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Chemistry of Nitrated Lipids: Remarkable Instability of 9-Nitrolinoleic Acid in Neutral Aqueous Medium and a Novel Nitronitrate Ester Product by Concurrent Autoxidation/Nitric Oxide-Release Pathways

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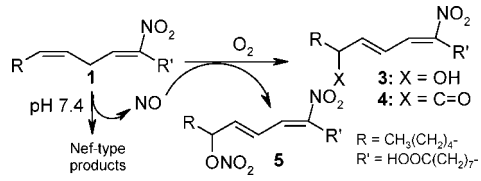

Despite the mounting interest in nitrolinoleic acids and related nitrated polyunsaturated fatty acids as a novel class of bioactive signaling lipids, their chemistry and metabolic fate have remained poorly elucidated. Herein, we report an expedient nitroselenenylation/oxidation route to 9-nitrolinoleic acid (**1**) and 10-nitrolinoleic acid (**2**), which enabled comparative product studies under physiologically relevant conditions. Under biomimetic conditions, **1** decayed at an unusually fast rate to give the hydroxy-, keto-, and nitronitrate ester derivatives **3**, **4**, and **5** as main products, identified by ESI-MS and 2D NMR spectroscopy, including $^{1}H,^{15}N$ HMBC experiments on the ^{15}N-labeled derivatives. The 13-nitrato functionality in **5** suggested partitioning of **1** between concurrent peroxidation and nitric oxide (NO)-release pathways. Lipid **2** decayed at a much slower rate giving only the hydroxynitro derivative **6** as an isolable product. Diphenylpicrylhydrazide (DPPH) radical quenching experiments and DFT computations concurred to support a higher H-atom donating ability of **1** versus **2**, due to more effective stabilization of the resulting pentadienyl radical by the terminal nitro group. The markedly different stability of isomeric nitrolinoleic acids disclosed in the present study may provide an explanation for the previous identification of **2**, but not **1**, in body fluids and offers a key for future insights into the biological activities of nitrated lipids.

Introduction

Nitrated derivatives of unsaturated fatty acids have emerged during the past decade as important products of lipid modification under (patho)physiological conditions associated with oxidative stress and elevated production of nitric oxide (NO).[1,2] Although the detailed structural features of the nitrated fatty acids generated in vivo remain to be defined, a significant proportion of them appears to be present in the form of nitroalkene derivatives, such as the nitrooleic acids and the nitrolinoleic acids.[3,4] These latter comprise four isomeric derivatives of (*Z*,*Z*)-9,12-octadecadienoic acid exhibiting a

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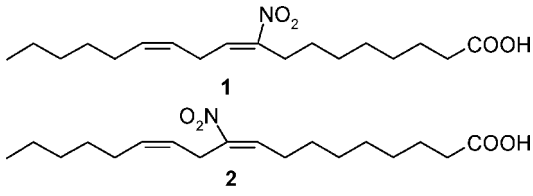
conjugated nitro group on the terminal (9- and 13-) or inner (10- and 12-) positions of the (*Z*,*Z*)-1,4-pentadienyl system. So far, 10-nitrolinoleic acid ((9*E*,12*Z*)-10-nitrooctadecadienoic acid, **2**) and the 12-nitro isomer have been detected in human plasma and urine by LC−MS techniques,[5−7] whereas 9-nitrolinoleic acid ((9*E*,12*Z*)-9-nitrooctadecadienoic acid, **1**) and its 13-nitro isomer have been described only as products of chemical nitration of linoleic acid in an organic solvent.[8,9]



A substantial body of evidence indicates that nitrolinoleic acids behave as pluripotent signaling molecules that transduce the actions of NO via multiple mechanisms,[7,10,11] which in part are receptor-mediated and in part reflect the electrophilic and NO-releasing behavior of the nitropentadiene reactive moiety in a physiological milieu. The electrophilic nature of nitrolinoleates is deduced by their ability to cause post-translational modifications of protein residues[9,12,13] and by the occurrence in vivo of a significant proportion of vicinal hydroxynitro derivatives,[7,10] suggesting addition of water onto the double bond. The ability of nitrolinoleic acids to release NO is the focus of much interest. Experimental evidence derived from different experiments (EPR, chemiluminescence, deoxy- and oxymyoglobin assays)[10,14−17] suggests the release of NO as well as nitrite,[15,16] but the detailed pathways remain to be definitively assessed. In the presence of other lipids or amphiphiles at levels above the critical micellar concentration, nitrolinoleic acids are stabilized and become highly resistant to NO-release. This finding suggests that nitrolinoleic acids provide a hydrophobically stabilized NO reserve and, in view of their accumulation to detectable levels in lipophilic biological compartments, represent the single largest pool of bioactive oxides of nitrogen in the vasculature.[10] Apart from the above studies, current knowledge of the chemical behavior and fate of these nitroalkene fatty acid derivatives under physiologically relevant conditions is scanty and limited to the decomposition routes supposedly associated to their NO donor abilities. Surprising is also the lack of general information about the free radical oxidation pathways of the nitro-1,4-pentadienyl system, the reactive core of nitrated polyunsaturated fatty acids. A detailed elucidation of the chemical properties of nitrolinoleic acids under physiologically relevant conditions is therefore of paramount importance for further progress toward an understanding of the signaling capabilities of these bioactive lipids. To this aim, access to the individual isomers is essential to advancing structure−activity knowledge and to rationally design new more active structural variants for therapeutic purposes. In this paper we report the first convenient access route to pure **1** and **2** as free acids and a comparative investigation of their chemical behavior in aqueous phosphate buffer at pH 7.4. By integrating product analysis with mechanistic experiments, we provide a comparative description of the basic chemistry of nitrolinoleic acids under physiologically relevant conditions. The remarkable position-dependent influence of the nitro group on the oxidation behavior of the 1,4-pentadienyl moiety is also addressed with the aid of a density functional theory (DFT) investigation.

