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Free radical oxidation of 15-(*S*)-hydroxyeicosatetraenoic acid with the Fenton reagent: characterization of an epoxy-alcohol and cytotoxic 4-hydroxy-2*E*-nonenal from the heptatrienyl radical pathway

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Abstract

The oxidation of (5*Z*,8*Z*,11*Z*,13*E*,15*S*)-15-hydroxy-5,8,11,13-eicosatetraenoic acid (15-(*S*)-HETE, **1a**) with the Fenton reagent (Fe^{2+}/EDTA/H_2O_2) was investigated. In phosphate buffer, pH 7.4, the reaction proceeded with 75% substrate consumption after 1 h to give a mixture of products, one of which was identified as (2*E*,4*S*)-4-hydroxy-2-nonenal (**3a**, 18% yield). Methylation of the mixture with diazomethane allowed isolation of another main product which could be identified as methyl (5*Z*,8*Z*,13*E*)-11,12-*trans*-epoxy-15-hydroxy-5,8,13-eicosatrienoate (**2a** methyl ester, 8% yield). A similar oxidation carried out on (15-^{2}H)-15-HETE (**1b**) indicated complete retention of the label in **2b** methyl ester and **3b**, consistent with an oxidation pathway involving as the primary event H-atom abstraction at C-10. Overall, these results support the recently proposed role of **1a** as a potential precursor of the cytotoxic γ-hydroxyalkenal **3a** and disclose a hitherto unrecognized interconnection between **1a** and the epoxy-alcohol **2a**, previously implicated only in the metabolic transformations of the 15-hydroperoxy derivative of arachidonic acid.

Keywords: Arachidonic acid; Fenton reagent; 4-hydroxynonenal; Lipid peroxidation; Deuterium labeling

1. Introduction

(5*Z*,8*Z*,11*Z*,13*E*,15*S*)-15-Hydroxy-5,8,11,13-eicosatetraenoic acid (15-(*S*)-HETE, **1a**) is a major product of lipid metabolism which is generated via the regiospecific and stereospecific oxidation of arachidonic acid catalyzed by 15-lipoxygenase, followed by a reduction step (Kühn, 1996). Additional routes to **1a** involve non-enzymatic peroxidation processes (Nemann and Khenkin, 1997) in which, however, the *R* enantiomer is also formed to a comparable extent, along with other positional isomers.

In mammalian cells **1a** acts as a physiological mediator, being involved in signal transduction and gene

Abbreviations: 15-(*S*)-HETE, (5*Z*,8*Z*,11*Z*,13*E*,15*S*)-15-hydroxy-5,8,11,13-eicosatetraenoic acid; PPAR, peroxysome proliferator-activated receptor; 13-(*S*)-HODE, (9*Z*,11*E*,13*S*)-13-hydroxy-9,11-octadecadienoic acid; EDTA, ethylendiaminetetraacetic acid; BSTFA, bis(trimethylsilyl)trifluoroacetamide; IBX, *o*-iodoxybenzoic acid; TMS, tetramethylsilane; COSY, correlation spectroscopy; HMQC, heteronuclear multiple quantum coherence; HMBC, heteronuclear multiple bond correlation; ESI(+)/MS, electrospray positive ion mode mass spectrometry; HR, high resolution; EI, electron impact

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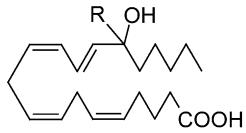
expression. It has been shown to be chemotactic and chemokinetic for polymorphonuclear leukocytes and vascular smooth muscle cells (Fischer et al., 1992). Additional biological activities of **1a** include stimulation of insulin secretion by pancreatic tissue (Laychock, 1985), induction of phosphorylation of cytoskeletal proteins, enhancement of surface expression of integrin receptors and platelet aggregation in tumor cells (Buchanan et al., 1998), and a role as second messenger in angiotensin-II induced aldosterone production (Natarajan et al., 1988). Moreover, **1a** is an important ligand to peroxysome proliferator-activated receptor (PPAR)-γ, (Nagy et al., 1998; Marx et al., 1999) a nuclear receptor that has been implicated in the modulation of critical aspects of development and homeostasis, including adipocyte differentiation, glucose metabolism, and macrophage development and function. Binding of **1a** to PPAR-γ nuclear receptors has been shown to induce apoptosis (Chen et al., 2003).

