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Biomimetic nitration of the linoleic acid metabolite 13-hydroxyoctadecadienoic acid: isolation and spectral characterization of novel chain-rearranged epoxy nitro derivatives

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Abstract

Nitration of unsaturated fatty acids is a (patho)physiologically important pathway of lipid modification induced by nitric oxide-derived species. We report herein on the unexpected chain rearrangement undergone by (13*S*,9*Z*,11*E*)-13-hydroxyoctadeca-9,11-dienoic acid (**1**), a linoleic acid metabolite, when exposed to nitrating agents of biological relevance. At pH 7.4 and at room temperature, reaction of **1** with peroxynitrite ($ONOO^-$) as well as Fe^{2+}-EDTA/H_2O_2/NO_2^- and horseradish peroxidase/H_2O_2/NO_2^- led to the formation of two nitration products, which could be isolated as the methyl esters and were identified as diastereoisomeric methyl (12*S*)-10,11-epoxy-12-hydroxy-9-nitromethylheptadecanoates by extensive ^1H, ^{13}C, ^{15}N NMR and MS analysis.

Keywords: Linoleic acid; Peroxynitrite; Fenton reagent; Lipid peroxidation

1. Introduction

Nitration of unsaturated fatty acids and related metabolites represents an increasingly important area of chemical research at the crossroads of biology and medicine. Bioactive nitrated derivatives of oleic acid and linoleic acid have been identified in bovine cardiac muscle, human plasma and red blood cells, and plasma from rats exposed to liver ischemia/reperfusion (Batthyany et al., 2006; Baker et al., 2004, 2005; Lima et al., 2002). They are robust ligands to peroxisome proliferator-activated receptor gamma (PPARγ) (Schopfer et al., 2005b; Alexander et al., 2006) and exhibit antiinflammatory, vasorelaxation and antiplatelet activities (Trostchansky et al., 2007; Lim et al., 2002; Coles et al., 2002a,b; Cui et al., 2006; Lima et al., 2005). Nitrated oleic and linoleic acid behave as hydrophobically stabilized nitric oxide (NO) donors which decay in polar milieus to act as lipid transducers of NO signaling (Schopfer et al., 2005a; Wright et al., 2006), and represent the single largest pool of bioactive oxides of nitrogen in the vasculature. Thus, products of fatty acid nitration have the potential to function as biomarkers and/or mediators of processes distinct from fatty acid peroxidation. Lipid nitration in vivo results from elevated production of NO and reactive nitrogen species (Lima et al., 2002; O'Donnell et al., 1999;

Abbreviations: PPAR, peroxisome proliferator-activated receptor; HRP, horseradish peroxidase; EDTA, ethylendiaminetetraacetic acid; 13-(*S*)-HODE, (9*Z*,11*E*,13*S*)-13-hydroxy-9,11-octadecadienoic acid; DEANO, *N*-ethylethaneamine:1,1-diethyl-2-hydroxy-2-nitrosohydrazine; BSTFA, bis(trimethylsilyl) trifluoroacetamide; TMS, tetramethylsilane; DMSO, dimethylsulfoxide; COSY, correlation spectroscopy; HMQC, heteronuclear multiple quantum coherence; HMBC, heteronuclear multiple bond correlation; NOESY, nuclear Overhauser exchange spectroscopy; TOCSY, total correlation spectroscopy; HPTLC, high performance thin layer chromatography; PICI-MS, positive chemical ionization-mass spectrometry; NICI-MS, negative chemical ionization-mass spectrometry; ESI+, electrospray ionization-positive ion; EI, electron impact; nOe, nuclear Overhauser effect.

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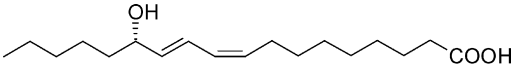
Rubbo and Freeman, 1996). NO can interact at diffusionally controlled rate with $O_2^{\bullet -}$ to give peroxynitrite, a mediator of biological nitrations and a strong oxidant for lipids (O'Donnell et al., 1999; Eiserich et al., 1998; Lancaster, 2006). The active oxidant in these processes is believed to be the unstable peroxynitrous acid (ONOOH) ($pK_a = 6.8$, $t_{1/2} < 1$ s) (Pryor and Squadrito, 1995) which is generated from the relatively stable peroxynitrite anion ($ONOO^-$) at physiological or lower pH values. Another metabolite of NO is nitrite (NO_2^-). Nitrite ions can be activated at acidic pH to form nitrous acid (Beake et al., 1994), or may interact with horseradish peroxidase (HRP)/H_2O_2 or iron complexes, e.g. the Fenton reagent (Fe^{2+}-EDTA/H_2O_2), to generate the powerful nitrating agent nitrogen dioxide (NO_2) (Bian et al., 2003; Thomas et al., 2002). An understanding of the mechanism underlying fatty acid nitration under physiomimetic conditions is therefore of fundamental importance if the generation and potential biological involvements of nitrated lipids are to be pursued in pathological settings of oxidative stress. Chemical research in this area is of central importance to aid elucidation of overall extent and patterns of fatty acid nitration in different metabolic or inflammatory states and to develop straightforward access routes to nitrated fatty acids for biological and pharmacological assays (Gorczynski et al., 2006). Previous studies from our laboratories have shown that ethyl linoleate reacts with nitrite ions in acidic media to form complex mixtures of nitration products (Napolitano et al., 2000, 2002b, 2004). Under the same conditions, (13*S*,9*Z*,11*E*)-13-hydroxyoctadeca-9,11-dienoic acid (13-(*S*)-HODE, **1**), the major 15-lipoxygenase metabolite of linoleic acid, an important mitogenic signaling molecule in certain tumors and a central mediator in inflammation, cell proliferation and differentiation (Jira et al., 1997; Reddy et al., 1997; Henricks et al., 1991; Nagy et al., 1998), reacts with nitrite ions in an acidic medium to give two major species that were identified as the 9-nitroderivative and an unusual oxidatively truncated product featuring a 4-nitro-2-oximinobutenal moiety (Napolitano et al., 2002a).



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We report now that **1** reacts with peroxynitrite and two NO_2^-/H_2O_2-based nitrating systems, i.e. HRP/H_2O_2/NO_2^- and Fe^{2+}-EDTA/H_2O_2/NO_2^-, to give two diastereoisomeric nitration products arising from an intriguing and unprecedented chain-rearrangement mechanism.

