In vitro production of long chain pyrrole fatty esters from carbonyl–amine reactions[1]

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Abstract The reaction of lipoxygenase pathway products methyl 9,10(*Z*)-epoxy-13-oxo-11(*E*)-octadecenoate (13-MZEO) and methyl 12,13(*Z*)-epoxy-9-oxo-10(*E*)-octadecenoate (9-MZEO), with butylamine and lysine was studied in order to investigate whether some oxidized fatty acids are able to react with amino groups, analogously to volatile aldehydes, and, therefore, contribute to the overall protein damage produced during oxidative stress. The reaction mixtures were incubated at room temperature or 37°C; the products were fractionated by semipreparative high performance liquid chromatography, and characterized by ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy and mass spectrometry. The products identified were methyl 9-hydroxy-9-(5-pentyl-*N*-alkylpyrrole-2-)nonanoate and *N*-alkyl-2-pentylpyrrole for the 13-MZEO, and methyl 8-[5-(1'-hydroxyhexyl)-*N*-alkylpyrrole-2-]octanoate and methyl 8-(*N*-alkylpyrrole-2-)octanoate for the 9-MZEO. Formation of these pairs of analogs suggested that compounds were produced by one mechanism with the loss of the 5-substituent as a short-chain aldehyde, which was identified for the 13-MZEO. A reaction mechanism that explains the formation of all these compounds is proposed and the biological significance of these findings in relation to low density lipoprotein oxidation is discussed. Although these results do not demonstrate the formation of long-chain pyrrole fatty acids in vivo, they suggest that these pyrroles could be produced as an ultimate step in the lipid peroxidation process.—**Hidalgo, F. J., and R. Zamora.** In vitro production of long chain pyrrole fatty esters from carbonyl–amine reactions. *J. Lipid Res.* 1995. **36:** 725–735.

Supplementary key words lipoxygenase pathway • oxidized lipids/proteins reactions • polyunsaturated fatty acid oxidation • oxidative stress • lysine modification • protein damage • LDL oxidation

It is now well established that free radicals (especially $O_2^{\cdot -}$) and other reactive oxygen species (such as H_2O_2) are continuously produced in vivo. Consequently, organisms have evolved not only antioxidant defense systems to protect against them, but also repair systems that prevent the accumulation of damaged molecules (1–4). Under physiological conditions, the range of defense systems available within cells is adequate to protect them against oxidative damage. However, the protective balance can be lost because of overproduction of free radicals that overwhelm the antioxidant defenses or by inadequate intake of nutrients that contribute to the defense system (5). This imbalance, namely oxidative stress, can produce major interrelated derangements of cell metabolism, including DNA-strand breakage, rises in intramolecular "free" Ca^{2+}, damage to membrane ion transporters and/or other specific proteins, and peroxidation of lipids (6–9).

Lipids are a highly vulnerable target for oxygen species due to the susceptibility of polyunsaturated fatty acids to free radical attack ultimately forming lipid hydroperoxides (10). These hydroperoxides can then be converted into a variety of secondary products including both nonvolatile fatty acid derivatives and volatile products. Although volatile products are only a small part of decomposition products of hydroperoxides, for example Fe(II)/Fe(III)-catalyzed linoleic acid hydroperoxide decomposition produced volatiles comprising less than 5 mol% of the total products (11), these volatile products have received much more attention than nonvolatile products. This is probably due to an easier availability of these compounds. The aldehydic lipid peroxidation products are biologically very active and can produce a number of deleterious effects in cells (12, 13). These aldehydes, and other products of lipid peroxidation, are more stable than free radical species and may more readily diffuse into cellular media, where they are available for facile reaction with various biomolecules. Modification of proteins and

Abbreviations: 9-LOOH, 9-hydroperoxy-10(*E*)-12(*Z*)-octadecadienoic acid; 9-MEEO, methyl 12,13(*E*)-epoxy-9-oxo-10(*E*)-octadecenoate; 9-MZEH, methyl 12,13(*Z*)-epoxy-9-hydroxy-10(*E*)-octadecenoate; 9-MZEO, methyl 12,13(*Z*)-epoxy-9-oxo-10(*E*)-octadecenoate; 13-LOOH, 13-hydroperoxy-9(*Z*)-11(*E*)-octadecadienoic acid; 13-MEEO, methyl 9,10(*E*)-epoxy-13-oxo-11(*E*)-octadecenoate; 13-MZEH, methyl 9,10(*Z*)-epoxy-13-hydroxy-11(*E*)-octadecenoate; 13-MZEO, methyl 9,10(*Z*)-epoxy-13-oxo-11(*E*)-octadecenoate; CC, column chromatography; GC–MS, gas chromatography coupled with mass spectrometry; HPLC, high performance liquid chromatography; HPLC–MS, HPLC coupled with mass spectrometry; LDL, low density lipoproteins; NMR, nuclear magnetic resonance; NMR signals multiplicity: *s*, singlet; *d*, doublet; *dd*, double doublet; *t*, triplet; *q*, quartet; *br*, broad; TLC, thin-layer chromatography.

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other biomolecules by lipid peroxidation products is believed to play a central role in many pathophysiological conditions often associated with free radical damage (14–16). The mechanisms and relative contributions of several potential reactions, however, are not well understood (17).

In this context, previous research from this laboratory has shown that volatile short-chain aldehydes having a 4,5-epoxy-1-oxo-2-pentene system, which have been detected in several systems (18, 19), are very reactive with amines, amino acids, and proteins producing brown color and fluorescence (20). The reaction of 4,5(*E*)-epoxy-2(*E*)-heptenal with lysine was studied and different 1-alkyl-2-(1'-hydroxypropyl)pyrroles and 1-alkylpyrroles were identified (21). These 1-alkyl-2-(1'-hydroxypropyl)pyrroles were the responsible for the color and fluorescence production via a polymerization reaction that was characterized, and this reaction has been suggested as an alternative mechanism for in vivo production of brown macromolecular pigments with fluorescent characteristics similar to lipofuscins (22).

Epoxyoxoene fatty acids with analogous 4,5-epoxy-1-oxo-2-pentene system are produced when fatty acid hydroperoxides are treated with different catalysts containing iron, such as $FeCl_3$/cysteine (23), hemoglobin (24), and lipoxygenase from various plant sources. Thus a crude soy extract converted 13-hydroperoxy-9(*Z*)-11(*E*)-octadecadienoic acid (13-LOOH), or linoleic acid, into numerous products, from which 12,13(*E*)-epoxy-9-oxo-10(*E*)-octadecenoic acid was isolated (25). This last fatty acid seems to be a final product in the degradation of the hydroperoxide, which involves the formation of an intermediate epoxyhydroperoxy fatty acid [12,13(*E*)-epoxy-9-hydroperoxy-10(*E*)-octadecenoic acid] (23). Analogous to the above short-chain epoxyaldehydes, epoxyoxoene fatty acids would also be able to react with the amino group of amines, amino acids, and proteins, producing similar pyrroles.