Results and Discussion

Preparation of Nitrolinoleic Acids. Unlike nitrooleic acids, for which simple access routes based on standard Henry condensation chemistry are available,[16,18] nitrolinoleic acids are difficult to synthesize. Their preparation currently relies on direct nitration of the parent fatty acid[4,6,8,9,19,20] because of the lack of synthetic routes assembling the C-18 nitrated fatty acid chain. Early methods based on acidic nitrite or NO_2^+-forming reagents led invariably to mixtures of regio- and stereoisomers in low yields, along with abundant side products, and only small amounts of **2** and the 12-nitro isomer as carboxylate esters could be obtained. More recently, a nitrophenylselenenylation/oxidation protocol has been reported to produce a statistical distribution of the four regioisomeric nitrolinoleic acids in good overall yields,[9] but their separation as free acids remained a difficult task. To provide access to individual nitrolinoleic acid isomers needed for probing the structural basis of their biological activities and fate, to determine if certain isomers are more reactive than others in competing autoxidation/NO-release and water addition pathways, and to understand why unsaturated lipids with the nitro group on terminal positions of the polyene systems (e.g., **1**) have so far eluded identification in vivo, it was necessary to devise expedient methods for preparation of representative isomeric nitrolinoleates as pure free acids. Nitrolinoleates **1** and **2** were thus selected for the purposes of the

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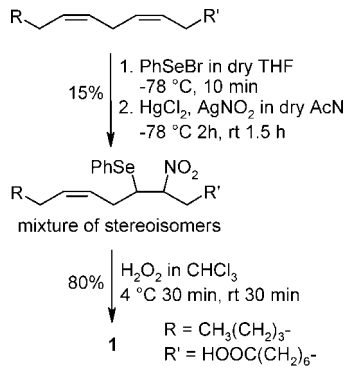
SCHEME 1. Preparation of 1



present study because of their appropriate positional isomerism. After an extensive search of reaction conditions with a variety of nitrating systems, it was found that the target nitroalkenes **1** and **2** could be conveniently obtained by a modification of the reported nitrophenylselenenylation/oxidation procedure[9] through a judicious combination of experimental conditions and protection strategies.

Compound **1** was prepared by a modified protocol in which linoleic acid was reacted with PhSeBr in THF at −78 °C and then with $HgCl_2$ and $AgNO_2$ in dry acetonitrile to produce regioisomeric nitrophenylselenenyl adducts (Scheme 1).[21] Preparative HPLC then afforded a main fraction containing a mixture of the appropriate stereoisomeric nitrophenylselenenyl adducts, which were treated with H_2O_2 to remove phenylseleninic acid and give the desired **1** in 12% overall yield. Complete spectral data of **1** are given in the Supporting Information. The proposed structural assignment was secured by TOCSY experiments showing cross peaks between the terminal methyl proton signal at δ 0.89 and resonances at δ 2.05, 5.35, and 5.52, due to H-14 allylic protons and H-12 and H-13 double bond protons, in that order. The same procedure was successfully extended to obtain ^{15}N-labeled **1** in 9% isolated yield using $Na^{15}NO_2$ in the place of $AgNO_2$. Preparation of **2** as free acid could not be achieved by the same protocol because of difficulties in the separation of the appropriate nitrophenylselenenyl intermediates or the final nitrolinoleates. Accordingly, an alternate strategy was devised which involved the use of the allylic ester of linoleic acid as the substrate. This ester group was preferred oversimple alkyl groups because of the facile removal by hydrogenolysis[18] and the presence of an additional double bond expected to improve chromatographic separation over silver nitrate-impregnated silica gel.[8] Indeed, nitrophenylselenenylation/oxidation of linoleic acid allylester as above followed by a chromatographic step gave pure **2** allyl ester, from which **2** was eventually obtained in ca. 10% overall yield after a deprotection step (Scheme 2). Spectral data of **2** are reported in the Supporting Information.

To summarize, the synthetic protocols described in Schemes 1 and 2 provide the first expedient access to single nitrolinoleic acids as free acids and in sufficient amounts for biological studies. They differ from previously published reports of nitrophenylselenenylation of fatty acids[9] in the modified experimental protocol, which relies on a low-temperature procedure favoring selective nitration of the Δ^9 double bond, and in the carboxyl protection and product separation strategies. In particular, access to **1** was made possible by preparative HPLC separation of the nitrophenylselelenyl adducts prior to H_2O_2 treatment, whereas preparation of **2** capitalized on the use of the linoleic acid allyl ester as the substrate of the nitrophenylselenenylation protocol, allowing better separation of the final nitrolinoleates and facile deprotection of the carboxyl group.

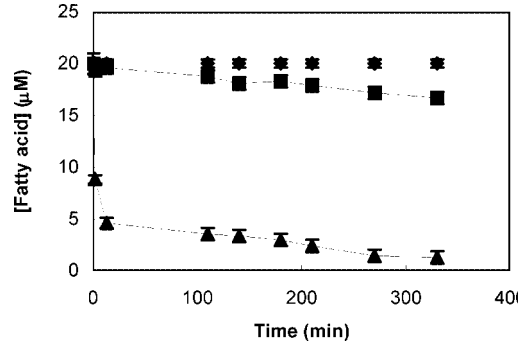
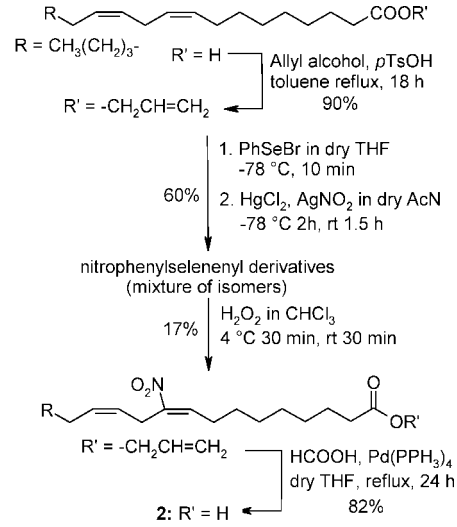


FIGURE 1. Consumption of linoleic acid (◆), **1** (▲) or **2** (■) with reaction time during incubation in 0.1 M phosphate buffer at pH 7.4. All experiments have been carried out in triplicate. Data are expressed as average ± SD.

SCHEME 2. Preparation of 2



Stability of 1 and 2 in Aqueous Phosphate Buffer at pH 7.4. Prior to product investigation, the relative stability of regioisomeric nitrolinoleic acids under physiologically relevant conditions was investigated. Accordingly, freshly prepared pure **1** and **2** at 20 μM concentration were dissolved in phosphate buffer at pH 7.4 containing 20% ethanol, under the conditions used in previous and related studies,[10,22] and their rates of decomposition at 20 °C were monitored by HPLC. Data in Figure 1 revealed a markedly faster decay of **1** relative to **2**: after 15 min nearly 80% **1** had decayed versus little or no **2**. Under the same conditions, linoleic acid remained virtually unchanged over more than 5 h.