In addition to being biologically active per se, **1a** is the key precursor to a range of potent lipid mediators, including the lipoxins, which arise by enzymatic oxidation mediated by 5-lipoxygenase (Chavis et al., 1992). This latter process has been extensively investigated following recognition of the key role of lipoxins in cell–cell interactions and the regulation of inflammatory signals. Very little is known, by contrast, about non-enzymatic oxidative transformations of **1a** induced by reactive oxygen species. Because such pathways may contribute to affect the biological activity of this important lipid mediator in settings of oxidative stress, an understanding of the nature of the products formed by oxidation of **1a** under biomimetic conditions is of considerable interest. A recent study (Schneider et al., 2004) has shown that **1a**, like (9*Z*,11*E*,13S)-13-hydroxy-9,11-octadecadienoic acid (13-(*S*)-HODE) (Sun and Salomon, 2004), can undergo autoxidative transformation at 37 °C as dry film to give cytotoxic 4-hydroxynonenal as a prominent polar product. The discovery of the unexpected tendency of ω6-hydroxy fatty acids to undergo oxidative fragmentation disclosed novel possible pathways of formation of bioactive aldehydes in vivo.

In the present paper, we have investigated the reaction of **1a** with Fe^{2+}/ethylendiaminetetraacetic acid (EDTA)/H_2O_2 (the Fenton reagent), which is widely used to model non-enzymatic oxidative processes. Aim of the work was to provide a complete spectral characterization of the main oxidation products of this important lipid mediator, and to assess its tendency to undergo oxidative fragmentation to cytotoxic γ-hydroxyalkenals under physiologically relevant free radical conditions different from those reported in previous studies (Schneider et al., 2004; Sun and Salomon, 2004).



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

2. Materials and methods

2.1. Materials

Arachidonic acid (99%) and soybean lipoxidase (linoleate oxygen reductase, E.C. 1.13.11.12) type IB were from Sigma. Hydrogen peroxide (water solution, 33%), 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. 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.

(5*Z*,8*Z*,11*Z*,13*E*,15*S*)-15-Hydroxy-5,8,11,13-eicosatetraenoic acid (**1a**, 15-(*S*)-HETE) was 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 (IBX) 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.

IR spectra were obtained using a Nicolet 5700 FT-IR spectrophotometer. Optical rotations were measured using a JASCO P-1010 polarimeter. NMR spectra were recorded in $CDCl_3$ with a Bruker DRX-400 MHz instrument. ^{1}H and ^{13}C NMR spectra were recorded at 400.1 and 100.6 MHz, respectively, using tetramethylsilane (TMS) as the internal standard. ^{1}H–^{1}H COSY, ^{1}H–^{13}C HMQC and ^{1}H–^{13}C HMBC NMR experiments were run at 400.1 MHz using standard pulse programs.

Analytical and preparative TLC analyses were performed on F_{254} silica gel plates (0.25 and 0.5 mm, respectively) using cyclohexane/ethyl acetate 70:30 (v/v)

(eluant A) and chloroform/methanol 95:5 (v/v) (eluant B). $Ce(SO_4)_2$ (0.05 M in 10% H_2SO_4) and iodine were used for product detection on TLC plates.

Electrospray positive ion mode mass spectrometry (ESI(+)/MS) were run on a Waters ZQ quadrupole mass spectrometer. High resolution (HR) ESI mass spectra were obtained with a Finnegan MAT 90 instrument. GC/MS was carried out on a Trace GC Ultra-ThermoFinnigan instrument coupled with a Trace DSQ-ThermoFinnigan mass spectrometer. Helium was the carrier gas with a 1 mL/min flow rate. Data were processed using QualBrowser software. The following analytical conditions were used: 30 m cross-bond 5% diphenylpolysiloxane column (0.25 mm i.d., 0.25 μm df). Temperature program: 110 °C, hold time 0.8 min; up to 280 °C, rate 5 °C/min, hold time 40 min. The inlet, transfer line, and ion source were taken at 280, 250, and 220 °C, respectively. The acquisition started 15 min after the injection (solvent delay 15 min), with scan time set at 0.62 s, sampling at 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 methyl ester 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 (5Z,8Z,11Z,13E)-15-oxo-5,8,11,13-eicosatetraenoic acid (*4*)

Compound **1a** (140 mg, 0.44 mmol) was dissolved in ethyl acetate (82 mL), treated with *o*-iodoxybenzoic acid (1.13 g, 4.1 mmol) and kept under reflux at 85 °C. After 1 h the mixture was filtered to remove unreacted *o*-iodoxybenzoic acid as a white solid and afforded pure **4** (133 mg, 95% yield).

4: FT-IR ($CHCl_3$) ν_{max} 1710 cm^{-1}; ^1H NMR (O'Flaherty et al., 1994); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 14.9 (CH_3), 23.4 (CH_2), 25.7 (CH_2), 27.4 (CH_2), 30.5 (CH_2), 32.3 (CH_2), 32.6 (CH_2), 34.2 (CH_2), 34.9 (CH_2), 41.9 (CH_2), 127.2 (CH), 129.2 (2 × CH), 130.1 (CH), 130.4 (2 × CH), 137.3 (CH), 140.4 (CH), 179.6 (C), 202.1 (C); GC/MS (methyl ester derivative) t_R 28.2 min; Electron impact (EI) *m/z* (relative intensity) 332 (M^+, 15), 317 (M^+-CH_3, 13), 261 (M^+-$CH_3(CH_2)_4$, 7), 205 (M^+-CH:$CH(CH_2)_3CO_2CH_3$, 22), 191 (M^+-CH_2CH:$CH(CH_2)_3CO_2CH_3$, 31), 181 (M^+-$CH_3(CH_2)_4$COCH:CHCH:CH, 37), 165 (M^+-CH:$CHCH_2$CH:$CH(CH_2)_3CO_2CH_3$, 53), 151 ($CH_3(CH_2)_4$COCH:CHCH:CH, 100).

