Autoxidation of Methyl Linolenate: Analysis of Methyl Hydroxylinolenate Isomers by High Performance Liquid Chromatography

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ABSTRACT

The mixture of methyl hydroxylinolenates obtained by reduction of the hydroperoxide isomers formed by autoxidation of methyl linolenate was resolved by high performance liquid chromatography into eight major components. These are positional isomers with the hydroxyl group at positions 9, 12, 13, and 16. Two geometrical isomers of each positional isomer are present; these differ in the configuration of the conjugated double bonds (*cis-trans* and *trans-trans*). Autoxidation of methyl linolenate is regioselective and favors the formation of positional isomers 9 and 16.

INTRODUCTION

The analysis by high performance liquid chromatography (HPLC) of the mixture of isomers of hydroperoxides formed by autoxidation of methyl linoleate and the corresponding mixture of hydroxy compounds (methyl hydroxylinoleates) derived (by $NaBH_4$ reduction) from them has been reported recently (1). Using adsorption chromatography on 5 μm silica particles, the hydroperoxides and hydroxy compounds were separated into individual components, thereby permitting a rapid and quantitative analysis of the composition of positional and geometrical isomers present in the mixture. Only four major isomers were produced by autoxidation of methyl linoleate and these were the 9- and 13-diene hydroperoxides having the double bonds in either *trans-trans* or *cis-trans* (*trans* double bond adjacent to the hydroperoxide group) configuration. Oxygenation therefore occurs at the ends of the 1,4-diene moiety of the linoleate molecule.

Autoxidation of methyl linolenate is more complex since two separate 1,4-diene systems are present. In a previous investigation, Frankel et al. (2), using countercurrent extraction and partition chromatography to isolate hydroperoxides, showed by chemical degradation that the major components in the mixture of hydroperoxides obtained from autoxidation of methyl linolenate were isomers having the hydroperoxide group in the 9, 12, 13, and 16 positions. Attack of oxygen occurs, therefore, at the ends of the two 1,4-diene systems, viz., between carbon atoms 9 & 13 and 12 & 16. In either case, the third double bond remains unaffected and autoxidation of linolenate is expected to be twice as complex as that of linoleate in terms of the number of isomers formed. The ability to separate the individual isomers of hydroperoxy and hydroxy dienes in the methyl linoleate series using HPLC suggested that this technique might have the resolution required for the analysis, as distinct components, of the corresponding mixtures obtained from linolenate autoxidation. This possibility has been investigated and the results are now reported.

MATERIALS AND METHODS

Methyl linolenate (98%) was obtained from Lipid Supplies (St. Andrews, UK) and Hiflosil from Applied Sciences Labs. Inc. (State College, PA).

Ultraviolet (UV) spectra were recorded in ethanol solution on a Pye-Unicam SP800 and infrared (IR) spectra (in CCl_4 solution) on a Pye-Unicam SP200 G spectrophotometer. Mass spectra were obtained from a GEC/AEI MS 902 mass spectrometer.

High Performance Liquid Chromatography (HPLC)

HPLC was carried out on 5 μm silica particles (Partisil-5) as previously described (1). For the isolation of quantities (1-3 mg) of individual components, ca. 1 mg of the mixture of methyl hydroxylinolenates was repeatedly injected. Chromatography of larger quantities resulted in substantial loss of resolution.

Preparation of Methyl Linolenate Hydroperoxide and Methyl Hydroxylinolenate Isomers

Methyl linolenate was autoxidized (40 C) in a Warburg apparatus until oxygen uptake reached ca. 5 mole % (20-40 hr). Iodometric titration (3) of the product yielded a peroxide to oxygen-uptake ratio of 0.82. The mixture of hydroperoxides was isolated by partition