2. Experimental procedures

2.1. General methods

Linoleic acid (99%), *m*-chloroperbenzoic acid, sodium nitrite, sodium [^{15}N]nitrite (>99%), hydrogen peroxide (water solution, 33%), *N*-ethylethaneamine:1,1-diethyl-2-hydroxy-2-nitrosohydrazine (1:1) (DEANO), $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, ethylenediaminetetraacetic acid (EDTA), sodium borohydride, sodium borodeuteride, D-mannitol, bis(trimethylsilyl)trifluoroacetamide (BSTFA) were used as obtained. Horseradish peroxidase (H_2O_2 oxidoreductase, E.C. 1.11.1.7) type II, catalase (H_2O_2:H_2O_2 oxidoreductase, E.C. 1.11.1.6) and soybean lipoxidase (linoleate oxygen reductase, E.C. 1.13.11.12) type IB were used. 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. (9*Z*,11*E*,13*S*)-13-Hydroxyoctadeca-9,11-dienoic acid (**1**) (Napolitano et al., 2002a), (13-^{2}H,9*Z*,11*E*)-13-hydroxy-9,11-octadecadienoic acid (**[13-^{2}H]1**) (Manini et al., 2005) and (9*E*,11*E*,13*S*)-13-hydroxy-9-nitrooctadeca-9,11-dienoate (**9**) (Napolitano et al., 2002a) were synthesized and purified as described. **1** methyl ester was obtained by treatment of the free acid with diazomethane. Peroxynitrite ($ONOO^-$) was synthesized from sodium nitrite ($NaNO_2$) and acidified hydrogen peroxide using a quenched-flow reactor as previously described (Koppenol et al., 1996) and its concentration determined spectrophotometrically ($\varepsilon_{302} = 1670\ M^{-1}\ cm^{-1}$). [^{15}N]$ONOO^-$ was prepared as reported above using $Na^{15}NO_2$ (O'Donnell et al., 1999). Residual hydrogen peroxide was removed by treatment with granular manganese dioxide. Peroxynitrite solutions was kept at −20 °C and used within 2 days. 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 (Vogel, 1951). *Caution! Diazomethane is explosive and must be kept at* −20 °C. IR spectra were obtained using a FT-IR spectrophotometer. Optical rotations were measured using a polarimeter. NMR spectra were recorded in $CDCl_3$ with a 400 MHz instrument. ^{1}H and ^{13}C NMR spectra were recorded at 400.1 and 100.6 MHz, respectively, using TMS as the internal standard. ^{15}N spectra were recorded at 40.5 MHz using [^{15}N]urea as the internal standard (76.97 ppm in DMSO relative to NH_3 (liquid, 298 K) 0.0 ppm (^{15}N NMR)) (Levy and Lichter, 1979). ^{1}H–^{1}H COSY, ^{1}H–^{13}C HMQC, ^{1}H–^{13}C HMBC, ^{1}H–^{15}N HMBC, NOESY and TOCSY experiments were run at 400.1 MHz using standard pulse programs. For all the products, except for compounds **5** and **6** methyl esters, ^{1}H and ^{13}C NMR resonances due to –OCH_3, *C*$OOCH_3$, C-2 (CH_2), C-3/C-4/C-5/C-6/C-7/C-14/C-15/C-16/C-17 (CH_2) and C-18 (CH_3) groups appear in the ^{1}H/^{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 (18H, m)/22–38, 0.89 (3H, m)/14.8 in the order. Analytical and preparative TLC were performed on F_{254} silica gel plates (0.25 and 0.5 mm, respectively) and high-performance TLC (HPTLC) plates using cyclohexane/ethyl acetate 70:30 (v/v) (eluant A). Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine in 5% phosphoric acid) (Sy and By, 1985), $Ce(SO_4)_2$ (0.05 M in 10% H_2SO_4), and iodine were used for product detection on TLC plates. Liquid chromatography was performed on silica gel (70–270 mesh). Purity (>90%) of isolated compounds was determined by ^{1}H NMR analysis.

2.2. GC–MS analyses

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) and negative ion chemical ionization-mass spectrometry (NICI-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, 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 50% BSTFA in pyridine and kept at 50 °C for 30 min to give the corresponding trimethylsilyl derivative.

2.3. LC–MS analyses

LC–MS analyses were carried out on a HPLC apparatus coupled with a quadrupole mass spectrometer. HPLC runs were performed on an octylsilane-coated column (4.6 mm × 150 mm, 5 μm) using the following elution conditions: 0.5% formic acid (solvent A) and acetonitrile (solvent B), from 30% to 50% solvent B gradient, for 20 min, from 50% to 70% solvent B gradient, for 30 min, and then from 70% to 50% solvent B gradient, for 10 min. Flow rates of 0.4 mL/min were used. Mass spectra were registered in the electrospray ionization-positive ion (ESI+) mode with the cone and the fragmentator voltages set at 4 kV and 70 V, respectively.

2.4. Reaction with peroxynitrite: general procedure

The appropriate substrate (0.084 mmol) dissolved in $CHCl_3$ (100 μL) was added to 0.1 M phosphate buffer pH 7.4 (17 mL) and treated dropwise with a 135 mM solution of peroxynitrite in NaOH (3 mL, 0.41 mmol) under vigorous stirring at room temperature. To keep the pH constant an equal volume of 0.25 M HCl was simultaneously added. After 3 h the reaction mixture was extracted with ethyl acetate (2× 6 mL) and the organic layers were combined, dried over anhydrous sodium sulfate, and evaporated to dryness under reduced pressure. The residue was taken up in ethyl acetate (1 mL) and treated with an ethereal solution of diazomethane (1 mL). After 15 min the mixture was evaporated to dryness and analyzed by TLC (cyclohexane/ethyl acetate 7:3 v/v, eluant A). Similar experiments were carried out in the presence of $NaHCO_3$ (5–20 mM).

2.5. Isolation of methyl (10R,11S,12S)-10,11-epoxy-12-hydroxy-9-nitromethylheptadecanoate (**5** methyl ester) and methyl (10S,11R,12S)-10,11-epoxy-12-hydroxy-9-nitromethylheptadecanoate (**6** methyl ester)

The reaction was carried out and worked up under the conditions described above using 100 mg of **1**. After treatment with diazomethane the mixture was fractionated by column chromatography (2.0 cm × 40 cm) using cyclohexane/ethyl acetate (8:2 to 6:4 v/v gradient mixtures) to afford, besides unreacted **1** methyl ester (16 mg, 15% yield, $R_f = 0.80$ eluant A), **2** methyl ester (31 mg, 30% yield, $R_f = 0.82$ eluant A), **3** methyl ester (17 mg, 15% yield, $R_f = 0.48$ eluant A), **5** methyl ester (3 mg, $R_f = 0.37$ eluant A), **6** methyl ester (8 mg, 6% yield, $R_f = 0.33$ eluant A), and a fraction at $R_f = 0.35$–0.40 which was further purified on HPTLC plates (eluant chloroform/methanol 98:2 v/v) to give **4** methyl ester (8 mg, 7% yield, $R_f = 0.35$ eluant A) and additional **5** methyl ester (2 mg, 4% overall yield).

5 methyl ester: FT-IR ($CHCl_3$) ν_{max} 3550–3300, 1732, 1550, 1370, 883 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ (ppm): 0.89 (3H, m, H-17), 1.3–1.4 (10H, m, H-4, H-5, H-6, H-15, H-16), 1.5–1.6 (8H, m, H-3, H-7, H-13, H-14), 1.94 (1H, ddd, $J = 14.8$, 11.6, 4.4 Hz, H-8), 2.31 (2H, t, $J = 7.6$ Hz, H-2), 2.49 (2H, m, H-8, H-9), 2.78 (1H, dd, $J = 3.2$, 2.4 Hz, H-11), 3.17 (1H, dd, $J = 5.2$, 2.4 Hz, H-10), 3.66 (3H, s, OCH_3), 3.80 (1H, m, H-12), 4.37 (1H, dd, $J = 18.0$, 7.6 Hz, CH_2NO_2), 4.86 (1H, dd, $J = 18.0$, 8.8 Hz, CH_2NO_2); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 14.8 (CH_3), 23.3 (CH_2), 24.0 (CH_2), 25.5 (CH_2), 25.7 (CH_2), 29.4 (CH_2), 29.5 (CH_2), 30.1 (CH_2), 32.5 (CH_2), 33.8 (CH_2), 34.8 (CH_2), 35.3 (CH_2), 43.2 (CH), 52.2 (CH), 52.3 (CH_3), 60.7 (CH), 70.6 (CH), 78.3 (CH_2NO_2), 175.0 ($COOCH_3$). GC–MS (*O*-TMS derivative) t_R 42.4 min; EI-MS *m/z* 445 (M^+), 399 ($M^+ - NO_2$); PICI-MS *m/z* 446 ($[M+H]^+$).

