JOC The Journal of Organic Chemistry

Article

pubs.acs.org/joc

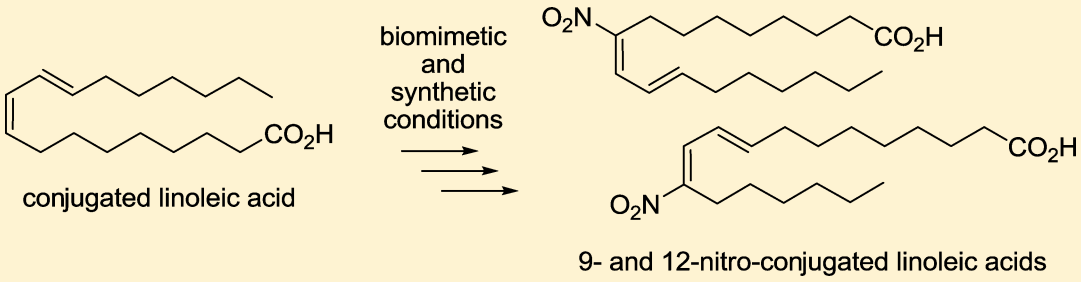
Biomimetic Nitration of Conjugated Linoleic Acid: Formation and Characterization of Naturally Occurring Conjugated Nitrodienes

Steven R. Woodcock,*[,†] Sonia R. Salvatore,[†] Gustavo Bonacci,[§] Francisco J. Schopfer,[†] and Bruce A. Freeman[†]

[†]Department of Pharmacology and Chemical Biology, University of Pittsburgh. Pittsburgh, Pennsylvania 15261, United States

[§]CIBICI-CONICET, Departamento de Bioquimica Clinica, Facultad de Ciencias Quimicas, Universidad Nacional de Cordoba, Cordoba X5000HUA, Argentina

Supporting Information



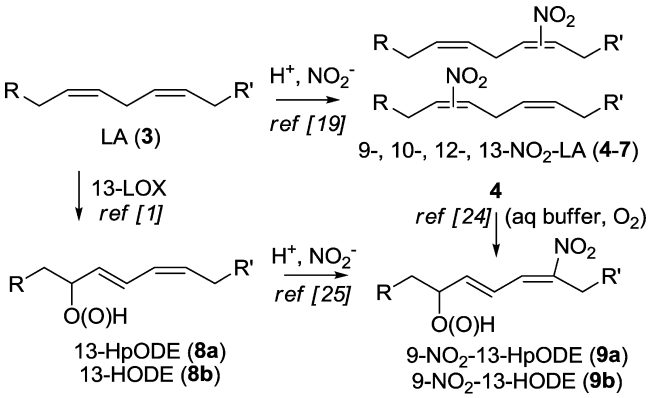
ABSTRACT: Nitro-conjugated linoleic acids (NO_2-cLA), endogenous nitrodiene lipids which act as inflammatory signaling mediators, were isolated and single isomers purified from the biomimetic acidic nitration products of conjugated linoleic acid (CLA). Structures were elucidated by means of detailed NMR and HPLC−MS/MS spectroscopic analysis and the relative double bond configurations assigned. Additional synthetic methods produced useful quantities and similar isomeric distributions of these unusual and reactive compounds for biological studies and isotopic standards, and the potential conversion of nitro-linoleic to nitro-conjugated linoleic acids was explored via a facile base-catalyzed isomerization. This represents one of the few descriptions of naturally occurring conjugated nitro dienes (in particular, 1-nitro 1,3-diene), an unusual and highly reactive motif with few biological examples extant.