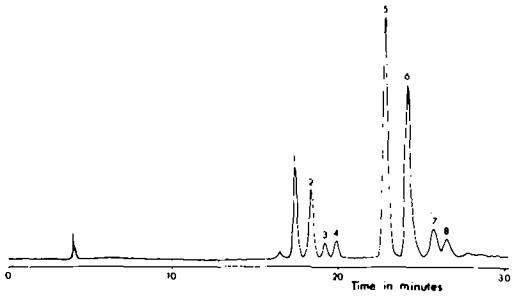


FIG. 1. High performance liquid chromatography of isomers of methyl hydroxylinolenate. Column packing: Partisil-5; eluting solvent: 0.75% ethanol in hexane; rate of solvent flow: 4 ml/min. Structures of isomers: 1, 13-*cis-trans;* 2, 12-*cis-trans;* 3, 12-*trans-trans;* 4, 13-*trans-trans;* 5, 16-*cis-trans*; 6, 9-*cis-trans;* 7, 16-*trans-trans;* 8, 9-*trans-trans.*

between petroleum ether (bp 40-60 C) and aqueous ethanol and purified by chromatography on Hiflosil as previously described for the hydroperoxides from autoxidation of methyl linoleate (1).

The mixture appeared as a single component on thin layer chromatography (silicic acid, developed with 30:70 ether:hexane) when detected by UV absorption, ferrous thiocyanate spray, and chromic acid charring. Its UV spectrum consisted of a single absorption band at 235 nm. Iodometric titration (3) of the mixture yielded a hydroperoxide to diene absorption ($\epsilon_{235\ nm}$ = 24,000 assumed) ratio of 1.03. Reduction ($NaBH_4$) to the mixture of methyl hydroxylinolenates was carried out as previously described (5).

Methyl 9-hydroperoxy-*trans*-10,*cis*-12,*cis*-15-octadecatrienoate and methyl 13-hydroperoxy-*cis*-9,*trans*-11,*cis*-15-octadecatrienoate were prepared using tomato and soybean lipoxygenases respectively as previously described for the oxidation of linoleate derivatives (4,5). When reduced to the methyl hydroxylinolenates, both preparations yielded a single isomer (the corresponding 9- and 13-hydroxy compounds) of over 90% isomeric purity (determined by HPLC).

Reduction to Methyl Hydroxystearates

The individual components of methyl hydroxylinolenate collected from HPLC of the mixture were reduced to the corresponding methyl hydroxystearates as previously described (5).

The mass spectra of the methyl hydroxystearates were examined for fragmentation ions resulting from isomers with the hydroxyl group in positions 8 to 16 (6). Only ions corresponding to isomers 9, 12, 13, and 16 were present to any appreciable extent (>30% of the base peak); the ions due to other isomers were in every case less than 1% of the base peak. The relative proportions of the intensities of the ions with m/e = 155, 197, 211, 253 (the 9, 12, 13, 16 isomers, respectively) from each component are listed in Table I.

RESULTS

In contrast to methyl linoleate hydroperoxides, the mixtures of the pure hydroperoxides obtained from the autoxidation of methyl linolenate was not resolved under the conditions of HPLC used but appeared as one composite peak. However, following reduction to the hydroxylinolenates, the mixture was resolved into eight major components (Fig. 1). The fact that the components are isomers of methyl hydroxylinolenate was confirmed by their individual UV spectra, all of which consisted of a single absorption band in the region 232-236 nm and by their IR spectra which were identical, apart from variations (see below) in

TABLE I

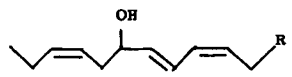
Ultraviolet (UV) and Infrared (IR) Spectral Data of Components of Methyl Hydroxylinolenate and Mass Spectrometry (MS) Data of the Derived Hydroxystearates

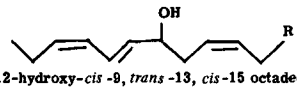
Component	% Composition (peak areas)[a]	UV (in ethanol) λ_{max} in nm	IR (in CCl_4) ν_{max} in cm^{-1}	MS of hydroxystearates (relative proportion of ions)			
				m/e =	155	197	211	253
				Isomer	9	12	13	16
1	10.2 ± 1.5	233	950, 989		–	5	95	–
2	8.2 ± 1.4	233	951, 990		–	94	6	–
3	1.9 ± 0.4	232	992		–	100	–	–
4	2.3 ± 0.4	232	992		–	4	96	–
5	38.1 ± 2.6	236	951, 989		10	–	–	90
6	30.0 ± 1.0	236	951, 988		100	–	–	–
7	5.8 ± 0.4	232	991		30	–	–	70
8	3.4 ± 0.5	232	992		100	–	–	–