The faster decay of **1** relative to **2** was also apparent from NMR analyses of the crude ethyl acetate extracts of the reaction mixtures from the nitrolinoleic acids suspended in 0.1 M phosphate buffer, pH 7.4. After 2 h incubation, complete conversion of **1** to a complex mixture of products was noted, whereas in the case of **2** a substantial proportion of unchanged

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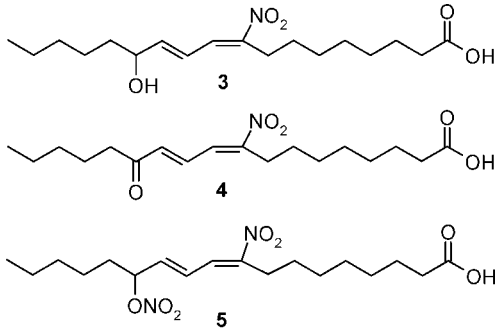
starting material was present. Similar data were obtained when the NMR analysis was run on an equimolar mixture of **1** and **2**. Notably, even when stored dry in the cold, **1** was unstable compared to **2**: whereas pure samples of the former underwent extensive degradation after a month or so, the latter remained virtually unaffected during the same period. In separate experiments, the NO-releasing abilities of **1** and **2** in phosphate buffer were investigated using the oxymyoglobin assay.[10,14,15] The results (see the Supporting Information) indicated a faster decrease in the 580 and 543 nm maxima (characteristic of the visible α- and β-band absorbance of oxymyoglobin) and a more rapid increase in the 630 and 503 nm maxima (characteristic of metmyoglobin) in the case of **1**, suggesting faster NO-release from this fatty acid. A detailed analysis of NO-release from nitrolinoleic acids was out of the scope of this paper and was not pursued further. Overall, these preliminary data highlighted a marked instability of **1** in phosphate buffer when compared to **2** and a consistently higher tendency to release NO. With this information available, the main reaction products generated under the same conditions were then investigated.

Product Studies. Incubation of **1** in 0.1 M phosphate buffer, pH 7.4, resulted in the generation of a very complex mixture of products. After 2 h, the mixture was worked up and chromatographed on silica gel plates to give three main UV−vis bands, A ($R_f = 0.20$), B ($R_f = 0.30$), and C ($R_f = 0.36$), positive to the Griess reagent for nitrite-releasing substances. The same products were also obtained by reacting ^{15}N-labeled **1** prepared as above. Compound A exhibited pseudomolecular ion peaks in the ESI(+)-MS spectrum at *m/z* 364 and 380 ($[M + Na]^+$ and $[M + K]^+$),[5] whereas ESI(−)/MS/MS analysis (see the Supporting Information) showed the pseudomolecular ion peak $[M - H]^-$ at *m/z* 340 and its daughter ions at *m/z* 322, 294, 293, due to loss of H_2O, NO_2, or HNO_2, and *m/z* 46 indicating $[NO_2]^-$. The compound was thus formulated as (9*E*,11*E*)-13-hydroxy-9-nitro-9,11-octadecadienoic acid (**3**) (15% isolated yield) on the basis of extensive 2D NMR experiments. The ^{15}N-labeled **3** consistently exhibited pseudomolecular ion peaks ($[M + Na]^+$ and $[M + K]^+$) at *m/z* 365 and 381 and distinct cross peaks between proton signals at δ 7.55 and 2.69 and the nitrogen resonance at δ 376.7 in the ^{1}H,^{15}N HMBC spectrum. Spectroscopic data were in good agreement with those reported in the literature for the corresponding methyl ester isolated from the reaction of 13-hydroxyoctadecadienoic acid with acidic nitrite.[23]



Compound B showed in the ESI(+) mass spectrum a pseudomolecular ion peak $[M + H]^+$ at *m/z* 340, whereas the ESI(−)/MS/MS analysis revealed the pseudomolecular ion peak $[M - H]^-$ at *m/z* 338 and its daughter ions at *m/z* 292 and 46 due to $[(M - NO_2) - H]^-$ and $[NO_2]^-$. The pattern of three olefin proton resonances in the ^{1}H NMR spectrum resembling that of **3** (δ 7.51, 7.28 and 6.64), with in addition a deshielded methylene proton signal at δ 2.79. A salient feature of the ^{13}C NMR spectrum was a signal at δ 199.2 suggestive of a conjugated keto group. The ^{1}H,^{15}N HMBC spectrum of the ^{15}N-labeled compound showed distinct cross peaks between the proton signals at δ 7.51 and 2.79 and a nitrogen resonance at δ 374.9, while the MS spectrum indicated a pseudomolecular ion peak $[M + H]^+$ at *m/z* 341. Based on these data, the compound was identified as (9*E*,11*E*)-9-nitro-13-oxo-9,11-octadecadienoic acid (**4**) (9% isolated yield). The least polar compound C displayed in the ^{1}H NMR spectrum, besides three olefinic proton signals, a double triplet at δ 5.41 giving a cross peak in the ^{1}H,^{13}C HMQC spectrum with a carbon signal at δ 83.0. Quite unexpectedly, the ^{1}H, ^{15}N HMBC spectrum of the labeled derivative revealed the presence of two nitrogen groups, one resonating at δ 375.3 and correlating with proton signals at δ 7.48 and 2.70, and the other resonating at δ 338.4, suggestive of a nitrate ester,[24,25] correlating with the proton signal at δ 5.41. Accordingly, the compound was identified as (9*E*,11*E*)-9-nitro-13-nitrate-9,11-octadecadienoic acid (**5**) (6% isolated yield). The ESI(+) mass spectrum of **5** showed peaks for the $[M + Na]^+$ and $[M + K]^+$ ions at *m/z* 409 and 425, as well as peaks at *m/z* 324, 346, and 362, suggesting loss of HNO_3 from the $[M + H]^+$, $[M + Na]^+$, and $[M + K]^+$ pseudomolecular ion peaks. ESI(−)/MS/MS analysis showed the pseudomolecular ion peak $[M - H]^-$ at *m/z* 385 and its daughter ion at *m/z* 62 due to $[NO_3]^-$. The ^{15}N-labeled derivative gave expected pseudomolecular ion peaks at *m/z* 411 and 427 ($[M + Na]^+$ and $[M + K]^+$) and peaks at *m/z* 325, 347, and 363 consistent with loss of $H^{15}NO_3$ from pseudomolecular ion peaks. ESI(−)/MS/MS experiments gave the pseudomolecular ion peak $[M - H]^-$ at *m/z* 387 and its daughter ion at *m/z* 63 assigned to $[^{15}NO_3]^-$. Although products **3**−**5** account for a modest mass balance, data refer to isolated yields and therefore underestimate actual product yields. Scrutiny of the ^{1}H and ^{13}C NMR spectra of the crude reaction mixtures after **1** had completely disappeared revealed the expected patterns of resonances for **3**−**5** and traces of other unknown species. The same products **3**−**5** were also formed by decomposition of 20 μM **1** (HPLC), suggesting that their formation is independent of substrate concentration. In a subsequent set of control experiments, it was found that: (a) in unbuffered water (pH around 5) formation of peroxidation products of **1** was markedly slower with no detectable **5**, (b) under an argon atmosphere formation of products **3**−**5** was inhibited, and (c) when **1** was left to decompose at room temperature as a dry film formation of **3** and **4** occurred but no detectable **5**.