Long-chain *N*-heterocyclic fatty acids have not been found in nature (26). However, long-chain pyrrole fatty acids have been detected when a furan fatty acid, 9-(5-butyl-2-furyl)nonanoic fatty acid, was incubated with bovine liver homogenate at pH 7.5 in the presence of cofactors known to stimulate fatty acid oxidation (27). The aim of this study was the isolation and characterization of the reaction products between the epoxyoxoene fatty acids formed in the lipoxygenase pathway, and amines and amino acids, in order to investigate reaction pathways between oxidized lipids and proteins. These studies should clarify whether epoxyoxoene fatty acids are, in fact, final products in the lipoxygenase pathway, and should allow synthesis of pure long-chain pyrrole fatty acids in milligram amounts so that they could be used in structural identifications and biological testings.

MATERIALS AND METHODS

Materials

Linoleic acid and soybean lipoxygenase were purchased from Fluka Chemie AG (Buchs, Switzerland). *n*-Butylamine and L-lysine were purchased from Aldrich Chemical (Milwaukee, WI). All other chemicals used were analytical grade and were purchased from reliable commercial sources.

Chromatographic and spectroscopic techniques

Thin-layer chromatography (TLC). TLC was performed on precoated silica gel $60F_{254}$ plates (Merck, Darmstadt, Germany) with detection with UV light (254 nm), Erlich reagent, picric acid as specific reagent for epoxides (28), and/or by charring with sulfuric acid.

Column chromatography (CC). CC was performed with silica gel 60 (70–230 mesh; Merck). Five milliliter fractions were collected and tested by TLC. Organic solutions were dried with anhydrous sodium sulfate and concentrated under vacuum at a temperature below 40°C.

High performance liquid chromatography (HPLC). The HPLC system consisted of a 126 Programmable Delivery Module and a 168 Diode Array Detector Module (Beckman, Fullerton, CA). Data acquisition and processing were carried out using Software System Gold 7.1 version (Beckman). Analytical separations were carried out on a Spherisorb ODS2 3-μm 25 × 0.46-cm column. A loop of 20 μl and a flow rate of 0.8 ml/min were used in the analytical experiments. Semipreparative separations were carried out on a Spherisorb ODS2 5-μm 25 × 1-cm column. A loop of 500 μl and a flow rate of 5 ml/min were used in these experiments. A temperature of 40°C and UV detection at 250 nm were selected in all the experiments. In the preparative separations, products were collected from successive injections and combined. Solutions were dried using nitrogen to remove acetonitrile, and were freeze-dried.

Gas chromatography coupled with mass spectrometry analyses (GC–MS). GC–MS analyses were conducted with a Hewlett-Packard 5890 Series II gas chromatograph (Hewlett-Packard, Palo Alto, CA) interfaced, via an open coupling system, to an AEI-MS/70VG mass spectrometer (VG Analytical, Manchester, UK). A DB-5 fused-silica capillary column (J & W Scientific, Folsom, CA), 30 m × 0.25 mm I.D. was used in all the experiments. The following column temperature conditions were programmed: method A, from 200 (9 min) to 250°C at 4°C/min; method B, from 100 (2 min) to 250°C at 2°C/min; method C, from 100 (2 min) to 250°C at 4°C/min; method D, from 200 (2 min) to 280°C at 4°C/min. The MS conditions were as follows: ionization by electron impact, 70 eV; accelerating voltage, 4 kV; emission current, 100 μA; ion source temperature, 220°C.

HPLC coupled with mass spectrometry analyses (HPLC-MS). An HPLC system analogous to the one used for analytical and semipreparative separations was used for HPLC-MS analyses. The HPLC column was a C_{18}, 5-μm particle size, 25 × 0.46 cm I.D. reversed-phase column. A 100 μl loop was used in the injector. The UV detector was in-line with the Universal Interface (Vestec, Houston, TX). Thus the total-ion current trace obtained from the MS data system could be directly compared with the UV chromatogram obtained from the UV detector. The Universal Interface was connected to the momentum separator via a Teflon tubing, and this separator was connected to a standard VG ion source of the AEI-MS/70VG mass spectrometer via a quartz tube. The temperature of the momentum separator was 135°C and the ion source temperature was maintained at 220°C. In a typical experiment the parameters of the Universal Interface were set as follows: input helium pressure, 1 bar; tip temperature, 160°C; separator temperature, 45°C; spray temperature, 70°C. The ion source temperature was 220°C, the electron energy was 70 eV, the accelerating voltage was 7.0 kV, and the trap current was 100 μA.

Nuclear magnetic resonance (NMR) experiments. ^{1}H and ^{13}C NMR at 300 and 75.4 MHz, respectively, were determined in a Bruker AC-300P (Karlsruhe, Germany), with Me_4Si as internal standard. For aqueous solutions, sodium 3-(trimethylsilyl)-1-propanesulfonate was used as internal standard. Two-dimensional NMR was used to assign ^{13}C NMR spectra.

Preparation of epoxyoxoene fatty esters

Chemical syntheses of methyl 9,10(*Z*)-epoxy-13-oxo-11(*E*)-octadecenoate (13-MZEO), methyl 9,10(*E*)-epoxy-13-oxo-11(*E*)-octadecenoate (13-MEEO), methyl 12,13(*Z*)-epoxy-9-oxo-10(*E*)-octadecenoate (9-MZEO), and methyl 12,13(*E*)-epoxy-9-oxo-10(*E*)-octadecenoate (9-MEEO) were carried out following a previously described method (29). Briefly, linoleic acid was oxidized by soybean lipoxygenase to produce mainly the corresponding 13-LOOH, and also a small quantity of the corresponding 9-LOOH. Reduction of the hydroperoxides with sodium borohydride followed by oxidation, esterification, and epoxidation yielded the corresponding epoxyoxoene fatty esters.

Reaction of epoxyoxoene fatty esters with butylamine

A solution of 9- and 13-MZEO (or 9- and 13-MEEO) (67.2 mg, 0.21 mmol) in chloroform (2 ml) was treated with butylamine (97 μl, 0.98 mmol) and stirred at 37°C. After overnight incubation, the organic solvent was evaporated, and 5 ml of acetonitrile-water 2:1 was added. The resulting solution was studied by GC-MS and fractionated by semipreparative HPLC using acetonitrile-water 2:1 as eluent. Isolated compounds were studied by ^{1}H and ^{13}C NMR and MS.

