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Free radical oxidation of coriolic acid (13-(*S*)-hydroxy-9*Z*,11*E*-octadecadienoic Acid)

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Abstract

The reaction of (13*S*,9*Z*,11*E*)-13-hydroxy-9,11-octadecadienoic acid (**1a**), one of the major peroxidation products of linoleic acid and an important physiological mediator, with the Fenton reagent (Fe^{2+}/EDTA/H_2O_2) was investigated. In phosphate buffer, pH 7.4, the reaction proceeded with >80% substrate consumption after 4 h to give a defined pattern of products, the major of which were isolated as methyl esters and were subjected to complete spectral characterization. The less polar product was identified as (9*Z*,11*E*)-13-oxo-9,11-octadecadienoate (**2**) methyl ester (40% yield). Based on 2D NMR analysis the other two major products were formulated as (11*E*)-9,10-epoxy-13-hydroxy-11-octadecenoate (**3**) methyl ester (15% yield) and (10*E*)-9-hydroxy-13-oxo-10-octadecenoate (**4**) methyl ester (10% yield). Mechanistic experiments, including deuterium labeling, were consistent with a free radical oxidation pathway involving as the primary event H-atom abstraction at C-13, as inferred from loss of the original *S* configuration in the reaction products. Overall, these results provide the first insight into the products formed by oxidation of **1a** with the Fenton reagent, and hint at novel formation pathways of the hydroxyepoxide **3** and hydroxyketone **4** of potential (patho)physiological relevance in settings of oxidative stress.

Keywords: Linoleic acid; Fenton reagent; Deuterium labeling; (1/2)D NMR; Lipid peroxidation

Abbreviations: 13-(*S*)-HODE, (13*S*,9*Z*,11*E*)-13-hydroxy-9,11-octadecadienoic acid; 13-(*S*)-HPODE, (13*S*,9*Z*,11*E*)-13-hydroperoxy-9,11-octadecadienoic acid; LDL, low-density lipoprotein; PPAR-γ, peroxysome proliferator-activated receptor; BSTFA, bis(trimethylsilyl)-trifluoroacetamide; EDTA, ethylendiaminotetraacetic acid; ABAP, 2,2′-azobis(2-amidinopropane); HRP, horseradish peroxidase; COSY, correlation spectroscopy; HMQC, heteronuclear multiple quantum coherence; HMBC, heteronuclear multiple bond correlation; TOCSY, total correlation spectroscopy; PICI/MS, positive chemical ionization/mass spectrometry; tetramethylsilane, TMS

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1. Introduction

(13*S*,9*Z*,11*E*)-13-Hydroxy-9,11-octadecadienoic acid (13-(*S*)-HODE, **1a**), commonly referred to as coriolic acid, is a major product of lipid metabolism which arises by 15-lipoxygenase-catalyzed oxidation of linoleic acid followed by reduction of the resulting hydroperoxide ((13*S*,9*Z*,11*E*)-13-hydroperoxy-9,11-octadecadienoic acid, 13-(*S*)-HPODE) (Kühn, 1996). Additional routes to **1a** involve cycloxygenase-catalyzed oxidation of linoleic acid (Hamberg and Samuelsson, 1980) as well as non-enzymatic peroxidation (Nemann and Khenkin, 1997). In these latter cases, however, the *R* enantiomer is also formed to comparable extents, along with the 9-hydroxy isomers, due to the lack of stereo- and regioselectivity of these reactions.

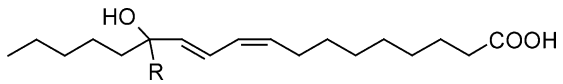
Both *S*- and racemic 13-HODE are produced by a variety of cell types, including polymorphonuclear leukocytes (Soberman et al., 1985), eosinophils (Engels et al., 1996), breast carcinoma cells (Reddy et al., 1997), and human dermal fibroblasts (Godessart et al., 1996). Racemic 13-HODE has been identified as a component of oxidized low-density lipoprotein (LDL) (Jira and Spiteller, 1996; Jira et al., 1997) and is the chief hydroxylated fatty acid in human psoriatic skin scales, where it occurs in levels of 115 ng/mg (Baer et al., 1990).

In mammalian cells **1a** acts as a physiological mediator, being involved in signal transduction and gene expression. It potentiates the mitogenic signal produced by epidermal growth factor in human breast carcinoma cells (Reddy et al., 1997), displays chemotactic properties toward polymorphonuclear leukocytes (Henricks et al., 1991), modifies inflammatory cell activity and is a ligand to peroxysome proliferator-activated receptor (PPAR-γ) (Nagy et al., 1998; Marx et al., 1999).

In the skin, **1a** has been ascribed antiinflammatory and antiproliferative properties, whereas in the vascular system it acts as a chemorepellant, reducing the adhesion of platelets (Buchanan et al., 1985; Haas et al., 1988). It also causes relaxation of coronary arteries (De Meyer et al., 1992) and may have both pro- and anti-atherogenic effects, depending on the rate of generation by the 15-lipoxygenase expressed in the macrophages recruited at sites of atherosclerotic lesions (Simon et al., 1989).