6 methyl ester: FT-IR ($CHCl_3$) ν_{max} 3550–3300, 1732, 1550, 1370, 883 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ (ppm): 0.89 (3H, m, H-17), 1.3–1.4 (10H, m, H-4, H-5, H-6, H-15, H-16), 1.5–1.6 (8H, m, H-3, H-7, H-13, H-14), 1.96 (1H, ddd, $J = 14.8$, 11.6, 4.4 Hz, H-8), 2.31 (2H, t, $J = 7.6$ Hz, H-2), 2.46 (1H, ddd, $J = 14.8$, 11.6, 4.4, H-8), 2.60 (1H, ddd, $J = 8.8$, 7.6, 4.4 Hz, H-9), 2.74 (1H, dd, $J = 4.0$, 2.4 Hz, H-11), 3.19 (1H, dd, $J = 4.0$, 2.4 Hz, H-10), 3.64 (1H, m, H-12), 3.66 (3H, s, OCH_3), 4.32 (1H, dd, $J = 18.0$, 7.6 Hz, CH_2NO_2), 4.83 (1H, dd, $J = 18.0$, 8.8 Hz, CH_2NO_2); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 14.8 (CH_3), 23.3 (CH_2), 24.0 (CH_2), 25.5 (CH_2), 25.7 (CH_2), 29.4 (CH_2), 29.5 (CH_2), 30.1 (CH_2), 32.5 (CH_2), 33.8 (CH_2), 34.8 (CH_2), 35.3 (CH_2), 43.2 (CH), 52.2 (CH), 52.3 (CH_3), 60.7 (CH), 70.6 (CH), 78.3 (CH_2NO_2), 175.0 ($COOCH_3$). GC–MS (*O*-TMS derivative) t_R 42.7 min; EI-MS *m/z* 445 (M^+), 399 ($M^+ - NO_2$); PICI-MS *m/z* 446 ($[M+H]^+$).

2.6. Reaction of **1** with $[^{15}N]ONOO^-$

The reaction was carried out as described above by treating **1** (100 mg) with a 130 mM solution of $[^{15}N]ONOO^-$ in NaOH. After 4 h reaction time the mixture was worked up and fractionated as described above to afford, besides **1** methyl ester (16 mg, 15% yield), **2** methyl ester (31 mg, 30% yield), **3** methyl ester (17 mg, 15% yield) and **4** methyl ester (8 mg, 7% yield), **[^{15}N]5** methyl ester (5 mg, 4% yield) and **[^{15}N]6** methyl ester (8 mg, 6% yield).

[^{15}N]5 methyl ester: GC–MS (*O*-TMS-derivative) t_R 42.4 min; EI-MS *m/z* 446 (M^+), 399 ($M^+ - {}^{15}NO_2$).

[^{15}N]6 methyl ester: GC–MS (*O*-TMS-derivative) t_R 42.7 min; EI-MS *m/z* 446 (M^+), 399 ($M^+ - {}^{15}NO_2$).

2.7. Reaction of **1** with the Fe^{2+}-EDTA/H_2O_2/$NaNO_2$ system

1 (25 mg, 0.084 mmol) dissolved in $CHCl_3$ (100 μL) was added to 0.1 M phosphate buffer pH 7.4 (17 mL) and treated under vigorous stirring at room temperature with $Fe(NH_4)_2(SO_4)_2{\cdot}6H_2O$ (16.4 mg, 0.042 mmol), EDTA (15.6 mg, 0.042 mmol) and sodium nitrite (29 mg, 0.42 mmol). The oxidation was started by addition of four aliquots of 1 M hydrogen peroxide (42 μL, 0.042 mmol each) over a period of 45 min. After 4 h the reaction mixture was worked up as above and analyzed by TLC (eluant A). Similar experiments were carried out: (i) as above but omitting EDTA or H_2O_2; (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.

2.8. Reaction of **1** with the horseradish peroxidase (HRP)/H_2O_2/$NaNO_2$ system

1 (25 mg, 0.084 mmol) dissolved in $CHCl_3$ (100 μL) was added to 0.1 M phosphate buffer pH 7.4 (17 mL) and treated under vigorous stirring at room temperature with sodium nitrite (58 mg, 0.84 mmol), horseradish peroxidase (52 U) and 1 M hydrogen peroxide, this latter 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 as above and analyzed by TLC (eluant A).

2.9. Reaction of **1** with DEANO

1 (20 mg, 0.068 mmol) dissolved in $CHCl_3$ (100 μL) was added to 0.1 M phosphate buffer pH 7.4 (14 mL) and treated under vigorous stirring at room temperature with a solution of DEANO (28 mg, 0.136) in 10 mM NaOH. After 4 h the reaction mixture was worked up as above and analyzed by TLC (eluant A).

2.10. Reaction of **1** methyl ester with nitrogen oxides

A solution of nitrite in water (1 M) was added to 10% sulfuric acid over 10 min. The red-orange gas which developed was conveyed with a flux of argon into a solution of **1** methyl ester (20 mg, 0.065 mmol) in ethyl acetate (4 mL). Three hours after the development of the red fumes was complete, the reaction mixture was worked-up as above and analyzed by TLC (eluant A).

2.11. Isolation of methyl (12-^{2}H,10R,11S)-10,11-epoxy-12-hydroxy-9-nitromethylheptadecanoate and methyl (12-^{2}H,10S,11R)-10,11-epoxy-12-hydroxy-9-nitromethylheptadecanoate (**[12-^{2}H]5** and **[12-^{2}H]6** methyl esters, mixture of diastereoisomers)

The reaction of **[13-^{2}H]1** (100 mg) with peroxynitrite was carried out as reported for **1**. After work up and treatment with diazomethane, the residue was subjected to column chromatography using cyclohexane/ethyl acetate (8:2 to 6:4 v/v gradient mixtures) to afford a fraction consisting of **[12-^{2}H]5/6** methyl esters.