Reaction of epoxyoxoene fatty esters with L-lysine

A solution of 9- and 13-MZEO (200 mg, 0.62 mmol) in 9 ml of acetonitrile-water 2:1 was treated with L-lysine (362 mg, 2.48 mmol) and stirred at room temperature for 6 days. After that time, the reaction mixture was diluted to 30 ml with acetonitrile-water 2:1 and then fractionated by semipreparative HPLC using analogous conditions as described above. Isolated compounds were studied by ^{1}H and ^{13}C NMR and MS.

RESULTS

Chemical synthesis of epoxyoxoene fatty esters

Oxidation of linoleic acid with soybean lipoxygenase mainly produced 13-LOOH. However, a small quantity of the corresponding 9-LOOH was also formed depending on the conditions used during the oxidation. For most of the reactions described in this study, this mixture of hydroperoxides was not fractionated and the subsequent

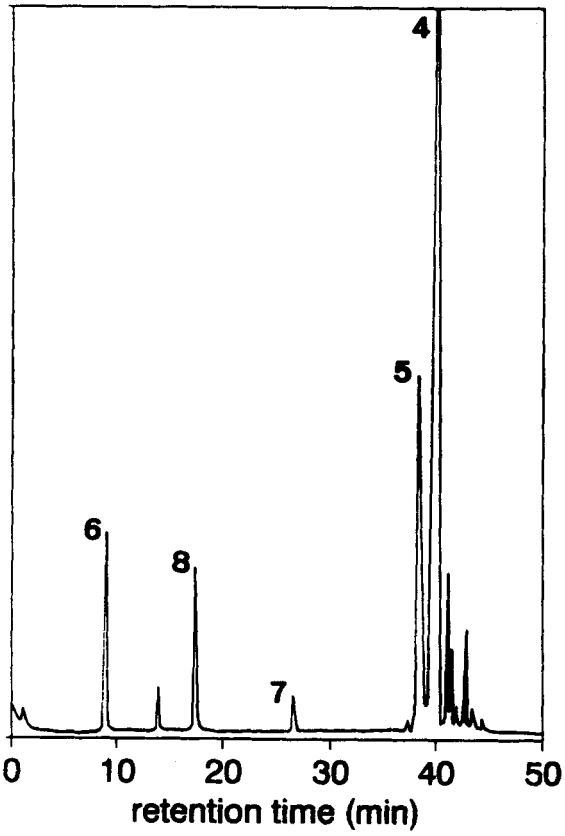


Fig. 1. Total ion chromatogram of GC-MS analysis for the reaction mixture of epoxyoxoene fatty esters (13-MZEO and 9-MZEO) and butylamine in chloroform after stirring overnight at 37°C. The reaction mixture (2 μl) was injected in the chromatograph and separated on a 30-m DB-5 fused silica capillary column using the following temperature conditions: from 100 (2 min) to 250°C at 4°C/min. Peaks are numbered according the structures of the different compounds which are collected in Fig. 2.

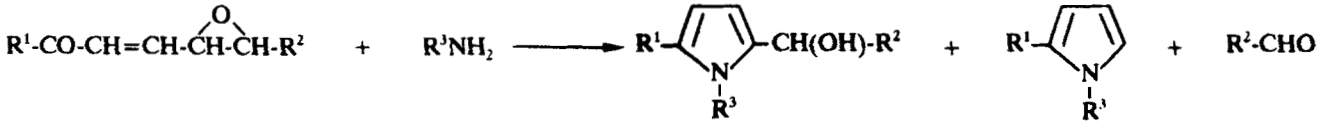
reduction of hydroperoxides, oxidation of hydroxyesters, esterification and epoxidation yielded a mixture of epoxyoxoene fatty esters that were fractionated by CC using hexane–diethyl ether 4:1 as eluent. Under these conditions two fractions were obtained, one containing 9- and 13-MZEO and the other containing 9- and 13-MEEO. Both mixtures could be easily identified by ^{1}H-NMR. Thus the coupling constants for epoxy ring protons ($J_{9,10}$ for the 13-MZEO and 13-MEEO, and $J_{12,13}$ for the 9-isomers) were 4.5 Hz for *cis* epoxy protons and 2.1 Hz for *trans* epoxy protons, in accordance with the literature (29). When the mixture of hydroperoxides was fractionated and only 13-LOOH was used in the preparation of epoxyoxoene fatty esters, the products obtained were diasteromerically pure 13-MZEO and 13-MEEO (29).

The obtained mixtures, one containing 9- and 13-MZEO and the other containing 9- and 13-MEEO, could not be easily fractionated. However, the presence of the two isomers in each mixture could be deduced from the ^{13}C NMR spectra that presented split signals for most of the carbons. The proportion of the two isomers could be obtained from the integral of the carbonyl signals in these spectra. The fractionation of each mixture by GC (method A) showed two peaks with analogous mass spectra. To confirm the assigned structures, the mixture of 9- and 13-MZEO was reduced with sodium borohydride and the corresponding epoxyhydroxyene fatty esters were sililated and studied by GC–MS (method B). Mass spectra of trimethylsilyl derivatives of methyl 9,10(*Z*)-epoxy-13-hydroxy-11(*E*)-octadecenoate (13-MZEH) and methyl 12,13(*Z*)-epoxy-9-hydroxy-10(*E*)-octadecenoate (9-MZEH) allowed distinguishing between them. Mass spectrometry of trimethylsilyl derivative of 13-MZEH yielded characteristic fragment ions at *m/e* 327, 259, and 173, according to a previous study (29). The most characteristic fragment ion of trimethylsilyl derivative of isomer 9-MZEH was at *m/e* 241. In addition, two decomposition products of 9- and 13-MZEH were observed by GC–MS. They were, respectively, methyl 12-oxo-8,10-decadienoate and methyl 9-oxononanoate. The ratio between these last two compounds was similar to the peak area ratio in the ^{13}C NMR spectra of 9- and 13-epoxyoxoene fatty esters, and provided an additional measurement of the ratio of isomers in the mixture.

The presence of two isomers in the starting epoxyoxoene fatty esters complicated the fractionation of their reaction products with amines and amino acids, but the use of these mixtures was preferred because it better resembled the actual mixtures present in nature for lipid peroxidation products.