Although the (patho)physiological routes leading to **1a** production in settings of oxidative stress now appear to be well established, much less is known about the effects of reactive oxygen species on the ultimate fate and biological activity of this important lipid mediator. Knowledge of the oxidative chemistry of **1a** is currently limited to formation of (9*Z*,11*E*)-13-oxo-9,11-octadecadienoic acid (**2**) by the action of a NAD(+)-dependent dehydrogenase (13-HODE dehydrogenase) (Bronstein and Bull, 1997), and no other oxidation product has been isolated in pure form and chemically characterized. Notably enough, **1a** and its congeners have been reported to be stable to various oxidizing systems, including air, air/Fe^{2+}/ascorbate, air/Fe^{2+}, air/Fe^{2+}/H_2O_2, air/Fe^{3+}, and have therefore gained the reputation of excellent markers of lipid peroxidation (Spiteller and Spiteller, 1997).

In the present paper, we have investigated the reaction of **1a** with Fe^{2+}/EDTA/H_2O_2 (the Fenton reagent), which is widely used to model non-enzymatic oxidative processes, as well as with other oxidizing systems of physiological relevance. Aim of the work was to assess whether **1a** displays patterns of reactivity other than conversion to the keto compound **2**, and to provide a detailed structural characterization of the oxidation products



1a: R = H
1b: R = ^{2}H

2. Materials and methods

2.1. Materials

Linoleic acid (99%), hydrogen peroxide (water solution, 33%), D-mannitol, 2-iodobenzoic acid, oxone ($2KHSO_5$–$KHSO_4$–K_2SO_4), sodium borohydride, sodium borodeuteride and bis(trimethylsilyl)trifluoroacetamide (BSTFA) were from Aldrich Chemie; $Fe(NH_4)_2(SO_4)_2{\cdot}6H_2O$ was from Carlo Erba; ethylenediaminetetraacetic acid (EDTA) was from Fluka; 2,2′-azobis(2-amidinepropane) (ABAP) chlorhydrate was from Polysciences. Horseradish peroxidase (HRP) (H_2O_2 oxidoreductase; E.C. 1.11.1.7) type II, catalase (H_2O_2:H_2O_2 oxidoreductase; EC

1.11.1.6) and soybean lipoxidase (linoleate oxygen reductase, E.C. 1.13.11.12) type IB were from Sigma. Organic solvents were HPLC quality; phosphate buffer (0.1 M, pH 7.4) and borate buffer (0.1 M, pH 9.0) were treated with Chelex-100 resin before use to remove transition metal contaminants.

(13*S*,9*Z*,11*E*)-13-Hydroxy-9,11-octadecadienoic acid (**1a**, 13-(*S*)-HODE) and (13*S*,9*Z*,11*E*)-13-hydroperoxy-9,11-octadecadienoic acid (13-(*S*)-HPODE) were synthesized and purified as described (Napolitano et al., 2002). **1a** methyl ester was obtained by treatment of the free acid with diazomethane. *o*-Iodoxybenzoic acid was freshly prepared from 2-iodobenzoic acid according to a reported procedure (Frigerio et al., 1999).

Diazomethane was prepared by reaction of *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide in ethanolic KOH and collected in peroxide-free ether in a dry ice/acetone bath. Caution! Diazomethane is explosive and must be kept at −20 °C.

UV and IR spectra were obtained using a Beckman DU 640 spechtrophotometer and a FT-IR Perkin Elmer 1760-X spectrophotometer, respectively. NMR spectra were recorded in $CDCl_3$ with a Bruker DRX-400 MHz instrument. ^1H and ^{13}C NMR spectra were recorded at 400.1 and 100.6 MHz, respectively, using TMS as the internal standard. ^1H–^1H COSY, ^1H–^{13}C HMQC, ^1H–^{13}C HMBC and TOCSY NMR experiments were run at 400.1 MHz using standard pulse programs.

For all oxidation products of **1a** isolated as methyl esters, resonances due to $-OCH_3$, $COOCH_3$, C-2 (CH_2), C-3/C-4/C-5/C-6/C-7/C-15/C-16/C-17 (CH_2) and C-18 (CH_3) groups appear in the $^1H/^{13}C$ NMR spectra at δ ($CDCl_3$) 3.66 (3H, s)/52.2, 175.0, 2.31 (2H, t $J = 7.6$ Hz)/34.8, 1.2–1.7 (16H, m)/22–38, 0.89 (3H, m)/14.8 in the order. Analytical and preparative TLC analyses were performed on F_{254} silica gel plates (0.25 and 0.5 mm, respectively) using 70:30 (v/v) cyclohexane–ethyl acetate (eluant A), 70:30 (v/v) cyclohexane–ethyl acetate plus 2% acetic acid (eluant B). $Ce(SO_4)_2$ (0.05 M in 10% H_2SO_4), and iodine were used for product detection on TLC plates. Column chromatography was performed on silica gel (70–270 mesh).

GC/MS was carried out on a GC instrument coupled with a quadrupole mass spectrometer. Helium was the carrier gas with a 1 mL/min flow rate. Positive chemical ionization/mass spectrometry (PICI/MS) measurements were carried out using methane as the reagent gas. Data were processed using G1701AA data analysis software. The following analytical conditions were used: 30 m cross-bond 5% diphenyl-95% dimethylpolysiloxane column (0.25 mm i.d., 0.25 μm d.f.). Temperature program: 40 °C, hold time 1 min; up to 280 °C, rate 5 °C/min. The inlet and detector were taken at 180 and 250 °C, respectively. The acquisition started 5 min after the injection (solvent delay 5 min), and was set in scan 50, and sampling was 1.6 scans/s.

Prior to GC/MS analysis, all the samples were treated with 200 μL of ethereal diazomethane solution to give the corresponding methylester derivative. When required, the samples were treated with 50 mL of a 50% solution of BSTFA in pyridine and kept at 50 °C for 30 min to give the corresponding trimethylsilyl derivative.