To gain a deeper insight into the products resulting from aqueous decomposition of **1**, the crude reaction mixtures obtained from both the unlabeled and ^{15}N-labeled substrates were investigated by LC−MS. Analysis of the LC traces at 2 h reaction time indicated the presence, besides **3**, **4**, and **5**, of several additional species, one of which exhibited distinct $[M + Na]^+$ and $[M + K]^+$ pseudomolecular ion peaks at *m/z* 380 and 396, suggesting the 13-hydroperoxy derivative of **1**. Another species displayed a $[M + H]^+$ pseudomolecular ion peak at *m/z* 295, compatible with a dienone in which the nitro group was

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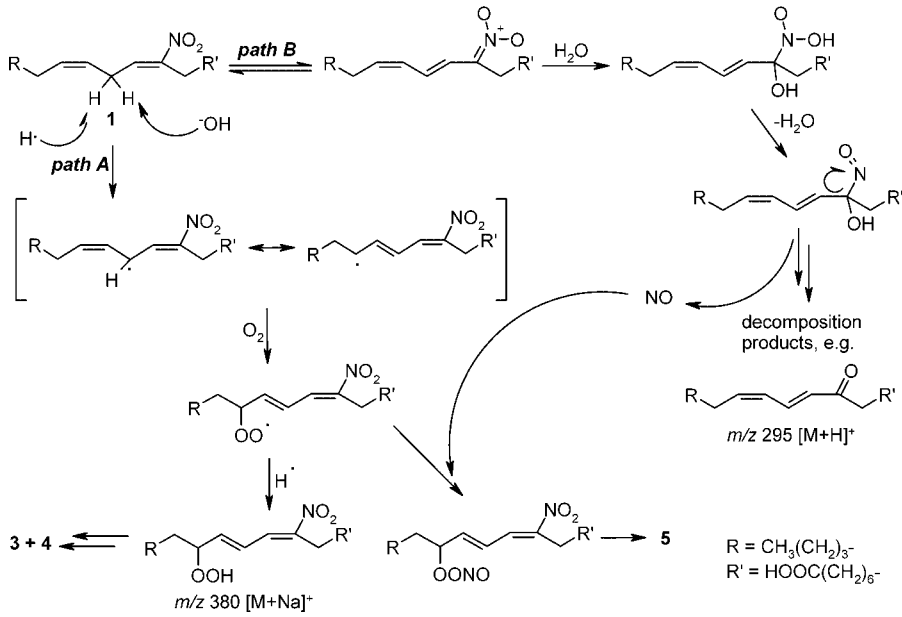
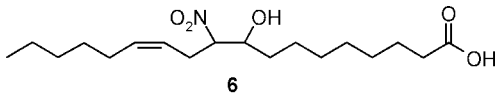
SCHEME 3. Mechanistic Pathway Proposed for the Formation of Compounds 3−5 from 1



evidently lost. The lack of nitrogen was corroborated by the analysis of the mixture from ^{15}N-labeled **1**, revealing the same pseudomolecular ion for the same peak. Similar species generated by decomposition of nitrolinoleic acids had been reported previously.[10] In contrast to **1**, decay of **2** in aqueous phosphate buffer at pH 7.4 gave after 2 h a single isolable species which was obtained by preparative TLC and was identified as (12*Z*)-9-hydroxy-10-nitrooctadec-12-enoic acid (**6**, 9% isolated yield) by extensive spectral characterization. Diagnostic features in the ^{1}H NMR spectrum were two sets of multiplets at δ 4.44−4.46 and 4.06/3.90, showing one-bond correlations with carbon resonances at δ 91.7/92.5 and at δ 72.4/71.5, respectively. These and ^{1}H,^{1}H COSY, ^{1}H,^{13}C HMQC and ^{1}H,^{13}C HMBC data indicated that the product was in fact a 1:1 mixture of diastereoisomers (pairs of enantiomers). This conclusion was confirmed by LC−MS analysis of the isolated band showing two species eluted under very close peaks (16.8 and 17.2 min) with pseudomolecular ion peaks $[M + H]^+$ at *m*/*z* 344.



6

Unreacted **2** accounted for some 80% of the mixture after 2 h reaction time. Although no other single product could be isolated from the mixture, NMR and LC−MS investigations indicated the formation, besides **6**, of several species all in minute amounts.

Mechanistic and Computational Studies. The product studies reported above revealed three main degradation channels of the nitropentadienyl system in neutral aqueous buffer, namely: (a) H-atom abstraction from the bis-allylic methylene group triggering classic peroxidation chain reactions, (b) NO-release, and (c) nucleophilic addition of water onto the nitroalkene moiety. Whereas the latter path accounts for the observed formation of **6** by decay of **2**, the first two routes may lead to **3**−**5**, as outlined in Scheme 3.

H-atom abstraction from the bis-allylic methylene group (path A) would result in a stabilized nitropentadienyl radical which would undergo coupling with oxygen to give **3** and **4** via the classic lipid peroxidation steps. This path is supported by the mass spectrometric identification of the hydroperoxy derivative and the substantial inhibition of product formation in the absence of oxygen. NO-release is apparently a pH-dependent route triggered by a deprotonation equilibrium and is involved in the formation of **5** at pH 7.4. A substantial deprotonation of nitrolinoleic acids can be predicted at pH 7.4 based on a suggested pK_a value of 5 for the allylic methylene group[10] and would be favored in the case of **1**, from which a highly conjugated, resonance-stabilized nitronate structure can be generated. One viable mechanism for NO-release is based on the recently proposed modified Nef sequence[10] and is sketched in path B. In this route, the first formed nitronate anion would be converted to a hydroxynitroso intermediate which can undergo homolytic cleavage to form NO and an allylic radical. The latter might then react with oxygen to give eventually a dienone product, as suggested by the presence of a species in the LC−MS chromatogram with a pseudomolecular ion peak at *m*/*z* 295. Alternative mechanisms, mutually nonexclusive, can account for NO-release, for example, nitroalkene rearrangement to a nitrite ester followed by N−O bond homolysis to form NO and a carbon-centered radical,[14] but they cannot be distinguished based on the present results. Fast and effective coupling of NO with peroxyl radicals ($k > 10^{10}$ M^{-1} s^{-1})[26] produced by peroxidation of **1** followed by rearrangement of the resulting peroxynitrite ester intermediate is likely to be involved in the formation of **5**. It may be relevant to notice, in this connection, that effective trapping of NO by the peroxyl radicals present in the mixture may bias estimation of NO release by the chemiluminescence and other assays. Product analysis suggested a higher oxidizability of **1** relative to **2**. Since H-atom abstraction from the 1,4-pentadiene system (C−H bond dissociation enthalpy (BDE) = 76.4 kcal mol^{-1})[27−29] dictates the oxidizability of polyunsaturated fatty acids, at least a rough estimate of the

