Nonenzymatic Oxidation Products of Methyl Arachidonate

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Received May 16, 1983

Six monohydroperoxide isomers were obtained on autoxidation of methyl arachidonate. In the isomeric mixture of monohydroperoxides, the amount ratio of outer isomers (5- and 15-isomers) was especially high. However, in the presence of 1.0% of α-tocopherol, the isomeric composition of monohydroperoxides was approximately homogeneous. Furthermore, no significant difference was observed in the susceptibility to decomposition between the isomers. From these results, it was suggested that the predominance of outer isomers was based on the difference in stability between their peroxy radicals.

With the accelerated oxidation of methyl arachidonate with ferrous ion-AsA catalyst, further oxygenated products, diOH, triOH and tetraOH compounds (after reduction and hydrogenation), were detected by GC-MS analysis.

Arachidonic acid is mainly present in the membrane phospholipids of mammalian tissues. This acid is metabolized by lipoxygenases\(^1\)\(^-\)\(^4\) to monohydroperoxyeicosatetraenoic acids, which are then converted to monohydroxy and dihydroxy acids, and by cyclooxygenases\(^5\)\(^-\)\(^6\) to PGs and thromboxans. Other oxygenated arachidonic acids, that is, epoxy acid,\(^7\) trihydroxy acid,\(^8\) and epoxy hydroxy acid,\(^9\) were also detected in the metabolized products of arachidonic acid.

On the other hand, arachidonic acid is considered to be very susceptible to nonenzymatic oxidation, because it has three pentadiene structures. Nugteren et al.\(^10\) found the formation of PGE-like compounds on the autoxidation of 8,11,14-eicosatrienoic acid. Pryor et al.\(^11\) suggested the formation of PG-like compounds as the precursors of TBA-reactive materials in the autoxidation of polyunsaturated fatty acids. Porter et al.\(^12\)\(^-\)\(^13\) detected monohydroperoxide regioisomers and their hydroxy derivatives produced by singlet oxygen oxygenation and autoxidation of arachidonic acid. Furthermore, they determined the rate of cyclization of peroxy free radicals derived from arachidonic acid by the use of a kinetic expression.\(^14\) Recently, Terao and Matsushita reported the results of GC-MS analysis of several oxygenated products formed by singlet oxygen oxidation\(^15\) and hemoprotein catalyzed peroxidation\(^16\) of arachidonic acid. However, the peroxidation pathways and products of arachidonic acid have been scarcely clarified because of the complexity of the reaction.

In this work, products of autoxidation and ferrous ion-catalyzed peroxidation of methyl arachidonate were investigated by GC-MS after reduction and hydrogenation.

MATERIALS AND METHODS

Materials. Methyl arachidonate (99% grade) was ob-
tained from Sigma Chemical Co., St Louis, Missouri. Before use, it was purified by preparative TLC to get rid of any peroxides. dl-α-Toc was provided by Eisai Co., Japan.

Autoxidation of AAMe. Five mg AAMe in a series of glass tubes was autoxidized by incubation with or without 1.0% of α-Toc at 37°C in the dark. A part of the autoxidized AAMe was taken from one of the glass tubes with a micro pipet at regular intervals.

Ferrous ion-AsA catalyzed oxidation. Emulsions of AAMe were prepared as follows; 2.0 ml of 0.1 M phosphate buffer (pH 6.2) containing 0.1% Tween 20 and AAMe (7.8 × 10⁻³ M) or its monoHPs (3.3~7.8 × 10⁻³ M) were mixed with a Vortex mixer for 1 min and then thoroughly emulsified by 1 min ultrasonic vibration. The reaction was initiated by the addition of catalysts, 50 μl of EDTA and 100 μl of AsA aqueous solution (final conc., FeSO₄, 10⁻⁵ M; EDTA, 10⁻⁵ M; AsA, 2 × 10⁻³ M). Incubation was at 25°C with continuous shaking.