[12-^{2}H]5^a/**6**^{b} methyl esters: ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 0.89 (3H, m, H-17), 1.3–1.4 (10H, m, H-4, H-5, H-6, H-15, H-16), 1.5–1.6 (8H, m, H-3, H-7, H-13, H-14), 1.94^a (1H, ddd, J = 14.8, 11.6, 4.4 Hz, H-8), 1.96^b (1H, ddd, J = 14.8, 11.6, 4.4 Hz, H-8), 2.31 (2H, t, J = 7.6 Hz, H-2), 2.46^b (1H, ddd, J = 14.8, 11.6, 4.4, H-8), 2.49^a (2H, m, H-8, H-9), 2.60^b (1H, ddd, J = 8.8, 7.6, 4.4 Hz, H-9), 2.74^b (1H, d, J = 2.4 Hz, H-11), 2.78^a (1H, d, J = 2.4 Hz, H-11), 3.17^a (1H, dd, J = 5.2, 2.4 Hz, H-10), 3.19^b (1H, dd, J = 4.0, 2.4 Hz, H-10), 3.66 (3H, s, OCH_3), 4.32^b (1H, dd, J = 18.0, 7.6 Hz, CH_2NO_2), 4.37^a (1H, dd, J = 18.0, 7.6 Hz, CH_2NO_2), 4.83^b (1H, dd, J = 18.0, 8.8 Hz, CH_2NO_2), 4.86^a (1H, dd, J = 18.0, 8.8 Hz, CH_2NO_2).

2.12. Synthesis of methyl (9Z,11R,12S,13S)-11,12-epoxy-13-hydroxy-9-octadecenoate (**7**) and methyl (9Z,11S,12R,13S)-11,12-epoxy-13-hydroxy-9-octadecenoate (**8**)

1 methyl ester (100 mg, 0.32 mmol) was dissolved in $CHCl_3$ (400 μL) and treated in a capped vial with a solution of *m*-chloroperbenzoic acid (70.4 mg, 0.41 mmol) in $CHCl_3$ (2.8 mL), added in seven portions (400 μL each) every 10 min. The reaction mixture was kept under stirring overnight and then extracted twice with 0.1 M phosphate buffer, pH 7.4 (2× 1 mL). The combined organic layers were dried over anhydrous sodium sulfate and evaporated under reduced pressure. The residue was fractionated by preparative TLC (eluant A) to afford pure **7** (12 mg, 11% yield, R_f = 0.60 eluant A) and **8** (20 mg, 19% yield, R_f = 0.50 eluant A), along with methyl (11*E*,13*S*)-13-hydroxy-9,10-epoxy-11-octadecenoate (33 mg, 31% yield, R_f = 0.42 eluant A).

7: $[\alpha]_D^{25}$ + 1.1° (*c* 0.71, $CHCl_3$); FT-IR ($CHCl_3$) ν_{max} 3547–3296, 1732, 891 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$), δ (ppm): 2.20 (2H, m, H-8), 2.92 (1H, dd, J = 2.8, 2.4 Hz, H-12), 3.67–3.71 (1H, m, H-11), 3.88 (1H, m, H-13), 5.08 (1H, t, J = 10.8 Hz, H-10), 5.73 (1H, dt, J = 10.8, 8.0 Hz, H-9); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 32.6 (CH_2, C-8), 51.3 (CH, C-11), 63.0 (CH, C-12), 69.1 (CH, C-13), 127.0 (CH, C-9), 138.0 (CH, C-10); GC–MS t_R 40.6 min; EI-MS *m*/*z* 326 (M^+), 308 ($M^+ - H_2O$); PICI-MS *m*/*z* 327 ($[M+H]^+$); NICI-MS *m*/*z* 326 (M^+).

8: $[\alpha]_D^{25}$ + 2.2° (*c* 0.22, $CHCl_3$); FT-IR ($CHCl_3$) ν_{max} 3547–3296, 1732, 891 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$), δ (ppm): 2.19 (2H, m, H-8), 2.88 (1H, dd, J = 4.8, 2.4 Hz, H-12), 3.54 (1H, m, H-13), 3.59 (1H, dd, J = 9.2, 2.4 Hz, H-11), 5.06 (1H, dd, J = 10.8, 9.2 Hz, H-10), 5.72 (1H, dt, J = 10.8, 7.6 Hz, H-9); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 32.6 (CH_2, C-8), 53.2 (CH, C-11), 63.7 (CH, C-12), 71.7 (CH, C-13), 126.8 (CH, C-9), 138.0 (CH, C-10); GC–MS t_R 41.4 min; EI-MS *m*/*z* 326 (M^+), 308 ($M^+ - H_2O$); PICI-MS *m*/*z* 327 ($[M+H]^+$); NICI-MS *m*/*z* 326 (M^+).

Methyl (11*E*,13*S*)-13-hydroxy-9,10-epoxy-11-octadecenoate: $[\alpha]_D^{25}$ +0.7° (*c* 0.45, $CHCl_3$); FT-IR ($CHCl_3$) ν_{max} 3547–3296, 1732, 968 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$), δ (ppm): 0.89 (3H, M, H-18), 1.6 (2H, m, H-8), 3.07 (1H, dt, J = 5.6, 4.8 Hz, H-9), 3.40 (1H, dd, J = 7.6, 4.8 Hz, H-10), 4.16 (1H, m, H-13), 5.55 (1H, dd, J = 15.6, 7.6 Hz, H-11), 5.95 (1H, dd, J = 15.6, 6.4 Hz, H-12); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 32.5 (CH_2), 57.3 (CH), 59.7 (CH), 73.0 (CH), 130.6 (CH), 140.4 (CH); GC–MS t_R 41.9 min; EI-MS *m/z* 326 (M^+), 308 ($M^+ - H_2O$); PICI-MS *m/z* 327 ($[M+H]^+$); NICI-MS *m/z* 326 (M^+).

2.13. Synthesis of methyl (9E,11R,12S,13S)-11,12-epoxy-13-hydroxy-9-nitro-9-octadecenoate (**10**) and methyl (9E,11S,12R,13S)-11,12-epoxy-13-hydroxy-9-nitro-9-octadecenoate (**11**)

Compound **9** (100 mg, 0.28 mmol) was dissolved in $CHCl_3$ (400 μL) and treated portionwise (7× 400 μL each, every 10 min) with a solution of *m*-chloroperbenzoic acid (61.4 mg, 0.36 mmol) in $CHCl_3$ (2.8 mL) in a capped vial. The reaction mixture was kept under stirring overnight and then worked up as described above. The residue was fractionated by preparative TLC (eluant A) which afforded pure **10** (36 mg, 35% yield, R_f = 0.53 eluant A) and **11** (53 mg, 51% yield, R_f = 0.50 eluant A).

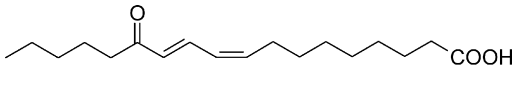
10: $[\alpha]_D^{25}$ +9.2° (*c* 0.26, $CHCl_3$); FT-IR ($CHCl_3$) ν_{max} 3547–3296, 1732, 1528, 1363 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$), δ (ppm): 2.73 (2H, m, H-8), 3.11 (1H, dd, J = 2.8, 2.0 Hz, H-12), 3.65 (1H, dd, J = 8.8, 2.0 Hz, H-11), 3.94 (1H, m, H-13), 6.70 (1H, d, J = 8.8 Hz, H-10); ^{13}C NMR (100 MHz, $CDCl_3$), δ (ppm): 32.5 (CH_2, C-8), 50.3 (CH, C-11), 63.3 (CH, C-12), 68.8 (CH, C-13), 133.0 (CH, C-10), 155.9 (C, C-9); GC–MS (*O*-TMS derivative) t_R 44.8 min; EI-MS *m/z* 443 (M^+); PICI-MS *m/z* 444 ($[M+H]^+$).

