Furan Fatty Acids Determined as Oxidation Products of Conjugated Octadecadienoic Acid

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ABSTRACT: The objective of this study was to identify oxidation products of conjugated linoleic acid (CLA), a series of octadecadienoic acids with conjugated double bonds, which have been reported to have antioxidant and anticarcinogenic properties. Reference materials of CLA were oxidized in different concentrations of water/methanol; for example, 0.5 g octadecadienoic acid was dissolved in 50 mL methanol, and 100 mL water was added; this suspension was heated at 50°C and continuously aerated. Aliquots of 5 mL were taken over time, extracted with ether, treated with diazomethane and examined by gas chromatography/mass spectrometry and/or gas chromatography with flame-ionization detection. Products identified included the following furan fatty acids (FFAs): 8,11-epoxy-8,10-octadecadienoic; 9,12-epoxy-9,11-octadecadienoic; 10,13-epoxy-10,12-octadecadienoic; and 11,14-epoxy-11,13-octadecadienoic. Conjugated dienes should be considered as a possible source of FFAs, and CLA may have products common to furans in their overall oxidative scheme.

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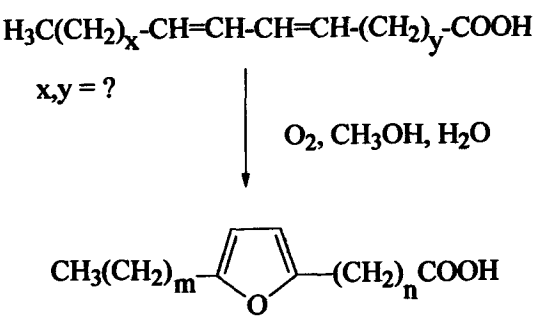
"Conjugated linoleic acid" (CLA) is a term used to describe a group of octadecadienoic acids which contain two conjugated double bonds. Interest in CLA has been growing as a result of recently reported antioxidant (1) and anticarcinogenic (1–3) properties of these compounds.

It is not apparent from the structure of CLA isomers how these compounds could be effective antioxidants. CLA does not have a tautomeric form nor does it seem to have the functional group necessary to chelate metal ions. The oxidation products of conjugated fatty acids have been studied much less than their methylene-interrupted counterparts (4).

Previous oxidation studies of conjugated acids do not indicate that hydroxylation occurs to the extent that significant stable products are formed (4). The work described here attempted to characterize oxidation products of CLA by oxidizing a commercially obtained reference, nominally 9,11-octadecadienoic acid, a mixture of *cis* and *trans* isomers of an octadecadienoic acid.

Initial attempts to oxidize CLA in a methanol solution were unsuccessful; the soluble CLA was remarkably stable, e.g., there were no detectable products after days of attempted oxidation at 45°C. When enough water was added to produce a suspension, oxidation commenced. The oxidation then proceeded at a controlled and reproducible rate. Four furan fatty acids (FFAs), 8,11-epoxy-8,10-octadecadienoic ($F_{8,11}$); 9,12-epoxy-9,11-octadecadienoic ($F_{9,12}$); 10,13-epoxy-10,12-octadecadienoic ($F_{10,13}$); and 11,14-epoxy-11,13-octadecadienoic ($F_{11,14}$), as shown in Scheme 1, were identified by gas chromatography/mass spectrometry (GC/MS) and GC/matrix isolation (MI)/Fourier transform–infrared spectroscopy (FT–IR) when methylated ether extracts of an oxidized, commercially obtained CLA reference were examined by GC.



	m	n
$F_{8,11}$	6	6
$F_{9,12}$	5	7
$F_{10,13}$	4	8
$F_{11,14}$	3	9

SCHEME 1

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Abbreviations: CLA, conjugated linoleic acid; EI, electron ionization; $F_{8,11}$, 8,11-epoxy-8,10-octadecadienoic acid; $F_{9,12}$, 9,12-epoxy-9,11-octadecadienoic acid; $F_{10,13}$, 10,13-epoxy-10,12-octadecadienoic acid; $F_{11,14}$, 11,14-epoxy-11,13-octadecadienoic acid; FAME, fatty acid methyl ester; FFA, furan fatty acid; FID, flame-ionization detection; FT–IR, Fourier transform–infrared spectroscopy; GC/MS, gas chromatography/mass spectrometry; MI, matrix isolation; PFK, perfluorokerosene; SFC, supercritical fluid chromatography.