*2.2. Synthesis of (9Z,11E)-13-oxo-9,11-octadecadienoic acid (**2**)*

1a (120 mg, 0.41 mmol) was dissolved in ethyl acetate (75 mL), treated with *o*-iodoxybenzoic acid (1.05 g, 3.7 mmol) and kept under reflux at 85 °C. After 90 min the mixture was filtered to remove unreacted *o*-iodoxybenzoic acid as a white solid. The filtrate was dried and purified on preparative TLC (eluant B) to give pure **2** (105 mg, 88% yield). FT-IR, ^1H NMR and ^{13}C NMR for compound **2** were in accord with those reported (Kuklev et al., 1997).

*2.3. Synthesis of (13-^2H,9Z,11E)-13-hydroxy-9,11-octadecadienoic acid (**1b**)*

2 (100 mg, 0.34 mmol) dissolved in $CHCl_3$ (1 mL) was added to 0.1 M borate buffer, pH 9.0 (50 mL) and treated under vigorous stirring at room temperature with sodium borodeuteride (20 mg, 0.48 mmol). After 5 min the mixture was acidified to pH 3 with HCl and extracted with $CHCl_3$ (3 × 30 mL). The organic layers were combined, treated with anhydrous sodium sulfate, and dried under reduced pressure to afford pure **1b** (70 mg, 70% yield).

1b: ^1H NMR (400 MHz, $CDCl_3$), δ (ppm): 1.40–1.55 (4H, m, H-8, H-14), 5.43 (1H, dt $J = 10.8$, 7.6 Hz, H-9), 5.65 (1H, d $J = 15.2$ Hz, H-12), 5.96 (1H,

t J = 10.8 Hz, H-10), 6.47 (1H, dd J = 15.2, 10.8 Hz, H-11).

2.4. Oxidation of **1a** with the Fe^{2+}/EDTA/H_2O_2 system

The reaction was carried out under conditions substantially similar to those reported (Spiteller and Spiteller, 1997) with some modifications. In brief, **1a** (15 mg, 0.05 mmol) was dissolved in $CHCl_3$ (100 μL) and added to 0.1 M phosphate buffer, pH 7.4 (22 mL) containing 0.2 M KCl (45 mL). The mixture was treated under vigorous stirring at room temperature with $Fe(NH_4)_2(SO_4)_2{\cdot}6H_2O$ (19.8 mg, 0.05 mmol) and EDTA (18.6 mg, 0.05 mmol). The oxidation was started by addition of four aliquots of 1 M hydrogen peroxide (12.5 μL, 0.013 mmol each) over a period of 45 min. After 4 h the reaction mixture was extracted with ethyl acetate (2 × 10 mL), and the organic layers were combined, dried over sodium sulfate and treated with an ethereal solution of diazomethane (2 mL). After 15 min the mixture was dried under reduced pressure and the residue was analyzed by TLC (eluant A).

Similar experiments were carried out (i) as above but omitting EDTA; (ii) treating the reaction mixture with D-mannitol (3.2 g, 16.8 mmol), or catalase (3100 U); (iii) under an oxygen depleted atmosphere, purging all the reagents with a flux of argon.

In other experiments the reaction was carried out under the general conditions described above but using 13-(*S*)-HPODE as the substrate.

2.5. Oxidation of **1a** with the HRP/H_2O_2 system

1a (25 mg, 0.084 mmol) was dissolved in $CHCl_3$ (100 μL) and added to 0.1 M phosphate buffer, pH 7.4 (17 mL). The mixture was treated under vigorous stirring at room temperature with HRP (52 U). One molar of hydrogen peroxide was then added in seven aliquots (84.3 μL, 84.3 mmol each) over a period of 90 min. After 4 h the reaction mixture was worked up and analyzed as above.

2.6. Oxidation of **1a** with ABAP

1a (15 mg, 0.05 mmol) was dissolved in $CHCl_3$ (100 μL) and added to 0.1 M phosphate buffer, pH 7.4 (13.5 mL). The mixture was treated under vigorous stirring and at 60 °C with ABAP (5.4 mg, 0.02 mmol). After 4 h the reaction mixture was worked up and analyzed as above.

2.7. Isolation of compounds **2–4** methyl ester

1a (250 mg, 0.84 mmol) was dissolved in $CHCl_3$ (1 mL) and emulsified in 0.1 M phosphate buffer, pH 7.4 (170 mL). The mixture was treated under vigorous stirring at room temperature with $Fe(NH_4)_2(SO_4)_2{\cdot}6H_2O$ (164 mg, 0.42 mmol) and EDTA (156 mg, 0.42 mmol). The oxidation was started by addition of four aliquots of 1 M hydrogen peroxide (420 μL, 0.56 mmol each) over a period of 45 min. After 4 h working up as above afforded a yellow oily residue which was fractionated by column chromatography (2.0 cm × 40 cm) using a cyclohexane/ethyl acetate gradient (starting from pure cyclohexane up to cycloesane/ethyl acetate 6:4 (v/v)) as the eluant to give, besides unreacted **1a** methyl ester (R_f = 0.80, eluant A, 50 mg, 20% yield), **2** methyl ester (R_f = 0.82, eluant A, 103 mg, 40% yield), **3** methyl ester (R_f = 0.48, eluant A, 41 mg, 15% yield) and **4** methyl ester (R_f = 0.35, eluant A, 27 mg, 10% yield). In other experiments the prepative scale reaction was carried out using **1b** as the substrate.