2.3. Synthesis of (15-^{2}H,5Z,8Z,11Z,13E)-15-hydroxy-5,8,11,13-eicosatetraenoic acid (*1b*)

A solution of **4** (100 mg, 0.31 mmol) in $CHCl_3$ (1 mL) was added to 0.1 M phosphate buffer, pH 7.4 (282 mL) and treated under vigorous stirring at room temperature with sodium borodeuteride (214 mg, 5.1 mmol). After 5 min the mixture was acidified to pH 3 with HCl and extracted with $CHCl_3$ (3 × 150 mL). The organic layers were combined, treated with anhydrous sodium sulfate, and dried under reduced pressure to afford pure **1b** (80 mg, 80% yield).

1b: ^1H NMR (400 MHz, $CDCl_3$), δ (ppm): 0.89 (3H, t J = 7.2 Hz, H-20), 1.2–1.5 (4H, m, H-18, H-19), 1.5–1.6 (4H, m, H-3, H-17), 1.71 (2H, t J = 7.2 Hz, H-16), 2.11 (2H, t J = 7.2 Hz, H-4), 2.32 (2H, t J = 7.2 Hz, H-2), 2.81 (2H, t J = 5.6 Hz, H-7), 2.96 (2H, t J = 6.4 Hz, H-10), 5.3–5.6 (5H, m, H-5, H-6, H-8, H-9, H-11), 5.70 (1H, d J = 15.2 Hz, H-14), 6.00 (1H, t J = 10.8 Hz, H-12), 6.58 (1H, dd J = 15.2, 10.8 Hz, H-13).

2.4. Isolation of compounds *2a* methyl ester and *3a*

Compound **1a** (100 mg, 0.31 mmol) was dissolved in $CHCl_3$ (1 mL) and emulsified in 0.1 M phosphate buffer, pH 7.4 (60 mL). The mixture was treated under vigorous stirring at room temperature with $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ (62 mg, 0.16 mmol) and EDTA (59 mg, 0.16 mmol), and the oxidation was started by addition of hydrogen peroxide (61 μL, 0.62 mmol). After 1 h the reaction mixture was extracted with ethyl acetate (2 × 30 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 fractionated by preparative thin layer chromatography (eluant A) to give, besides unreacted **1a** methyl ester (R_f = 0.56, eluant A, 26 mg, 25% yield), **2a** methyl ester (R_f = 0.40, eluant A, 8 mg, 8% yield) and **3a** (R_f = 0.32, eluant A, 8 mg, 18% yield) (Gioacchini et al., 1999).

2a methyl ester: FT-IR ($CHCl_3$) ν_{max} 3500–3200, 1732, 968, 870 cm^{-1}; $[\alpha]_D^{25}$ = +4.2° (*c* 1.34, $CHCl_3$); ^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 32.4 min; EI *m/z* (relative intensity) 422 (M^+, 15), 407 (M^+-CH_3, 11), 349 (M^+-$Si(CH_3)_3$, 78), 281 (M^+-CH_2CH:$CH(CH_2)_3CO_2CH_3$, 22), 249 (M^+-$C_6H_{12}OSi(CH_3)_3$, 100), 173 ($^\bullet C_6H_{12}OSi(CH_3)_3$, 44). ESI(+)/HRMS for $C_{21}H_{35}O_4$ calcd. 351.2535 $[M+H]^+$, found 351.2529

Table 1
Spectral data of compound **2a** methyl ester ($CDCl_3$)

