives supported cyclization of the internal 12- and 13-hydroperoxides of linolenate. The results reported here provide more direct evidence for cyclization of the 12- and 13-hydroperoxides into hydroperoxy-cyclic peroxides. The 1,3-cyclization scheme previously formulated by Pryor et al. (24) can be advanced for the formation of 9- and 16-hydroperoxy-cyclic peroxides identified in this work and the 16-hydroperoxy-cyclic peroxide reported by Chan et al. (7) (Scheme I). This type of cyclization requires the presence of a *cis* double bond homo-allylic to the internal 12- or 13-hydroperoxides. Formation of 5-membered cyclic peroxides is consistent with the model studies of Porter et al. (15) and the rules of cyclization reported by Baldwin (25).

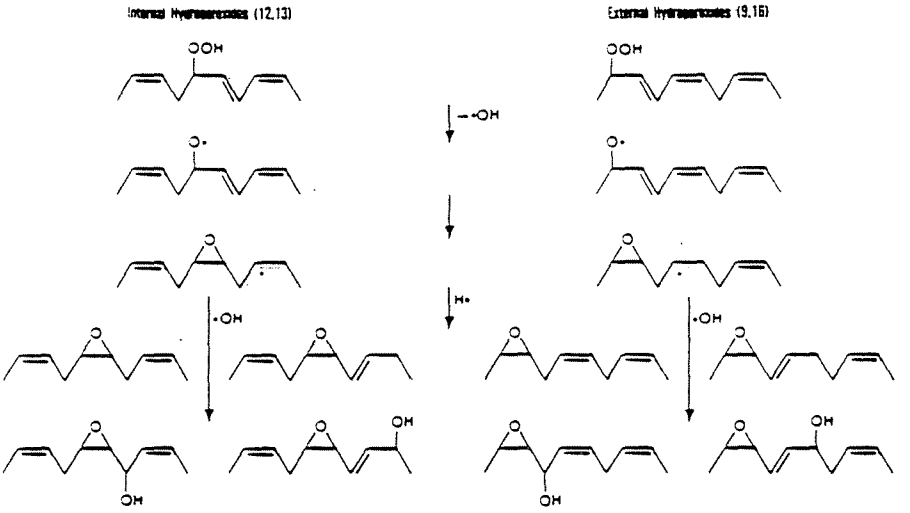
The minor dihydroperoxides identified in this study are those expected from the secondary oxidation of the terminal 9- and 16-hydroperoxides of methyl linolenate. According to Scheme II, abstraction of hydrogen from the doubly allylic methylene groups 11 and 14 produces pentadienyl radicals. Oxidation at either end of these pentadienyl radicals form the 9,12- and 13,16-dihydroperoxides with conjugated diene-triene systems and the 9,16-dihydroperoxides with conjugated triene system. The structures of these dihydroperoxides are consistent with those of the dihydroxy



SCHEME I.



SCHEME II.



SCHEME III.

esters identified after reduction and of the dihydroperoxides identified directly. Because these dihydroperoxides are minor products, they would not be expected to affect significantly the relative proportion of the 9- and 16-hydroperoxides in autoxidized linolenate.

The formation of minor epoxy and epoxy-hydroxy esters can be explained by a mechanism similar to that previously advanced for autoxidized linoleate (19,20,26). 1,2-Cyclization of linolenate hydroperoxides would produce nonconjugated 12-epoxydienes from the internal isomers and a mixture of 9- and 16-epoxydienes from the external isomers (Scheme III). Further hydroxylation (or hydroperoxidation) would produce the corresponding epoxy-hydroxy (or epoxy-hydroperoxy) dienes. The structures of these epoxy esters are supported by the chromatographic and spectral data. Epoxy-hydroxy diene structure is further supported by MS data, but additional evidence is necessary to establish position of double bonds and epoxy groups.

The possible contribution of secondary oxidation products to flavor deterioration in unsaturated fats has been discussed previously (19-21). Allylic epoxy aldehydes recently identified in oxidized butterfat (27) and trilinolein (28) can be derived from epoxy-hydroperoxides identified by Gardner et al. (29) among the secondary oxidation products of methyl linoleate hydroperoxides. Similarly, the hydroperoxy-cyclic peroxides were suggested as precursors of volatile compounds produced by hydroperoxides from autoxidized and photosensitized-oxidized methyl linolenate (21). The dihydroperoxides identified in this study can also serve as precursors of volatile compounds by carbon-carbon cleavage on either side of the alkoxy radicals postulated as intermediates in the decomposition of monohydroperoxides (21,30). Current research is aimed at determining more directly the contribution of these secondary oxidation products of linolenate to flavor deterioration. The biological role of these secondary oxidation products may also become important if some of the symptoms of intracellular lipid oxidation can originate from the prostaglandin-related cyclic peroxides identified in this and other studies (4,5,7,24).

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