11: $[\alpha]_D^{25}$ +11.5° (*c* 0.61, $CHCl_3$); FT-IR ($CHCl_3$) ν_{max} 3547–3296, 1732, 1528, 1363 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$), δ (ppm): 2.72 (2H, m, H-8), 3.10 (1H, dd, J = 3.6, 2.0 Hz, H-12), 3.60 (1H, dd, J = 8.8, 2.0 Hz, H-11), 3.68 (1H, m, H-13), 6.68 (1H, d, J = 8.8 Hz, H-10); ^{13}C NMR (100 MHz, $CDCl_3$), δ (ppm): 32.5 (CH_2, C-8), 51.8 (CH, C-11), 63.6 (CH, C-12), 70.5 (CH, C-13), 132.6 (C-10), 155.9 (C-9); GC–MS (*O*-TMS derivative) t_R 45.4 min; EI-MS *m/z* 443 (M^+); PICI-MS *m/z* 444 ($[M+H]^+$).

3. Results and discussion

3.1. Reaction of **1** with biomimetic nitrating systems: isolation and characterization of nitration products

Reaction of **1** (5 mM) with peroxide-free peroxynitrite, prepared according to a standard procedure (Koppenol et al., 1996), in 0.1 M phosphate buffer at pH 7.4, followed by extraction with ethyl acetate and methylation with diazomethane revealed the formation of products **2**–**4**, previously obtained by free radical oxidation of **1** with the Fenton reagent (Manini et al., 2005), and two additional products (R_f = 0.37 and 0.33), positive to the Griess reagent for nitroso/nitro compounds. Trace amounts of other Griess-positive products could also be detected. A very similar product distribution was observed with the HRP/H_2O_2/NO_2^- and the Fe^{2+}-EDTA/H_2O_2/NO_2^- systems.



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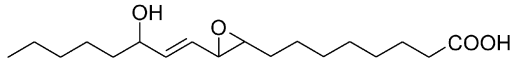

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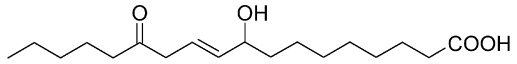

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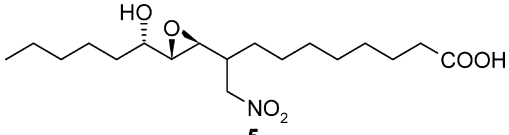
After careful silica gel column chromatography followed by high-performance thin layer chromatography (HPTLC) fractionation, the products were eventually obtained in pure form and were formulated as the novel methyl (10*R*,11*S*,12*S*)-10,11-epoxy-12-hydroxy-9-nitromethylheptadecanoate (**5** methyl ester, R_f = 0.37) and methyl (10*S*,11*R*,12*S*)-10,11-epoxy-12-hydroxy-9-nitromethylheptadecanoate (**6** methyl ester, R_f = 0.33) by extensive spectral analysis coupled with chemical and mechanistic evidence.



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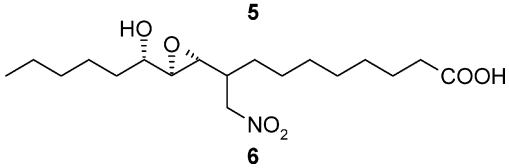

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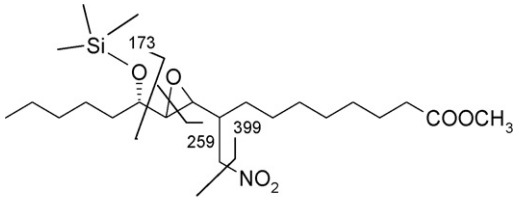
The products lacked the conjugated diene chromophore of **1**. They showed on FT-IR analysis bands at 3550–3300 cm^{-1}, due to the –OH group, as well as at 1550 and 1370 cm^{-1} indicating aliphatic nitro groups. PICI (positive ion chemical ionization)-MS analysis of the *O*-TMS derivatives of **5** and **6** methyl esters gave a pseudomolecular ion peak at *m/z* 446. A weak, barely detectable molecular ion peak at *m/z* 445 was noted in the EI-MS spectrum along with a fragmentation peak at *m/z* 399, reflecting loss of NO_2. A possible interpretation of the fragmentation peaks is given in Fig. 1.



***O*-TMS derivative of 5 methyl ester**

Fig. 1. Interpretation of the fragmentation pattern observed in the EI-MS spectrum of the *O*-TMS derivative of **5** methyl ester.

NMR analysis, summarized in Table 1, revealed similar patterns of resonances, suggesting that the products were diastereoisomers. The characteristic $>CHCH_2NO_2$ moiety was readily apparent from the sets of signals in the ^1H NMR spectra at δ 4.86 and 4.37, for **5** methyl ester, and at δ 4.83 and 4.32, for **6** methyl ester. These signals showed one-bond correlations with a carbon resonance at δ 78.3 and gave cross-peaks in the ^1H–^1H COSY spectrum with proton signals at δ 2.49 (**5** methyl ester) and 2.60 (**6** methyl ester) indicating methylene groups linked to tertiary CH carbons.

Furthermore, when **1** was allowed to react with ^{15}N-labeled peroxynitrite, ^1H–^{15}N HMBC analysis of the compound corresponding to **6** methyl ester revealed a ^{15}N signal at δ 385.6, typical of nitro groups linked to sp^3 carbons (Napolitano et al., 2002b), giving cross-peaks with the methylene proton resonance at δ 4.32, the CH proton signal at δ 2.60, and the methylene protons at δ 1.96 and 2.46. This data provides conclusive evidence for the $–CH_2NO_2$ group at the chain branching point. The epoxyalcohol moiety was revealed by proton resonances at δ 3.80, 3.17 and 2.78 (**5** methyl ester) and 3.64, 3.19 and 2.74 (**6** methyl ester), correlating with carbon signals at δ 70.6, 52.2 and 60.7, in that order. The *trans* configuration of the epoxy functionality was deduced from the coupling constant of 2.4 Hz and was corroborated by a band in the FT-IR spectrum at 883 cm^{-1} typical of the C–H stretching of *trans* epoxides (Gardner and Kleiman, 1981). From ^1H–^{13}C HMBC data it was further deduced that the epoxyalcohol functionality was directly linked to the branching site, while TOCSY experiments revealed positioning of the C*H*OH proton closer to the methyl terminus of the chain as in the parent **1**. The configuration of the epoxy ring relative to the hydroxyl group in the α-epoxyalcohol moiety was assigned on the basis of the different nOe contacts between the epoxy ring and the C*H*OH protons in the *threo* and *erythro* isomers (Pettersson et al., 1992), and of chemical shift data (Mihelich, 1979). In particular, **5** methyl ester showed distinct cross-peaks in the NOESY experiment between the resonances at δ 3.80 and δ 3.17 and at δ 3.80 and δ 2.78, with this latter more intense, and was accordingly assigned the *erythro* configuration at the α-epoxyalcohol moiety, whereas **6** methyl ester displayed com-

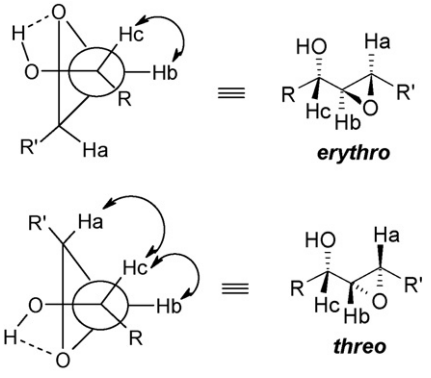


Fig. 2. nOe contacts between the –C*H*OH proton Hc and the epoxy protons Ha and Hb in the *erythro* and *threo* α-epoxyalcohol moieties of **5** and **6** methyl esters.