INTRODUCTION

Nitrated derivatives of unsaturated fatty acids are products of the reaction of nitrogen oxides with unsaturated lipids.[1] Nitrated fatty acids are generated by cardiac tissue following ischemia/reperfusion[2] and ischemic preconditioning,[3] oxidative stress and hypoxia,[4] and acid-catalyzed reactions in the gastric compartment.[5] Nitrated lipids[6] are a representative subset of electrophilic lipid mediators,[7] a class that includes a number of oxidized and nitrated lipids produced and detected in vivo that exert signaling actions[8] via Michael addition to thiol-containing proteins. Through this reversible Michael addition[9] they have been shown to mediate reversible post-translational modification of key proteins[10] regulating inflammation[11] and metabolism[10,12] and to modulate the function of inflammatory and cytoprotective signaling mediators including Keap1/Nrf2,[13] HSF-1,[14] and NF-κB,[11] and PPARγ.[15−17]

Acidic nitration replicates the environmental conditions that occur in biological compartments including mitochondrial membranes, lysosomes, tissues subjected to ischemic conditions, and digestion. Initial work in this field demonstrated the nitration of bis-allylic linoleic acid (LA (**3**), Scheme 1) via biomimetic acidic nitration[18,19] (as well as phenylselenation[20] for larger scale). Both methods are nonregiospecific, producing a mixture of positional isomers of NO_2-LA with nitration at the 9-, 10-, 12-, and 13-positions (**4**−**7**). Studies that isolate and compare individual isomers,[21] synthesize regiospecific iso-

Scheme 1. Nitration and Oxidation of Bis-allylic Linoleic Acid (3)



Received: March 1, 2013
Published: December 19, 2013

   dx.doi.org/10.1021/jo4021562 | J. Org. Chem. 2014, 79, 25−33

mers[22] (**5** and analogs[23]), and characterize the degradation manifolds of particular isomers[24] (**4**) have been reported. These initial reports focused on understanding the nitration of LA because of its natural abundance and the presence of a reactive bisallylic methylene that favors hydrogen abstraction reactions.

In a related study, the conjugated diene-containing product of enzymatic lipid oxidation 13-hydro(per)oxyoctadeca-9,11-dienoic acid (13-HpODE, **8a**, and 13-HODE, **8b**)[25] was nitrated in a biphasic reaction to provide the corresponding 9-nitro-13-hydro(per)oxyoctadeca-9, 11-dienoic acid (**9a** or **9b**); this product was later shown to be also produced by a base-catalyzed rearrangement/oxidation of 9-NO_2-LA (**4**). These were the first reports of conjugated nitrodienes[26] derived from biological lipids.

In a recent work,[5] we demonstrated that the direct reaction of nitrating species with conjugated double-bond-containing lipids is highly favored when compared to mono- or polyunsaturated fatty acids (e.g., LA), occurring at a faster and more extensive rate and yielding higher observed concentrations in vivo, and is potentially responsible for most if not all endogenous nitration. Conjugated double-bond-containing lipids are available from dietary sources[27,28] and are normally present in membranes, plasma, and tissues. 9*Z*,11*E*-Conjugated linoleic acid (**10**) has been identified[29] as the primary isomer available in vivo (estimated at >80%). CLA was found[5] to be nitrated in vivo, and the products were shown to display unique potency and signaling activity. In particular, two predominant positional isomers of naturally occurring nitrated conjugated linoleic acid (Figure 1), collectively referred to as

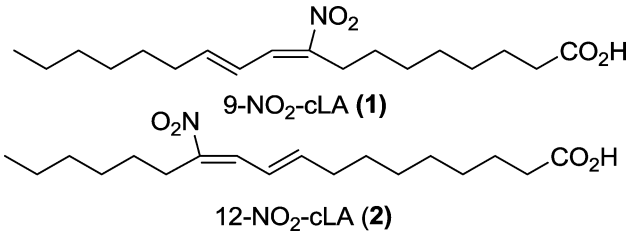


Figure 1. Nitro-conjugated linoleic acids: (9*E*,11*E*)-9-nitrooctadeca-9,11-dienoic acid (9-NO_2-cLA, **1**) and (9*E*,11*E*)-12-nitrooctadeca-9,11-dienoic acid (12-NO_2-cLA, **2**).

NO_2-cLA (9- and 12-nitro-conjugated linoleic acid, **1** and **2**), were formed from CLA and act as biologically relevant electrophilic signaling mediators. Multiple biological conditions including exposure of CLA to acidified nitrite, myeloperoxidase/hydrogen peroxide/nitrite, peroxynitrite, and gaseous nitrogen dioxide (Scheme 2, a–d) were shown[5] to form NO_2-cLA at a micromolar scale. However, there has not been detailed characterization or report of a useful synthetic method for production of these lipids to encourage progress in the field. In the present work, we report the synthesis, isolation, and spectral characteristics of the biologically relevant nitrodiene-containing products of the reaction of **10** with nitrite.

