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# Identification of Radical Adducts Formed in the Reactions of Unsaturated Fatty Acids with Soybean Lipoxygenase Using Continuous Flow Fast Atom Bombardment with Tandem Mass Spectrometry@

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Structures of α-(4-pyridyl-1-oxide)-*N-tert*-butylnitrone (4-POBN) radical adducts formed in the reactions of soybean lipoxygenase with linoleic acid, linolenic acid, and arachidonic acid were determined using continuous flow fast atom bombardment (CF-FAB) combined with tandem mass spectrometry. The radical adducts of these lipoxygenase-dependent reactions were: n-octanoic acid radical, 12,13-dihydroxylinoleic acid radical, 12,13-epoxylinoleic acid radical, and n-pentyl radical from linoleic acid; n-octanoic acid radical, ethyl radical, and *cis/trans* and/or positional isomers (1- and 3-pentenyl) of pentenyl radical from linolenic acid; and 14,15-epoxyarachidonic acid radical and n-pentyl radical from arachidonic acid. Of these radical adducts, the n-octanoic acid radical from linoleic and linolenic acid, the ethyl radical from linolenic acid, and the 12,13-dihydroxylinoleic acid radical are identified for the first time in the reactions of soybean lipoxygenase. Thus the CF-FAB combined with tandem mass spectrometry employed here, by which both radical adducts and their fragment ions can be detected, is shown to be a powerful tool in the structural identification of free radicals.

*Keywords:* Linoleic acid, Linolenic acid, Arachidonic acid, Lipid peroxidation, HPLC/EPR, spin trapping, continuous flow fast atom bombardment combined with tandem mass spectrometry

## INTRODUCTION

There is a continuing and increasing interest in elucidating the mechanism of cell damage induced by free radicals.[1–3] Determination of the structure of the free radicals becomes a starting point for clarification of the mechanism by which cells are damaged by free radicals. The direct detection and identification of the free radicals have been studied primarily by electron paramagnetic resonance (EPR). The spin trapping technique, which employs spin trap reagents to form relatively stable radical adducts with free

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radicals, has often been used to detect short-lived free radicals.[4–7]

Using spin trapping, the structures of the radicals have been determined based on EPR hyperfine coupling constants.[8] However, similar hyperfine coupling constants were often observed for different radical adducts, which makes determination of radical adduct structure difficult. Thus, in order to obtain comprehensive knowledge of the molecular structures of the radical adducts, information other than EPR hyperfine coupling constants is necessary.

Mass spectrometry is a technology which can provide significant amounts of structural information. Direct probe mass spectrometry and gas chromatography/mass spectrometry (GC/MS) with electron impact (EI) or chemical ionization (CI) have been employed for the identification of some radical adducts.[9–17] Unfortunately, the molecular ions of many radical adducts produced with EI and CI are often of low abundance, or even absent. Furthermore, many radical adducts are stable only in solution and cannot be isolated for direct probe analysis or are too thermally labile for GC separation.

We and other researchers have recently employed high performance liquid chromatography/electron paramagnetic resonance (HPLC / EPR) and liquid chromatography/mass spectrometry (LC/MS) to determine the structures of radical adducts. Soft ionization methods, such as thermospray ionization (TSP),[18] continuous flow fast atom bombardment (CF-FAB),[19] and electrospray ionization (ESI),[20] which are utilized in LC/MS, have been shown to be powerful techniques to obtain molecular ions of the radical adducts.[21–25]

Unfortunately, these soft ionization methods often do not yield structurally-informative fragment ions. Tandem mass spectrometry (MS/MS), based on the coupling of two mass analyzers, is one mass spectrometric technique that can provide the missing information. In an MS/MS experiment, an ion selected by MS-I is induced to fragment, with the fragments being mass-analyzed and detected by MS-II. This technique has been used extensively to provide structural information under soft ionization conditions.[26,27]

In the present study, ESI, CF-FAB[28–34] and CF-FAB in combination with tandem mass spectrometry[35–37] are utilized to obtain both molecular weight and structural information for the α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (4-POBN) radical adducts formed in the reactions of soybean lipoxygenase with the unsaturated fatty acids linoleic acid, linolenic acid, and arachidonic acid (a major precursor of the free radicals formed in living systems). The molecular structures of the following new free radicals were determined: 12,13-dihydroxylinoleic acid radical, n-octanoic acid radical, and ethyl radical. In addition the already established pentyl radical,[17] pentenyl radical,[17] 12,13-epoxylinoleic acid radical,[21] and 14,15-epoxyarachidonic acid radical[21] were also detected. This is the first report describing the use of tandem mass spectrometry in spin trapping.

## MATERIALS AND METHODS

### Materials

Arachidonic acid (5,8,11,14-eicosatetraenoic acid) was purchased from Biomolecular Research Laboratories, Inc. (Plymouth Meeting, PA, USA). Linoleic acid (9,12-octadecadienoic acid), linolenic acid (9,12,15-octadecatrienoic acid), soybean lipoxygenase (type I), and α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (4-POBN) were obtained from Sigma Chemical Co. (St Louis, MO, USA). Sep-pak $C_{18}$ cartridges were from Waters (Milford, MA, USA). All other chemicals used were commercial products of the highest grade available.

### Reaction Conditions

Reaction mixtures contained 20 ml of 0.05 M borate buffer (pH 9.0), 2 ml of 1 M 4-POBN, 0.2

ml of a 25 mg/ml solution of the fatty acid (linoleic acid, linolenic acid, or arachidonic acid) in ethanol, and 0.06 ml of soybean lipoxygenase ($1.4 \times 10^6$ units/ml). The pH employed here optimizes the overall reaction rate. The reaction was allowed to proceed at 25 °C in the absence of light. The reaction mixture rapidly became anaerobic, since we used a large amount of soybean lipoxygenase and the unsaturated fatty acid was added in excess over the dissolved oxygen. After 24 hr, 4 ml of each reaction mixture was mixed with 0.4 ml of 0.2 M boric acid, filtered, and then injected onto a Waters μBondapak $C_{18}$ semi-preparative column [Waters (Milford, MA, USA)] [30 mm long × 10 mm id] with a Varian EPR spectrometer (Varian Associates, Palo Alto, CA, USA) as the detector. In order to produce large amounts of radical adducts, the long, 24-hr, incubation was employed. For the time-course studies, shorter reaction times were used.

## HPLC/EPR

High performance liquid chromatography/electron paramagnetic resonance (HPLC/EPR) analyses were done with an HPLC system equipped with an EPR spectrometer as a detector.[38–41] The HPLC/EPR was performed by using a Waters model 6000A solvent-delivery system with a Varian E-104 EPR spectrometer. The EPR spectrometer was connected to the HPLC system with a Teflon tube that passed through the EPR tube cavity. The magnetic field of the EPR spectrometer was fixed at the third EPR peak of the 4-POBN radical adduct. The EPR settings were: microwave power, 20 mW; modulation amplitude, 2.0 G; modulation frequency, 100 kHz; time constant, 2 s. The HPLC conditions were as follows: flow rate, 2.0 ml/min; injection volume, 4.4 ml; gradient elution [solvent A, 10 mM ammonium acetate; solvent B, 10 mM ammonium acetate/80% (v/v) acetonitrile] from 0% B to 80% B in 30 min.

In order to purify the 4-POBN radical adducts, peaks from five HPLC/EPR chromatograms were collected and combined. Chromatography was performed again using the same HPLC/EPR conditions after the volume of the combined sample had been reduced to about 4 ml in a Jouan vacuum concentrator/evaporator (Jouan, Inc., Winchester, VA, USA). Respective peak fractions on the second chromatogram showed the same retention times as those on the initial HPLC/EPR chromatogram indicating that the radical adducts were stable during the isolation procedures.

## Mass Spectrometry

*Electrospray.* The purified spin adducts were analyzed using a VG 12-250 quadrupole mass spectrometer (VG Masslab, Altrincham, Cheshire, UK) equipped with a Vestec electrospray source, Model 611B (Vestec Corp., Houston, TX, USA). Typical operating conditions for the VG 12-250 quadrupole mass spectrometer were: needle voltage, 3.08 kV; spray current, 0.234 mA; block temperature, 262 °C; chamber temperature, 51 °C; skimmer voltage, 14 V. In order to have sufficient sensitivity to detect the radical adducts, the resolution was set so that the peak-width at half-height was approximately 1.5–2.0 Da.