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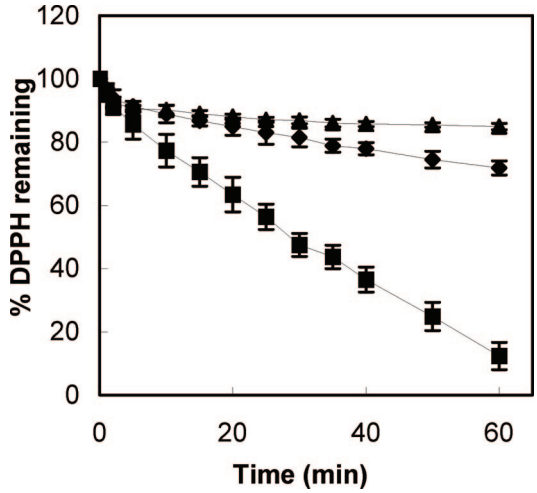
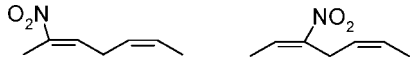


FIGURE 2. Absorbance decay at 515 nm of a 100 μM solution of DPPH in ethanol after addition of: **1** (■), **2** (◆), and linoleic acid (▲). Final fatty acid concentration: 7.7 mM. All experiments have been carried out in triplicate. Data are expressed as average ± SD.

relative abilities of nitrolinoleic acids to act as H-atom donors was desirable. Accordingly, the activity of **1** and **2** in the diphenylpicryl hydrazide (DPPH) radical quenching spectrophotometric test was determined against linoleic acid as a reference fatty acid.[30] This test, which is run in ethanol and is therefore independent of pH-related effects, enables measurement of the radical-scavenging properties of a given substance and is often used in the food industry to establish the rank order of antioxidants. The results in Figure 2 indicated more effective H-atom transfer to the DPPH radical from **1** than from **2** or linoleic acid. This trend can be taken to suggest a larger radical-stabilizing effect of the nitro group on the terminal pentadienyl positions, making **1** more prone to H-atom loss and free radical formation.

To support this conclusion and to investigate in some detail the position-dependent influence of the nitro group on radical stability, a computational (DFT) investigation of the isomeric nitro-1,4-pentadienyl systems was undertaken. A theoretical description of the 1,4-pentadiene system and the corresponding radical, and a discussion of how theoretical methodologies perform in modeling these systems, including determination of bond dissociation enthalpies (BDEs), has appeared recently.[28] For the purposes of the present study two simplified truncated structures featuring the isomeric nitropentadiene moieties typical of **1** and **2** were investigated, namely **7** and **8**, respectively.



7 **8**

Geometry optimizations were based on the "hybrid" PBE0 functional,[31] which has provided quite satisfactory energies and geometries for a wide range of organic and biological systems (including notably radical species) in combination with the 6-31+G(d,p) basis set.[32,33] The unrestricted formulation was used to describe radical species. The conformational spaces of

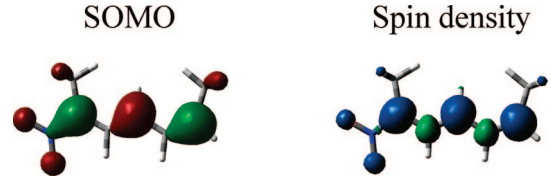


FIGURE 3. Isodensity surfaces of the singly occupied molecular orbital and total spin density of the main conformer of **7R**, computed at the UPBE0/6-31+G(d,p) level.

7 and **8**, as well as the corresponding bis-allylic radicals (dubbed **7R** and **8R**), were sampled by relaxed grid searches of the C3−C4 and C4−C5 dihedrals (methyl rotations were not systematically explored). For both **7** and **8**, four minima were identified, arranged in enantiomeric pairs which, within each compound, are separated by 0.2−0.4 kcal mol^{-1}: the energy difference between the absolute minimum of **8** with respect to **7** is also rather small (0.5 kcal mol^{-1}). The bis-allylic radical **7R** gives rise to six minima (two enantiomeric pairs, and two structures of C_s symmetry), one of the planar conformers being definitely more stable than the others (by at least 3.9 kcal mol^{-1}). By contrast, planar conformers were not found among the stable conformers of **8R**, which features instead four pairs of enantiomeric minima: even in those conformers in which the carbon skeleton is approximately planar, the nitro group is significantly distorted out of the molecular plane. A first indication of the relative stabilities of the two radicals can be gained by comparison of the energies of their most stable conformers: then, **7R** is favored by 9.0 kcal mol^{-1} with respect to **8R**. Such a direct comparison is significant, since, as stated before, **7** and **8** are almost isoenergetic at this level. Moreover, the overall picture is confirmed when more refined treatments are adopted, including single point energy evaluations with the recently developed M05-2X density functional,[34] which has shown remarkable performance for energetics involving radicals,[35] use of a large basis set, and introduction of rotational/harmonic vibrational contributions (see the Supporting Information): in terms of computed enthalpies at 298.15 K, **8** is more stable than **7** by 0.4 kcal mol^{-1}, while **8R** is less stable than **7R** by 8.4 kcal mol^{-1}. In view of the relatively apolar nature of the models, it is not surprising that introduction of the aqueous environment by means of an implicit model (namely the polarizable continuum model)[36−39] involves only minor changes in computed geometries and relative stabilities (see the Supporting Information). Figure 3 depicts the SOMO and the total spin density for the main conformer of **7R**: the 2, 4, and 6 positions appear almost equivalent, with significant involvement of the nitro group.

A more quantitative comparison (see the Supporting Information) based on SOMO localization and on use of a larger basis set shows a slightly lower relative weight at position C-6 with respect to C-2 and C-4. A note of caution here is in order

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concerning the validity of the truncated structure to model lipid peroxidation. In particular, steric interactions between the long tails of the real lipids are evidently ill reproduced by the small methyl groups of the models. However, for both **7** and **8**, the most stable conformer is characterized by a skew arrangement of the terminal C-CH_3 units, which should be compatible even with the complete chains. The chain termini are almost co-oriented in the secondary conformers identified, but again this holds for both **7** and **8**. The situation is slightly more involved for the radical species: in the most stable conformer of **7R**, the C-CH_3 units are coplanar and point in the same direction; in the conformer which comes next, the backbone is still planar, but the methyl groups are now on opposite sides (however, as hinted above, this conformer is significantly less stable). The most stable conformer identified for **8R** corresponds, with minor distortions, to this latter chain fold; conversely, the second conformer of **8R** resembles the geometry of the absolute minimum of **7R** (but is much closer in energy to its own minimum). While the relative orientation of the terminal C-CH_3 groups can admittedly provide only a rough indication as to the stability of the untruncated compounds, overall it would appear that longer terminal tails could be better accommodated in the floppier **8R** than in **7R**; in turn, this would imply a smaller energy separation between the two radicals, and would to some extent qualify the rather drastic reactivity differences predicted by use of the heptadiene models.