Carbon	δ_C	δ_H(mult. J (Hz))	^1H–^1H COSY	^1H–^{13}C HMBC
1	175.2	–	–	–
2	34.2	2.32 (2H, t 7.2)	1.70	27.4, 25.5
3	25.5	1.70 (2H, t 7.2)	2.11, 2.32	27.4, 34.2, 130.0
4	27.4	2.11 (2H, dt 7.2, 5.6)	1.70, 5.38	25.5, 34.2, 129.3, 130.0
5	129.3	5.38 (1H, m)	2.11	25.5
6	130.0	5.38 (1H, m)	2.78	27.4
7	32.6	2.78 (2H, t 5.6)	5.38	129.3, 130.0
8	130.0	5.38 (1H, m)	2.78	30.5
9	129.3	5.38 (1H, m)	2.40	32.6
10	30.5	2.40 (2H, m)	2.88, 5.38	58.2, 60.4, 123.1, 129.3, 130.0
11	60.4	2.88 (1H, dt 5.6, 2.4)	2.40, 3.15	30.4, 129.3
12	58.2	3.15 (1H, dd 7.6, 2.4)	2.88, 5.43	123.1, 129.3, 136.4
13	123.1	5.43 (1H, dd, 15.6, 7.6)	3.15, 5.94	58.2, 60.4, 72.7
14	136.4	5.94 (1H, dd 15.6, 6.4)	4.12, 5.43	58.2, 72.7
15	72.7	4.12 (1H, m)	1.30, 1.53, 5.94	–
16	37.9	1.30 (1H, m)	1.53, 4.12	25.8
		1.53 (1H, m)	1.30, 4.12	25.8, 136.4
17	26.6	1.53 (2H, m)	1.30	25.8
18	25.8	1.30 (2H, m)	1.53	14.8, 23.4, 37.9
19	23.4	1.30 (2H, m)	0.89	14.8, 25.8
20	14.8	0.89 (3H, t 7.2)	1.30	23.4, 25.8
$-OCH_3$	52.3	3.67 (3H, s)	–	–

2.5. Isolation of compound **2b** methyl ester and **3b**

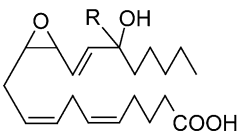
Compound **1b** was subjected to oxidation with the Fenton reagent under the same conditions adopted for **1a**. After work up and treatment with an ethereal solution of diazomethane, preparative thin layer chromatography (eluant A) afforded, besides unreacted **1b** methyl ester ($R_f = 0.56$, eluant A, 26 mg, 25% yield), **2b** methyl ester ($R_f = 0.40$, eluant A, 8 mg, 8% yield) and **3b** ($R_f = 0.32$, eluant A, 8 mg, 18% yield).

2b methyl ester: ^1H NMR (400 MHz, $CDCl_3$), δ (ppm): 0.89 (3H, t $J = 7.2$ Hz, H-20), 1.30 (5H, m, H-16, H-18, H-19), 1.53 (3H, m, H-16, H-17), 1.70 (2H, t $J = 7.2$ Hz, H-3), 2.11 (2H, dt $J = 7.2$, 5.6 Hz, H-4), 2.32 (2H, t $J = 7.2$ Hz, H-2), 2.40 (2H, m, H-10), 2.78 (2H, t $J = 5.6$ Hz, H-7), 2.88 (1H, dt $J = 5.6$, 2.4 Hz, H-11), 3.15 (1H, dd $J = 7.6$, 2.4 Hz, H-12), 3.67 (3H, s, $-OCH_3$), 5.38 (4H, m, H-5, H-6, H-8, H-9), 5.43 (1H, dd $J = 15.6$, 7.6 Hz, H-13), 5.94 (1H, d $J = 15.6$ Hz, H-14); GC/MS (TMS derivative) t_R 32.4 min; EI m/z 423 (M^+). ESI(+)/HRMS for $C_{21}H_{34}{}^2HO_4$ calcd. 352.2598 $[M+H]^+$, found 352.2602.

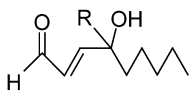
3b: ^1H NMR (400 MHz, $CDCl_3$), δ (ppm): 0.89 (3H, t $J = 7.2$ Hz, H-9), 1.2–1.4 (6H, m, H-6, H-7, H-8), 1.5–1.7 (2H, m, H-5), 6.34 (1H, dd $J = 15.6$, 7.6 Hz, H-2), 6.82 (1H, d $J = 15.6$ Hz, H-3), 9.60 (1H, d $J = 7.6$ Hz, H-1); ESI(+)/MS m/z (relative intensity) 173 ($[M+CH_3]^+$, 24), 158 ($[M+H]^+$, 100), 140 ($[M+H-H_2O]^+$, 6).

3. Results

The reaction of **1a** with the Fenton reagent (0.5 mequiv of the Fe^{2+}/EDTA complex and 2 mequiv of H_2O_2) was typically carried out with the lipid emulsified in 0.1 M phosphate buffer, pH 7.4 (Spiteller and Spiteller, 1997). After 1 h, TLC analysis of the reaction mixture extracted with ethyl acetate after methylation with diazomethane revealed the presence of a complex mixture of species, two of which, eluted at $R_f = 0.40$ (A, positive to $Ce(SO_4)_2$) and $R_f = 0.32$ (*B*, $\lambda = 254$ nm, positive to $Ce(SO_4)_2$), appeared to be relatively more abundant. Preparative TLC of a mixture obtained by preparative scale oxidation of **1a** eventually afforded the products in sufficient amounts and pure form for spectral characterization.