*CF-FAB.* The CF-FAB data were acquired using a VG ZAB-4F tandem mass spectrometer (VG Analytical, Manchester, UK). The ZAB-4F is of $B_1E_1$–$E_2B_2$ design and is operated at 8 kV and a resolution of approximately 1000. An Ion Tech atom gun and a standard VG continuous flow fast atom bombardment (CF-FAB) source heated at 40–60 °C were used. The samples were bombarded with 8 keV xenon atoms.

A coaxial continuous flow FAB interface, in which a fused silica analytical capillary is threaded inside a sheath capillary, was used in this work.[32–37] The sample solution flows through the inner column (typically 10 μm id, 150 μm od) while the matrix simultaneously flows through the outer column (160 μm id, 350 μm od). Sample introduction was performed using a stainless steel pressure injection vessel.[35] All of the coaxial CF-FAB experiments were acquired using flow injection analysis.

A microliter syringe pump (Isco, Inc., Lincoln, NE) was used to pump the matrix through the outer capillary at a flow rate of 0.3 mL/min. The matrix composition was 25% glycerol in water (5 mM heptafluorobutyric acid). The flow rate of the inner column was ~70 nL/min which resulted in an elution time of ~1.40 minutes. Full-scan MS data were acquired using an exponential scan, typically from 850 to 85 amu at 3 sec/decade in the centroided mode. Ions were detected in the third field-free region using a photomultiplier. The MS/MS experiments were carried out by focusing the parent ion through MS-I. Fragment spectra were obtained by collisional activation of the parent ion at 8 keV with helium gas (50% beam attenuation) in the collision cell located in the third field-free region. Collisional activated decomposition spectra of the resulting fragment ions were obtained by a linear $E_2B_2$ linked scan of MS-II (unit resolution). The MS/MS data were acquired in the continuum mode. The data acquisition system used was a VG Analytical 11-250 data system.

## RESULTS

### HPLC/EPR Analyses

The 24-hr reaction mixtures of linoleic acid, linolenic acid, and arachidonic acid with soybean lipoxygenase were analyzed using HPLC/EPR spectrometry (Figures 1A, 1B, and 1C, respectively). Retention times and percent peak areas of all the peaks for all three reactions are shown in Table 1. The largest peak in Figure 1A by area (fraction 10) could be the 4-POBN/pentyl radical adduct, which has already been identified as the major radical adduct under the reaction conditions employed here.[17] Fraction 14 of the linolenic acid reaction (Fig. 1-B) could correspond to one of the isomers of the 4-POBN/pentenyl radical adduct.[17] The retention time of fraction 21 in the HPLC/EPR elution profile of arachidonic acid (Fig. 1-C) is almost the same as that of fraction 10 in linoleic acid (Fig. 1-A), and also could be the 4-POBN/pentyl radical adduct.[17]

The 4-POBN/pentyl and 4-POBN/pentenyl radical adducts have long retention times compared to the other unsaturated fatty acid-derived radical adducts on the reverse phase HPLC elution profiles. The absence of a carboxyl group in the 4-POBN/pentyl and 4-POBN/pentenyl radical adducts increases the retention times as expected for a reversed phase column.

### Mass Spectrometric Analyses

Mass spectra of the purified 4-POBN spin adducts with linoleic acid-, linolenic acid-, and arachidonic acid-derived radicals were obtained using continuous flow FAB mass spectrometry. The masses of the protonated molecular ions observed for each fraction in the HPLC/EPR analyses are shown in Table 1.

#### *Fraction 10 of Linoleic Acid and Fraction 21 of Arachidonic Acid*

The protonated molecular ions of fraction 10 of linoleic acid (Figure 1A) and of fraction 21 of arachidonic acid (Fig 1C) were observed at m/z 267 which corresponds to the protonated molecular ion of the reduced form of the 4-POBN/pentyl radical adduct (Figure 2). This radical adduct apparently undergoes reduction during the ionization process of fast atom bombardment. A fragment ion at m/z 251, which corresponds to the loss of an oxygen atom from the protonated molecular ion of the reduced form, was observed in the CF-FAB mass spectra of fraction 10 of linoleic acid and of fraction 21 of arachidonic acid (data not shown).

The CF-FAB/MS/MS spectrum of the m/z 267 ion of fraction 10 of the linoleic acid reaction mixture is shown in Figure 3. The fragment ion at m/z 179 may correspond to cleavage **b**, cleavage **a-$O_2$**, or cleavage **d-OH**. All of these ions are con-

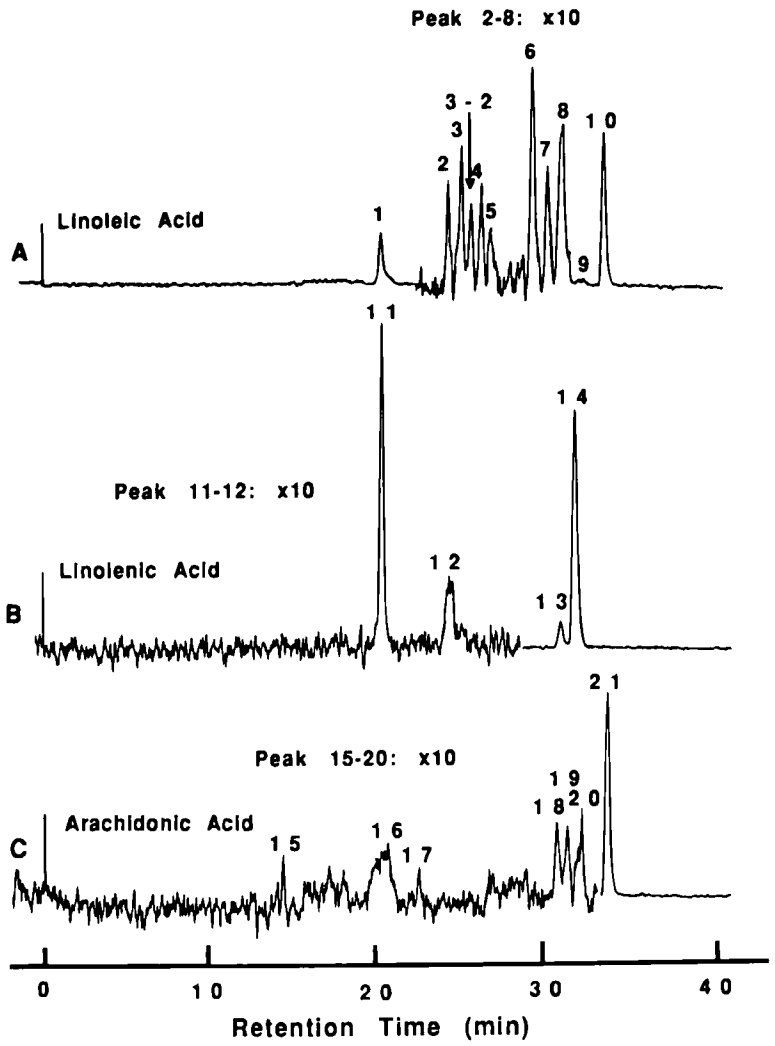



FIGURE 1 HPLC/EPR analyses of the reaction mixtures of A) linoleic acid; B) linolenic acid; and C) arachidonic acid with soybean lipoxygenase.



sistent with the 4-POBN/pentyl radical adduct structure. The CF-FAB/MS/MS spectrum of the m/z 267 ion from fraction 21 of arachidonic acid (data not shown) is identical with that shown in Figure 3 for linoleic acid.