Reaction of epoxyoxoene fatty esters with butylamine

The reaction of epoxyoxoene fatty esters 9- and 13-MZEO, or 9- and 13-MEEO, with butylamine produced different compounds. **Figure 1** shows the total ion gas chromatogram (method C) obtained after overnight incubation of the mixture. After this time, the original epoxyoxoene fatty esters were not detected and the forma-



1-2	3	4-5	6-7	8-9

Compound	R^1	R^2	R^3
1	$(CH_2)_4CH_3$	$(CH_2)_7CO_2CH_3$	-
2	$(CH_2)_7CO_2CH_3$	$(CH_2)_4CH_3$	-
3	-	-	$(CH_2)_3CH_3$
4	$(CH_2)_4CH_3$	$(CH_2)_7CO_2CH_3$	$(CH_2)_3CH_3$
5	$(CH_2)_7CO_2CH_3$	$(CH_2)_4CH_3$	$(CH_2)_3CH_3$
6	$(CH_2)_4CH_3$	-	$(CH_2)_3CH_3$
7	$(CH_2)_7CO_2CH_3$	-	$(CH_2)_3CH_3$
8	-	$(CH_2)_7CO_2CH_3$	-
9	-	$(CH_2)_4CH_3$	-

Fig. 2. Structure of the compounds identified in the reaction between epoxyoxoene fatty esters and butylamine. Most compounds were isolated by semipreparative high performance liquid chromatography using the conditions described in the Methods section, and identified by ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy and mass spectrometry. Compound **8** was identified as its butylimino derivative. Compound **9** was not isolated in this study, possibly because headspace GC analysis was not used.

TABLE 1. ^{1}H NMR chemical shifts and coupling constants (in Hz) of selected signals (pyrrole moieties and characteristic substituents) of the compounds described in this study

Compound	H-9	H-10	H-11	H-12	H-13	H-1'[a]
4	4.56*t* J = 6.8		6.04*d* $J_{11,12}$ = 3.6	5.83*d* $J_{11,12}$ = 3.6		3.88*m* (2H)
5		5.83*d* $J_{10,11}$ = 3.6	6.04*d* $J_{10,11}$ = 3.6		4.56*t* J = 6.8	3.88*m* (2H)
7		5.86*ddd* $J_{8,10}$ = 0.8 $J_{10,11}$ = 3.1	6.06*t* J = 3.1	6.57*dd* $J_{10,12}$ = 1.9 $J_{11,12}$ = 2.6		3.77*t* (2H) $J_{1',2'}$ = 7.4
11	4.57*t* J = 7.1		5.94*d* $J_{11,12}$ = 3.6	5.73*d* $J_{11,12}$ = 3.6		3.84*m* (2H)
17		5.75*m*	5.92*t* J = 3.1	6.57*dd* $J_{10,12}$ = 1.8 $J_{11,12}$ = 2.7		3.83*t* (2H) $J_{1',2'}$ = 7.2
18		5.75*m*	5.96*t* J = 3.1	6.77*dd* $J_{10,12}$ = 1.8 $J_{11,12}$ = 2.7		4.43*dd* (1H)

[a]Signal H-1' includes the integral to distinguish between α- and ε-derivatives of lysine in compounds **11**, **17**, and **18**. When numbering the protons, the products are considered fatty acid derivatives and, therefore, it conserves the numbering of the fatty acid chain.

tion of several new compounds was observed. More than 80% of the total area corresponded to peaks 4 and 5 in the chromatogram which were lately identified as two C_{18} pyrrole fatty esters produced by reaction of 13- and 9-MZEO (or MEEO), respectively, and butylamine. Two other peaks (peaks 6 and 7) corresponded to short chain pyrroles produced by cleavage of the carbon chain. The peak number in the total ion gas chromatogram is in accordance with the numbering and structures for the different identified compounds that are collected in **Fig. 2.** Major compounds of the reaction were isolated by semipreparative HPLC and studied by ^{1}H and ^{13}C NMR and MS. Minor compounds were studied only by GC–MS and/or HPLC–MS.

The major product of the reaction was identified as methyl 9-hydroxy-9-(5-pentyl-*N*-butyl-1H-pyrrole-2-)nonanoate (**4**). GC, R_t 40.3 min (method C). Semipreparative HPLC, R_t 16.8 min. ^{1}H NMR ($CDCl_3$)[3] showed the selected signals listed in **Table 1.** Other signals appeared at δ 0.92 *t* (3H, $J_{17,18}$ = 6.8 Hz, H18), 0.96 *t* (3H, $J_{3',4'}$ = 7.3 Hz, H4'), 1.35 *m* (14H, H4-H7, H16, H17, and H3'), 1.62 *m* (6H, H3, H15, and H2'), 1.87 *m* (2H, H8), 2.30 *t* (2H, J = 7.5 Hz, H2), 2.51 *t* (2H, J = 7.9 Hz, H14), and 3.66 *s* (3H, OCH_3). ^{13}C NMR ($CDCl_3$) showed the selected signals listed in **Table 2.** Other signals appeared at δ 13.91 *q* (C4'), 14.11 *q* (C18), 20.34 *t* (C3'), 22.61 *t* (C17), 24.97 *t* (C3), 26.44 *t* (C14), 26.50 *t* (C7), 28.33, 29.13, 29.24 and 29.41 (4*t*, C4, C5, C6, and C15), 31.92 *t* (C16), 33.91 *t* (C2'), 34.12 *t* (C2), 36.53 *t* (C8), 51.52 *q* (OCH_3), and 174.39 *s* (C1). GC–MS *m/e* (relative intensity, ion structure): 361 (57, M^+-H_2O), 304 (100, 361-butyl), 232 (88, 361-methoxycarbonylpentyl). HPLC–MS *m/e* (relative intensity, ion structure): 379 (5, M^+), 361 (61, M^+-H_2O), 330 (13, M^+-H_2O-methoxy), 304 (100, 361-butyl), 232 (98, 361-methoxycarbonylpentyl), 222 (91, M^+-methoxycarbonylheptyl). MS of its trimethylsilyl derivative (70 eV) *m/e* (relative intensity, ion structure): 451 (6, M^+), 380 (8, M^+-pentyl), 294 (74, M^+-methoxycarbonylheptyl), 138 (59), 80 (100).

The corresponding C_{18} pyrrole fatty ester of the 9-isomer was identified as methyl 8-[5-(1'-hydroxyhexyl)-*N*-butyl-1H-pyrrole-2-]octanoate (**5**). GC, R_t 38.6 min (method C). Semipreparative HPLC, R_t 16.3 min. This compound yielded essentially the same ^{1}H NMR spectrum as that of compound **4,** and only slight differences were observed in its ^{13}C NMR spectrum. ^{13}C NMR ($CDCl_3$) showed the selected signals listed in Table 2. Other signals appeared at δ 13.82 *q* (C4'), 14.06 *q* (C18), 20.29 *t* (C3'), 22.63 *t* (C17), 24.94 *t* (C3), 26.46 *t* (C8), 26.22, 28.50, 29.13, 29.21 and 29.45 (5*t*, C4-C7 and C15), 31.81 *t* (C16), 33.89 *t* (C2'), 34.11 *t* (C2), 36.50 *t* (C14), 51.53 *q* (OCH_3), and 174.35 *s* (C1). GC–MS *m/e* (relative intensity, ion structure): 361 (46, M^+-H_2O), 318 (51, 361-propyl), 218 (100, 361-methoxycarbonylhexyl). HPLC–MS *m/e* (relative intensity, ion structure): 379 (4, M^+), 361 (37, M^+-H_2O), 318 (44, 361-propyl), 218 (100, 361-methoxycarbonylhexyl).