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



3a: R = H
3b: R = ^{2}H

The ^{1}H NMR spectrum of product A displayed as most significant features a group of signals centered at δ 5.38, due to the protons on the skipped *cis* double bonds, and two signals at δ 5.43 and 5.94, the latter showing coupling with a signal at δ 4.12 (1H, m), suggesting an allylic alcohol moiety with an *E* configuration at the double bond (J = 15.6 Hz).

The ^{1}H–^{1}H COSY spectrum revealed correlation of the olefin proton at δ 5.43 with a resonance at δ 3.15 which in turn gave a cross peak with a signal at δ 2.88. The latter two signals were strongly suggestive of protons on a *trans* epoxide functionality (J = 2.4 Hz). Moreover, the proton signal at δ 2.88 correlated with a –CH_2 resonance at δ 2.40 which was coupled to one of the *cis* olefin protons at δ 5.38. In the high field region of the spectrum two signals at δ 2.78 and 2.11 were clearly ascribable to bisallylic- and allylic-CH_2 protons, respectively.

The ^{1}H–^{13}C HMBC spectrum displayed correlations between the *trans* double bond proton resonances at δ 5.43 and 5.94 and the carbon resonances at δ 58.2, due to the epoxide ring, and δ 72.7, relative to the alcoholic functionality; the epoxide proton at δ 3.15 and the carbon resonances at δ 123.1 and 136.4 belonging to the *trans* double bond; between the methylene protons at δ 2.11, 2.40, and 2.78 and the carbon signals at δ 129.3 and 130.0 of the *cis* double bonds. On the basis of these data, the product was assigned the structure of methyl (5*Z*,8*Z*,13*E*)-11,12-*trans*-epoxy-15-hydroxy-5,8,13-eicosatrienoate (**2a** methyl ester). Structural assignments for **2a** methyl ester based on extensive 2D NMR spectral analysis are reported in Table 1.

In accord with this conclusion, the FT-IR spectrum displayed intense bands in the range 3500–3200 cm^{-1} consistent with an hydroxyl group; at 968 cm^{-1} (C–H bending of trans olefins) and at 870 cm^{-1} typical of the 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* 422. Diagnostic fragmentation peaks in the EI/MS spectrum corroborated the structural conclusions based on NMR analysis (see Fig. 1).

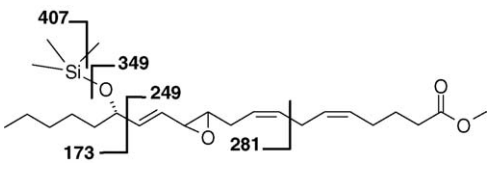
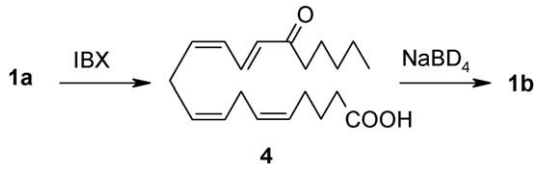


Fig. 1. Origin of salient fragmentation peaks in the EI/MS spectrum of the *O*-TMS derivative of compound **2a** methyl ester.



Scheme 1. Preparation of **1b** from **1a**.

The compound exhibited optical activity ($[\alpha]_D^{25}$ = +4.2° (*c* 1.34, $CHCl_3$)). Close inspection of the ^{1}H NMR spectrum of **2a** methyl ester indicated a single species, ruling out the presence of a mixture of diastereoisomeric *trans* epoxide derivatives. Unfortunately, attempts to define the stereochemical features of **2a** met with failure, not was it possible to isolate other diastereoisomers. However, evidence for the formation of trace amounts of other diastereoisomeric 11,12-epoxide derivatives was obtained by ^{1}H NMR analysis of the crude reaction mixture which revealed the presence of a species featuring two signals at δ 2.93 and 3.15 (J = 2.4 Hz), typical of a trans epoxide functionality. These signals gave in the ^{1}H–^{1}H COSY spectrum distinct correlations with an adjacent allylic alcohol moiety with an *E* configuration at the double bond closely similar to those observed for **2a** methyl ester.

Product B was identified as (2*E*)-4-hydroxy-2-nonenal (**3a**) by comparison of the spectral properties with literature data (Gioacchini et al., 1999). The identity of compound B with **3a** was also secured by comparing its chromatographic and spectral properties with those of an authentic sample prepared by a synthetic procedure (Gioacchini et al., 1999).

Overall yields of products **2a** methyl ester and **3a** accounted for about 35% of reacted **1a**; ^{1}H NMR analysis of other fractions of the mixture indicated that the remainder of the mixture consisted mainly of hydroxy-, oxo- and epoxy-derivatives.