The electrospray ionization mass spectra of fraction 10 of linoleic acid and fraction 21 of arachidonic acid give a protonated molecular ion at m/z 266 (data not shown). This ion corresponds to the protonated molecular ion of the radical form of the 4-POBN/pentyl radical adduct (Figure 2). Unlike the FAB ionization process, reduction of the radical adduct did not occur during the electrospray ionization process. Ions at m/z 531 and m/z 445 were also observed in the electrospray mass spectra, and these ions corre-

TABLE I HPLC/EPR Retention Times and Molecular Ion Mass Numbers of the Reduced 4-POBN Radical Adducts Derived from Linoleic Acid, Linolenic Acid, and Arachidonic Acid

| Linoleic Acid | | | | |
|---|---|---|---|---|
| Fraction No | Retention Time (min) | $(M+H)^+$ m/z | % Peak Area | Radical |
| 1 | 20.5 | 339 | 16.2 | n-octanoic acid |
| 2 | 24.7 | 509 | 3.5 | dihydroxylinoleic acid |
| 3 | 25.5 | 509 | 4.6 | 12, 13-dihydroxylinoleic acid |
| 3-2 | 26.1 | 509 | 2.8 | dihydroxylinoleic acid |
| 4 | 26.7 | 509 | 3.3 | dihydroxylinoleic acid |
| 5 | 27.4 | 491 | 1.9 | 12, 13-epoxylinoleic acid |
| 6 | 29.8 | 491 | 6.8 | 12, 13-epoxylinoleic acid |
| 7 | 30.8 | 491 | 3.7 | epoxylinoleic acid |
| 8 | 31.8 | 491 | 6.3 | 12, 13-epoxylinoleic acid |
| 9 | 33.0 | 491 | 2.3 | epoxylinoleic acid |
| 10 | 34.2 | 267 | 48.5 | n-pentyl |
| Linoleic Acid | | | | |
| Fraction No | Retention Time (min) | $(M+H)^+$ m/z | % Peak Area | Radical |
| 11 | 20.4 | 339 | 8.1 | n-octanoic acid |
| 12 | 24.6 | 225 | 3.8 | ethyl |
| 13 | 31.4 | 265 | 8.9 | pentenyl |
| 14 | 32.2 | 265 | 79.2 | pentenyl |
| Arachidonic Acid | | | | |
| Fraction No | Retention Time (min) | $(M+H)^+$ m/z | % Peak Area | Radical |
| 15 | 15.7 | | 1.0 | |
| 16 | 20.6 | | 9.5 | |
| 17 | 22.5 | | 0.7 | |
| 18 | 31.0 | 515 | 3.0 | 14, 15-epoxyarachidonic acid |
| 19 | 31.4 | 515 | 3.0 | epoxyarachidonic acid |
| 20 | 32.3 | 515 | 3.8 | epoxyarachidonic acid |
| 21 | 34.1 | 267 | 79.1 | n-pentyl |



spond to the protonated dimer ion, $(2M+H)^+$, and the loss of $[(CH_3)_3C(O)NH]$ from the protonated dimer, $[2M + H - (CH_3)_3C\,(O)\,NH]^+$, respectively.

### *Fractions 13 and 14 of Linolenic Acid*

CF-FAB mass analyses of fractions 13 and 14 of linoleic acid give molecular ions at m/z 265, which corresponds to the protonated molecular ion of the reduced form of the 4-POBN/pentenyl radical adduct. The reduction of the spin adduct probably occurs during the FAB ionization process. A fragment ion at m/z 249 corresponding to the loss of an oxygen atom from the protonated molecular ion is observed in the CF-FAB mass spectra (data not shown).

The CF-FAB/MS/MS spectra of the m/z 265 ion from fraction 13 of linolenic acid is shown in Figure 4. Structurally-significant ions are observed at m/z 196, m/z 179, and m/z 123. These ions correspond to the **d** cleavage, **d-OH** cleavage, and **a-c-OH** cleavage, respectively. These ions indicate that the analyte is the 4-POBN/pentenyl radical adduct. The CF-FAB/MS/MS spectra of the fractions 13 and 14 are nearly identical, indicating that they are isomers

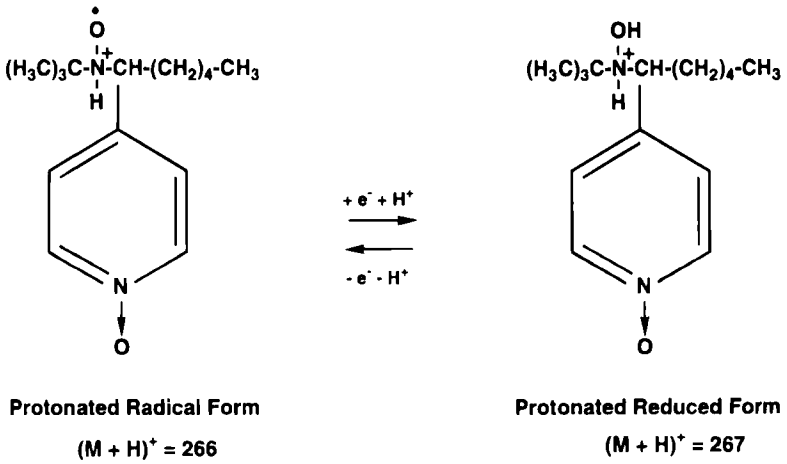



FIGURE 2 Structures of reduced and radical forms of 4-POBN/pentyl radical adduct.

of the 4-POBN/pentenyl radical adduct (data not shown). The data do not permit differentiation of the isomers as to whether they are *cis/trans* and/or positional isomers (1- and 3-pentenyl).

Electrospray ionization of fractions 13 and 14 of linolenic acid gives molecular ions at m/z 264, which corresponds to the protonated radical form of 4-POBN/pentenyl radical adducts. Reduction of the radical adduct did not occur during the electrospray ionization process.

### *Fraction 1 of Linoleic Acid and Fraction 11 of Linolenic Acid*

The CF-FAB mass spectra of fraction 1 of linoleic acid and fraction 11 of linolenic acid, which have similar retention times in the HPLC/EPR elution profiles (Figure 1), show identical protonated molecular ions at m/z 339 (Table 1). This ion corresponds to the protonated molecular ion of the reduced form of the 4-POBN/octanoic acid radi-

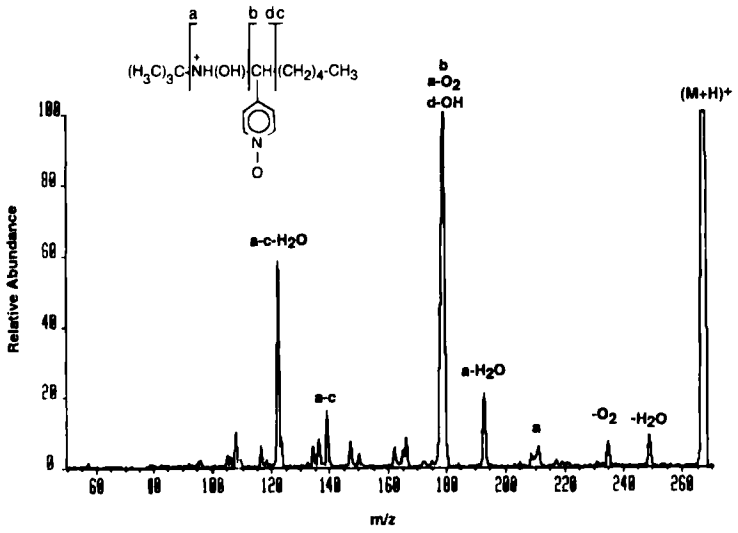



FIGURE 3 Coaxial CF-FAB/MS/MS spectrum of the m/z 267 ion from fraction 10 of linolenic acid.

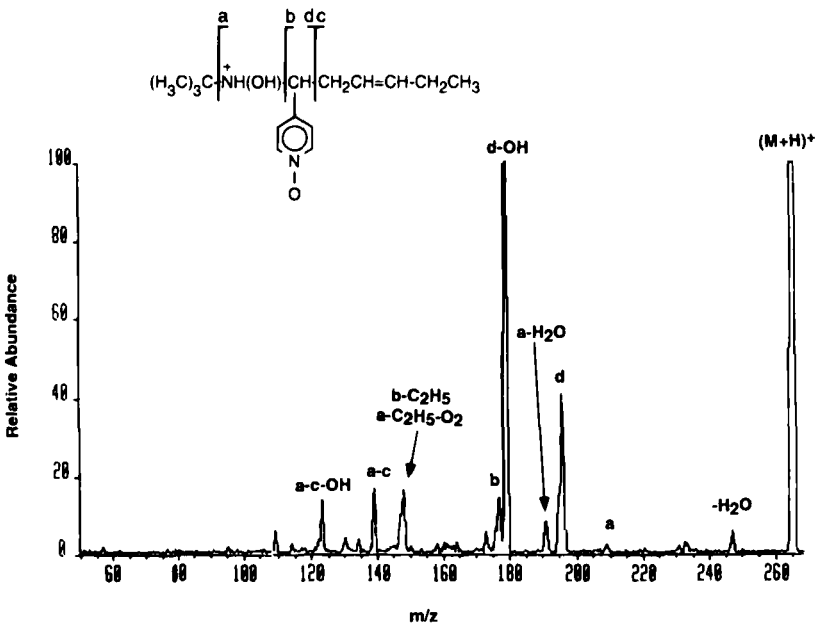



FIGURE 4 Coaxial CF-FAB/MS/MS spectrum of the m/z 265 ion from fraction 13 of linoleic acid.

cal adduct. A fragment ion indicating loss of an oxygen atom is also observed in the mass spectra of the two fractions.