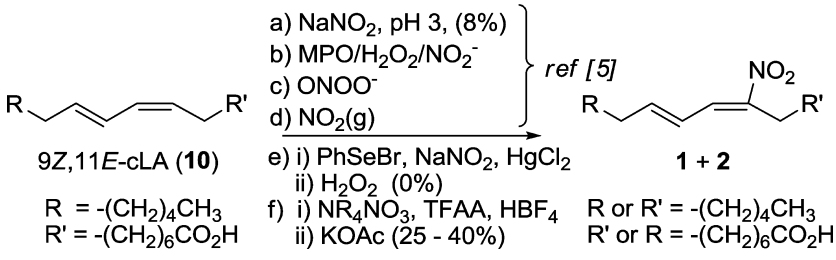
RESULTS AND DISCUSSION

Acidic nitration of CLA on a preparatory scale (Scheme 2, a) was investigated using a modification of previous reports of LA nitration.[18,19] As the primary isomer available in vivo, only 9*Z*,11*E*-conjugated linoleic acid (**10**) was used for this study. Reactions of **10** were typically conducted in a biphasic mixture with an aqueous acidified nitrite solution in the absence of oxygen. The reaction was followed by TLC until products were observed to be UV-active and positive to Griess reagent. Upon workup, the specific product NO_2-cLA (**1** + **2**) could be isolated chromatographically in modest yield (8%) from the resulting mixture of unreacted starting material (45–75%) and other nitrated and oxidized species and was characterized by HPLC–MS/MS as a mixture of two regioisomers. Isotopically labeled sodium nitrite ($Na^{15}NO_2$) was also used for production of labeled nitro-group products for mass spectrometric standards.

After single-isomer isolation (see below) and MS analysis, structures were assigned on that basis as well as NMR, UV–vis, and FTIR analysis. On the basis of the formation of specific fragments upon LC–MS/MS,[5,30] it was confirmed that nitration of **10** occurred only at the 9- and 12-positions. The products displayed strong UV–vis absorbance as a distinct chromophore with a significantly higher molar absorptivity in methanol at longer wavelengths (λ_{max} 312 nm) relative to isolated nitroalkene-containing NO_2-LA (λ_{max} 257 nm), as well as strong FTIR bands of 1317 and 1510 cm^{-1}, distinct from nitroalkene (1334, 1522 cm^{-1} in NO_2-LA)[31] or nitroalkane (1379, 1550 cm^{-1} in nitrostearic acid)[32] stretching frequencies. Aside from features common to all lipids, the ^1H NMR spectrum of each isomer featured in the low-field olefinic region a doublet at δ 7.52 ppm and two correlated multiplets in the δ 6.0–6.5 ppm region (J = 15 Hz indicating a *trans* alkene) (Figure 2). The δ 6.20 signal was coupled to the δ 7.52 doublet (*s-trans*) and the δ 6.34 to the methylene quadruplet at δ 2.24; ^{15}N labeling of the nitro group demonstrated coupling to the low-field doublet and the δ 2.65 methylene triplet by HMQC. Proton–proton and proton-carbon correlations were confirmed by 2-D COSY and HMQC. Overall, this resonance spectrum suggested two nearly identical isomers containing mutually symmetric, conjugated, electron-deficient diene regions, consistent with an (*E*,*E*)-conjugated nitrodiene. On the basis of this evidence, NO_2-cLA was formulated as an approximately equimolar mixture of **1** and **2**.

Note that *Z*-nitroalkene protons[33] have a significantly lower chemical shift, ~5.6 ppm vs 7.1 ppm for *E*-nitroalkenes.