[3]When assigning signals in NMR spectra, we have preferred to number the compounds as fatty ester derivatives for an easier interpretation, and to consider pyrrole protons and carbons as belonging to the carbon chain of the fatty esters. Therefore, numbers in the assignation of the spectra and in systemic names of compounds are not necessarily equivalent.

TABLE 2. ^{13}C NMR chemical shifts of selected signals (pyrrole moieties and characteristic substituents) of the compounds described in this study

Compound	C-9	C-10	C-11	C-12	C-13	C-1'
4	66.48*d*	134.46*s*	104.33*d*	104.02*d*	133.93*s*	43.42*t*
5	134.00*s*	104.01*d*	104.30*d*	134.26*s*	66.51*d*	43.42*t*
7	133.04*s*	104.93*d*	106.40*d*	119.60*d*		46.17*t*
11	66.94*d*	134.49*s*	105.88*d*	105.31*d*	134.55*s*	44.12*t*

When numbering the carbons, the products are considered fatty acid derivatives and, therefore, it conserves the numbering of the fatty acid chain.

Peak 7 in the total ion gas chromatogram could also be isolated by semipreparative HPLC and it was identified as methyl 8-(*N*-butyl-1H-pyrrole-2-)octanoate (7). GC, R_t 26.7 min (method C). Semipreparative HPLC, R_t 13.7 min. ^1H NMR ($CDCl_3$) showed the selected signals listed in Table 1. Other signals appeared at δ 0.94 *t* (3H, $J_{3',4'}$ = 7.3 Hz, H4'), 1.35 *m* and 1.63 *m* (14H, H3–H7, H2', H3'), 2.31 *t* (2H, $J_{2,3}$ = 7.5 Hz, H2), 2.51 *t, br* (2H, $J_{7,8}$ = 7.8 Hz, H8), and 3.67 *s* (3H, OCH_3). ^{13}C NMR ($CDCl_3$) showed the selected signals listed in Table 2. Other signals appeared at δ 13.79 *q* (C4'), 20.06 *t* (C3'), 24.92 *t* (C3), 26.18 *t* (C8), 28.79, 29.09, 29.13 and 29.39 (4*t*, C4–C7), 33.52 *t* (C2'), 34.09 *t* (C2), 51.49 *q* (OCH_3), and 174.34 *s* (C1). GC–MS *m/e* (relative intensity, ion structure): 279 (23, M^+), 248 (13, M^+–methoxy), 236 (6, M^+–propyl), 136 (100, M^+–methoxycarbonylhexyl). HPLC–MS *m/e* (relative intensity, ion structure): 279 (15, M^+), 248 (8, M^+–methoxy), 150 (16, M^+–methoxycarbonylpentyl), 136 (100, M^+–methoxycarbonylhexyl).

Peaks 6 and 8 in the total ion gas chromatogram could not be isolated by semipreparative HPLC and they could only be studied by GC–MS. They were identified as *N*-butyl-2-pentylpyrrole (**6**) and methyl 9-butyliminonanoate (**8**), respectively. Compound **6** had an R_t of 9.1 min in GC (method C), and its GC–MS *m/e* (relative intensity, ion structure) was: 193 (60, M^+), 178 (3, M^+–methyl), 164 (3, M^+–ethyl), 150 (33, M^+–propyl), 136 (100, M^+–butyl), 122 (33, M^+–pentyl). Compound **8** had an R_t of 17.6 min in GC (method C), and its GC–MS *m/e* (relative intensity, ion structure) was: 241 (0.6, M^+), 240 (1, M^+–1), 210 (10, M^+–methoxy), 198 (8, M^+–propyl), 112 (34, M^+–methoxycarbonylpentyl), 99 (44), 84 (100, M^+–methoxycarbonylheptyl), 57 (91, butyl).

Reaction of epoxyoxoene fatty esters with lysine

The reaction of epoxyoxoene fatty esters with lysine was analogous to the above described with butylamine. However, the reaction rate was slower and the number of products was higher because epoxyoxoene fatty esters reacted with both α- and ϵ-amino groups of lysine. Unlike the reaction with butylamine, this reaction could not be followed by GC–MS because of the low volatility of the reaction products. However, the formation of the products could be easily deduced by monitoring the reaction using NMR spectroscopy (**Fig. 3** shows the ^1H spectrum obtained after 2 days of reaction). During the reaction, a gradual disappearance of olefinic protons (signals a) was observed together with the appearance of heterocyclic signals. Two types of pyrrolic signals were observed analogous to the reaction with butylamine. The main products were 1,2,5-trisubstituted pyrroles (signals b), and the formation of 1,2-disubstituted pyrroles was also produced (signal c). Isolation of major compounds was carried out by semipreparative HPLC. A characteristic chromatogram of the reaction mixture is shown in **Fig. 4.** Two main fractions were isolated (A and B). The other fractions were much less important quantitatively, in spite of their high absorbance at 250 nm. Fraction A consisted mainly of methyl 9-hydroxy-9-[5-pentyl-*N*-(5'-amino-5'-carboxypentyl)-1H-pyrrole-2-]nonanoate (**11**). However, a minor presence of methyl 9-hydroxy-9-[5-pentyl-*N*-(5'-amino-1'-carboxypentyl)-1H-pyrrole-2-]nonanoate (**12**),

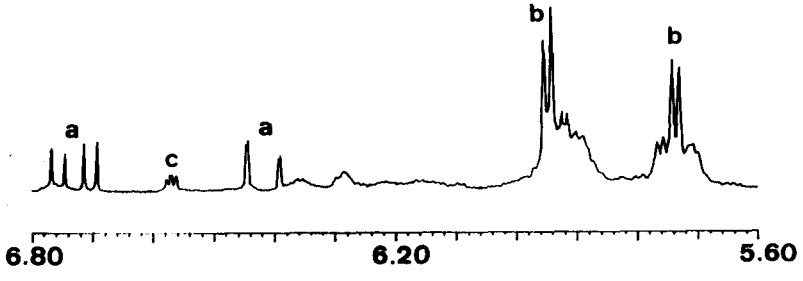


Fig. 3. ^1H Nuclear magnetic resonance spectrum (olefinic and pyrrolic protons portion) of the reaction mixture of epoxyoxoene fatty esters (13-MZEO and 9-MZEO) and lysine in acetonitrile–water 2:1 after 2 days of reaction at room temperature. A 10% solution of the reaction mixture in deuterated methanol was acquired in a Bruker AC-300P spectrometer using a 45° excitation pulse, and a 2.28 s acquisition time. A total of 64 scans were collected. The spectrum showed the signals corresponding to unreacted epoxyoxoene fatty esters (signals a), 1,2,5-trisubstituted pyrroles (signals b), and 1,2-disubstituted pyrroles (signal c).