Derivatization. Reduction with NaBH₄ and hydrogenation with palladium on carbon were done in the same manner as described previously. Then, the sample was silylated with trimethylchlorosilane in pyridine solution.

GLC analysis. After derivatization, the reaction products were analyzed by GLC with a Shimadzu GC-9A apparatus equipped with a glass column packed with 2% OV-1 on Neopak 2A, 60/80 mesh. The flow rate of nitrogen gas was 60 ml/min and the column over temperature was programmed from 180 to 260°C (3°C/min). A flame ionization detector was used. The quantities were calculated from the ratio to the internal standard, methyl stearate.

GC-MS analysis. GC-MS was carried out with a PAC 300 system, consisting of a Shimadzu LKB-9000 spectrometer and an OKITAC 4300S minicomputer. The column used was a glass tube (2 m × 3 mm), packed with 2% OV-1 on Chromosorb W, 60/80 mesh. Helium gas was used at 30 ml/min. The column temperature was set at 200~260°C (3°C/min). Operation conditions were as follows; ion source temperature of 290°C, separator temperature of 280°C, ionizing electron energy of 22 eV, trap current of 60 μA, and accelerator voltage of 3.5 kV. The isomeric composition of HP was calculated by computer summation of the peak areas of fragment ions due to the α-cleavage of the trimethylsilyloxy group of each isomer in the mass chromatogram.

Preparation of monoHPs. AAMe was autoxidized at room temperature for 1~2 days (PV 2500~4000). MonoHPs were separated from autoxidized samples by preparative TLC (Merck silica gel 60) using n-hexane-diethyl ether = 8:7 as the solvent system. UV spectra. UV spectra were taken in methanol with a Shimadzu UV 200 spectrophotometer.

RESULTS

Autoxidation of methyl arachidonate

Autoxidation products of AAMe were analyzed by GC-MS. Especially, the effect of α-Toc, a radical scavenger, on the composition of autoxidation products of AAMe was examined. The changes in the amounts of AAMe and monoHP during autoxidation are shown in Fig. 1. When α-Toc was not added (Fig. 1(A)), AAMe was reduced to about 24 mol% of the original after 24 hr incubation. However, the accumulation of monoHP was less than 10 mol% in the course of the reaction. On the other hand, addition of α-Toc remarkably depressed the rate of AAMe de-

Fig. 1. Changes in the Amounts of MonoHP (•) and AAMe (○) on Autoxidation of AAMe with (B) or without (A) α-Toc.

Fig. 2. TLC Patterns of Autoxidation Products of AAMe after 12 hr (without α-Toc) or 120 hr (with α-Toc) Incubation. Development was performed with n-hexane-diethyl ether (8:7, v/v). The plate was sprayed with 50% H₂SO₄ in saturated K₂CrO₇ solution and heated for 10 min.
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crease (Fig. 1(B)). In this case, most consumed AAMe accumulated as monoHP, and the amount of monoHP reached 18 mol% after 120 hr incubation.

When AAMe was autoxidized with or without 1.0% α-Toc for 12 or 120 hr, respectively, it was reduced approximately to the same level, and the reaction products were subjected to TLC (Fig. 2). Autoxidized AAMe in the presence of α-Toc gave mainly two spots, (a) and (b). The UV spectrum of fraction (a) gave a $\lambda_{\text{MeOH}}^\text{max}$ at 236.5 nm due to conjugated diene (Fig. 3). The spectrum of fraction (b) showed absorption peaks due to conjugated triene ($\lambda_{\text{MeOH}}^\text{max}$ = 260, 270, and 280 nm). Both spots, (a) and (b), were positive for a solution of potassium iodide, a peroxide-detecting reagent.