The CF-FAB/MS/MS mass spectra of fraction 1 of linoleic acid and fraction 11 of linolenic acid show several structurally informative fragment ions. Fragment ions corresponding to loss of water, loss of water and $C_4H_8$, and loss of $(CH_3)_3C(O)NH$ are all observed (data not shown). These ions indicate that the radical adduct is the 4-POBN/octanoic acid radical adduct.

Electrospray ionization spectra of these same fractions show protonated molecular ions at m/z 338 which corresponds to the protonated molecular ion of the 4-POBN/octanoic acid radical adduct. Ions at m/z 360 and m/z 251 are also observed, corresponding to $(M + Na)^+$ and $(M + H - N(O)C(CH_3)_3)]^+$, respectively.

### *Fractions 5, 6, 7, 8, and 9 of Linoleic Acid*

CF-FAB analyses of fractions 5, 6, 7, 8, and 9 of linoleic acid give protonated molecular ions at m/z 491. This ion corresponds to the protonated reduced form of the 4-POBN/epoxylinoleic acid radical adduct and/or the 4-POBN/alkoxylinoleic acid radical adduct. It again appears that reduction is occurring during the FAB ionization process. A fragment ion at m/z 475, loss of an oxygen atom, is also observed in these spectra.

The CF-FAB/MS/MS spectra of the m/z 491 ions from fractions 5, 6, 7, 8, and 9 are shown in Figures 5A–E. Several structurally informative fragment ions are observed. The fragment ions corresponding to cleavages of **a-g-$H_2O$** (m/z 304), **b-g-O** (m/z 275), **b-e** and/or **a-e-$O_2$** (m/z 264, and **b-f** and/or **a-f-$O_2$** (m/z 260) are indicative of a 4-POBN/12,13-epoxylinoleic acid radical adduct where the radical center is at carbon-9. It would be difficult to assign structures to these ions if the radical adducts were derived from the 4-POBN/13- (or 9-) alkoxylinoleic acid radicals. Fractions 5 and 6 are probably due to the *cis*- and *trans*-isomers of the 4-POBN/12,13-epoxylinoleic acid radical adducts.

In Figure 5D, the MS/MS spectrum of fraction 8 shows ions corresponding to cleavage of **a-g-$H_2O$** (m/z 304) and **a-g-$O_2$** (m/z 289), which indi-

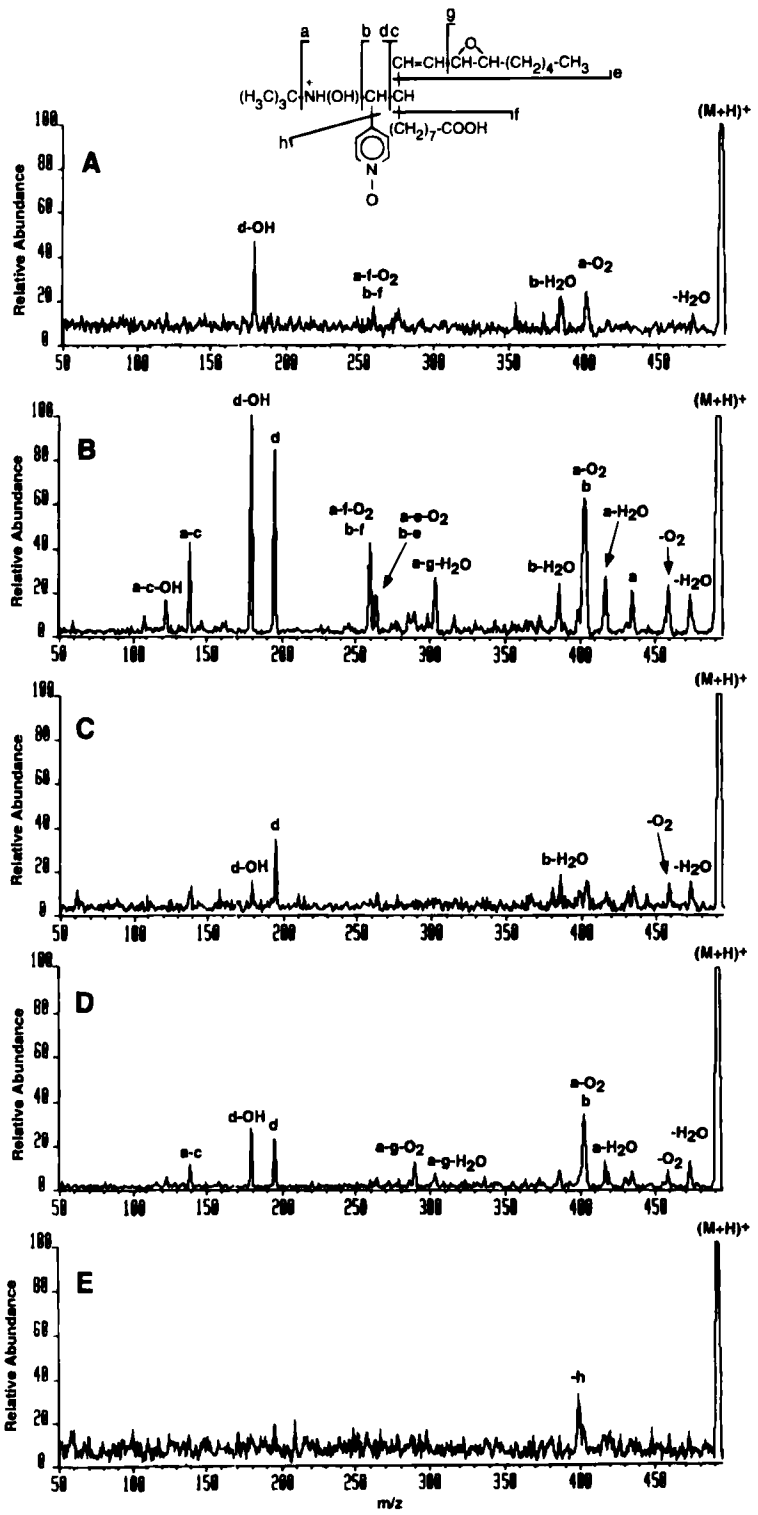



FIGURE 5 Coaxial CF-FAB/MS/MS spectrum of the m/z 491 ion from A) fraction 5; B) fraction 6; C) fraction 7; D )fraction 8; E) fraction 9 of linoleic acid.

cates that this compound is the 4-POBN/12,13-epoxylinoleic acid radical. No ions which give information about the radical center were detected for this fraction. Since a radical center at C-11 is also possible in the 12,13-epoxylinoleic acid radical (see Scheme 1), this compound may correspond to the 4-POBN/C-11 carbon-centered 12,13-epoxylinoleic acid radical adduct. In this case, it would also be difficult to assign structures to the m/z 304 and m/z 289 ions based on a 4-POBN/13- (or 9-) alkoxylinoleic acid radical adduct.

The CF-FAB/MS/MS spectra of fractions 7 and 9 (Figure 5C and E) did not give any fragment ions which revealed information about the structure of the epoxylinoleic acid moiety. Possible structures of these fractions are the *cis*- and *trans*-isomers of 4-POBN/9,10-epoxylinoleic acid radical adducts. In a previous spin-trapping experiment, in which nitrosobenzene and 2-methyl-2-nitrosopropane were used as spin trapping reagents, the formation of the carbon-centered 12,13-epoxylinoleic acid radical was also observed.[21]

Electrospray ionization mass analysis of fractions 6, 7, and 8 of linoleic acid also give ions at m/z 491 which correspond to the protonated molecular ion of the 4-POBN/epoxylinoleic acid radical adducts. These m/z 491 ions appear to be a protonated reduced form of the 4-POBN / epoxylinoleic acid radical adduct. We cannot determine whether reduction occurs during the ESI process or not because of the relatively poor mass resolution (1.1 or 1.2) necessary to have sufficient sensitivity.