To inquire into the mechanism of the Fenton-induced oxidation and to determine whether the asymmetric center at C-15 was affected in the conversion of **1a** to **2a** and **3a**, the reaction was carried out on the 15-^{2}H derivative of 15-HETE (**1b**). The latter compound was synthesized by reaction of **1a** with *o*-iodoxybenzoic acid (IBX) to give (5*Z*,8*Z*,11*Z*,13*E*)-15-oxo-5,8,11,13-eicosatetraenoic acid (**4**) followed by reduction with $NaBD_4$, according to a previously reported procedure (Manini et al., 2005) (Scheme 1).

^{1}H NMR analysis of the main reaction products indicated complete retention of the label, consistent with structures **2b** methyl ester and **3b** (Figs. 2 and 3). This was apparent from (a) the lack of detectable –C*H*OH sig-

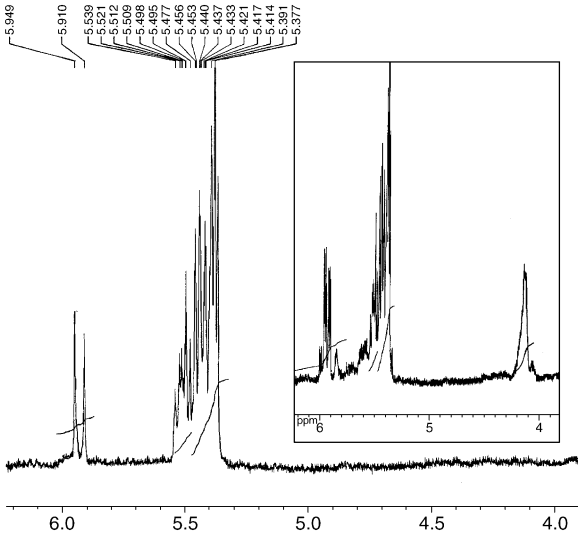


Fig. 2. Selected region of the ^{1}H NMR spectrum of **2b** methyl ester. Inset shows the same spectral region for the unlabelled compound.

nals in the region of the ^{1}H NMR spectrum comprised between δ 4.0–4.5; (b) the multiplicity of the signals at δ 5.94 (d, J = 15.6 Hz) due to the H-14 proton of **2b** methyl ester and at δ 6.82 (d, J = 15.6 Hz) due to the H-3 of **3b**; (c) the mass spectrometric analysis of the products, giving pseudomolecular ion peaks consistent with the presence of one deuterium atom.