Scheme 2. Nitration of Conjugated Linoleic Acid by Multiple Biomimetic and Synthetic Conditions



dx.doi.org/10.1021/jo4021562 | J. Org. Chem. 2014, 79, 25–33

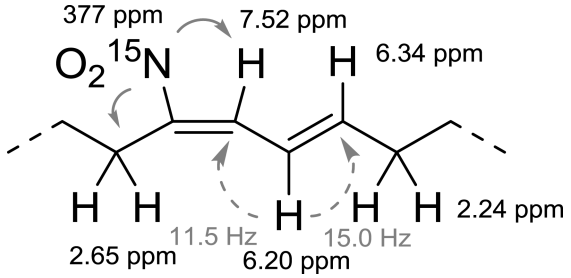


Figure 2. NMR chemical shifts and correspondences of the nitro diene moiety of [^{15}N]-NO_2-cLA. The double-bond configuration shown (*E,E*) based on these coupling values is reproduced in both isomers (**1** and **2**).

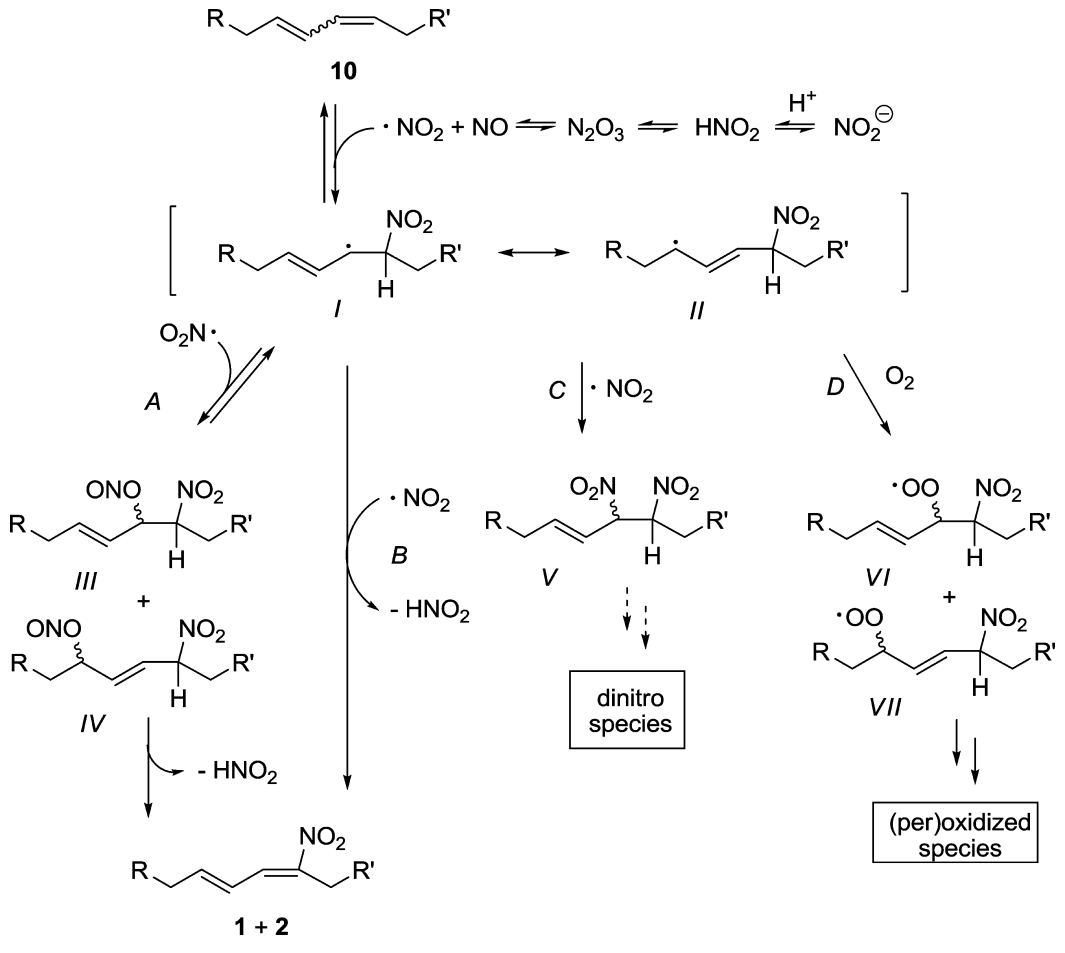
Energetically, nitroalkenes adopt the *E*-configuration to minimize allylic repulsion, and *E*-configuration is also thermodynamically preferred for a 1,2-disubstituted alkene such as the *γ,δ*-double bond. Two isolated *E,E*-isomers implied that this is a thermodynamic product and that alternative (possibly kinetic) products were not observed.