### *Fraction 18, 19, and 20 of Arachidonic Acid*

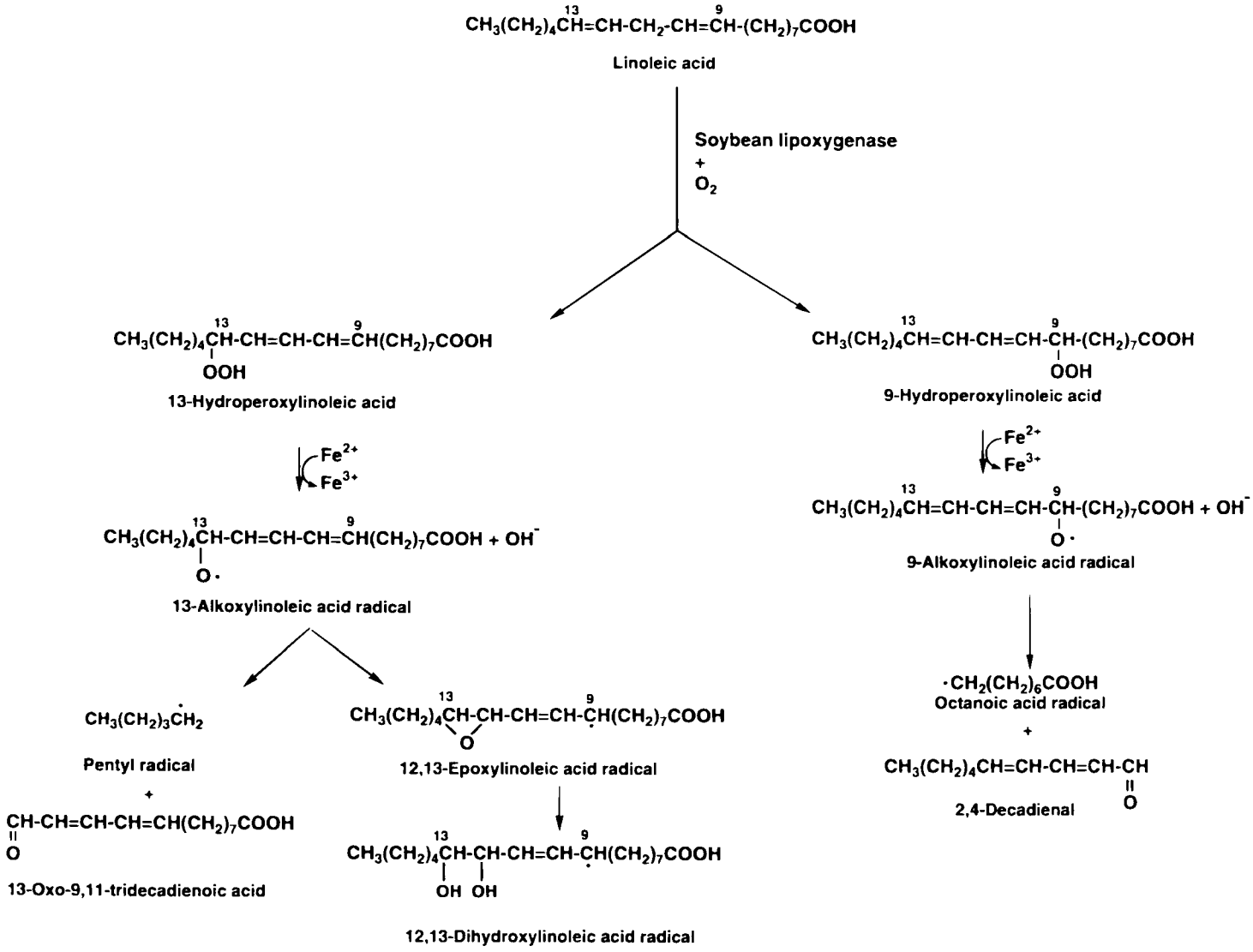
The CF-FAB mass analyses of fractions 18, 19, and 20 from arachidonic acid give protonated molecular ions at m/z 515 in all three cases. This molecular ion corresponds to the protonated reduced form of 4-POBN/epoxyarachidonic acid radical adducts and/or 4-POBN/alkoxyarachidonic acid radical adducts. These species were also reduced during the FAB ionization process. Fragment ions at m/z 499, which correspond to the loss of an oxygen atom from the m/z 515, are also observed in these data.

The CF-FAB/MS/MS spectra of the m/z 515 ions of fraction 18, 19, and 20 of arachidonic acid are shown in Figures 6A-C, respectively. The fragment ions corresponding to cleavages of **b-e** and/or **a-e-$O_2$** at m/z 288 and cleavages of **b-f** and/or **a-f-$O_2$** at m/z 260 indicate that fraction 18 is the 4-POBN/14,15-epoxyarachidonic acid radical adduct where the radical center is at C-11 (Figure 6A). It would be very difficult, on the other hand, to assign these fragment ions to the 4-POBN/15- (or 11-) alkoxyarachidonic acid radical adduct. We, therefore, have assigned the m/z 515 ion to the 4-POBN/14,15-epoxyarachidonic acid radical adduct. In a previous spin-trapping experiment, in which nitrosobenzene and 2-methyl-2-nitrosopropane were used as the spin trapping reagent, the formation of the carbon-centered 14,15-epoxyarachidonic acid radical was also observed.[21]

The CF-FAB/MS/MS spectra of fractions 19 and 20 are of significantly lower abundance (Figure 6B and C) with fewer fragment ions being observed. These fractions are presumably *cis/trans* and/or positional isomers of fraction 18, but there is insufficient information in the MS/MS spectra to establish the structure of the epoxyarachidonic acid moiety.

### *Fractions 2, 3, 3-2, and 4 of Linoleic Acid*

The CF-FAB analyses of fractions 2, 3, 3-2, and 4 from linoleic acid show protonated molecular ions at m/z 509. This mass corresponds to the protonated molecular ion of the reduced form of the 4-POBN/dihydroxylinoleic acid radical adduct. Again, the radical adducts were apparently reduced during the FAB ionization process. Ions at m/z 491 were also observed in the mass spectra of fractions 3 and 4. These ions correspond to the loss of water from the protonated molecular ion. An additional fragment ion at




Scheme 1. Proposed scheme for the formation of pentyl radical, 12, 13-dihydroxylinoleic acid, and n-octanoic acid radical from linoleic acid.

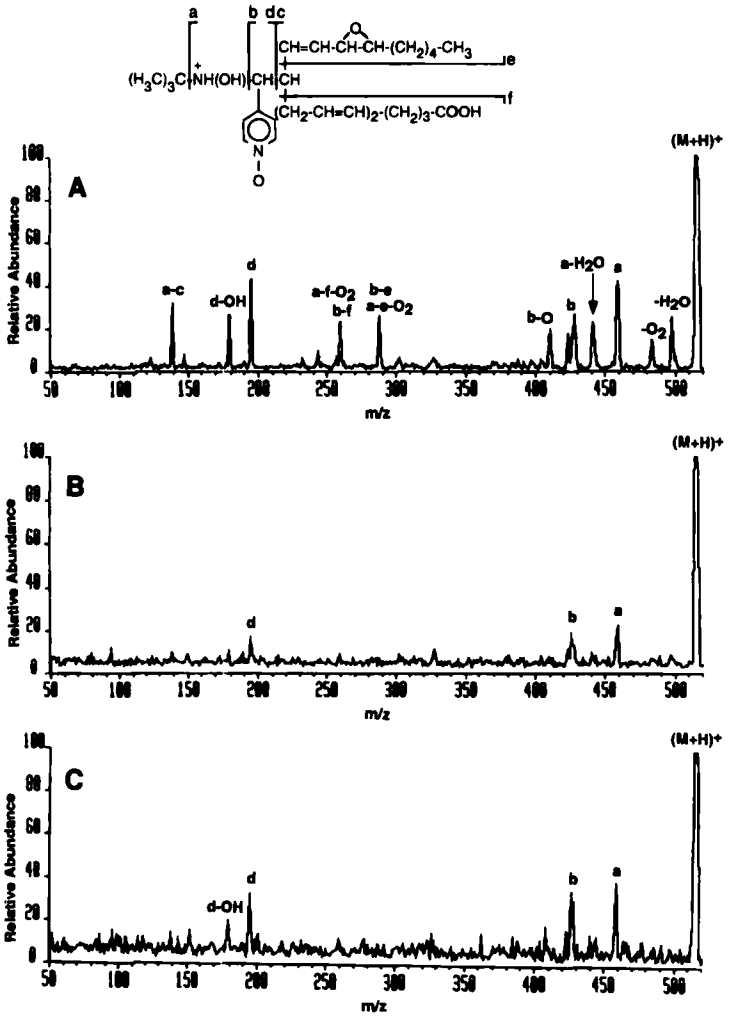



FIGURE 6 Coaxial CF-FAB/MS/MS spectra of the m/z 515 ion from A) fraction 18; B) fraction 19; and C) fraction 20 of arachidonic acid.

m/z 475, which corresponds to loss of an oxygen atom from the ion of m/z 491, is observed in the CF-FAB spectrum of fraction 3 of linoleic acid.