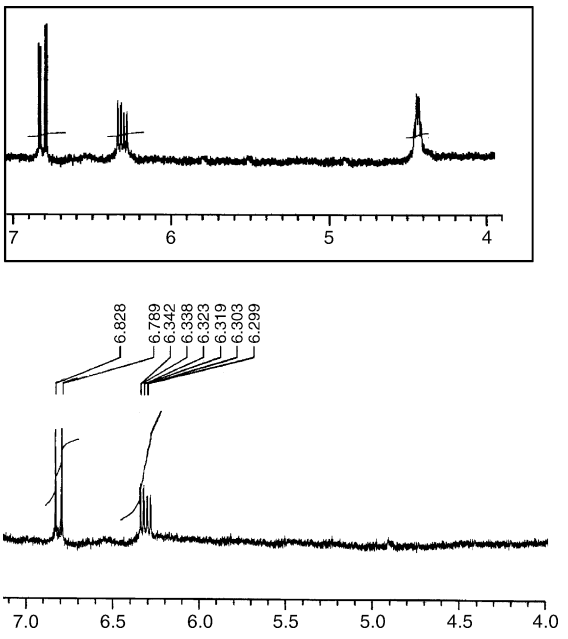


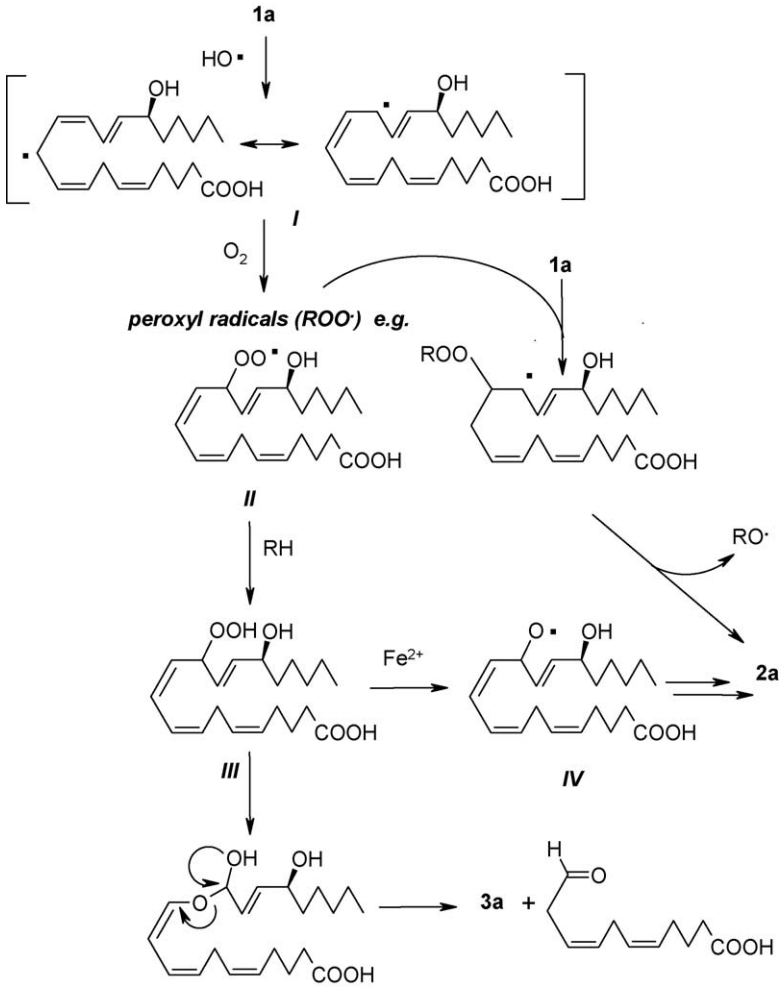
Fig. 3. Selected region of the ^{1}H NMR spectrum of **3b**. Inset shows the same spectral region for the unlabelled compound.

4. Discussion

Formerly regarded as relatively stable products of lipid peroxidation (Spiteller and Spiteller, 1997), the ω6-hydroxy derivatives of linoleic acid (13-(*S*)-HODE) and arachidonic acid (15-(*S*)-HETE, **1a**) have recently been proposed to play a role as potential precursors of biologically active lipid compounds (Schneider et al., 2004; Sun and Salomon, 2004). In a previous study we showed that 13-(*S*)-HODE can undergo free radical oxidation induced by the Fenton reagent to give 13-oxo-9,11-octadecadienoic acid, 9,10-epoxy-13-hydroxy-11-octadecenoic acid and 9-hydroxy-13-oxo-octadecenoic acid (Manini et al., 2005). We have now extended the study to **1a** and have shown that this important inflammatory mediator can likewise undergo free radical oxidation when exposed to the Fenton reagent to give a collection of products, the major of which could be isolated and spectrally characterized.

A major outcome of the present study is the identification of the cytotoxic γ-hydroxyalkenal **3a**, as the main fragmentation product of **1a** under the conditions of the Fenton-mediated oxidation. Compound **3a** is one of the most prominent cytotoxic products generated during lipid peroxidation, which may contribute to various disease processes secondary to oxidative stress through covalent adduct formation with proteins and nucleic acids (Benedetti et al., 1980; Cohn et al., 1996; Comporti, 1998; Ji et al., 2001; Nair et al., 1999). The generation of **3a** from **1a** may therefore represent a pathologically significant process that contributes to the accumulation of cytotoxic aldehydes subsequent to non-enzymatic oxidative lipid transformation in vivo.

Several epoxy alcohols have been described from the oxidation of arachidonic acid and/or its hydroperoxides (Chang et al., 1996; Pfister et al., 1998; Pfister et al., 2003), but only two have so far been structurally characterized by NMR techniques, namely (13*R*,14*R*,15*S*)-13,14-epoxy-15-hydroxy-5*Z*,8*Z*,11*Z*-eicosatrienoic acid and (11*S*,12*R*,15*S*)-11,12-epoxy-15-hydroxy-5*Z*,8*Z*,13*E*-eicosatrienoic acid (Hamberg et al., 1986); this latter, which was isolated by oxidative conversion of arachidonic acid by the fungus *Saprolegnia parasitica*, differs from **2a** only in the *cis* geometry of the epoxy functionality as apparent from the H11-H12 coupling constant (J = 4.2 Hz versus 2.4 Hz in the case of **2a**). For these epoxy alcohols a biosynthetic origin via lipoxygenase-mediated conversion of arachidonic acid to the 15-hydroperoxy derivative was suggested, followed by the action of a hydroperoxide isomerase (Pfister et al., 1998). Formation pathways of epoxy



Scheme 2. Proposed mechanism of formation of compounds **2a** and **3a**.

alcohol derivatives by oxidation of **1a** have not so far been reported.

Plausible mechanisms for the free radical oxidation of **1a** to **2a** and **3a** are outlined in Scheme 2.