A mechanism for nitration of dienes by acidified nitrite is proposed in Scheme 3, beginning with formation of nitrogen dioxide. In the presence of strong acid, nitrite (NO_2^- pK_a = 3.4)[34] is rapidly protonated to nitrous acid (HNO_2), which rapidly equilibrates with dinitrogen trioxide (N_2O_3) which may disproportionate favorably into nitric oxide (·NO) and nitrogen dioxide radical (·NO_2). The latter is a powerful nitrating agent, which may react with available double bonds by allylic hydrogen abstraction and direct addition.

Direct addition of nitrogen dioxide to the electron-rich diene proceeds rapidly, forming a resonance-stabilized allylic radical intermediate[35,36] (*I* and *II*). Addition at the terminal positions is favored by enhanced electron density at the 1,4-positions in the diene HOMO and maximizes the stability of the resulting delocalized radical; hence, nitration of 9*Z*,11*E*-cLA (**10**) produced only species nitrated at the 9- or 12-position. Upon formation of *I* and *II*, free carbon−carbon bond rotation is allowed and the original bond configuration is lost.

Radical intermediates *I* and *II* may proceed to form multiple products. Nitrodiene formation has been proposed[19,37−39] from either addition/elimination of a second nitrogen dioxide (path A) or a one-step hydrogen abstraction (path B). Path A is a two-step route, in which formation of unstable nitrito-nitro species (*III* and *IV*) leads to loss of HNO_2 and formation of the conjugated nitroalkenes **1** and **2**. Path B is a one-step radical hydrogen abstraction, by either nitrogen dioxide or secondary radicals present. This pathway requires no leaving group but could potentially produce *Z*-isomers or nitroalkane−alkenes (not observed). In plausible pathway C, a second nitration event may also lead to unproductive dinitro species (*V*), which have been previously reported[39] in alkene nitration. Low yields of isolated product (**1** + **2**) support a number of unproductive paths being active, perhaps even favored. In the presence of oxygen pathway D becomes viable, with sequential nitration/peroxidation leading to formation of multiple oxidation

Scheme 3. Proposed Mechanism of cLA Nitration via Radical Addition



dx.doi.org/10.1021/jo4021562 | *J. Org. Chem.* 2014, 79, 25−33

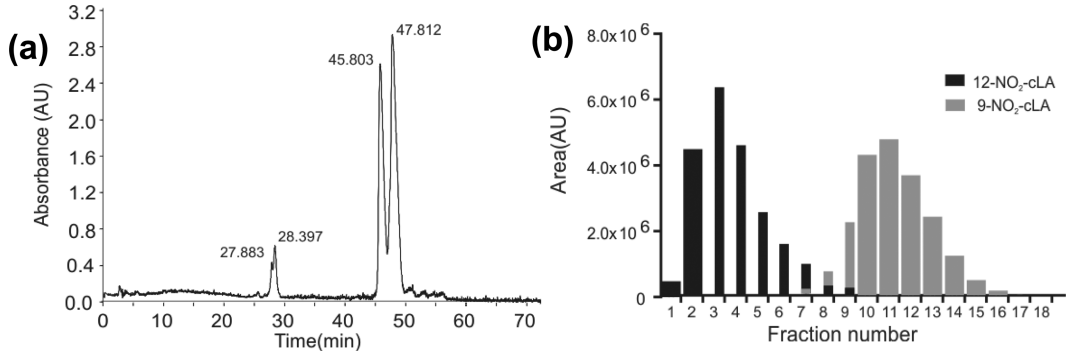


Figure 3. (a) Elution profile of individual isomers 12- and 9-NO_2-cLA (**2** and **1**) by preparatory HPLC. Absorbance monitored at 210 nm. (b) Isomer enrichment of individual fractions collected, as measured by LC/MS (bar thickness proportional to size of fraction). Fractions 1−6 were combined to afford single-isomer **2** (28.8% recovery of injected material, 8.2% yield based on starting material), and fractions 10−16 likewise pooled to afford **1** (41.7% recovery, 12.3% from SM).

products (*VI*, *VII*) identified previously[5] but outside the scope of this present study.