3 methyl ester: FT-IR ($CHCl_3$) ν_{max} 3547–3296, 1732, 968, 883 cm^{-1}; $[\alpha]_D^{25}$ = 0.0 (*c* 2.7, $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$), δ (ppm): see Table 1; ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): see Table 1; GC/MS (tetramethylsilane (TMS) derivative) t_R 40.1 min; EI *m/z* (relative intensity) 398 (M^+, 3), 383 ($M^+ - CH_3$, 15), 327 ($M^+ - C_5H_{11}$, 6), 241 ($M^+ - (CH_2)_7CO_2CH_3$, 8), 199 ($M^+ - C_8H_{14}OSi(CH_3)_3$ and $M^+ - C_9H_{16}OCO_2CH_3$, 100) (see Fig. 1); PICI *m/z* 399 ($[M+H]^+$).

4 methyl ester: FT-IR ($CHCl_3$) ν_{max} 3547–3296, 1732, 1715 cm^{-1}; $[\alpha]_D^{25}$ = 0.0 (*c* 2.0, $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$), δ (ppm): see Table 1; ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): see Table 1; GC/MS (TMS derivative) t_R 41.0 min; EI *m/z* (relative intensity) 398 (M^+, 2), 383 ($M^+ - CH_3$, 3), 367 ($M^+ - OCH_3$, 2), 308 ($M^+ - (CH_3)_3SiOH$, 3), 299 ($M^+ - C_5H_{11}CO$, 4), 285 ($M^+ - C_5H_{11}COCH_2$, 5), 259 ($M^+ - C_9H_{15}O$, 6), 241 ($M^+ - C_9H_{17}O_2$, 100), 179 ($308 - C_7H_{13}O_2$, 6), 157 ($M^+ - C_{13}H_{25}O_2Si$, 4), 129 ($^{\bullet}C_7H_{13}O_2$, 3), 99 ($^{\bullet}C_5H_{11}CO$, 78), 71 ($^{\bullet}C_5H_{11}$, 18) (see Fig. 1); PICI *m/z* 399 ($[M+H]^+$).

Table 1
Selected spectral data of compounds **3** and **4** methyl esters

Carbon	**3**		**4**	
	δ_C	δ_H (mult. *J* (Hz))	δ_C	δ_H (mult. *J* (Hz))
8	32.7	1.53–1.59 (2H, m)	37.9	1.53 (2H, m)
9	61.4	2.81 (1H, dt 5.6, 2.4)	73.4	4.10 (1H, m)
10	58.7	3.09 (1H, dd 7.6, 2.4)	138.2	5.57 (1H, dd 15.2, 6.4)
11	128.9	5.41 (1H, dd 15.6, 7.6)	124.0	5.76 (1H, dt 15.2, 6.8)
12	138.8	5.92 (1H, dd 15.6, 6.4)	46.9	3.15 (2H, d 6.8)
13	72.9	4.13 (1H, m)	210.0	–
14	37.9	1.53–1.59 (2H, m)	43.5	2.42 (2H, t 7.2)

3. Results

The reaction of **1a** with the Fenton reagent (Fe^{2+}/EDTA/H_2O_2, 1 molar equivalent) was typically carried out with the lipid emulsified in 0.1 M phosphate buffer, pH 7.4 (Spiteller and Spiteller, 1997). After 4h, HPLC analysis showed ca. 80% substrate consumption and the formation of a defined pattern of products. TLC analysis of the mixture after methylation with diazomethane revealed the presence of three main products at $R_f = 0.88$ (A, $\lambda = 254$ nm), 0.48 (B, positive to $Ce(SO_4)_2$), and 0.35 (C, weak UV absorption, positive to $Ce(SO_4)_2$). Column chromatography (eluant cyclohexane/ethyl acetate gradient) afforded the products in pure form.

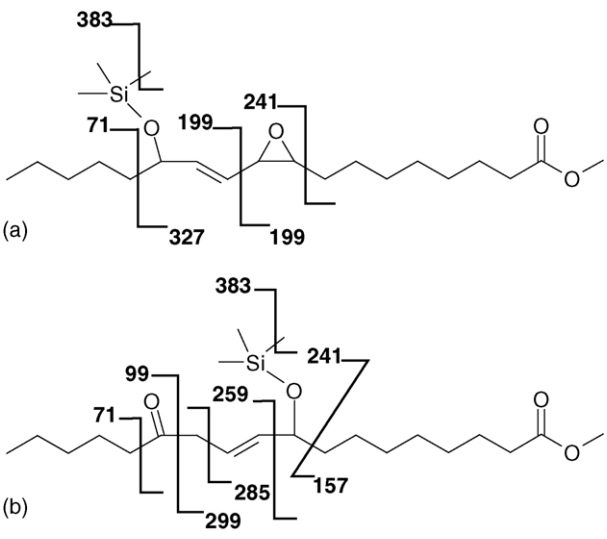


Fig. 1. Origin of salient fragmentation peaks in the EI/MS spectrum of the *O*-TMS derivative of compounds **3** (a) and **4** (b) methyl esters.

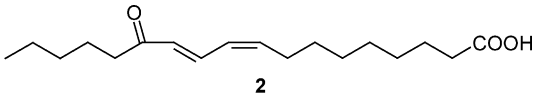
Compound A (103 mg, about 40% yield) was identified as (9*Z*,11*E*)-13-oxo-9,11-octadecadienoate (**2**) methyl ester by straightforward spectral analysis.