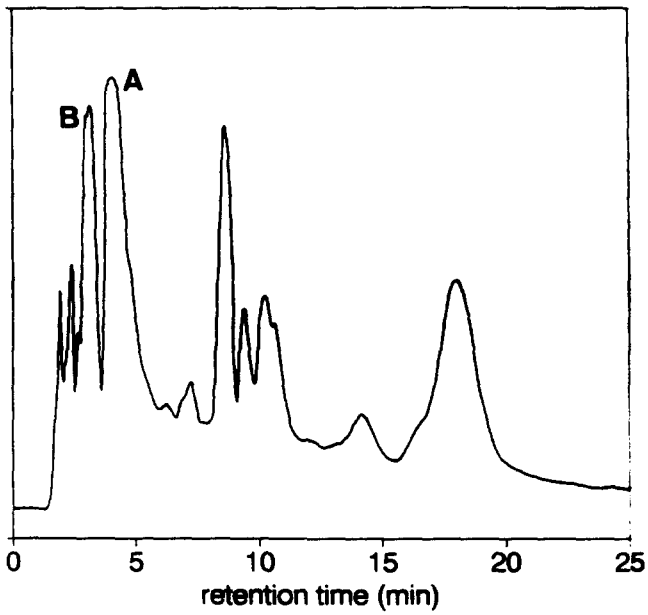
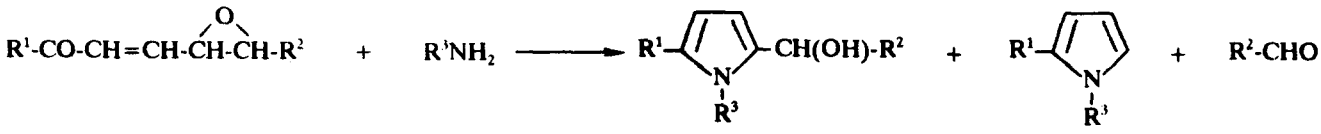


Fig. 4. Semipreparative high performance liquid chromatogram obtained for the reaction mixture of epoxyoxoene fatty esters (13-MZEO and 9-MZEO) and lysine in acetonitrile–water 2:1 after 6 days at room temperature. The reaction mixture was diluted and fractionated on a Spherisorb ODS2 5 μm 25 × 1-cm column at 40°C. A loop of 500 μl, a flow rate of 5 ml/min, and UV detection at 250 nm were used. Two main fractions were isolated (A and B). The other fractions were much less important quantitatively, in spite of their high absorbance exhibited at 250 nm. Fraction A mainly consisted of compound **11.** However minor presence of compounds **12–14** was also detected in this fraction by HPLC–MS. Fraction B was mainly a mixture of compounds **17** and **18.** In addition, presence of compounds **15** and **16** in fraction B was also detected by GC–MS of the methyl esters obtained by reaction with diazomethane. Structures for compounds identified in the reaction of epoxyoxoene fatty esters with lysine are collected in Fig. 5.

and methyl 8-[5-(1′-hydroxyhexyl)-*N*-(5′-amino-5′ or 1′-carboxypentyl)-1H-pyrrole-2-]octanoate (**13** and **14,** respectively), was also detected in this fraction by HPLC–MS. Fraction B was mainly a mixture of methyl 8-[*N*-(5'-amino-5' or 1'-carboxypentyl)-1H-pyrrole-2-]octanoate (**17** and **18,** respectively). In addition, the presence of 2-pentyl-*N*-(5'-amino-5' or 1'-carboxypentyl)-1H-pyrrole (**15** and **16,** respectively) was also detected in fraction B by GC–MS of the methyl esters obtained by reaction with diazomethane. Major isolated compounds were studied by ^{1}H and ^{13}C NMR and/or HPLC–MS. They were also esterified with diazomethane and studied by GC–MS. Structure for compounds identified in the reaction of epoxyoxoene fatty esters with lysine are collected in **Fig. 5.**

Compound **11** had an R_t of 4.27 min by semipreparative HPLC. ^{1}H NMR (CD_3OD) showed the selected signals listed in Table 1. Other signals appeared at δ 0.92 *t* (3H, H18), 1.34, 1.50 and 1.60 (3 *m*, 18H, H3–H7, H15–H17, H3'), 1.85 *m* (6H, H8, H2', H4'), 2.31 *t* (2H, $J_{2,3}$ = 7.4 Hz, H2), 2.53 *t* (2H, $J_{14,15}$ = 7.6 Hz, H14), 3.50 *m* (1H, H5'), and 3.64 *s* (3H, OCH_3). ^{13}C NMR (CD_3OD) showed the selected signals listed in Table 2. Other signals appeared at δ 14.47 *q* (C18), 23.18 *t* (C17), 23.66 *t* (C3'), 25.99 *t* (C3), 27.35 *t* (C14), 27.53 *t* (C7), 29.89, 30.08, 30.12 and 30.35 (4*t*, C4–C6, C15), 32.47 *t* (C2'), 32.54 *t* (C4'), 32.87 *t* (C16), 34.47 *t* (C2), 37.61 *t* (C8), 52.00 *q* (OCH_3), 56.11 *d* (C5'), 176.00 *s* (C1), and 181.05 *s* (C6'). HPLC–MS *m/e* (relative intensity, ion structure): 434 (100, M^+-H_2O), 390 (18, $M^+-H_2O-CO_2$),



1-2 **10** **11-14** **15-18** **8-9**

Compound	R^1	R^2	R^3
10	-	-	$(CH_2)_4CH(COOH)NH_2$
11	$(CH_2)_4CH_3$	$(CH_2)_7CO_2CH_3$	$(CH_2)_4CH(COOH)NH_2$
12	$(CH_2)_4CH_3$	$(CH_2)_7CO_2CH_3$	$CH(COOH)(CH_2)_4NH_2$
13	$(CH_2)_7CO_2CH_3$	$(CH_2)_4CH_3$	$(CH_2)_4CH(COOH)NH_2$
14	$(CH_2)_7CO_2CH_3$	$(CH_2)_4CH_3$	$CH(COOH)(CH_2)_4NH_2$
15	$(CH_2)_4CH_3$	-	$(CH_2)_4CH(COOH)NH_2$
16	$(CH_2)_4CH_3$	-	$CH(COOH)(CH_2)_4NH_2$
17	$(CH_2)_7CO_2CH_3$	-	$(CH_2)_4CH(COOH)NH_2$
18	$(CH_2)_7CO_2CH_3$	-	$CH(COOH)(CH_2)_4NH_2$

Fig. 5. Structure of the compounds identified in the reaction between epoxyoxoene fatty esters and lysine. Major compounds were isolated by semipreparative high performance liquid chromatography using the conditions described in the Methods section, and identified by ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy and mass spectrometry. Structures for compounds **1, 2, 8,** and **9** are given in Fig. 2.