The CF-FAB/MS/MS spectra of the $(M + H)^+$ ion at m/z 509 and the $(M + H - H_2O)^+$ ion at m/z 491 of fraction 3 are shown in Figures 7A and B, respectively. These data are consistent with the structure being that of the reduced 4-POBN/ 12,13-dihydroxylinoleic radical adduct. In Figure 7B, the ions of m/z 276, m/z 265, and m/z 260 arising from the cleavage of **a-f-O**, **b-e** and/or **a-e-$O_2$**, and **b-f** and/or **a-f-$O_2$**, respectively, indicate that the radical center of the reduced 4-POBN/ 12,13-dihydroxylinoleic acid radical spin adduct is the carbon at C-9.

The CF-FAB/MS/MS spectra of fractions 2, 3-2, and 4 were of significantly lower abundance. These peaks are presumably *cis/trans* and/or positional isomers of fraction 3, but there was insufficient information in the MS/MS spectra to establish the structure of the dihydroxylinoleic acid moiety.

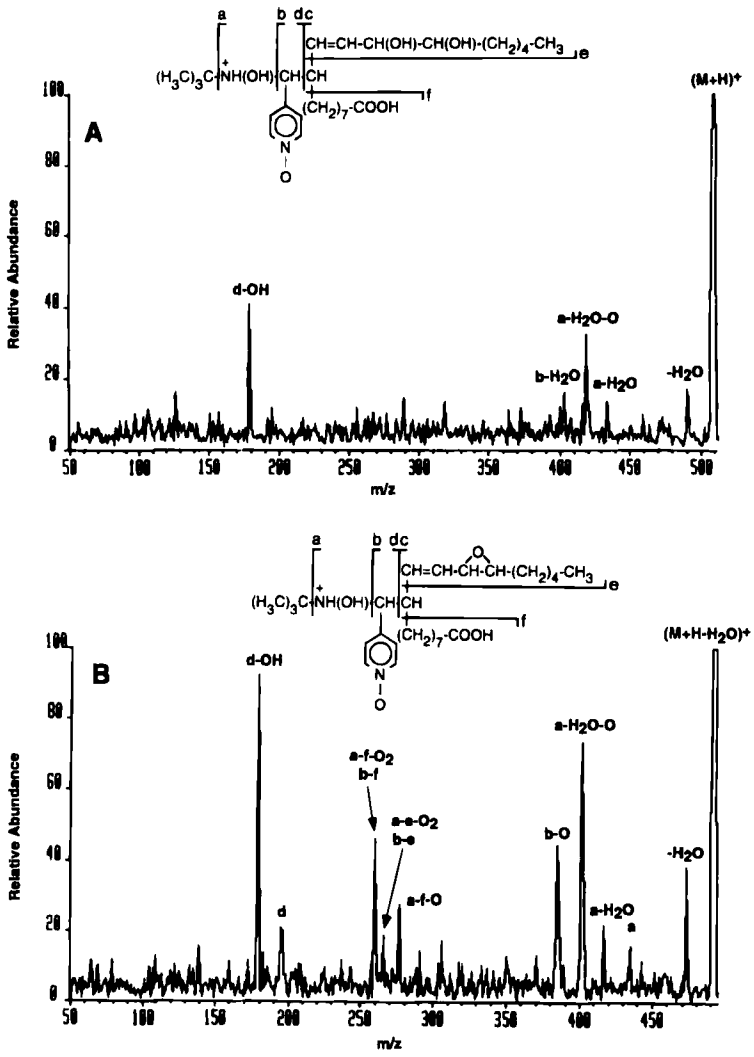



FIGURE 7 Coaxial CF-FAB/MS/MS spectra of the A) m/z 509 ion and B) m/z 491 ion (m/z 509 - $H_2O$) from fraction 3 of linoleic acid.

### *Fraction 12 of Linolenic Acid*

The CF-FAB analysis of the fraction 12 of the linolenic acid reaction mixture gives a protonated molecular ion at m/z 225. This molecular ion corresponds to the protonated reduced form of the 4-POBN/ethyl radical adduct. This radical adduct is also reduced during the FAB ionization process.

The CF-FAB/MS/MS spectra of the ion of m/z 225 of fraction 12 of linoleic acid is shown in Figure 8. Observation of the ions at m/z 179 and m/z 139 is consistent with the structure being the 4-POBN/ethyl radical adduct. These fragment ions correspond to the cleavages **d-OH** and **a-c**, respectively.

### Time Course Experiments

Time course experiments of linoleic acid, linolenic acid, and arachidonic acid were per-