The ^{1}H NMR spectrum of product B (41 mg, 15% yield) displayed as most significant features two signals at δ 5.41 (1H, dd $J = 15.6$, 7.6 Hz) and 5.92 (1H, dd $J = 15.6$, 6.4 Hz), the latter showing coupling with a signal at δ 4.13 (1H, m), suggesting an allylic alcohol moiety with an *E* configuration at the double bond. The ^{1}H–^{1}H COSY spectrum revealed correlation of the olefin proton at δ 5.41 with a resonance at δ 3.09 which in turn gave a cross peak with a signal at δ 2.81. These latter two signals were strongly suggestive of protons on a *trans* epoxide functionality.

The ^{1}H–^{13}C HMQC spectrum showed cross peaks between the epoxide proton resonances at δ 2.81 and 3.09 and carbon resonances at δ 61.4 and 58.7, respectively; between the olefin proton signals at δ 5.92 and 5.41 and two carbon signals at δ 138.8 and 128.9; and between the multiplet at δ 4.13 and a carbon signal at δ 72.9. The ^{1}H–^{13}C HMBC spectrum displayed well apparent correlations between the proton at δ 4.13 and carbon resonances at δ 37.9 (CH_2), 138.8 and 128.9, corroborating the allylic alcohol functionality. Additional correlations between the olefin proton resonance at δ 5.41 and a carbon signal a δ 58.7; and between the epoxide proton signal at δ 3.09 and the carbon resonance at δ 61.4 indicated that the epoxide ring was close to the carbon–carbon double bond (see Table 1).

Finally, a TOCSY experiment, showing a well apparent correlation between the terminal methyl group at δ 0.89 and the proton alpha to the OH group at δ 4.13, provided conclusive evidence that the position of the OH was unchanged with respect to **1a**.

On the basis of these data, the product was assigned the structure of (11*E*)-9,10-epoxy-13-hydroxy-11-octadecenoate (**3**) methyl ester.



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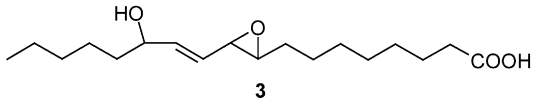

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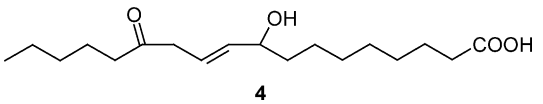


4

In accord with this conclusion, the FT-IR spectrum displayed intense bands in the range 3547–3296 cm^{-1}, consistent with a hydroxyl group; at 968 cm^{-1} (C–H bending of trans olefins) and at 883 cm^{-1} typical for C–H stretching of trans epoxides (Gardner and Kleiman, 1981). The EI/MS spectrum of the *O*-TMS derivative gave a molecular ion peak at *m/z* 398, in line with the PICI (positive ion chemical ionization) spectrum showing pseudo-molecular ion peak at *m/z* 399. Diagnostic fragmentation peaks in the EI/MS spectrum corroborated the structural conclusions derived from NMR analysis (see Fig. 1). The proton and mass spectrum of the compound bore considerable resemblance to those reported for fractions containing methyl 13-/9-hydroxy-9,10/12,13-epoxyoctadec-11/10-enoates obtained by decomposition of 13-(*S*)-HPODE (Frankel et al., 1977; Neff et al., 1978; Blee et al., 1993; Gardner and Kleiman, 1981; Spiteller and Spiteller, 1998; Spiteller, 1998). Interestingly, the product was optically inactive, suggesting that the stereogenic centre was affected during the oxidation process.

The most polar product (C) exhibited a diagnostic band in the FT-IR spectrum at 1715 cm^{-1}, indicating a C=O group. The ^1H NMR spectrum (Gardner et al., 1974) displayed a characteristic pattern of resonances at δ 5.76 (1H, dt, $J = 15.0$, 6.8 Hz), 5.57 (1H, dd, $J = 15.0$, 6.4 Hz), and 4.10 (1H, m), suggestive of an allylic alcohol moiety with *E* configuration. The signal at δ 5.76 was moreover coupled with a doublet at δ 3.15 ascribed to a dieshielded CH_2 group. Two triplets for additional methylene groups at δ 2.42 and 2.31 (2H each) were also observed.

Noticeable feature of the ^{13}C NMR spectrum was a signal at δ 210.0 ascribed to a keto group, which displayed cross peaks in the ^1H–^{13}C HMBC spectrum with the methylene resonances at δ 2.42 and 3.15. Furthermore, the TOCSY spectrum indicated correlation between the terminal methyl group at δ 0.89 and the methylene protons at δ 2.42, adjacent to the carbonyl functionality, as well as between the resonance of the proton alpha to the OH group at δ 4.10 and the signal at δ 1.53 and the chain resonances in the region at δ 1.2–1.8. These latter data clearly denoted that the allylic OH group was on the 9-position and, hence, that the former alcoholic functionality at the 13-position was replaced by the carbonyl group.

On this basis, the compound was assigned the structure of the hydroxyketone **4** methyl ester. A closely similar proton spectrum was reported for a mixture of positional isomers (13-/9-oxo-9-/13-hydroxy-*trans*-10/11-octadecenoic acids) obtained by Fe(II)-catalyzed decomposition of 13-HPODE (Gardner et al., 1974).