Figure 4 shows the GLC pattern of derivatized products obtained from the autoxidized (with or without α-Toc) AAMe preparation at the same level. After reduction and derivatization of the products, their mass spectra were measured. From their mass spectra, peaks 1 and 2 were identified as methyl eicosanoate derived from unoxidized AAMe and an isomeric monoHP mixture, respectively. AAMe has three pentadiene structures, and six kinds of monoHPs based on hydrogen atom abstraction at carbons 7, 10 and 13 are formed. From the fragment ions due to the α-cleavage of the TMS group in the mass spectra of monoOH, it was confirmed that the 5-, 8-, 9-, 11-, 12- and 15-isomers were present in peak 2. Fraction (a) (Fig. 2) was also identified as an isomeric monoHP mixture.

The isomeric composition of monoHPs produced during autoxidation of AAMe with or without 1% α-Toc was calculated from the peak areas of their mass chromatograms and the results are shown in Fig. 5. Without α-Toc (12 hr incubation), the amount ratio of monoHP isomers increased in the order of 8-, 12- (middle isomers) < 9-, 11- (inner isomer) < 5-, 15- (outer isomers). In contrast, with α-Toc (120 hr incubation), there was little difference among the amount ratios of monoHP isomers, and they were approximately equal.

Peaks 3, 4 and 5 in Fig. 4 were supposed to be derived from secondary oxygenated products of AAMe. When AAMe was autoxidized alone (Fig. 4(A)), a broad peak appeared behind monoOH. The mass spectra of peak 3 indicated that it consisted of a complicated mixture of diOH, triOH, and PG-like com-
pounds possessing a dihydroxycyclopentane ring. In addition, peak 4 was identified from its mass spectrum as an isomeric mixture of methyl monohydroxyheptadecanoate, and its isomeric composition is shown in Fig. 6. The ratio of 5- and 12-isomers was remarkably high, and the presence of these compounds in the derivatized products supports the formation of PGG₂-like endoperoxides during autoxidation of AAMe.²,16)

On the other hand, with α-Toc, a sharp peak appeared behind monoOH (Fig. 4(B)). The mass spectrum of peak 5 (Fig. 7) showed

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**Fig. 5.** The Isomeric Compositions of MonoHP on Autoxidation of AAMe with or without α-Toc. (A), without α-Toc, after 12 hr incubation; (B), with α-Toc, after 120 hr incubation.

The m/z values used for monitoring the monoHP isomers (TMS derivatives) on mass chromatography were 5-isomer, 203 and 313; 6-, 217 and 299; 7-, 231 and 285; 8-, 245 and 271; 9-, 259 and 257; 10-, 273 and 243; 11-, 287 and 229; 12-, 301 and 215; 13-, 315 and 201; 14-, 329 and 187; and 15-, 343 and 173, respectively.

**Fig. 6.** The Isomeric Composition of Methyl Monohydroxyheptadecanoate Derived from Autoxidation of AAMe after 12 hr Incubation.

The m/z values used for monitoring positional isomers (TMS derivatives) were 5-isomer, 203 and 271; 6-, 217 and 257; 7-, 231 and 243; 8-, 245 and 229; 9-, 259 and 215; 10-, 273 and 201; 11-, 287 and 187; and 12-, 301 and 173, respectively.

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**Fig. 7.** Mass Spectrum of DiOH Obtained on Autoxidation of AAMe with α-Toc after Derivatization (Fig. 4, peak 5).

An isomeric mixture of 5,15-diOH, 5,12-diOH and 8,15-diOH.
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characteristic ions at m/z 487 [M−15, loss of CH₃]. Fragment ions due to the α-cleavage of the TMS group appeared at m/z 431, 401, 389, 359, 341 [431−90, loss of TMSOH], 311 [401−90], 299 [389−90], 269 [359−90], 245, 215, 203 and 173. The above spectrum indicates that peak 5 is an isomeric mixture of diOH. Thus, it is likely that fraction (b) (Fig. 2) is an isomeric mixture of diHP in which hydroperoxy groups are located at C-5, 15, C-5, 12, and C-8, 15. Fragment ions due to the α-cleavage of the TMS group are shown in the Appendix. Peak 5 was monitored by fragment ions m/z 191 and 217, characteristically appearing in PG-like compounds possessing a dihydroxy cyclopentane ring, but no PG-like compounds were detected.