Conclusions

The preparation of two isomeric nitrolinoleic acids in pure form is reported for the first time (only the ester derivative of **2** was obtained previously),[8] and their markedly different chemical behavior is disclosed. Compound **1**, bearing a nitro group on a terminal position of the pentadiene moiety, is markedly unstable when exposed to phosphate buffer at pH 7.4, and partitions mainly between two concurrent degradation pathways, one resulting in NO-release presumably via a base-dependent Nef-type reaction, and the other involving a typical peroxidation process. A remarkable outcome is formation of the nitronitrate ester **5** involving probably trapping of NO by transient peroxyl radicals. Compound **2** decays at slower rate and gives mainly the stereoisomeric hydroxynitro derivatives **6** by nucleophilic addition of water. The differential behavior of **1** and **2** can be rationalized on the basis of DPPH radical quenching experiments and DFT calculations, indicating a higher H-atom donating ability and a larger radical stabilization when the nitro group is located on the terminal rather than inner positions of the 1,4-pentadienyl core. These results provide an underpinning for ongoing investigations of the physiological properties of nitrolinoleic acids and may orient the design of new pharmacologically active nitrated lipids. They also offer a plausible explanation as to why **1** and the isomeric 13-nitrolinoleic acid have so far eluded detection in biological systems.

Experimental Section

For all isolated compounds, ^{1}H and ^{13}C NMR resonances due to 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 ^{1}H/^{13}C NMR spectra at δ ($CDCl_3$) 2.34 (2H, t, J = 7.4 Hz)/33.6, 1.2−1.6 (16H, m)/22−32, 0.89 (3H, t, J = 7.2 Hz)/14.0, in that order.

Synthesis of (9*E*,12*Z*)-9-Nitroottadecadienoic Acid (1). The title compound was prepared according to a procedure reported in the literature with modifications.[4,6,9] In brief, a solution of **1** (1 g, 3.6 mmol) in dry THF (6 mL) was added to a solution of PhSeBr (843 mg, 3.6 mmol) in dry THF (6 mL) at −78 °C under an argon atmosphere. After 10 min, $HgCl_2$ (1.26 g, 4.6 mmol) was added to the mixture followed by a solution of $AgNO_2$ (550 mg, 3.6 mmol) in dry acetonitrile (12 mL, aliquots of 1 mL over 30 min). After 2 h, the reaction mixture was taken at room temperature for 1.5 h, then filtered on Celite and washed extensively with diethyl ether. The surnatant was evaporated to dryness, taken up in $CHCl_3$, extracted with brine, and evaporated under reduced pressure after drying over anhydrous sodium sulfate. The residue was subjected to preparative HPLC (eluant I) to afford two fractions, **I** (t_R = 18−20 min) and **II** (t_R = 21−23 min). Fractions **I** and **II** were evaporated under reduced pressure and worked up as described above. The residues were taken up in $CHCl_3$ and treated separately with H_2O_2 (4 molar equiv) under vigorous stirring for 30 min at 4 °C and for additional 30 min at room temperature. After workup as above, residues were subjected to preparative TLC (eluant a) to afford from fraction **I** a 1:1 mixture of **1** and **2** (264 mg) and from fraction **II** pure **1** (135 mg, 12% yield, R_f = 0.50, eluant a).

1: ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 2.05 (2H, m, H-14), 2.59 (2H, t, J = 7.6 Hz, H-8), 2.95 (2H, t, J = 7.6 Hz, H-11), 5.34 (1H, m, H-12), 5.52 (1H, m, H-13), 7.02 (1H, t, J = 7.6 Hz, H-10); $_{13}$C NMR (100 MHz $CDCl_3$) δ (ppm) 26.9 (CH_2), 27.1 (CH_2), 28.2 (CH_2), 123.3 (CH), 133.3 (CH), 134.4 (CH), 151.8 (C) 180.0 (C); LC−MS t_R = 43 min; ESI(+)-MS m/z 326 ([M + H]$^+$), 348 ([M + Na]$^+$), 364 ([M + K]$^+$); ESI(+)-HRMS for $C_{18}H_{31}NO_4$ calcd 325.2253 [M + H]$^+$, found 325.2249.

Synthesis of [^{15}N]-(9*E*,12*Z*)-9-Nitroottadecadienoic Acid ([^{15}N]1). The title compound was prepared as described for **1**, but using $Na^{15}NO_2$ (250 mg, 3.6 mmol) instead of $AgNO_2$. Two fractions were isolated, one consisting of pure [^{15}N]**1** (198 mg, 17% yields) and the other of a 1:1 mixture of [^{15}N]**1** and [^{15}N]**2** (105 mg).

[^{15}N]**1**: ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 2.05 (2H, m, H-14), 2.59 (2H, dt, J = 7.6, 3.6 Hz, H-8), 2.95 (2H, t, J = 7.6 Hz, H-11), 5.34 (1H, dt, J = 7.2, 10.0 Hz, H-12), 5.52 (1H, dt, J = 7.2, 10.5 Hz, H-13), 7.02 (1H, dt, J = 7.6, 3.6 Hz, H-10); LC−MS t_R = 43 min; ESI(+)-MS m/z 327 ([M + H]$^+$), 349 ([M + Na]$^+$), 365 ([M + K]$^+$); ESI(+)-HRMS for $C_{18}H_{31}{}^{15}NO_4$ calcd 326.2223 [M + H]$^+$, found 326.2220.

Synthesis of allyl (9*E*,12*Z*)-10-Nitrooctadecadienoate (2 Allyl Ester). The title compound was prepared following the nitrophenylselenenylation protocol described for **1** with allyl (9*Z*,12*Z*)-octadecadienoate as the substrate. After workup, the residue was fractionated on silica gel using cyclohexane/ethyl acetate (gradient mixture from pure cyclohexane to cyclohexane/ethyl acetate 98:2) to afford the mixture of nitrophenylselenenyl-adducts of allyl (9*Z*,12*Z*)-octadecadienoate (1.0 g, 60% yield). This latter was taken up in $CHCl_3$ and treated with H_2O_2 (4 molar equiv) under vigorous stirring at 4 °C. After 30 min, the mixture was taken at room temperature for additional 30 min. The reaction mixture was then extracted with brine, and the organic layers were dried over anhydrous sodium sulfate, filtered, and evaporated. The residue was fractionated on silver-impregnated silica gel (eluant cyclohexane) to afford two fractions, one consisting of a mixture of **1** allyl ester and **2** allyl ester (98 mg) and the other of pure **2** allyl ester (119 mg, 17% yield, R_f = 0.62, eluant b).