Consistent with proposed structure **4** the EI/MS spectrum of the *O*-TMS derivative showed a molecular ion peak at *m/z* 398, and the PICI spectrum a pseudo-molecular ion peak at *m/z* 399. The diagnostic EI fragmentation pattern (see Fig. 1) confirmed the structural formulation proposed for **4** and its positional isomers identified as constituents of complex mixtures obtained under different conditions (Sessa et al., 1977; Spiteller and Spiteller, 1998).

Overall yields of products **2**–**4** methyl esters accounted for about 80% of reacted **1a**.

Other minor components of the reaction mixture could not be obtained in sufficient amounts for structural characterization. Thus, for example, a fraction ($R_f = 0.70$–0.80, eluant A) which resisted at all attempts of purification of preparative value, and on NMR analysis proved to consist of the starting material and a minor constituent featuring an allylic alcoholic group, a trans double bond and a trans epoxide functionality. Based on the mapping of the spin system as deduced by the ^1H–^1H COSY spectrum of the gross fraction, the compound was tentatively formulated as a diastereomer of **3**.

Depletion of oxygen by running the reaction under an argon atmosphere markedly slowed down the rate of substrate consumption and product formation, whereas omission of H_2O_2 or EDTA resulted in complete prevention of the reaction, in line with previous observations (Spiteller and Spiteller, 1997).

In separate experiments it was found that products **2**–**4** were also formed by HRP/H_2O_2 oxidation of **1a** as well as by vigorously stirring **1a** in air-equilibrated phosphate buffer at pH 7.4 in the presence of the free radical initiator ABAP. Under the latter conditions the reaction rate was much slower than that observed in the Fenton reaction (40% consumption at 4 h reaction time) and the overall product yield was about 70% with respect to reacted **1a**.

For comparative purposes, the hydroperoxide 13-(*S*)-HPODE was allowed to react with the Fenton reagent under the typical reaction conditions used for **1a**. After 4 h substrate consumption was >90% and a much more complex product pattern was observed compared to the case of **1a**. Following methylation, compounds **2**–**4** methyl esters could be identified by TLC analysis under different elution conditions and NMR analysis of the reaction mixture.

Oxidation of **1a** by the Fenton reagent (Fe^{2+}/EDTA/H_2O_2) conceivably involves the generation of the OH radical as the species triggering substrate conversion. The role of this radical was supported by experiments run in the presence of OH radical scavengers, such as D-mannitol and DMSO, which inhibited both substrate consumption and product formation. Typically, a >95% recovery of **1a** was obtained using 100-fold molar excess of D-mannitol at 4 h reaction time.

To inquire into the mechanism of the Fenton-induced oxidation, a competition experiment was devised in which an equimolar mixture of **1a** and (13-^{2}H,9*Z*,11*E*)-13-hydroxy-9,11-octadecadienoic acid (**1b**) was allowed to react with the Fenton reagent as described above. Analysis of unreacted **1a**/**b** at about 50% consumption indicated a virtually 1:1 ratio, indicating a comparable extent of oxidation. In other experiments **1b** was oxidized with the Fenton reagent and the reaction products were isolated and analyzed for deuterium content at about 50% substrate consumption. No detectable signal for the proton alpha to the OH group was observed in the spectrum of the recovered substrate. However, isolated product **3** methyl ester featured in the ^{1}H NMR spectrum a well discernible resonance at δ 4.13 with an integrated area of ca. 0.15H taking the protons of the double bond as reference (see Fig. 2, bottom panel). The splitting of the proton signal at δ 5.92, due to the presence of either ^{1}H or ^{2}H on the vicinal carbon, provided additional evidence that the isolated product was indeed a mixture of 13-^{1}H/^{2}H **3** methyl ester.

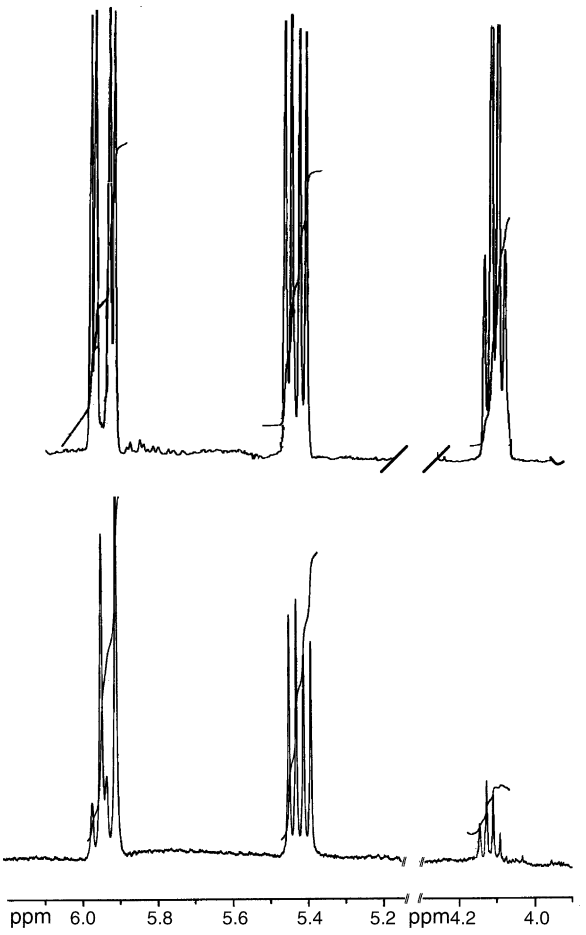


Fig. 2. Selected region of the ^{1}H NMR spectrum of compound **3** methyl ester obtained from oxidation of **1a** (top plot) and **1b** (bottom plot).