One route involves HO•-induced H-atom abstraction at C-10 carbon, leading to formation of the highly delocalized heptatrienyl radical I. Subsequent coupling with oxygen would give rise to peroxyl radicals. The factors that govern the partitioning of oxygen to the several possible reactive sites of the heptatrienyl radical I and the relevance of such factors to product distribution have been addressed recently (Pratt et al., 2003). Based on theoretical analysis and model studies, it was shown that a heptatrienyl radical related to I would display the greatest spin densities on the inner positions, corresponding to the C-10 and C-12 positions in I. This suggests that products resulting from oxygen addition at these positions such as the peroxyl radical II would be mainly the kinetic products, formed under the relatively high H atom donor conditions of reaction ensured by the presence of unreacted **1a** during oxidation. H-atom abstraction from a donor converts peroxyl radical II to the corresponding hydroperoxide III that could be engaged with two main reaction routes. A Hock-type rearrangement of hydroperoxide III seems likely and is invoked here to account for the generation of **3a** (Schneider et al., 2004; Frimer, 1979). The other route involves the Fe^{2+}-mediated reduction of III to the alkoxyl radical IV by a Fenton-type mechanism, followed by intramolecular cyclization and hydrogen abstraction to give the epoxy-derivative **2a**. This latter is akin to that proposed for the formation of 14,15-leukotriene A_4 by cyclization of the 15-alkoxy derivative generated in the hematin-catalyzed decomposition of 15-hydroperoxy

derivative of arachidonic acid (Schreiber et al., 1986). Other evolution pathways of the radical IV should also be considered involving coupling with oxygen to give eventually α-or γ-hydroxyl/oxo epoxides by mechanisms similar to those described for the iron- (Gardner and Kleiman, 1981) and hematin- (Dix and Marnett, 1985) induced decomposition of 13-hydroperoxy-9,11-octadecadienoic acid (HPODE). Evidence for the formation of epoxy and hydroxy derivatives of **1a** as minor constituents of the Fenton oxidation mixture was obtained by NMR analysis.

An alternative route to **2a** involves homolytic attack of peroxyl radical intermediates to **1a** followed by decomposition of the resulting radical. Such processes are rather common and have been invoked in the epoxidation of the unsaturated components of plasmalogens (Murphy, 2001; Stadelmann-Ingrand et al., 2001) and of cholesterol linoleate (Spiteller, 2002). The preferential formation of the 11,12 epoxide **2a** observed under the reaction conditions examined may be accounted for in terms of the higher stability of the allylic radical resulting from addition at the 11- position. Despite the rather poor mass balance, **2a** and **3a** represent the most significant components of the oxidation mixture of **1a** and are likely to reflect the main reaction routes. All other components of the reaction mixture were present in very low amounts and could not be identified despite several efforts.

The reaction paths proposed in Scheme 2 are consistent with the observed retention of deuterium in products **2b** and **3b** derived from oxidation of **1b**. This finding clearly indicates a significant change of mechanism with respect to the oxidation of 13-(*S*)-HODE: whereas in the latter case H-atom abstraction at the allylic -CHOH center was the prevailing reaction channel, as evidenced from appreciable proton–deuterium exchange and loss of optical activity of the epoxy alcohol product (Manini et al., 2005), in the case of **1a** this route would hardly compete with oxidation at the bis-allylic C-10 center. This could be explained considering that the C–H bond dissociation enthalpy (BDE) associated with the formation of a heptatrienyl radical such as I is ca. 9 kcal/mol lower than that associated with the formation of a pentadienyl radical such as that centered on the C-8 of 13-(S)-HODE (Pratt et al., 2003).

On this basis, it is concluded that all products isolated by oxidation of **1a** retain the original *S* configuration at C-15 of the parent lipid. This is in accord with the results of the previous study on the generation of **3a** by oxidation of **1a**, in which carbon chain cleavage was shown to leave the stereochemistry at C-15 unaffected (Schneider et al., 2004).

5. Conclusions

This study represents the first investigation of the reactivity of **1a** with the Fenton reagent. We have shown that **1a** may be an important source of the cytotoxic aldehyde **3a** by free radical oxidation processes under conditions of physiological relevance, thus confirming previous observations relating to the transformation of **1a** under autoxidative conditions (Schneider et al., 2004). In addition, we have been able to demonstrate that **1a** can be converted to a signficant extent to the epoxy alcohol **2a**. Although there is a vast literature on the formation of epoxy alcohols in mammalian systems by both enzymatic and non-enzymatic routes from fatty acid hydroperoxides (Chang et al., 1996; Pfister et al., 1998; Pfister et al., 2003), these compounds have never been related to the oxidative metabolism of **1a**. The physiological significance of **2a** and its isomers is also an attractive issue for future studies. It may be relevant to mention here the identification of 11,12,15-trihydroxyeicosatrienoic acid, produced by hydrolysis of the corresponding epoxy alcohols, as endothelium derived relaxing factor of rabbit aorta (Pfister et al., 1998; Pfister et al., 2003), whereby a possible involvement of the transformation products of **2a** in the mechanisms of regulation of the vascular tone may be suggested.

Acknowledgments

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