377 (25, M^+-H_2O-butyl), 305 (52, M^+-methoxycarbonylpentyl), 84 (84). GC-MS of the methyl ester obtained by reaction with diazomethane, R_t 27.4 min (method D), *m/e* (relative intensity, ion structure): 448 (100, M^+-H_2O), 417 (100, M^+-H_2O-methoxy), 319 (40, M^+-H_2O-methoxycarbonylpentyl).

Although compound **11** was the major product in the reaction, the presence of three other isomers in fraction A was observed by HPLC-MS. They were tentatively identified as compounds **12, 13,** and **14.** The mass spectrum of isomer **12** was very similar to that described for compound **11.** However, MS of compounds **13** and **14** was slightly different. The presence of 391 and 291 *m/e* ions in the spectra was diagnostic of a pyrrole derived from 9-MZEO.

In addition to the above described formation of 1,2,5-trisubstituted pyrroles, the formation of two types of 1,2-disubstituted pyrroles was observed in fraction *B.*

Compounds **17** and **18** had an R_t of 3.29 min by semipreparative HPLC. The mixture obtained was a 7:3 ratio of ϵ- and α-amino derivatives of lysine as deduced from the integral of heterocyclic protons in the ^1H NMR spectrum. ^1H NMR (CD_3OD) showed the selected signals listed in Table 1. Other signals appeared at δ 1.2-2 *br,m* (16H, H3-H7, H2'-H4'), 2.31 (*t,* 2H, H2), 2.49 and 2.51 (2*t,* 2H, H8), 2.85 *t* (H5' of α-isomer), 3.45 *m* (H5' of ϵ-isomer), and 3.63 and 3.64 (2*s,* OCH_3 of α- and ϵ-isomers, respectively). GC-MS of the methyl esters obtained by reaction with diazomethane, R_t 16.1 min (method D), *m/e* (relative intensity, ion structure): 366 (100, M^+), 334 (49, M^+-CH_3OH), 307 (33, M^+-methoxycarbonyl), 290 (39, M^+-methoxycarbonyl-NH_3), 278 (86, $M^+-CH(NH_2)CO_2CH_3$). These compounds did not give good spectra by HPLC-MS under the assayed conditions, but the *m/e* 334 ion (M^+-H_2O) was observed.

Compounds **15** and **16** were only detected by GC-MS of the methyl esters obtained by reaction with diazomethane, R_t 4.4 min (method D), *m/e* (relative intensity, ion structure): 280 (78, M^+), 221 (19, M^+-methoxycarbonyl), 204 (80, M^+-methoxycarbonyl-NH_3), 192 (90, $M^+-CH(NH_2)CO_2CH_3$), 82 (100).

DISCUSSION

The above results show that epoxyoxoene fatty acids are not necessarily final products in the lipoxygenase pathway, but they are able to react with the amino groups of amines, amino acids, and, possibly, proteins, to produce *N*-substituted long-chain pyrrole fatty acids. Two types of pyrroles have been isolated and characterized in the reaction of 9- and 13-MZEO (and 9- and 13-MEEO) with butylamine and lysine. The major product was a 1,2,5-trisubstituted pyrrole. The production of 1,2-disubstituted pyrroles was also detected. These results are analogous to those obtained in the reaction between 4,5(*E*)-epoxy-2(*E*)-heptenal and amines and amino acids (21, 22), and, therefore, the reaction seems to follow a mechanism similar to the one previously found for short-chain epoxyaldehydes. A possible mechanism that explains the different compounds isolated and characterized in this study is suggested in **Fig. 6.** It implies the formation of an imine at a first stage that would produce the pyrrole ring by intramolecular attack of the nitrogen at one of the epoxy carbons. A simple rearrangement of electrons with transfer of one proton would produce the isolated 1,2,5-trisubstituted pyrroles. The transfer of this proton would probably take place in two steps to avoid the strain involved in the formation of the 4-membered ring needed for a concerted transfer. An alternative pathway would also produce the isolated 1,2-disubstituted pyrroles, which would be accompanied by the formation of an aldehyde in the leaving group. Analogous to the reaction of 4,5(*E*)-epoxy-2(*E*)-heptenal with amines and amino acids, which is accompanied by the formation of propanal (21), the reaction of epoxyoxoene fatty esters with amines and amino acids should also be accompanied by the formation of methyl 9-oxononanoate when starting from the 13-isomer, and hexanal when starting from the 9-isomer. Although hexanal has not been found in this study, possibly because headspace GC analyses have not been carried out, the identification of methyl 9-butyliminonanoate among the products of the reaction between 9- and 13-MZEO and butylamine confirms the proposed pathway. Therefore, this reaction constitutes a new route for formation of short chain aldehydes from oxidized lipids, and can contribute to the overall production of these compounds.