4. Discussion

Oxidative modifications of polyunsaturated fatty acids and their derivatives represent important post-synthetic pathways of lipid metabolism and transformation, and have been the focus of extensive chemical and biochemical literature (Dix and Marnett, 1985; Aikens and Dix, 1993; Schneider et al., 2001). In this frame, the isolation and complete spectral characterization of the oxidation products of **1a** reported in this

paper offers an improved basis to look at the biological fate and roles of **1a** under oxidative stress conditions.

Compound **3** and structurally related epoxides were described in the reaction of linoleic and linolenic acid or their hydroperoxides with lipoxygenases from different sources (Hamberg and Hamberg, 1990; Hamberg, 1989). In these processes the epoxidation is the result of an epoxidase activity of the enzymes that use hydroperoxides as oxygen donor, as demonstrated by ^{18}O labeling experiments (Blee et al., 1993). On the other hand, the ketone **4** was identified among the constituents of soybean phosphatidylcholines based on MS analysis (Sessa et al., 1977). Compounds **3** and **4** were also identified among the products formed in the decomposition of 13-(*S*)-HPODE as promoted by Fe^{2+} (Spiteller and Spiteller, 1998; Gardner et al., 1974) or the cysteine–$FeCl_3$ catalyst (Gardner and Jursinic, 1981) or in the autooxidation of methyl linoleate (Frankel et al., 1977; Neff et al., 1978). In all these studies, however, compounds **3** and **4** could not be distinguished from their regioisomers, i.e. 12,13-epoxy-9-hydroxy-10-octadecenoate and 9-oxo-13-hydroxy-11-octadecenoate.

Product **1a** was postulated as one of the products or an intermediate in the iron(II) catalyzed decomposition of the corresponding hydroperoxide (Mlakar and Spiteller, 1996; Spiteller and Spiteller, 1998). Further transformation of this species under such reaction conditions to give hydroxy/hydroperoxides derivatives was also proposed (Mlakar and Spiteller, 1996). Yet, in no case **1a** was considered as a direct precursor of products **3** and **4**. Moreover, the specific conditions of the Fenton system including EDTA–chelated iron(II) and hydrogen peroxide have never been examined as the oxidizing agent toward either **1a** or 13-(*S*)-HPODE.

Based on the mechanistic experiments including deuterium labeling, it could be argued that formation of products **2**–**4** by free radical oxidation of **1a** proceeds by an oxygen-dependent mechanism outlined in Scheme 1.

According to this scheme, the primary event would be H-atom abstraction from the 13-position to give the resonance-stabilized pentadienyl radical I (Fig. 3). Formation of this radical would be favored over the alternative H-atom abstraction route from the 8-position, leading to the pentadienyl radical II, because of the lower energy of the C–H bond adjacent to the OH group, as reported in the case of alcohols and polyols

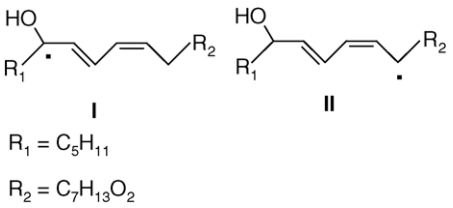


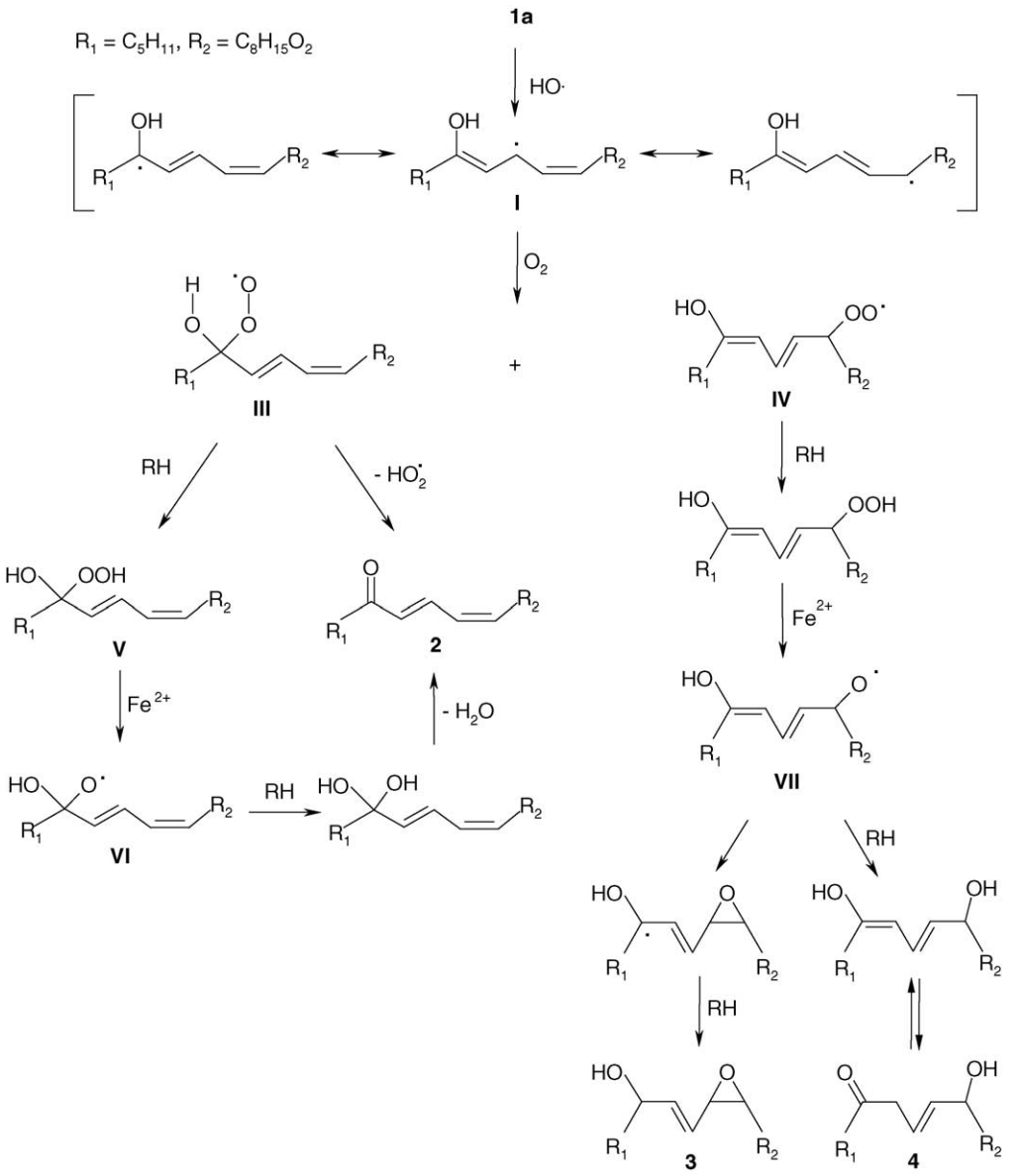
Fig. 3. Possible pentadienyl radicals generated by H-atom abstraction from **1a**.