Unfortunately, this method, while a useful model of biological nitration, was hampered by low (and inconsistent) yields and difficulty in purification from multiple similar nitration/oxidation products. This method was intended to provide a biomimetic model for the production of nitrodienes in vivo and not otherwise developed in an attempt to increase yields. Reaction products included unreacted CLA **10** (45−75%), smaller amounts of nitroalkyl addition products (possibly *III*−*V* which had not reacted further), and NO_2-cLA. Overall yields of **1** and **2** were similar to NO_2-LA[19] nitration. This work focused on the electrophilic products of nitration that were observed to exert biological effects, and products which were nonactive in biological milieu were not characterized further. The limitations of the acidic nitration approach encouraged our search for a scalable NO_2-cLA synthetic method that would closely mimic the nitrated products derived from human samples (Scheme 2, e,f).

There are limited methods for diene nitration in the literature.[26] Phenylselenation/nitration methods, previously used for synthesis of nitroalkenes, produced only complex mixtures consistent with prior reports of nitration−epoxidation products.[40] Instead, the most promising approach developed from a report[41,42] of a two-step trifluoroacetoxy nitration method. This method was adaptable to a small scale, producing significantly larger and more consistent quantities (25% yield) of desired nitro dienes **1** and **2** than acidic nitration. In addition, the products could be more easily purified, producing fewer chromatographically similar side products than the multiplicity of radical nitration products. Subsequent modification of the method by exchanging ammonium nitrate for isotopically labeled ammonium nitrate ($NH_4{}^{15}NO_3$) gave similar yields, while more soluble tetrabutylammonium nitrate improved yield (40%). Analysis by LC/MS and NMR showed products identical to those obtained by acid nitration and human derived products, a main goal of the present work. In particular, LC/MS/MS analysis again showed only 9- and 12-nitration products **1** and **2** from **10**, indicating no isomerization or double-bond migration.

Individual isomers which had been initially identified by LC/MS were isolated via preparatory HPLC (Figure 3a). Elution of a product mixture prepurified by PTLC was monitored by UV−vis and individual fractions collected. After individual LC/MS analysis to determine relative isomer amounts and purity (Figure 3b), fractions were pooled, subjected to a second PTLC, and quantified by gravimetric and UV−vis methods. Recovery of isolated material in this case afforded milligram quantities of single isomers **2** and **1** each in greater than 90% purity by LC/MS.

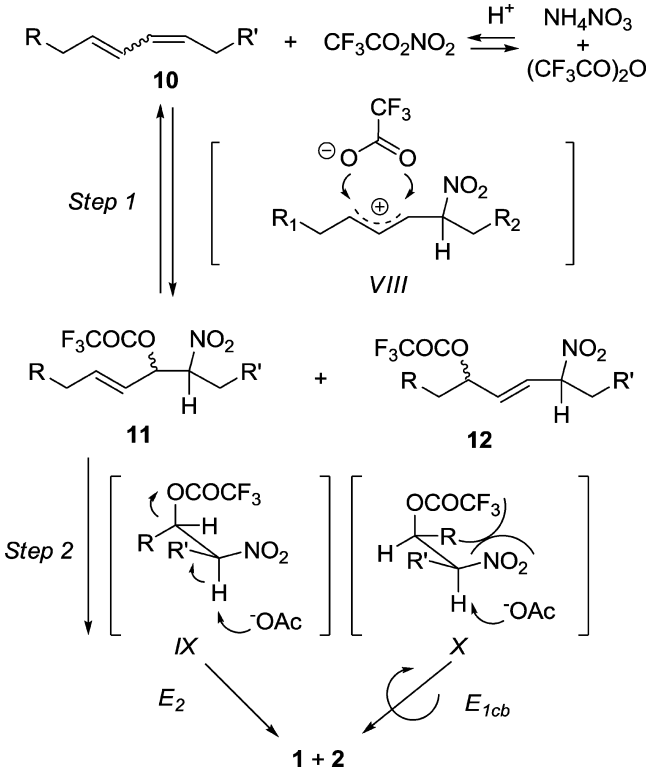
Both methods of diene nitration allow for facile incorporation of an ^{15}N-nitro group, as labeled sodium nitrite and ammonium nitrate are both readily available. Isotopic labeling of the NO_2-cLA allows convenient and efficient standards for detailed mass spectrometric analyses as well as biological probes. In addition, it provides the first synthetic route to obtain isotopically labeled standards for analytical determination of biological samples containing conjugated nitrated fatty acids, and given that biological production is expected to include both regioisomers in the same proportion, the [^{15}N]-containing standards are appropriate for analysis and quantitation of all natural product lipids. Labeling by a one-step procedure[43] is preferable economically as well as practically, and isotopic substitution of the nitro group allows facile monitoring of product ions via MS/MS where scanning for charged loss of NO_2^- is used.