Ferrous catalyzed peroxidation of methyl arachidonate

The GLC pattern of derivatized products obtained on ferrous ion-AsA catalyzed peroxidation of AAMe is shown in Fig. 8. From the mass spectra, peak 1 was identified as an isomeric mixture of monoOH derived from monoHP. Fragment ions due to the α-cleavage of the TMS group in its mass spectra, confirmed that 5-, 8-, 9-, 11-, 12- and 15-isomers were present in AAMe monoHP. The isomeric composition of monoHP was calculated from the peak areas of its mass chromatogram, and the data are shown in Table I. It was the same as that obtained for autoxidation.

The mass spectrum of peak 3 agreed with that obtained for autoxidation or hemoprotein catalyzed peroxidation of AAMe. Thus, peak 3 was identified as an isomeric mixture of methyl trihydroxyprostanoates. The mass chromatogram of peak 3 shows that four kinds of isomers were present and the ratios of 5- and 15-isomers were particularly high compared with other isomers (Fig. 9).

<table>
<thead>
<tr>
<th>Table I. Isomeric Composition of MonoOH Obtained on Fe²⁺-Catalyzed Peroxidation of AAMe after Derivatization</th>
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<tbody>
<tr>
<td>Relative percent (%)</td>
</tr>
<tr>
<td>5-OH</td>
</tr>
<tr>
<td>2 hr</td>
</tr>
<tr>
<td>4 hr</td>
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<td>6 hr</td>
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Incubation was carried out at 25°C for 6 hr.
Figure 10 shows the mass spectrum of peak 4, in which characteristic ions were present at \( m/z \) 485 [M\-(15+90), loss of CH\(_3\) and TMSOH], 469 [M\-(31+90), loss of CH\(_3\)O and TMSOH], and \( \alpha \)-cleavage ions at \( m/z \) 301, 271, 203 and 173. Therefore, peak 4 was identified as an isomeric mixture of triOH in which hydroxy groups are located at C-5, 6 and 8, and C-12, 14 and 15.

The mass spectrum of peak 5 is shown in Fig. 11. Although characteristic ions did not appear, the intense \( \alpha \)-cleavage ions at \( m/z \) 259, 243, 229, 213, 203 and 173 suggest that peak 5 is an isomeric mixture of tetraOH. The isomeric composition of this compound is not clear, but it can be assumed that four isomers are present, that is, 5,8,9,11-, 5,12,14,15-, 9,11,12,15- and 5,6,8,15-tetraOH.

The peaks behind peak 5 in Fig. 8 could not be identified, because characteristic ions did not appear.

The GLC pattern (Fig. 8) shows a discern-
ible shoulder, 2, with a shorter retention time on the side of peak 3. In the mass spectrum of this shoulder 2, a characteristic ion was present at m/z 487 \([M - 15, \text{loss of CH}_3]\). Thus, it is likely that this shoulder 2 contains diOH. However, the isomeric composition is not clear, because the \(\alpha\)-cleavage ions overlap those of peak 3.

All the products described above were similar to those produced by autoxidation. Therefore, the reaction is deduced to proceed by a radical reaction.

Figure 12 shows the changes in the amounts of monoHP and AAMe during the peroxidation catalyzed by ferrous ion-AsA. AAMe was consumed rapidly being about 25 mol% after 6 hr incubation. The amount of monoHP reached 10 mol% after 2 hr, and then gradually decreased.