Table 1
Salient NMR data ($CDCl_3$) for compounds **5** and **6** methyl esters

Carbon	^{13}C	^1H (mult., J (Hz))	^1H–^1H COSY	^1H–^{13}C HMBC	TOCSY[a]	NOESY
5 methyl ester						
C-8	29–30	1.94 (ddd, 14.8, 11.6, 4.4)	1.5–1.6, 2.49	–	1.5–1.6	1.3–1.4, 2.49, 3.17
		2.49 (m)	1.5–1.6, 1.94	52.2, 78.3	1.3–1.4, 1.5–1.6	1.94, 2.78, 3.17, 4.37, 4.86
C-9	43.2	2.49 (m)	1.5–1.6, 1.94, 3.17, 4.37, 4.86	52.2, 78.3	1.3–1.4, 1.5–1.6	1.94, 2.78, 3.17, 4.37, 4.86
C-10	52.2	3.17 (dd, 5.2, 2.4)	2.49, 2.78	43.2, 78.3	2.78, 3.80, 4.37, 4.86	1.94, 2.49, 3.80, 4.37
C-11	60.7	2.78 (dd, 3.2, 2.4)	3.17, 3.80	70.6	1.3–1.4, 1.5–1.6, 3.17, 3.80, 4.37, 4.86	2.49, 3.80, 4.37
C-12	70.6	3.80 (m)	1.5–1.6, 2.78	–	0.89[b], 1.3–1.4, 1.5–1.6, 2.78, 3.17	2.78, 3.17
CH_2NO_2	78.3	4.37 (dd, 18.0, 7.6)	2.49, 4.86	43.2, 52.2	2.78, 3.17, 4.86	2.49, 2.78, 3.17, 4.86
		4.86 (dd, 18.0, 8.8)	2.49, 4.37	–	2.78, 3.17, 4.37	2.49, 4.37
6 methyl ester						
C-8	29–30	1.96 (ddd, 14.8, 11.6, 4.4)	1.5–1.6, 2.46	–	1.5–1.6	1.3–1.4, 2.46, 3.19
		2.46 (ddd, 14.8, 11.6, 4.4)	1.5–1.6, 1.96	52.2, 78.3	1.3–1.4, 1.5–1.6	1.96
C-9	43.2	2.60 (ddd, 8.8, 7.6, 4.4)	3.19, 4.32, 4.83	52.2, 78.3	1.96, 2.31	2.74, 3.19, 4.32, 4.83
C-10	52.2	3.19 (dd, 4.0, 2.4)	2.60, 2.74	43.2, 78.3	2.74, 3.64, 4.32, 4.83	1.96, 2.60, 2.74, 3.64, 4.32
C-11	60.7	2.74 (dd, 4.0, 2.4)	3.19, 3.64	70.6	1.3–1.4, 1.5–1.6, 3.19, 3.64, 4.32, 4.83	2.60, 3.19, 3.64, 4.32
C-12	70.6	3.64 (m)	1.5–1.6, 2.74	–	0.89[b], 1.3–1.4, 1.5–1.6, 2.74, 3.19	2.74, 3.19
CH_2NO_2	78.3	4.32 (dd, 18.0, 7.6)	2.60, 4.83	43.2, 52.2	2.74, 3.19, 4.83	2.60, 2.74, 3.19, 4.83
		4.83 (dd, 18.0, 8.8)	2.60, 4.32	–	2.74, 3.19, 4.32	2.60, 4.32

[a] Run in acetone-d_6.
[b] Protons on C-17.

parable cross-peaks between the proton resonance at δ 3.64 and at δ 2.74 and 3.19, consistent with a *threo* configuration (Fig. 2).

The configuration at the CHOH center was assessed by checking whether an H-atom abstraction step takes place at that site during product formation. To address this issue, (13-^{2}H,9*Z*,11*E*)-13-hydroxy-9,11-octadecadienoic acid (**[13-^{2}H]1**) (Manini et al., 2005) was used as a mechanistic probe. When **[13-^{2}H]1** was reacted with peroxynitrite or the Fe^{2+}-EDTA/H_2O_2/NO_2^- system, the two diastereoisomeric nitrated products corresponding to **5/6** methyl esters displayed complete retention of the label. This observation indicates that the stereogenic CHOH center was unaffected and thus retained the original configuration as in **1**. Determination of relative configuration at C-9 by spectral analysis was difficult because of the very similar proton and carbon chemical shifts and poorly informative coupling constants. Scrutiny of the region between δ 4 and 5 in the ^{1}H NMR spectrum of the whole reaction mixture revealed, besides the characteristic pairs of double doublets due to the CH_2NO$_2$ resonances of **5** and **6**, two similar, albeit much less intense, pairs of signals, suggesting the presence in the mixture of two minor stereoisomers of **5** and **6**. Attempts to isolate these minor diastereoisomers were thwarted by the exceedingly low formation yields, the unfavorable chromatographic behavior (they migrated as impurities of the main bands) and the lack of chromophore hampering HPLC fractionation. Isolated yields of **5** and **6** methyl esters were about 4% and 6%, respectively, which, together with **2**–**4**, account for about 73% of reacted **1**.