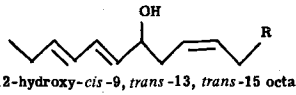
[a]Average of three autoxidation experiments.

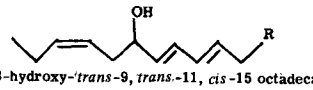
the region 950-1000 cm^{-1}, with that of a sample of methyl 13-hydroxy-*cis*-9,*trans*-11,*cis*-15-octadecatrienoate. Acid dehydration (7) of the individual isomers in ethanol solution yielded in every case the spectrum of a pure tetraene. Based on the value (7) of 49,200 for the molar extinction coefficient of the mixture of geometrical isomers of tetraenes, the yields of the conversion fall within the range 100 ± 15%. The geometrical and positional isomerism of each isomer was established by its IR spectra in the region 950-1000 cm^{-1} and by the mass spectra of the corresponding hydroxystearates (Table I). The mass spectra indicated that each component consisted essentially of one single positional isomer with a small degree of contamination from adjacent isomer(s). The apparently low isomeric purity of component 7 (a 16-isomer) is possibly due to the fact that it is a component positioned between two 9-isomers and that the 9-isomer gives rise to ions with higher intensity (approximately threefold) (6) than the 16-isomer. The purity of component 7 is therefore higher than that indicated by its mass spectrum. The geometrical isomerism of the double bonds that are in conjugation in each isomer was designated by analogy with the *cis-trans* and *trans-trans* isomers of methyl linoleate hydroperoxide (1) using the 950-1000 cm^{-1} region of the IR spectrum, i.e., a *cis-trans* isomer has two bands (950 and 989 cm^{-1}) while a *trans-trans* isomer has a single band (992 cm^{-1}). The absence of an absorption band at 970 cm^{-1} in their IR spectra confirmed that the double bond that is not in conjugation is, in each case, in a *cis* configuration. This allows the isomers 1-8 (Fig. 1) to be assigned the following structures:

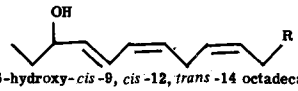
R = $-(CH_2)_6COOMe$

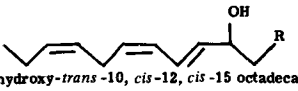
1.  (13-*cis-trans*)
methyl 13-hydroxy-*cis*-9, *trans*-11, *cis*-15 octadecatrienoate

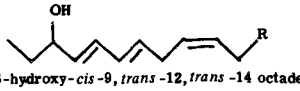
2.  (12-*cis-trans*)
methyl 12-hydroxy-*cis*-9, *trans*-13, *cis*-15 octadecatrienoate

3.  (12-*trans-trans*)
methyl 12-hydroxy-*cis*-9, *trans*-13, *trans*-15 octadecatrienoate

4.  (13-*trans-trans*)
methyl 13-hydroxy-*trans*-9, *trans*-11, *cis*-15 octadecatrienoate

5.  (16 *cis-trans*)
methyl 16-hydroxy-*cis*-9, *cis*-12, *trans*-14 octadecatrienoate

6.  (9-*cis-trans*)
methyl 9-hydroxy-*trans*-10, *cis*-12, *cis*-15 octadecatrienoate

7.  (16-*trans-trans*)
methyl 16-hydroxy-*cis*-9, *trans*-12, *trans*-14 octadecatrienoate

8.  (9-*trans-trans*)
methyl 9-hydroxy-*trans*-10, *trans*-12, *cis*-15 octadecatrienoate

The identities of isomers 1 and 6 were further established by co-injection of authentic samples obtained from lipoxygenase oxidations. For convenience, the isomers of methyl hydroxylinolenate and methyl linolenate hydroperoxide are also designated by the position of the hydroxyl or hydroperoxy group and the geometrical isomerism of the double bonds that are in conjugation (e.g., 13-*cis-trans* etc.).