2 allyl ester: ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 2.12 (dt, J = 7.2 Hz, H-14), 2.23 (2H, dt, J = 7.6 Hz, H-8), 3.33 (2H, d, J = 7.2 Hz, H-11), 4.56 (2H, d, J = 6.0 Hz, −OCH−), 5.2−5.3 (1H, m, H-12), 5.23 (1H, dd, J = 10.5, 0.9 Hz, −OCH_2CH=CH_2), 5.31 (1H, dd, J = 17.1, 0.9 Hz, −OCH_2CH=CH_2), 5.48 (1H, m, H-13), 5.90 (1H, ddt, J = 17.1, 10.5, 6.0 Hz, −OCH_2CH=CH_2), 7.09 (1H, dt, J = 7.6, 3.9 Hz, H-9); ESI(+)-HRMS for $C_{21}H_{35}NO_4$ calcd. 365.2566 [M + H]$^+$, found 325.2563.

Deprotection of 2 Allyl Ester. A solution of **2** allyl ester (119 mg, 0.3 mmol) in dry THF (5.84 mL) was treated with HCOOH (123 μL, 3.3 mmol) and $Pd(PPh_3)_4$ (18.8 mg, 16.3 μmol) under an argon atmosphere at 80 °C. After 24 h, the reaction mixture was

filtered on Celite, evaporated to dryness, and fractionated by preparative TLC (eluant a) to afford **2** (87 mg, 82% yield, R_f = 0.48, eluant a).

2: ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 2.12 (2H, dt, J = 7.2 Hz, H-14), 2.21 (2H, dt, J = 7.6 Hz, H-8), 3.33 (2H, d, J = 6.9 Hz, H-11), 5.26 (1H, m, H-12), 5.48 (1H, m, H-13), 7.08 (1H, dt, J = 7.6, 3.9 Hz, H-9); ^{13}C NMR (50 MHz $CDCl_3$) δ (ppm) 25.1 (CH_2), 27.9 (CH_2), 28.0 (CH_2), 123.8 (CH), 132.7 (CH), 136.2 (CH), 150.8 (C) 179.3 (C); LC−MS t_R = 42 min; ESI(+) m/z 326 ([M + H]$^+$), 348 ([M + Na]$^+$), 364 ([M + K]$^+$).

Isolation of (9*E*,11*E*)-13-Hydroxy-9-nitro-9,11-octadecadienoic Acid (3), (9*E*,11*E*)-9-Nitro-13-oxo-9,11-octadecadienoic Acid (4), and (9*E*,11*E*)-9-Nitro-13-nitrate-9,11-octadecadienoic Acid (5). A solution of **1** (75 mg) in ethanol (44 mL) was added to 0.1 M phosphate buffer, pH 7.4 (178 mL) and kept at 37 °C and under vigorous stirring. After 2 h, the reaction mixture was acidified to pH 3 with HCl 3 M and extracted with chloroform. After drying over anhydrous sodium sulfate and evaporation of the solvent, the residue was fractionated by preparative TLC (eluant a) to afford **3** (11 mg, 15% yield, R_f = 0.20, eluant a), **4** (7 mg, 9% yield, R_f = 0.30, eluant a), and **5** (5 mg, 6% yield, R_f = 0.36, eluant a).

3: ^{1}H NMR (400 MHz,$CDCl_3$) δ (ppm) 1.2−1.6 (2H, m, H-14), 2.69 (2H, t, J = 7.6 Hz, H-8), 4.33 (1H, m, H-13), 6.32 (1H, dd, J = 14.8, 5.2 Hz, H-12), 6.45 (1H, dd, J = 14.8, 11.6 Hz, H-11), 7.55 (1H, d, J = 11.6 Hz, H-10); ^{13}C NMR (100 MHz $CDCl_3$) δ (ppm) 27.2 (CH_2), 37.8 (CH_2), 72.7 (CH), 122.8 (CH), 133.4 (CH), 150.0 (CH), 151.6 (C), 178.6 (C); LC-MS t_R = 20 min; ESI(+)-MS^5 m/z 324 ([M − H_2O + H]$^+$), 364 ([M + Na]$^+$), 380 ([M + K]$^+$); ESI(−)/MS/MS m/z 340 ([M − H]$^-$), 322 ([(M − H_2O) − H]$^-$), 294 ([(M − NO_2) − H]$^-$), 293 ([(M − HNO_2) − H]$^-$), 46 ([NO_2]$^-$).

4: ^{1}H NMR (400 MHz,$CDCl_3$) δ (ppm) 2.62 (2H, t, J = 7.6 Hz, H-14), 2.79 (2H, t, J = 7.6 Hz, H-8), 6.64 (1H, d, J = 15.2 Hz, H-12), 7.28 (1H, dd, J = 15.2, 12.0 Hz, H-11), 7.51 (1H, d, J = 12.0 Hz, H-10); ^{13}C NMR (125 MHz $CDCl_3$) δ (ppm) 27.2 (CH_2), 42.3 (CH_2), 129.6 (CH), 137.3 (CH), 156.1 (CH), 158.2 (C), 177.9 (C), 199.2 (C); LC−MS t_R = 26 min; ESI(+)-MS m/z 340 ([M + H]$^+$), 362 ([M + Na]$^+$), 378 ([M + K]$^+$); ESI(−)/MS/MS: m/z 338 ([M − H]$^-$), 292 ([(M − NO_2) − H]$^-$), 46 ([NO_2]$^-$); ESI(+)-HRMS for $C_{18}H_{29}NO_5$ calcd. 339.2046 [M + H]$^+$, found 339.2049.

5: ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 1.80 (2H, m, H-14), 2.70 (2H, t, J = 7.6 Hz, H-8), 5.41 (1H, dt, J = 7.2, 6.8 Hz, H-13), 6.17 (1H, dd, J = 15.2, 7.2 Hz, H-12), 6.49 (1H, dd, J = 15.2, 11.6 Hz, H-11), 7.48 (1H, d, J = 11.6 Hz, H-10); ^{13}C NMR (100 MHz $CDCl_3$) δ (ppm) 27.2 (CH_2), 26.4 (CH_2), 83.0 (CH), 127.6 (CH), 129.3 (CH), 132.6 (CH), 153.8 (C), 179.0 (C); LC-MS t_R = 39 min; ESI(+)-MS m/z 324 ([M − HNO_3 + H]$^+$), 409 ([M + Na]$^+$), 346 ([M − HNO_3 + Na]$^+$), 425 ([M + K]$^+$), 362 ([M − HNO_3 + K]$^+$); ESI(−)/MS/MS: m/z 385 ([M − H]$^-$), 62 ([NO_3]$^-$); ESI(+)-HRMS for $C_{18}H_{30}N_2O_7$ calcd 386.2053 [M + H]$^+$, found 386.2055.