**DISCUSSION**

AAMe is susceptible to nonenzymatic oxidation, because it has three pentadiene structures. First, 5-, 8-, 9-, 11-, 12- and 15-peroxy radicals are formed on each of the pentadiene radicals which are produced by the elimination of hydrogen atom from active methylene at C-7, C-10 and C-13, respectively.\(^{13}\) Next, six isomeric monoHPs are produced from each peroxo radical by abstraction of a hydrogen atom. These six monoHPs can be classified into three groups based on the position of each hydroperoxy group as follows; 5-, 15-isomers (outer isomers), 8-, 12-isomers (middle isomers), and 9-, 11-isomers (inner isomers). The isomeric composition of methyl linolenate monohydroperoxides reported by Frankel et al.\(^{19,20}\) indicates that outer isomers (hydroperoxy group located outside the triene structure) have higher yields than inner isomers (hydroperoxy group located inside the triene structure). A similar tendency was found in the isomeric composition of monoHPs obtained on autoxidation (Fig. 4(A)) and ferrous ion-AsA catalyzed peroxidation (Table I) of AAMe.

The predominance of outer isomers (5- and 15-monoHPs) may be explained either (1) by the selective attack of an oxygen molecule, or (2) by differences in stability between isomers.\(^{20,21}\) However, in the presence of 1.0% \(\alpha\)-Toc, the isomeric composition of monoHPs produced in the autoxidation of AAMe was
homogeneous (Fig. 4B)). This result suggests that the attack of an oxygen molecule on the pentadiene radical is nonspecific. And the monoHP formation pathway is shown in Scheme 1.

The theory of methyl linolenate autoxidation proposed by Pryor et al.\textsuperscript{11} suggested that the peroxyl radicals which have double bonds at $\beta-\gamma$ from the carbon attached peroxy group can be cyclized to form PGG-like endoperoxides. According to this theory, the 8-, 9-, 11- and 12-peroxy radicals in AAMe are able to be cyclized. On the other hand, in the presence of 1.0% $\alpha$-Toc, the formation of monoHPs occurred predominantly at every peroxyl radical, because $\alpha$-Toc acts as a strong hydrogen donor. Actually no PG-like compounds were detected in the products derived on autoxidation of AAME with $\alpha$-Toc. Consequently, it is assumed that the predominance of outer isomers was based on the differences in stability between the peroxyl radicals, outer, middle and inner.

It is well known that monohydroperoxides are produced in the early stage of nonenzymatic oxidation of polyunsaturated fatty acids. However, monoHP did not accumulate remarkably in the autoxidation or ferrous ion-AsA catalyzed peroxidation of AAME (Fig. 1). Because monoHP themselves have one or two pentadiene structures, they are susceptible to further oxidation. By the accelerated oxidation with ferrous ion-AsA catalyst, further oxygenated products, diOH, triOH and tetraOH compounds, were detected after reduction and hydrogenation of the reacted mixture. The mechanisms of the further oxygenation will be described in the following paper.

### APPENDIX

The mass fragmentation of TMS derivatives of methyl hydroxyeicosanoate. (X = TMS)

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\begin{align*}
\text{CH}_3\text{(CH}_2\text{)}_4\text{CH}_3&\rightarrow\text{CH}_2\text{CH}_3\text{-CH-CH}_2\text{-CH-CH}_2\text{-COOCH}_3 \\
\text{173} \text{ (341)} &\rightarrow \text{401} \text{ (311)} \\
\text{CH}_3\text{(CH}_2\text{)}_4\text{CH}_3&\rightarrow\text{CH}_2\text{CH}_3\text{-CH-CH}_2\text{-CH-CH}_2\text{-COOCH}_3 \\
\text{215} \text{ (299)} &\rightarrow \text{401} \text{ (311)} \\
\text{CH}_3\text{(CH}_2\text{)}_4\text{CH}_3&\rightarrow\text{CH}_2\text{CH}_3\text{-CH-CH}_2\text{-CH-CH}_2\text{-COOCH}_3 \\
\text{173} \text{ (341)} &\rightarrow \text{359} \text{ (389)} \\
\text{CH}_3\text{(CH}_2\text{)}_11\text{CH}_3&\rightarrow\text{CH}_2\text{CH}_3\text{-CH-CH}_2\text{-CH-CH}_2\text{-COOCH}_3 \\
\text{271} \text{ (321)} &\rightarrow \text{387} \text{ (297)} \\
\end{align*}
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### REFERENCES

Autoxidation of Arachidonate