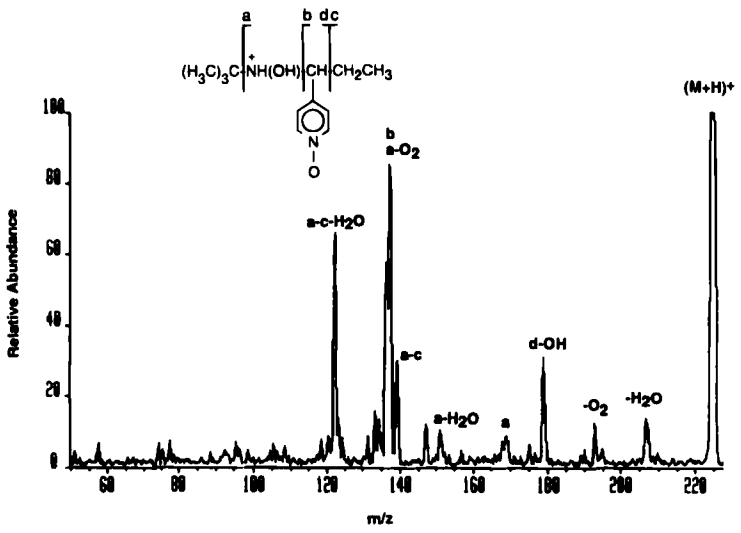


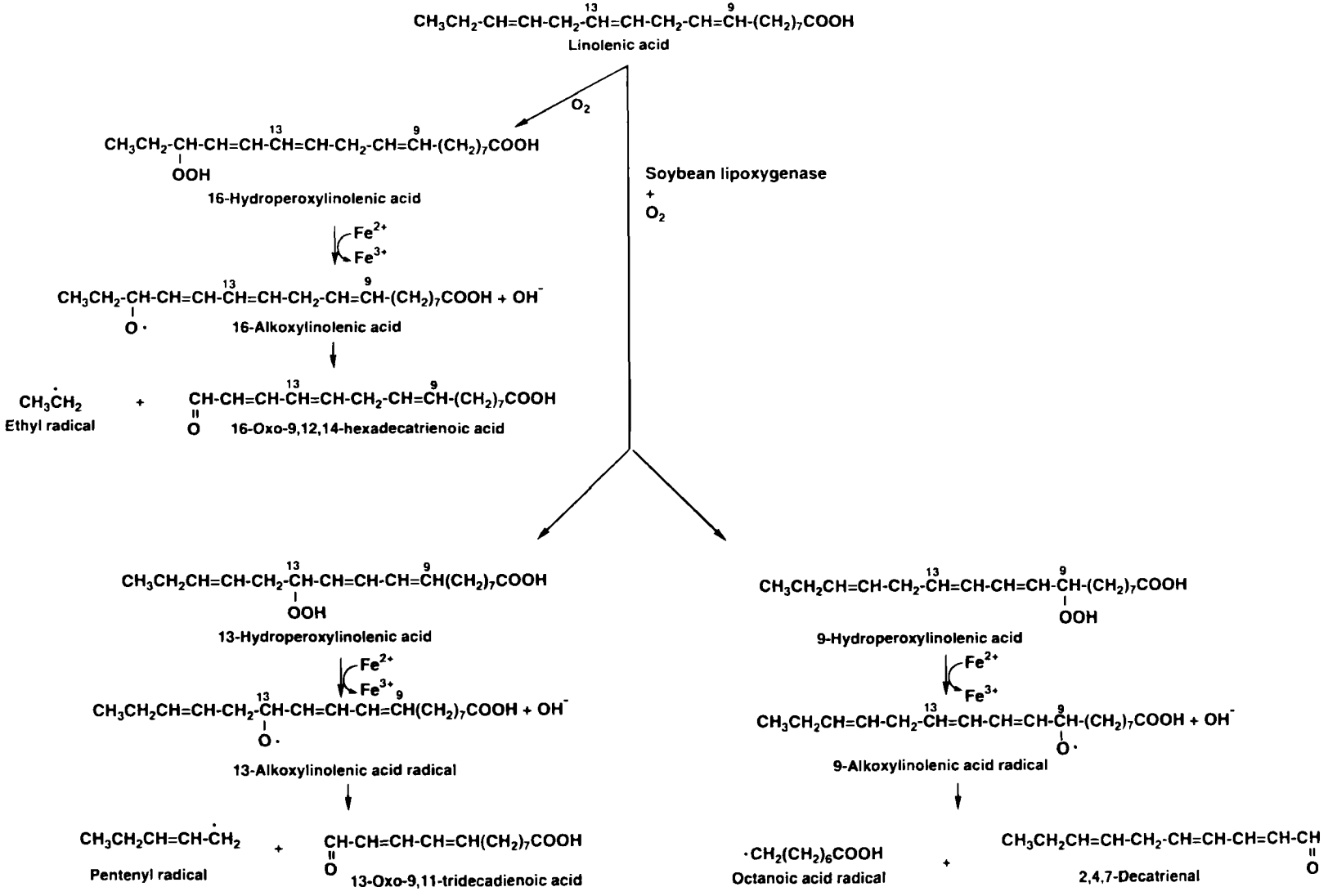

FIGURE 8 Coaxial CF-FAB/MS/MS spectrum of the m/z 225 ion from fraction 12 of linolenic acid.

formed and monitored by HPLC/EPR (Table 2). The ratio of fraction 10 (pentyl radical) peak area to fraction 1 (octanoic acid radical) peak area in linoleic acid is almost constant throughout the experiment: 3.5 (0.17 hr), 3.4 (1 hr), 3.9 (4 hr), 3.7 (8 hr), and 3.0 (24 hr). The average ratio of these peak areas is 3.5, which indicates that 3.5 times as much of the pentyl radical is formed as the octanoic acid radical if the trapping efficiencies and stabilities are the same for the two 4-POBN radical adducts. The pentyl radical and octanoic acid radical are formed from 13-hydroperoxylinoleic acid and 9-hydroperoxylinoleic acid, respectively (Scheme 1). The relative ratio of formation of the pentyl radical over the octanoic acid radical is quite reasonable, because soybean lipoxygenase peroxidizes predominantly at the C-13 position of linoleic acid.[42–44] If regiospecificity is estimated from our ratio, the 13-hydroperoxylinoleic acid:9-hydroperoxylinoleic acid = 78:22, which is in the range of reported values.[42–44]

Fractions 2, 3, 3-2, and 4 in linoleic acid were very weak at 0.17 hr (Table 2). The area of these fractions relative to the area of fractions 5, 6, 7, 8, and 9 gradually increased over time. This time course experiment may indicate that the 4-POBN/12,13-dihydroxylinoleic acid radical adducts (fractions 2, 3, 3-2, and 4) may be formed by addition of water to the epoxide in the 4-POBN/12,13-epoxylinoleic acid radical adducts (fractions 5, 6, 7, 8, and 9). The formation of dihydroxyl compounds from epoxides was described by Chan and Coxon.[45]

Fractions 13 and 14 of linolenic acid are *cis/trans* and/or positional (1-pentenyl or 3-pentenyl radical) isomers of pentenyl radicals. The ratio of fraction 13 peak area to fraction 14 peak area in linolenic acid decreased with time (Table 2): 0.40 (0.017 hr), 0.35 (1 hr), 0.19 (4 hr), 0.14 (8 hr) and 0.11 (24 hr). The time course of the relative fraction areas, therefore, probably indicates that fraction 14 radical adduct is the more stable isomer, i.e., the *trans* isomer in the case of *cis/trans* isomers or the 3-pentenyl radical in the case of positional isomers.

The pentenyl radical and octanoic acid radical are formed from 13-hydroperoxylinolenic acid and 9-hydroperoxylinolenic acid, respectively (Scheme 2). The ratio of the sum of fractions 13 and 14 (pentenyl radical) peak areas to fraction 11 (octanoic acid radical) in linolenic acid is




Scheme 2. Proposed scheme for the formation of ethyl radical, pentenyl radical, and n-octanoic acid radical from linolenic acid.

TABLE II Time Course of HPLC/EPR Peak Area

| Time (hr) | Linoleic Acid Fraction Number | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 3-2 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| 0.17 | 2.8 | 1.1 | 0 | 1.0 | 0 | 0 | 2.4 | 1.7 | 4.6 | 2.6 | 9.9 |
| 1 | 5.6 | 1.1 | 0 | 1.1 | 0.8 | 0.5 | 3.4 | 2.0 | 3.8 | 2.3 | 19.0 |
| 4 | 9.0 | 2.1 | 1.2 | 2.2 | 1.1 | 1.2 | 6.0 | 2.0 | 4.0 | 2.1 | 35.0 |
| 8 | 13.6 | 1.6 | 2.8 | 1.1 | 1.7 | 1.7 | 4.1 | 5.2 | 4.0 | 3.3 | 50.2 |
| 24 | 16.2 | 3.5 | 4.6 | 2.8 | 3.3 | 1.9 | 6.8 | 3.7 | 6.3 | 2.3 | 48.5 |

Linolenic Acid

| Time (hr) | Fraction Number | | | |
|---|---|---|---|---|
| | 11 | 12 | 13 | 14 |
| 0.017 | 0.0 | 0.0 | 4.0 | 10.1 |
| 1 | 11.0 | 0.8 | 2.6 | 7.5 |
| 4 | 4.2 | 2.1 | 3.5 | 18.5 |
| 8 | 4.4 | 3.0 | 4.2 | 29.9 |
| 24 | 8.1 | 3.8 | 8.9 | 79.2 |

Arachidonic Acid

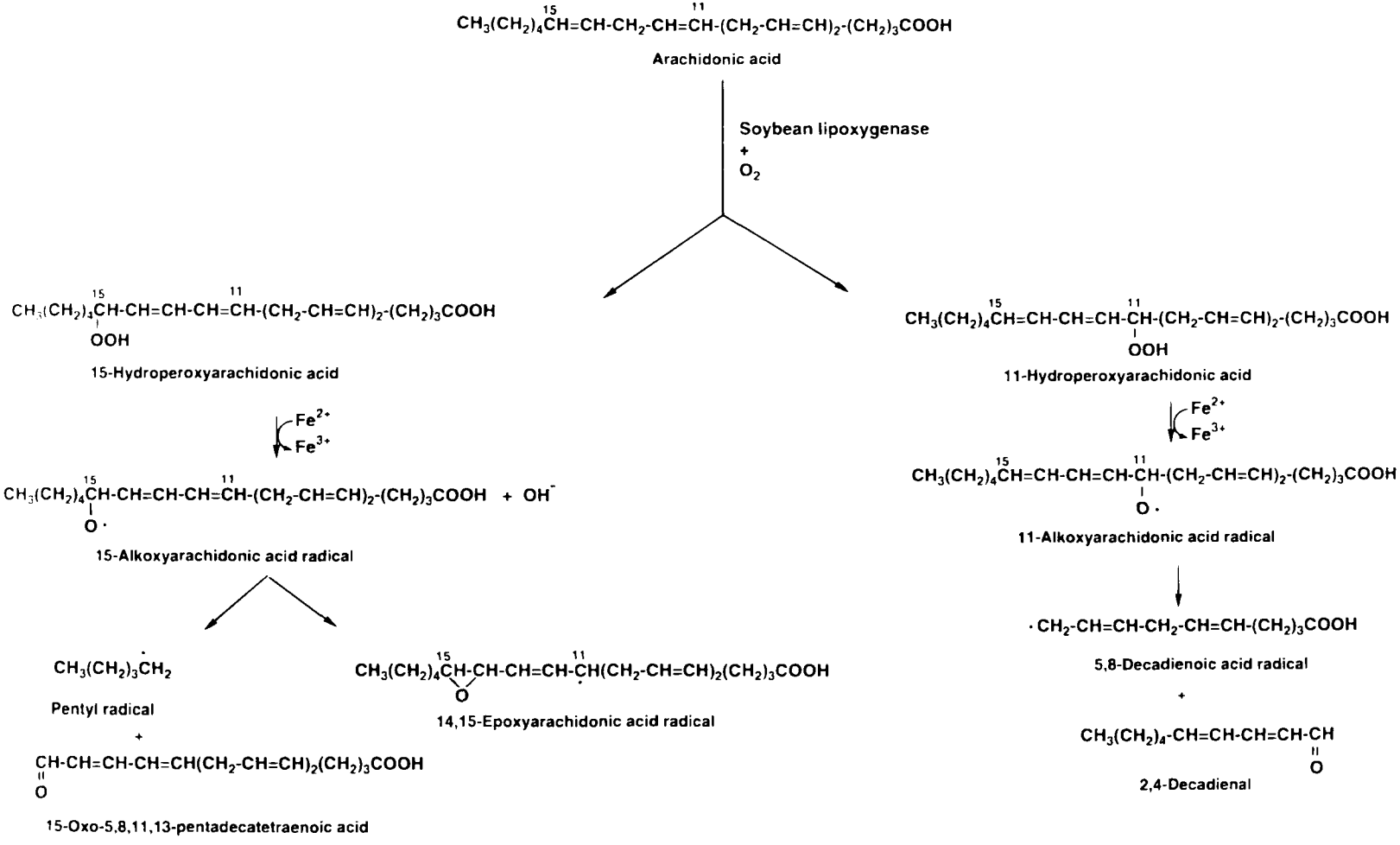
| Time (hr) | Fraction Number | | | |
|---|---|---|---|---|
| | 18 | 19 | 20 | 21 |
| 0.17 | 1.5 | 1.4 | 1.1 | 14.6 |
| 1 | 1.6 | 1.1 | 2.4 | 16.1 |
| 4 | 1.6 | 1.1 | 1.9 | 29.6 |
| 8 | 2.5 | 1.9 | 2.7 | 44.5 |
| 24 | 3.0 | 3.0 | 3.8 | 79.1 |