The compound $F_{9,12}$ has been found in *Exocarpus cupressiformis* as a plant lipid (5), and its oxidation and that of $F_{10,13}$ have been studied (6,7). FFAs with alkyl ring substitution have been found in latex (8), fish (9,10), plants (11), and bovine liver (12). The origin (13) and biological function (14,15) of their occurrence are active areas of current research. Although many compounds may result from the oxidation of FFAs (6) and undoubtedly other oxidation products of CLA, this work presents analytical data related only to the identification of the FFAs shown in Scheme 1.

MATERIALS AND METHODS

Diazomethane was prepared from Diazald (*N*-methyl-*N*-nitroso-*p*-toluenesulfonamide) according to Technical Information Bulletin No. AL-180, Aldrich Chemical Company (Milwaukee, WI). CLA, 9,11-octadecadienoic acid (manufacturer's assay: *c*-9,*t*-11/*t*-9,*c*-11 41%; *t*-10,*c*-12 44%; *c*-10,*c*-12 9.5%; *t*-9,*t*-11/*t*-10,*t*-12 1.3%; *c*-9,*c*-11 1%; *c*-9,*c*-12 0.7%) and other fatty acids and methyl esters were obtained from Nu-Chek-Prep, Inc. (Elysian, MN). CLA produced from alkali isomerization of safflower oil (assay: palmitic/oleic/stearic 30%; linoleic 0.5%; *c*-9,*t*-11-$C_{18:2}$/*t*-9,*c*-11-$C_{18:2}$ 29%; *t*-10,*c*-12-$C_{18:2}$ 32%; *c*-9,*c*-11-$C_{18:2}$ 2.4%; *c*-10,*c*-12-$C_{18:2}$ 2.7%; *t*-9,*t*-11-$C_{18:2}$/*t*-10,*t*-12-$C_{18:2}$ 4.8%) was obtained from Sou F. Chin (Food Research Institute, University of Wisconsin, Madison, WI). Organic solvents were redistilled-from-glass. Water was distilled and deionized. Cylinder air was breathing quality. Methyl $F_{9,12}$ was synthesized by a previously published procedure (16).

CLA oxidation. Stearic acid ($C_{18:0}$) or heptadecanoic acid ($C_{17:0}$) was sometimes used as the internal reference for the oxidations described below. CLA acids were exposed to varying amounts of air, water, and methanol. Several oxidations were performed similarly, with the following minor variations: *Oxidation 1* (OX_1): Fixed quantities of 75 mg CLA and 3.38 mg $C_{17:0}$ were dissolved in 1 mL methanol in test tubes; 0.0, 0.1, 0.2, 0.5, 1.0, or 2 mL water was added and the individual tubes, which contained air, were sealed and immersed in an oil bath and held at 45°C for 46 h. *Oxidation 2* (OX_2): 0.5 g CLA was dissolved in 50 mL methanol in a 250-mL Erlenmeyer flask with a stirring bar and air flowing through a pipette under the liquid surface at 100–200 mL/min. Then 100 mL water was added, and the suspension was heated on a stirrer-hot plate at temperatures varying from 48 to 69°C. The heat was turned off after 7.5 h, but restored 16 h later. Methanol/water (1:2) was added as needed to maintain a volume between 100 and 150 mL. Aliquots (5 mL) were taken hourly and examined after methylation. *Oxidation 3* (OX_3): Fixed quantities (e.g., 44.2 mg CLA and 3.22 mg $C_{18:0}$) were dissolved in 2 mL methanol in test tubes; a micro stirring bar and 4 mL water were added to each tube. The tubes were placed in an oil bath on a stirrer-hot plate and held at 40°C. Tubes were continuously stirred and aerated (2–5 mL/min). Methanol/water (1:2) was added as needed to maintain a volume between 4 and 6 mL. Individual tubes were removed from the oil bath at different times and examined after methylation.