Isolation of [^{15}N]-(9*E*,11*E*)-13-Hydroxy-9-nitro-9,11-octadecadienoic ([^{15}N]3), [^{15}N]-(9*E*,11*E*)-9-Nitro-13-oxo-9,11-octadecadienoic ([^{15}N]4) and [^{15}N]-(9E,11E)-9-Nitro-13-nitrate-9,11-octadecadienoic Acids ([^{15}N]5). Compounds [^{15}N]**3**, [^{15}N]**4**, and [^{15}N]**5** were prepared from [^{15}N]**1** following the same procedure described above for **1**.

[^{15}N]**3**: ^{1}H NMR (400 MHz,$CDCl_3$) δ (ppm) 1.2−1.6 (2H, m, H-14), 2.69 (2H, dt, J = 7.6, 3.6 Hz, H-8), 4.34 (1H, m, H-13), 6.32 (1H, dd, J = 14.2, 5.2 Hz, H-12), 6.45 (1H, dd, J = 14.8, 11.6 Hz, H-11), 7.55 (1H, dd, J = 11.6, 3.6 Hz, H-10); LC−MS t_R = 20 min; ESI(+)-MS^5 m/z 325 ([M − H_2O + H]$^+$), 365 ([M + Na]$^+$), 381 ([M + K]$^+$).

[^{15}N]**4**: ^{1}H NMR (400 MHz,$CDCl_3$) δ (ppm) 2.61 (2H, t, J = 7.6 Hz, H-14), 2.79 (2H, dt, J = 7.6, 3.6 Hz, H-8), 6.64 (1H, d, J = 15.2 Hz, H-12), 7.28 (1H, dd, J = 15.2, 12.0 Hz, H-11), 7.51 (1H, dd, J = 12.0, 3.6 Hz, H-10); LC−MS t_R = 26 min; ESI(+)-MS m/z 341 ([M + H]$^+$), 363 ([M + Na]$^+$), 379 ([M + K]$^+$); ESI(+)-HRMS for $C_{18}H_{29}{}^{15}NO_5$ calcd 340.2016 [M + H]$^+$, found 340.2020.

[^{15}N]**5**: ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 1.80 (2H, m, H-14), 2.70 (2H, dt, J = 7.6, 3.6 Hz, H-8), 5.41 (1H, ddt, J = 7.2, 6.8, 3.6 Hz, H-13), 6.17 (1H, dd, J = 15.2, 7.2 Hz, H-12), 6.49 (1H, dd, J = 15.2, 11.6 Hz, H-11), 7.48 (1H, dd, J = 11.6, 3.6 Hz, H-10); LC−MS t_R = 39 min; ESI(+)-MS m/z 325 ([M − H$^{15}NO_3$ + H]$^+$), 411 ([M + Na]$^+$), 347 ([M − H$^{15}NO_3$ + Na]$^+$), 427 ([M + K]$^+$), 363 ([M − H$^{15}NO_3$ + K]$^+$); ESI(−)/MS/MS m/z 387 ([M − H]$^-$), 63 (^{15}[NO_3]$^-$); ESI(+)-HRMS for $C_{18}H_{30}{}^{15}N_2O_7$ calcd 388.1994 [M + H]$^+$, found 388.1996.

Isolation of (12*Z*)-9-hydroxy-10-nitro-12-ottadecenoic Acid (6) (Mixture of Stereoisomers). The reaction for compound **2** was carried out as described for **1**. After workup, preparative TLC afforded **6** (mixture of stereoisomers) (7 mg, 9% yield, R_f = 0.35, eluant a).

6: ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) (the superscripts a and b are referred to the two diastereoisomers) 1.2−1.6 (2H, m, H-8), 2.02 (2H, m, H-14), 2.51^b (1H, m, H-11), 2.60^a (1H, m, H-11), 2.78^b (1H, m, H-11), 2.91^a (1H, m, H-11),4.44−4.46^{a,b} (1H, m, H-10), 3.90^b (1H, m, H-9), 4.06^a (1H, m, H-9), 5.29 (1H, m, H-12), 5.57 (1H, m, H-13); ^{13}C NMR (100 MHz $CDCl_3$) δ (ppm) 25.0 (CH_2), 26.4^a (CH_2), 28.2 (CH_2), 28.8^b (CH_2), 71.5^b (CH), 72.4^a (CH), 91.7^a (CH), 92.5^b (CH), 121.9 (CH), 136.0 (CH), 179.1 (C); LC−MS t_R = 16.8 e 17.2 min; ESI(+)-MS m/z 344 ([M + H]$^+$), 366 ([M + Na]$^+$), 382 ([M + K]$^+$); ESI(+)-HRMS for $C_{18}H_{33}NO_5$ calcd 343.2359 [M + H]$^+$, found 343.2355.

Computational Methods. All calculations were performed with the Gaussian package of programs.[40,41] Geometries were optimized at the DFT level of theory using the PBE0 functional with the 6-31+G(d,p) basis set.[32,33] The PBE0 (also referred to as PBE1PBE) is a hybrid functional obtained by combining a predetermined amount of exact exchange with the Perdew−Burke−Ernzerhof exchange and correlation functionals.[31] In control calculations, the polarizable continuum model (PCM)[36−39] was used to simulate the aqueous environment. In view of the faster convergence, a scaled Van der Waals cavity based on universal force field (UFF) radii[42] was used, and surface elements were modeled by spherical Gaussian

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functions.[43,44] All minima were checked by computing the harmonic vibrational frequencies. Single-point energy evaluations were also performed with the recently developed M05-2X density functional[34] and the large 6-311+G(2df,p) basis set.

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Supporting Information Available: General methods and experimental procedures. NMR spectra of compounds **1**, [^{15}N]**1**, **2**, **3**, [^{15}N]**3**, **4**, [^{15}N]**4**, **5**, [^{15}N]**5**, **6**, allyl (9*Z*,12*Z*)-octadecadienoate, and **2** allylester. ESI(+) mass spectra of compounds **1**, **2**, **3**, [^{15}N]**3**, **4**, [^{15}N]**4**, **5**, [^{15}N]**5**, **6**, (9 *E*,11 *E*)-13-hydroperoxy-9-nitrooctadecadienoic acid, and (10 *E*,12 *Z*)-9-oxooctadecadienoic acid. ESI(−)/MS/MS analysis of compounds **3**, **4**, **5**, and [^{15}N]**5**. LC−MS profile of the autoxidation mixture of **1**. UV−vis spectra for oxymyoglobin assay of compounds **1** and **2**. Computational data for compounds **7** and **8** and the corresponding bis-allylic radicals **7R** and **8R**. This material is available free of charge via the Internet at http://pubs.acs.org.

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