almost constant throughout the time course experiment (Table 2): 9.2 (1 hr), 5.3 (4 hr), 7.8 (8 hr) and 11 (24 hr). The average ratio of the sum of fractions 13 and 14 (pentenyl radical) peak areas to fraction 11 (octanoic acid radical) peak area is 8.3, which indicates that 8.3 times as much pentenyl radical is formed as octanoic acid radical (assuming that trapping efficiencies and stabilities are the same for the two 4-POBN radical adducts). These results are quite reasonable because soybean lipoxygenase is known to peroxidize predominantly at the C-13 position of linolenic acid.[46,47]

Fraction 12 (ethyl radical) in linolenic acid only appeared at 1 hr after the reaction started (Table 2). The ethyl radical, therefore, seems to be formed in a secondary process or minor enzymatic process. The 16-hydroperoxylinolenic acid, which is the precursor of the ethyl radical (Scheme 2), could be formed from auto-oxidation or minor soybean lipoxygenase-catalyzed peroxidation of linolenic acid. Because of the lag time, it is unlikely that soybean lipoxygenase directly catalyzes the formation of the 16-hydroperoxylinoleic acid. More likely, oxidizing free radicals produced during the reaction of linolenic acid hydroperoxide with lipoxygenase cause this co-oxidation. The ethyl radical was not detected in the linolenic acid reaction mixture without lipoxygenase, excluding auto-oxidation.

The 5,8-decadienoic acid radical, which could be formed from 11-hydroperoxyarachidonic acid, was not detected under these reaction conditions (Scheme 3). This result is quite reasonable because soybean lipoxygenase is known to catalyze peroxidation predominantly at the 15-carbon of arachidonic acid (Scheme 3).[46,47]




Scheme 3. Proposed scheme for the formation of pentyl radical and 14, 15-epoxyarachidonic acid radical from arachidonic acid.

## DISCUSSION

In this study we have identified the 4-POBN radical adducts of n-octanoic acid radical, 12,13-epoxylinoleic acid radical, 12,13-dihydroxylinoleic acid radical, pentyl radical, ethyl radical, *cis/trans* or positional isomers of pentenyl radical, and 14,15-epoxyarachidonic acid radical in the reaction mixtures of linoleic acid, linolenic acid, and arachidonic acid with soybean lipoxygenase. Possible reaction paths for the formation of these radicals are shown in Schemes 1-3. The pentyl radical (linoleic acid and arachidonic acid) and the ethyl radical (linolenic acid) could be precursors of pentane and ethane. Pentane and 13-oxo-9,11-tridecadienoic acid which form from the 13-alkoxylinoleic acid radical (Scheme 1) were detected in the reaction mixture of soybean lipoxygenase with linoleic acid.[48] Garssen *et al.* proposed the mechanism of the formation of pentane via the pentyl radical as shown in Scheme 1.[49] 13-Oxo-9,11-tridecadienoic acid, which could form concurrently, was also detected by Garssen *et al.*[48] On the other hand, formation of ethane and pentane has been proposed as a sensitive index for lipid peroxidation in toxicological studies.[50,51] The 12,13-epoxylinoleic acid radical detected here could be the precursor of the following various kinds of linoleic acid-related compounds. The products found include: linoleic acid dimers containing epoxide from the reaction mixture of linoleic acid and its hydroperoxide with soybean lipoxygenase,[49] methyl 11-(2,2,5,7,8-pentamethyl-6-oxychroman)-*cis*-12,13-epoxy-*trans*-9-octadecenoate and methyl 11-(2,2,5,7,8-pentamethyl-6-oxychroman)-*trans*-12,13-epoxy-*trans*-9-octadecenoate from the reaction mixture of methyllinoleate hydroperoxide and α-tocopherol model compound,[52] 9-oxo-*trans*-12, 13-epoxy-*trans*-10-octadecenoic acid, 9-oxo-*cis*-12,13-epoxy-*trans*-10-octadecenoic acid, 11-hydroxy-*trans*-12,13-epoxy-*cis*-9-octadecenoic acid, 11-hydroxy-*trans*-12,13-epoxy-*trans*-9-octadecenoic acid from the reaction mixtures of 13-hydroperoxylinoleic acid with $FeCl_3$-cysteine,[53] hemoglobin,[54] and soybean lipoxygenase,[55] respectively, and *trans*-12,13-epoxy-9-hydroperoxy-*trans*-10-octadecenoic acid from the reaction mixture of 13-hydroperoxylinoleic acid with cysteine-$FeCl_3$.[56] Octanoic acid radical and 2,4-decadienal could be concomitantly formed.

During the reaction process, some oxygen-centered radicals should be formed (Scheme 1-3), but under the reaction conditions employed here, 4-POBN/oxygen-centered radical adducts were not detected. The 4-POBN/oxygen-centered radical adducts are known to be very unstable.[7]

It is thought that linoleic acid radical, linolenic acid radical, and arachidonic acid radical, formed by abstraction of a hydrogen atom from C-11 in linoleic acid and linolenic acid, and from C-13 in arachidonic acid, respectively, could be formed during the initial step of peroxidation. These carbon-centered radicals, however, were not detected under our reaction conditions. All of the other 4-POBN/carbon-centered radical adducts such as n-octanoic acid radical, 12,13-epoxylinoleic acid radical, 12,13-dihydroxylinoleic acid radical, pentyl radical, ethyl radical, pentenyl radicals, and the 14,15-epoxyarachidonic acid radical are relatively stable, as shown in this paper.

The CF-FAB analyses of the 4-POBN radical adducts gave characteristic fragment ions in which an oxygen atom is eliminated from protonated molecular ions. This fragment ion was detected for almost all of the 4-POBN radical adducts analyzed here. The loss of 16 amu appears to be a characteristic of 4-POBN radical adducts. One possible candidate for the oxygen atom is the nitroxide group in 4-POBN. The fragment ions which are observed from the MS/MS experiments give important structural information allowing identification of the 4-POBN radical adducts. The tandem MS includes a kind of purification process in its measurement, i.e., a molecular ion selected by MS-I is forced to fragment, with the fragment ions being mass-analyzed by MS-II. We believe

that MS/MS may give sufficient information to identify the radical adducts in other complicated biological systems where the radical adducts are sufficiently stable and are produced in high enough concentrations.

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