Methylation procedure. To each aliquot in a test tube were added 5 mL saturated NaCl/water and 5 mL ethyl ether. The tube was vortexed for 30–60 s. The ether portion was dried over Na_2SO_4; 1 mL diazomethane in ether solution was carefully and slowly added to the dried ether portion. After 20 min, 1–2 drops of acetic acid were added to react with the remaining diazomethane. The solution was injected into the gas chromatograph without further cleanup.

Instrumentation. For GC, a Hewlett-Packard 5890A instrument was used under the following conditions: column, 50 m × 0.25 mm i.d. CP Sil 88 capillary (Chrompack, Raritan, NJ); helium carrier gas; flame-ionization detector (FID); temperatures, injector 220°C, detector 280°C, column 75°C for 2 min, then raised 20°C/min to 185°C and held for 33 min, then raised at 4°C/min to 225°C. Samples were run in both split and splitless modes.

Low-resolution electron ionization (EI) GC/MS analyses were obtained with a Hewlett-Packard 5890 series II gas chromatograph coupled to a Fissons VG (Wytheshawe, United Kingdom) Autospec Q mass spectrometer and OPUS 2000 data system. The GC/MS system used version 1.6C software.

The same capillary GC column was used to obtain FID data and GC/MS data. Adjusting the capillary GC column head pressure to 10 psi gave chromatography results comparable to the GC/FID data. The GC/MS conditions were as follows: splitless injection with helium sweep restored 1 min after injection; injector and transfer lines 250°C; oven 75°C for 2 min after injection, then 20°C/min to 185°C, hold 185°C 15 min, 4°C/min to 225°C, hold 225°C 5 min.

The mass spectrometer was tuned to a resolution of 1000 (5% valley) by observing *m/z* 305 in the EI mass spectrum of perfluorokerosene (PFK). The mass scale was calibrated with PFK for magnet scans from 440 to 44 daltons at 1 s per decade. Filament emission was 200 μA at 70 eV. Ion source temperature was 250°C. A TSQ-46 instrument was used for direct probe MS/MS by a procedure that has been described (17).

A Mattson Instruments Model Sirius 100 FT-IR spectrometer equipped with an MI Cryolect interface operated at 10 K under vacuum was used with GC to obtain IR spectra. This system, which was used with a CP-Sil-88 capillary column, has been described in detail (18).

The supercritical fluid chromatography (SFC)/FT–IR system has been previously described in detail (19). Spectra were obtained by using a 25% cyanopropylsiloxane column, 100 μm i.d., with the following density (CO_2) gradient: 0.18 g/mL, hold for 20 min, then from 0.18 to 0.80 g/mL at 0.02 g/mL/min, hold for 5 min. Test solutions were injected from the OX_2 experiment without prior methylation.

RESULTS AND DISCUSSION

The oxidized CLA was treated with diazomethane to obtain methyl esters prior to GC analysis. In initial experiments, it was observed that CLA was very stable in methanol solutions

and the experiment OX_1 was performed to determine if the addition of water would speed up oxidation. No FFAs or other volatile compounds were detected when methanol or 10% water/methanol was used as the solvent. The amounts of FFA produced with increasing percentages of water are as follows: 1.9 mg FFA with 20% water; 2.8 mg FFA with 33% water; 3.0 mg FFA with 50% water; and 2.3 mg FFA with 67% water. It was observed that the better the suspension of CLA acid was, the greater the yield of FFAs.