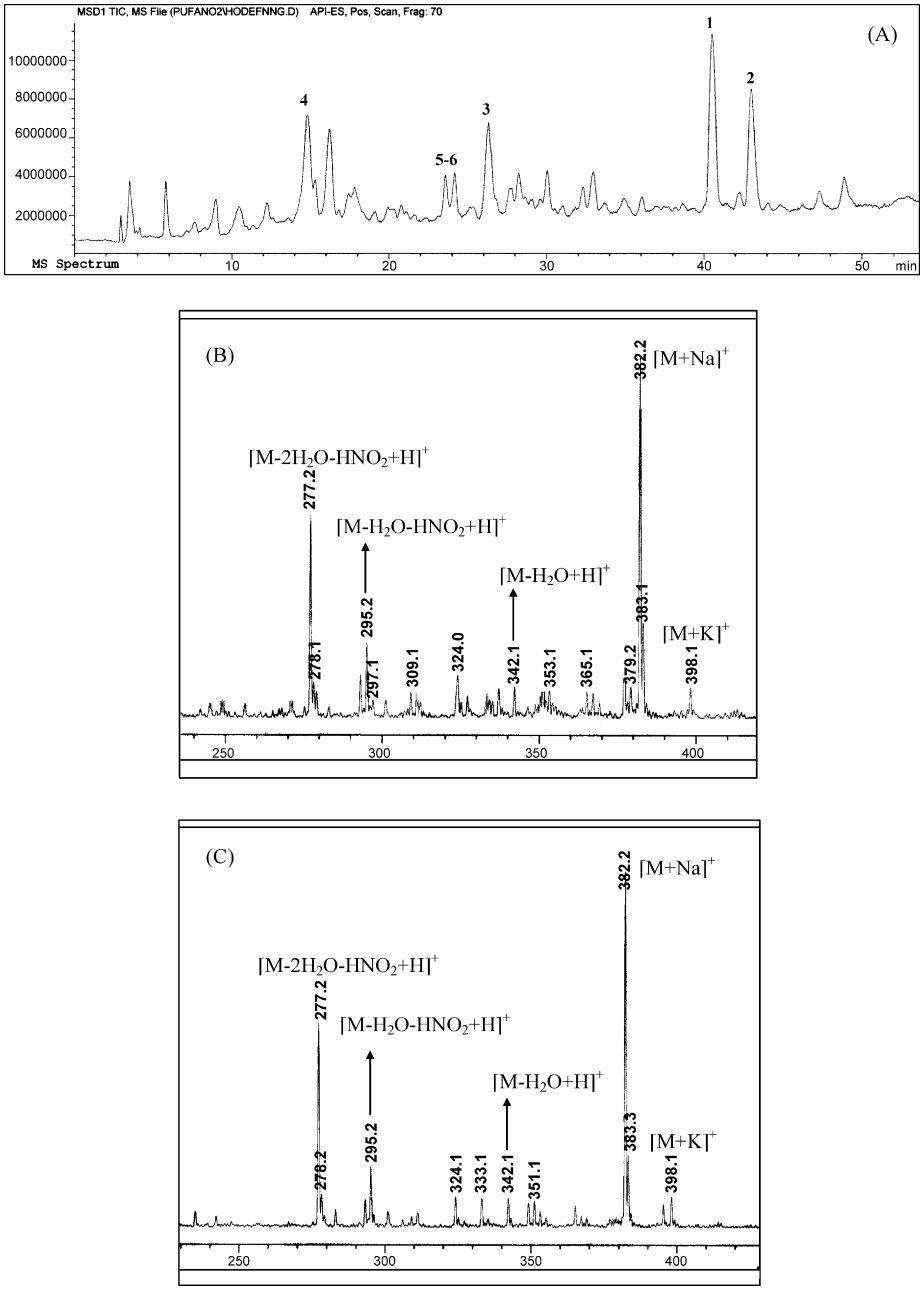


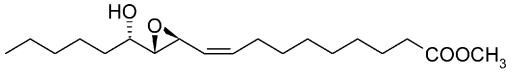
Fig. 3. LC–MS profile of the reaction mixture of **1** with $ONOO^-$ (A) and ESI(+)-MS spectra of compounds **5** (B) and **6** (C).

The remainder of the reaction mixture consisted of other products that could not be identified, including possibly the minor isomers of **5/6**.

LC–MS analysis of crude ethyl acetate-extracts from reaction mixtures prior to derivatization and silica gel purification provided a reliable and selective means to monitor the generation of **5** and **6** as free fatty acids during the reaction course. After 4 h reaction time the LC(ESI+)–MS profile (Fig. 3A) revealed the presence, besides unreacted **1**, of three main products which were identified as the oxidation products **2**, **3** and **4** by analysis of their mass spectra. Among the other components of the mixtures, two products eluted under two peaks at 23.6 and 24.3 min showed pseudomolecular ions at *m/z* 382 $[M+Na]^+$ and *m/z* 398 $[M+K]^+$, consistent with **5** and **6**, together with peaks due to subsequent losses of water and HNO_2 (Fig. 3B and C). Identification of these compounds was secured by parallel analysis of the reaction mixtures obtained using ^{15}N labeled nitrite ions (data not shown).

3.2. Mechanistic issues

All of the nitrating systems reported in this study behaved similarly to give the same oxidation and nitration products. The HRP/H_2O_2/NO_2^- and Fe^{2+}-EDTA/H_2O_2/NO_2^- systems are known to generate NO_2 as the actual oxidizing/nitrating species (Bian et al., 2003; Brennan et al., 2002). In the Fenton system, this species would result from NO_2^- oxidation (E° NO_2/NO_2^- = +1.04 V) by the $HO^\bullet$ radical or an equivalent primary product of the Fenton reaction, since $HO^\bullet$ radical scavengers such as mannitol inhibited the formation of **5** and **6** (data not shown). A similar inhibition of the Fenton oxidation/nitration processes was observed in the absence of EDTA or H_2O_2, and under an argon atmosphere. The presumption of similar nitration mechanisms with all the systems examined would lead to the conclusion that NO_2 is also involved in the peroxynitrite-mediated nitration. This conclusion, however, needs to be assessed in the light of what is currently known about the chemistry of peroxynitrite (or peroxynitrous acid, hydrogen oxoperoxonitrate) which is characterized by epoxidation, nitration and oxidative cleavage reactions, and is largely dictated by the nature of the substrate and experimental conditions (Eiserich et al., 1998; Niles et al., 2006; Ducrocq et al., 1999; Nonoyama et al., 2001; Grossi et al., 2001). A number of papers in the literature report that peroxynitrous acid can undergo homolytic fission to give NO_2 and OH radicals (see, e.g. Bartesaghi et al., 2006; Botti et al., 2004), though this view has been questioned on the basis of kinetic and thermodynamic data (Koppenol and Kissner, 1998). In particular, it was suggested that peroxynitrous acid itself is a strong oxidant and the actual oxidizing species is a twisted form of *trans* peroxynitrous acid related to the transition state for the isomerization to nitrate (Ramezanian et al., 1996). In physiological environments, peroxynitrite reactivity is modulated by the levels of CO_2 which reacts with $ONOO^-$ to give $ONOOCO_2^-$ ($k = 6 \times 10^4\ M^{-1}\ s^{-1}$ at 37 °C) (Uppu et al., 2000). This species generates NO_2 and the potent oxidant $CO_3^{\bullet-}$ (E° $CO_3^{\bullet-}/CO_3^{2-}$ = +1.5 V) (Uppu et al., 2000) which may induce H-atom abstraction or one-electron oxidation processes. When the reaction was carried out with $NaHCO_3$ in concentration up to 20 mM, no appreciable increase in the formation of nitration products was observed, thus ruling out a critical role of $CO_3^{\bullet-}$ in oxidation/nitration steps (Bartesaghi et al., 2006). In separate experiments it was found that neither the nitrogen oxides separately generated by acidification of a nitrite solution and bubbled through a solution of **1** in ethyl acetate by way of an argon stream, nor a solution of the NO-donor, *N*-ethylethaneamine: 1,1-diethyl-2-hydroxy-2-nitrosohydrazine (1:1) (DEANO), in phosphate buffer at pH 7.4 were able to convert **1** to detectable **5/6**. In both cases other products were observed that were not characterized. No trace of the rearranged nitration products **5/6** could be observed with acidic nitrite under previously reported conditions (Napolitano et al., 2002a). In another set of experiments, we prepared a number of compounds by oxidation and/or nitration of **1** and investigated their behavior to the various nitration systems tested under the usual reaction conditions, to assess their possible intermediacy *en route* to **5/6**. Tested compounds include **3**, methyl (9*Z*,11*R*,12*S*,13*S*)-11,12-epoxy-13-hydroxy-9-octadecenoate (**7**), methyl (9*Z*,11*S*,12*R*,13*S*)-11,12-epoxy-13-hydroxy-9-octadecenoate (**8**), methyl (9*E*,11*E*,13*S*)-13-hydroxy-9-nitrooctadeca-9,11-dienoate (**9**) (Napolitano et al., 2002a), methyl (9*E*,11*R*,12*S*,13*S*)-11,12-epoxy-13-hydroxy-9-nitro-9-octadecenoate (**10**) and methyl (9*E*,11*S*,12*R*,13*S*)-11,12-epoxy-13-hydroxy-9-nitro-9-octadecenoate (**11**). All these products were then allowed to stand in phosphate buffer at pH 7.4 both in the presence and in the absence of the oxidizing/nitrating systems, but in no case could any detectable conversion to **5** and **6** methyl esters be observed, thus ruling out their involvement in the formation of **5/6**.