The average percentage composition of the peak areas of the components obtained from three separate autoxidation preparations are shown in Table I. However, there was insufficient material to enable a gravimetric determination of the molar extinction coefficients (ϵ-values) of all the components and thus any quantitation of the amounts of the components would require assumptions concerning their ϵ-values, especially the differences between *cis-trans* and *trans-trans* forms. The inaccuracies that arise from such assumptions are, however, minimized if not removed by summing the areas of both forms of a positional isomer. The relative proportions of the four positional isomers thus obtained were: 9-isomer, 33.4 ± 1.0; 12-isomer, 10.1 ± 1.4; 13-isomer, 12.5 ± 1.5; and 16-isomer, 43.9 ± 2.6.

DISCUSSION

Despite its complexity, the mixture of methyl hydroxylinolenates was separated into its constituent components by chromatography on Partisil-5. The components may be divided into two types, viz., the "outer" and "inner" isomers depending on whether the hydroxyl group is positioned outside the system of the three

double bonds (9 and 16 positional isomers) or whether it is placed within the double bond system so that it is sandwiched between a conjugated diene and an allyl function, i.e., it occupies an allylic and homo-allylic position (12- and 13-isomers). The two types are well separated by chromatography with the "inner" isomers having the shorter retention times. Analysis of the components confirmed the previous observation (2,6) that only four positional isomers (9, 12, 13, and 16) were formed from autoxidation of methyl linolenate. Like the autoxidation of methyl linoleate, two major geometrical isomers of each positional isomer were formed, viz., the *cis-trans* and the *trans-trans*. In the case of linoleate oxidation, the *cis-trans* isomer has the *trans* double bond adjacent to the hydroperoxide group. That this is also the case in linolenate autoxidation was indicated by cochromatography of lipoxygenase-produced isomers which have this configuration.

There are, however, differences between linoleate and linolenate autoxidation. Unlike linoleate oxidation, which yields equal proportions of 9 and 13 positional isomers (1), the relative proportions of the four positional isomers obtained from linolenate autoxidation are not equal but favor the 9 and 16 (i.e., "outer") isomers by a ratio of over 3 to 1. Frankel et al. (2) also obtained higher proportions of 9 and 16 isomers in their degradation studies. They attributed this either to the preferential attack of oxygen at positions 9 and 16 or to the higher rate of decomposition of the 12- and 13-hydroperoxides. The latter possibility can be discounted since we found that the 9 and 13 methyl linolenate hydroperoxides (obtained from lipoxygenase oxidations) decompose at similar rates. We have also ruled out the further possibility that the distribution of isomers observed is a result of the isomerization (8) of hydroperoxides after their formation; the pure 9- and 13-isomers did not undergo appreciable isomerization when heated at 40 C for 16 hr (Chan and Levett, unpublished data). Autoxidation of methyl linolenate is therefore regioselective, oxygenation occurring preferentially at positions 9 and 16 to favor the formation of "outer" isomers. The distribution of geometrical isomers is also significantly different for linolenate autoxidation which yields proportions of *cis-trans* isomers substantially higher than those of linoleate autoxidation (1).

The "inner" and "outer" isomers of methyl hydroxylinolenate also have minor differences in their UV spectra. While the "outer" isomers (9- and 16-) have a substantial difference in the wavelength of the absorption maxima of *cis-trans* (236 nm) and *trans-trans* (232 nm) isomers as is the case with the corresponding linoleate derivatives (1), the difference is much less marked for the "inner" isomers (12- and 13-) (Table I). This may be a consequence of the fact that the hydroxyl group is in a homoallylic position as well as adjacent to a conjugated diene in an "inner" isomer.

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