The chromatogram in Figure 1 shows the FFAs produced after 26.5 h of oxidation by the OX_1 procedure. The major components eluting before CLA include aldehydes, methylated short-chain fatty acids and aldehyde-acids. The FFA esters were identified by mass spectrometry. The general scheme for identifying FFA methyl esters has been reported (20). The molecular ion, *m/z* 308, and the furanyl ring with 0, 1, or 2 methylene groups, *m/z* 67, 81, and 95, respectively, are characteristic of the fatty acid methyl esters (FAMEs) of all the FFAs shown in Scheme 1. The ions characteristic of the specific furan isomers, $[M^+\text{-}(CH_2)_{n-1}CO_2CH_3]$ and $[M^+\text{-}(CH_2)_{m-1}CH_3]$, respectively, and their intensities in parentheses are as follows: $F_{8,11}$, *m/z* 179(100), *m/z* 223(20); $F_{9,12}$, *m/z* 165(100), *m/z* 237(18); $F_{10,13}$, *m/z* 151(100), *m/z* 251(18); and $F_{11,14}$, *m/z* 137(100), *m/z* 265(14). The identity of the FFA methyl ester $F_{9,12}$ was confirmed by comparison of its GC retention time and mass spectrum with those of a synthesized reference material (16).

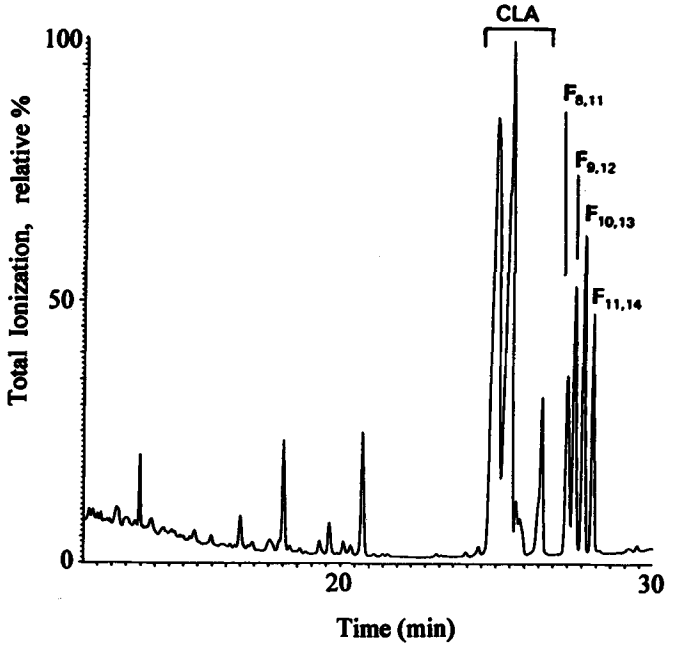

FIG. 1. Chromatogram with total ion mass spectrometry detection after 26.5 h of oxidation of conjugated linoleic acid (CLA) isomers by the Oxidation 2 method (details in Materials and Methods section). Retention regions where methyl esters of CLA and furan fatty acids elute are indicated; earlier responses are due to aldehydes and short-chain fatty acid methyl esters (FAMEs) and aldehyde-acid FAMEs. Methyl esters of $F_{8,11}$, 8,11-epoxy-8,10-octadecadienoic acid; $F_{9,12}$, 9,12-epoxy-9,11-octadecadienoic acid; $F_{10,13}$, 10,13-epoxy-10,12-octadecadienoic acid; $F_{11,14}$, 11,14-epoxy-11,13-octadecadienoic acid.

GC/MI/FT–IR spectra were similar for the CLA oxidation products and the synthesized $F_{9,12}$. The frequencies (4 cm^{-1} resolution) characteristic of the furan that we observed were assigned as follows: 3111, 3031, and 3000 cm^{-1} for =C–H stretch; 1574 cm^{-1} for C=C stretch; 1013 cm^{-1} for C–O stretch; and 780 cm^{-1} for =C–H out-of-plane bend.

The increase in FFA and the decrease in CLA concentration with time (OX_3) are shown in Figure 2. At 46 h, the total of the furan FAMEs amounted to 3.2% of the starting amount of CLA used in this experiment. It is clear from the plot (Fig. 2) that the majority of the mass balance is not accounted for by formation of furans and loss of CLA alone. A plausible explanation, which we intend to investigate, is that under these oxidative conditions the furans are less stable than the CLA isomers from which they may arise. Aldehydes and esters of short- and medium-chain fatty acids and aldehyde-acids may account for a majority of the remaining unaccounted mass, but no effort was made to recover them from the oxidation mixtures.