These results may be extended to other oxidized fatty acids having the same 4,5-epoxy-1-oxo-2-pentene system and to other biological amino groups and, therefore, may constitute a new general reaction pathway of amino group modification by long-chain lipid peroxidation products. Epoxyoxoene fatty acids are not uncommon in nature. They are produced from the corresponding fatty acid hydroperoxides via alkoxyl radicals (30). These alkoxyl radicals are able to rearrange intramolecularly by addition to the α,β double bond affording epoxyallylic radicals. Epoxyallylic radicals, which have been spin-trapped (31) and shown to be produced in the oxidation of many polyunsaturated fatty acids including arachidonic acid (30, 32), then combine with oxygen to form epoxyhydroperoxy fatty acids and, in a succeeding step, epoxyoxoene and epoxyhydroxyene fatty acids. The reaction follows a specific mechanism and only certain isomers are produced. Thus, when starting from 13-LOOH, the epoxyoxoene obtained was 12,13-epoxy-9-oxo-10(*E*)-octadecenoic acid. Because the present reaction with amino groups is also specific, the products expected would be the pyrroles derived from 9-MZEO and hexanal. Analogously, if the substrate is arachidonic acid oxidized via the 15-lipoxy-

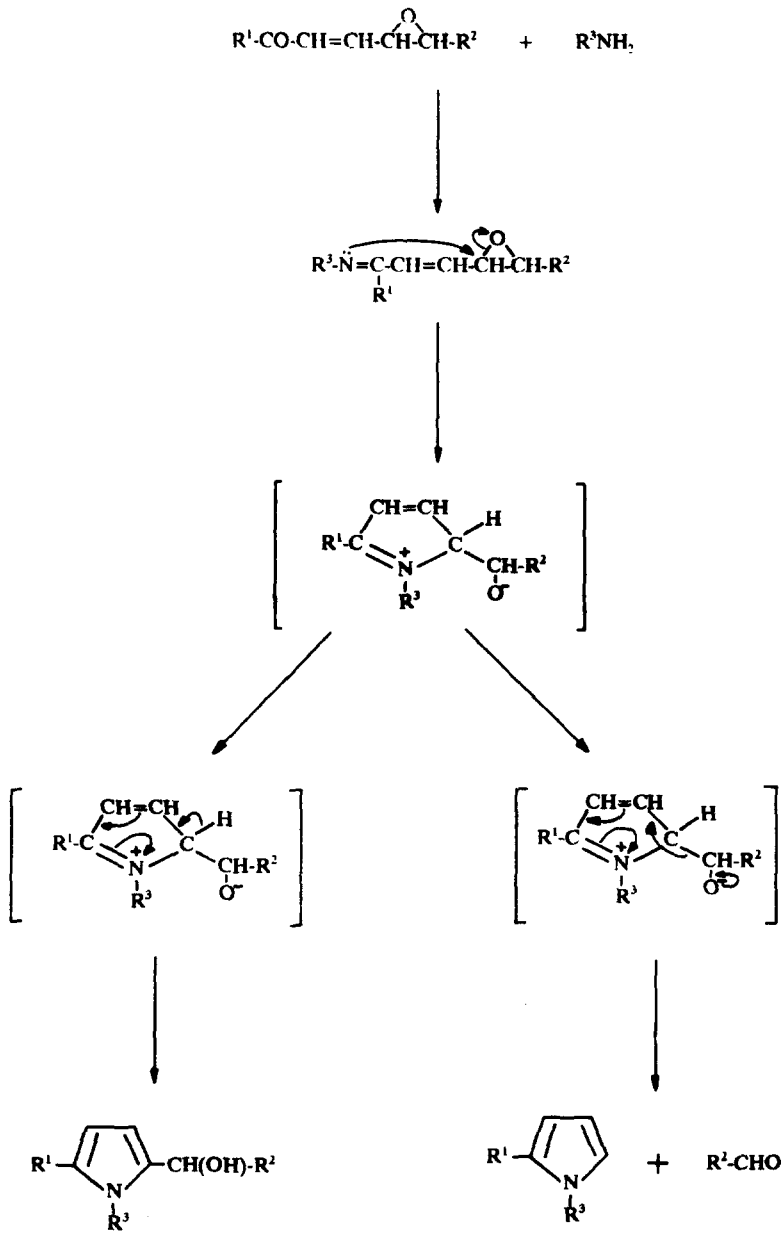


Fig. 6. Proposed mechanism of pyrrole formation in the reaction of epoxyoxoene fatty esters with butylamine and lysine. The major pathway (about 80% of the reaction in the conditions used in this study) was the formation of the C_{18} pyrrole fatty esters. However, the formation of 1,2-disubstituted pyrroles was always observed.

genase reaction, as supposedly takes place during modification of the low density lipoproteins (LDL) induced by endothelial cells (33), the corresponding hydroperoxide produced would be 15-hydroperoxy-5(*Z*),8(*Z*),11(*Z*),13(*E*)-eicosatetraenoic acid. Following the previous mechanism, the epoxyoxoene fatty acid produced in the next step would be 14,15-epoxy-11-oxo-5(*Z*),8(*Z*),12(*E*)-eicosatrienoic acid. This oxidized fatty acid would be able to react with biological amino groups yielding two types of pyrroles 10-[5-(1'-hydroxyhexyl)-*N*-alkylpyrrole-2-]-5(*Z*),8(*Z*)-decadienoic acid and 10-(*N*-alkylpyrrole-2-)-5(*Z*),8(*Z*)-decadienoic acid. The production of this last compound would be parallel to the formation of hexanal.

In membranes and lipoproteins, lipids are very close to proteins and the products of lipid peroxidation can interact with the amino groups on amino acid side-chain on proteins thus altering their charge and nature. This has been proposed, for example, as a contributory mechanism to the oxidative modification of LDL which facilitates their recognition by scavenger receptors on macrophages (34). Although it is not known how LDL oxidation is initiated, during this oxidative process the LDL undergoes many physicochemical changes which have been shown to be a consequence of the peroxidation of the LDL lipids

(35). Oxidized LDL lipids are able then to decompose, and the resulting aldehydes form covalent bonds with the lysine ϵ-amino group of apoB, via Schiff's base formation, and thus generate a negatively charged apolipoprotein, which appears to be the ligand recognized by the macrophage receptor (36, 37). Although these effects have been related almost exclusively to the reaction with short- or medium-chain aldehydes, several studies suggest that these compounds are not the only ones responsible for LDL modification. Steinbrecher et al. (38) found that the reactive products that modify LDL did not appear to be simple saturated or unsaturated aldehydes, but more complex oxygen-containing compounds. In addition, the contribution of long-chain lipid peroxidation products to modification of LDL has also been suggested by Fruebis, Parthasarathy, and Steinberg (39). The present paper constitutes, to our knowledge, the first evidence that oxidized long-chain fatty acids are able to react with amino groups producing stable compounds and, therefore, modifying the charge and nature of these amino groups. Additional studies should be carried out to investigate the role that these compounds might play in the modification of LDL upon oxidation. In addition, the reactions described in this study might also be implicated in some of the age-related diseases that are consequence of free radical damage to cellular function (40).

All these results provide the basis for future in vivo studies to assess the role of protein modification by long-chain oxidized fatty acids, which are more abundant than short-chain aldehydes, in certain diseases, pathophysiological conditions, and aging. Although these results do not demonstrate the formation in nature of long-chain pyrrole fatty acids, they strongly suggest that these pyrroles could be produced as an ultimate step in the lipid peroxidation process, and methods should be developed for their detection. The methods described in this study may be useful in the preparation of standards.

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