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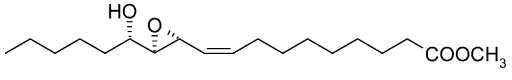

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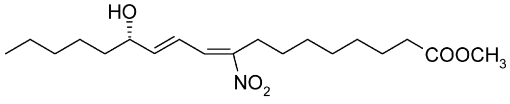

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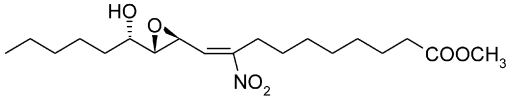

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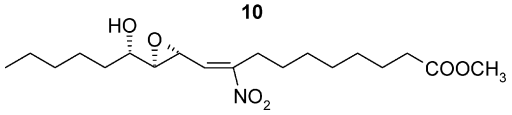

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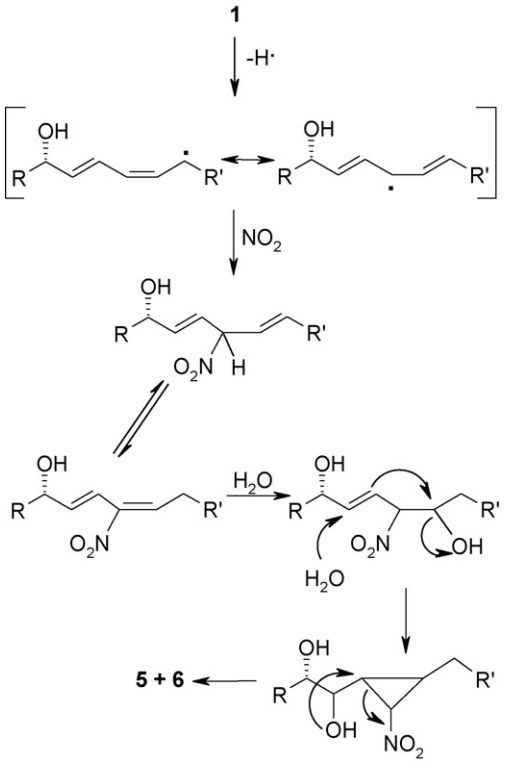
On the basis of available evidence it is not possible to draw any substantiated mechanism for product formation. A tentative reaction sequence for the formation of **5/6** is given in Scheme 1.

In this scheme, H-atom abstraction at C-8 would be the initiating step, possibly carried out by peroxyl radicals, $HO^\bullet$ and/or

NO_2 (Pryor and Lightsey, 1981; Pryor et al., 1982; Gallon and Pryor, 1994). Coupling of the resulting radical with NO_2 at C-10 would then lead to a nitrodiene intermediate. It should be noted that allylic H-atom abstraction at C-8 followed by free radical coupling at C-10 is an established degradative pathway of 13-hydroperoxyoctadecadienoic acid, the hydroperoxy analog of **1** (Schneider et al., 2001). Tautomerization of the initially formed 3-nitro-1,4-diene would lead to a reactive 2-nitro-1,3-diene intermediate which would undergo water addition to give a hydroxylated nitrocyclopropane intermediate. It is known that 2-nitro-1,3-dienes are highly reactive toward nucleophiles (Bajpai and Bhaduri, 1996) and that nitrocyclopropanes may be susceptible to ring opening under a variety of conditions (Ballini et al., 2007; Budynina et al., 2006; Vettiger and Seebach, 1990). The ring opening process would be driven by the ability of the nitro group to sustain an incoming negative charge on the α-carbon thus assisting heterolytic cleavage of the adjacent carbon–carbon bond (Yurdakul et al., 1998; Panzella et al., 2006).

The critical step invoking hydroxide anion as leaving group is perhaps unusual, but in aqueous solution proton exchanges are quite frequent even under mildly basic conditions and it is possible that the internal substitution leading to the nitrocyclopropane intermediate is favored by solvation of the hydroxy group.

The above free radical nitration mechanism seems to be applicable with all nitrating systems examined, including peroxynitrite, based on similar product patterns, but this conclusion remains speculative since all attempts to detect putative intermediates in the proposed pathway by NMR analysis were unsuccessful despite considerable efforts, and alternative routes cannot be ruled out.



Scheme 1. Proposed key steps in the formation of **5/6**.

4. Conclusions

This paper represents an extension of previous studies from our laboratory (Napolitano et al., 2000, 2002a,b) focused on the reaction chemistry of linoleic acid and its major metabolite, **1**, with nitrating systems of biological relevance. The results have shown that **1** is susceptible of nitration by three different nitrating systems modeling those that may occur in pathophysiological settings of oxidative and nitrosative stress, and undergoes a remarkable chain rearrangement reaction. To the best of our knowledge, this is the first report dealing with the isolation and structural characterization of shortened, branched-chain derivative by nitration of a bioactive fatty acid under mild conditions. This work may be of interest because it discloses a novel, intriguing facet of fatty acid chemistry under conditions of physiological relevance. Moreover, it furnishes analytical and spectroscopic data of new compounds that may provide guidance in elucidating novel pathways of polyunsaturated fatty acid modification under inflammatory conditions. Although no evidence currently exists for concomitant oxidative and nitrative metabolism of fatty acids, our data suggest that tissue damage and/or alteration of lipid signaling in inflammatory pathways may derive at least in part by the observed NO_2^--mediated reactions targeted to hydroxy derivatives of unsaturated lipids. These would be tertiary metabolic events (nitration of already oxidized fatty acids) that would be deserving of further attention considering the current interest into the oxidative transformations of hydroxy derivatives of fatty acids, the observed reactivity of the conjugated diene moiety of **1** toward reactive nitrogen species, and the typically high bioactivities of these metabolites even when formed in trace amounts.

Unlike the parent **1**, compounds **5/6** lack double bonds and allylic alcoholic functionalities, which would somewhat blunt their susceptibility to further oxidation. Moreover, at variance with known nitrooleates and nitrolinoleates, the new nitrated lipids described in this paper possess a nitro group bound to an isolated methylene group, which would not be expected to impart effective NO donating and signaling properties. Based on the above lines of reasoning, **5/6** would represent minor end-products of lipid peroxidation in an inflammatory process, but these predictions await verification in future chemical studies.

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