The CLA produced from alkali isomerization of safflower oil, when oxidized (OX_2), produced only $F_{9,12}$ and $F_{10,13}$. The compositions of the two CLA reference materials that we oxidized differ in the distribution of octadecadienoic acid *cis/trans* isomers. Both reference materials were produced by alkali isomerization of the linoleic fatty acid moiety. The Nu-Chek-Prep assay was performed for only one lot and may not necessarily represent the actual isomeric distribution in the test portion that we examined. It is possible that the FFAs produced may be better indicators of the original CLA composition; hence, in Scheme 1, m = x and n = y. At this time, we do not know how the furans are formed, and the results of these experiments should not be freely extrapolated to other matrices containing CLA.

Researchers have previously shown that FFAs may arise from precursor compounds (e.g., methyl-9,12-dioxo-stearate) when the precursor is injected into a gas chromatograph (10).

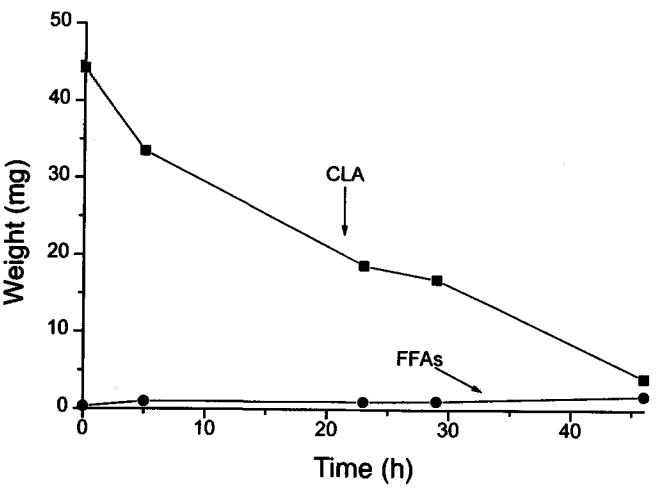

FIG. 2. Abundance of conjugated linoleic acid (CLA) and furan fatty acid (FFA) methyl esters as a function of oxidation time at 40°C, Oxidation 3 method (details in Materials and Methods section); weight is based on stearic acid, which was included in the oxidation.

Furans are also known to arise as a result of dehydration of endoperoxides (21). We do not know the extent to which such precursors may be present in the CLA oxidation mixtures; however, the presence of the furans in methylated and unmethylated CLA oxidation mixtures was confirmed. The presence of furan acids in the unmethylated OX_2 mixture was confirmed by SFC/FT–IR; the major bands expected for the furan acids were observed, and the –C=C– stretch at 1574 cm^{-1} was especially characteristic. The methyl esters of the four FFAs shown in Figure 1 were also confirmed to be present in the methylated, oxidized mixture by direct probe MS/MS. The ions *m/z* 137, 151, 165, and 179 were determined to be daughter ions of the *m/z* 308 methyl FFA parent ion.

It turns out that the same reaction products described in the above experiments are produced by merely exposing neat CLA or CLA methyl esters to ambient air. The greatly improved stability of CLA in solution may be related to its antioxidant properties, i.e., it will readily react, when solvated, only with oxidants that are more reactive than those found in ambient air. The formation of FFAs from conjugated diene systems is known to occur when singlet oxygen is used as a reactant (21). It was surprising that FFAs were produced in the experiments described here, because singlet oxygen was not deliberately introduced into any of the systems described. Oxidation products of conjugated dienes should be considered as a source of FFAs in biological systems.

Experiments are ongoing to establish the mass balance in the described oxidations, and to determine the relationship of the FFAs produced to the specific isomers of octadecadienoic acid used as starting materials.

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