Trifluoroacetoxy nitration/deprotonation is reported[42] to occur by an ionic mechanism (Scheme 4) with trifluoroacetyl nitrate generated in situ from nitrate ion and trifluoroacetic anhydride. This reactive species rapidly adds a nitronium ion to the terminus of an electron-rich diene. The resonance-stabilized intermediate ion pairs (*VIII*) then combine to form a 1,2- or 1,4-nitrotrifluoroacetoxy ester (intermediate mixtures **11** and **12**). For comparison, the ratio of 1,2- to 1,4-addition for 1,3-butadiene was reported to be approximately 3:2,[42] but LC/MS analysis of the positional preferences of intermediates **11** and **12** proved inconclusive due to facile elimination of labile trifluoroacetoxy groups upon collision-induced fragmentation that did not produce informative product ions. However, hydrolysis of the trifluoroacetoxy nitro intermediates to the corresponding hydroxy nitro compounds proved successful, and these were identified as 9- and 12-nitro species based on in-source fragmentation (see the Supporting Information). Unfortunately, the position of the hydroxyl was lost on fragmentation, and thus the ratio of 1,4- to 1,2-addition was not defined using MS/MS data.

Subjecting esters **11** and **12** to mild base eliminated trifluoroacetate and formed the same regioisomers in the same double-bond configuration as the acidic nitration-derived nitrodiene. Base-catalyzed elimination of trifluoroacetate from