(Mlakar and Spiteller, 1996). The comparable rate of consumption of **1a** and **1b** indicated that H-atom abstraction at C-13 was not rate-determining in the oxidation/oxygenation process.

The proposed route would also be consistent with the observed oxidation or loss of configuration at the C-13 stereogenic centre in products **2**–**4** as indicated in the case of **3** by ORD measurements. Whereas with the Fenton reagent the critical H-atom abstraction step would be brought about by the OH radical, in the case of the HRP/H_2O_2 system this could be done by the enzyme forms in which iron is at higher oxidation states, in particular compound I. This is two oxidizing equivalents above the resting ferrous state and consists of a haem derived ferryl intermediate (Fe^{IV}=O) and porphyrin π cation radical which make it competent to oxidize a broad spectrum of organic substrates by sequential one-electron transfer mechanisms. Alkylperoxyl radicals generated in the aerobic decomposition of the free radical initiator ABAP can likewise be envisaged to account for the initial H-atom abstraction.

Subsequent coupling of radical I with oxygen at the 9- or 13-positions would give rise to peroxyl radicals III and IV. Radical III could be engaged with two main reaction routes. The first involves a rearrangement step with loss of hydroperoxyl radical $HO_2^{\bullet}$ to give ketone **2**. Similar mechanisms are usually reported to account for the generation of α,β-unsaturated ketones by conversion of hydroxy- and hydroperoxy derivatives produced during lipid peroxidation (Spiteller, 1998, 2001).

The other route involves conversion of III to the hydroperoxide V which in the presence of a hydrogen donor may be reduced by Fe^{2+} to give the alkoxyl radical VI, by a mechanism similar to that of the Fenton reaction. Radical VI then would be converted to **2** by H-atom abstraction from a donor and dehydration of the resulting geminal diol. Formation of peroxyl radicals of type III and their subsequent decomposition to



Scheme 1. Proposed mechanism of formation of compounds **2**–**4**.

alkoxy radicals was proposed in the aerobic oxidation of alcohols to carbonyl compounds mediated by transition metal salts (Iwahama et al., 2000; Minisci et al., 2003, 2004).

Through an analogous reaction pathway, radical IV could evolve to give the alkoxyl radical VII. This latter could be converted to the corresponding alcohol, and then to **4** by tautomerization, or undergo intramolecular cyclization to **3**. The mechanistic route to **3** involves in the final stage an exchange at the C-13 with a H-atom donor. It is likely that the starting alcohol substrate could primarily serve this function at least in the initial stages of the reaction. Thus, the observed partial (15%) gain of hydrogen at the C-13 site in product **3** obtained from **1b** could be interpreted as due to the contribution to this latter step of H-atom donors other than the

starting material. Plausible candidates are hydroperoxide intermediates e.g. that deriving from peroxyl radical IV. It is clear however that the OH bearing C-13 site remains the main source of $^{1}H/^{2}H$ atom.

5. Conclusions

This study represents the first investigation of the reactivity of **1a**, one of the major metabolic product of linoleic acid peroxidation, toward the Fenton reagent under biomimetic conditions. Although generally regarded as a convenient model for free radical oxidations of (patho)physiological relevance, to the best of our knowledge the reactivity of the Fenton system toward polyunsaturated lipids and products thereof has remained so far uncharted. Highlights of the present study include: (a) the first isolation and complete structural characterization of products **3** and **4** previously described among the constituents of the mixtures generated by decomposition of 13-(*S*)-HPODE or obtained by lipoxygenase-catalyzed oxygenation of linoleic acid, but not unambigously discriminated from other regioisomers; (b) provide the first experimental evidence for a prominent reactivity of **1a** toward oxidation as promoted by OH radicals in contrast with previous reports. Overall the results presented hint at novel (patho)physiologically relevant pathways of linoleic acid oxidation in oxidative stress settings.

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