dx.doi.org/10.1021/jo4021562 | J. Org. Chem. 2014, 79, 25−33

Scheme 4. Proposed Mechanism of cLA Nitration via Trifluoroacetoxy Nitration

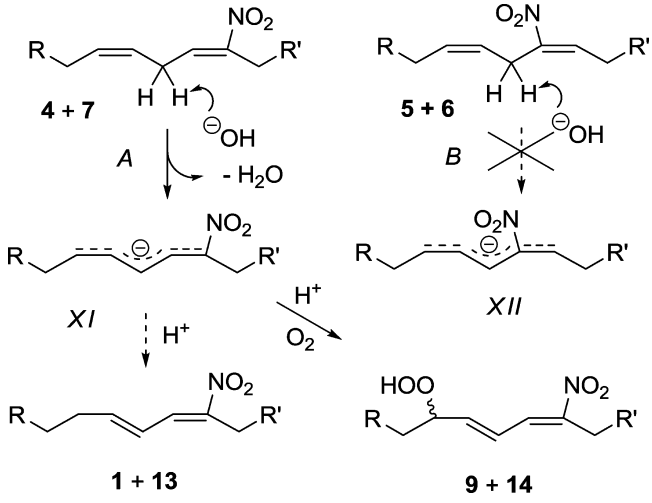


1,2-addition product **11** is analogous to the well-studied[44] formation of nitroalkenes, in which steric considerations allow a facile E2 mechanism for diastereomers in which the proton and trifluoroacetate are antiperiplanar (*IX*) while the nitro group is opposite the methylene; for the other diastereomers (*X*) steric repulsion either results in a kinetic product (*Z*-nitroalkene) or a slower $E1_{cb}$ mechanism and internal rotation. The lack of observed *Z*-isomers suggests that the slower $E1_{cb}$ is favored in this case. For either diastereomer of 1,4-addition product **12** elimination is likely to be facile, as deprotonation at an allylic position (lower pK_a) would lead to a stabilized intermediate (again $E1_{cb}$), that would in turn allow elimination with minimal steric repulsion. Based on these observations we can only speculate whether a similar mechanism confers the (*E*,*E*) configuration in the acidic nitration mechanism (Scheme 3).

To further explore the relationship of the NO_2-cLA in this study with NO_2-LA, the UV–vis spectrum of NO_2-cLA was compared to nonconjugated NO_2-LA. Prior determinations of the UV–vis spectra of nonconjugated NO_2-LA (**4**–**7**) had employed a base addition step[20,45] to simplify and amplify the poorly defined spectra of the parent NO_2-LA to form a noncharacterized product (Scheme 5). Addition of base to NO_2-LA yielded a new species with λ_{max} 330 nm (Figure 4a) and significantly higher absorptivity indicative of a significantly more conjugated species. Subsequent acidic quenching of the conjugated intermediate resulted in a double-peak with maxima at 302 and 312 nm (see the Supporting Information). This new peak was similar but not identical to the spectrum of NO_2-cLA (λ_{max} 312 nm).

Rapid deprotonation at the methylene bridge position of NO_2-LA affords a highly conjugated intermediate. For lipids with nitro groups located at the 9- or 13- (outer) positions (**4** and **7**), deprotonation is facile and proceeds to conjugated

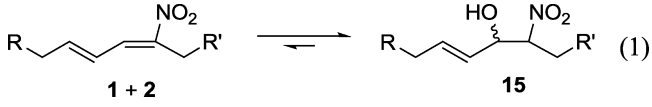
Scheme 5. Base-Promoted Conjugation and Oxidation of Bis-allylic Nitrolinoleic Acids (4–7)



intermediate *XI* stabilized by the terminal nitro group. For nitro groups located at the 10- or 12- (inner) positions (**5** and **6**), deprotonation would be slower and would produce intermediate *XII*; however, inspection of the reaction products by ^{1}H NMR indicated no reaction of **5** and **6**.

Acidic quenching of *XII* would result in a conjugated diene with the nitro group at the first position, but oxygen insertion was evidently more rapid than protonation, as only hydroperoxy **9a**[24] and regioisomer **14** were observed along with unreacted **5** and **6** (Figure 4b). Thus, the observed spectrum was a result of the mixture of products (**5**, **6**, **9a**, and **14**). Despite the suggestively similar maxima we found no evidence of formation of **1** or **13** under these conditions. Base-catalyzed conversion of NO_2-LA to conjugated species provides some context for the structural ambiguity of previous biological descriptions of NO_2-LA, but oxidation is evidently highly preferred.

Finally, the nitrodiene motif[25,42] is highly reactive to both acids and bases and displays potential for intramolecular electrocyclic rearrangement[46] and intermolecular polymerization.[47] Synthesis may be done at scales appropriate to rapid usage, as long-term storage can be problematic. After synthesis, neat nitro dienes decompose at ambient temperatures (rapidly at elevated temperatures) but can be stable for up to a week at −20 °C. The NO_2-cLA preparations reported herein were stable for at least 2 months at −80 °C when solvated in DMSO or methanol (Figure 5a), and these solvents are preferred for long-term storage. Aqueous buffer (phosphate or PBS, pH 7.4) solutions below the critical micelle concentration were stable for several hours (Figure 5b). Samples were monitored by UV–vis and analyzed by LC/MS. The appearance of hydroxy or keto species suggested a parallel to the hydration reaction of nitroalkenes,[24] which are in equilibrium with α-hydroxy (**15**) nitro species in aqueous environment (eq 1). Note that the hydrophobic membranes



dx.doi.org/10.1021/jo4021562 | J. Org. Chem. 2014, 79, 25–33

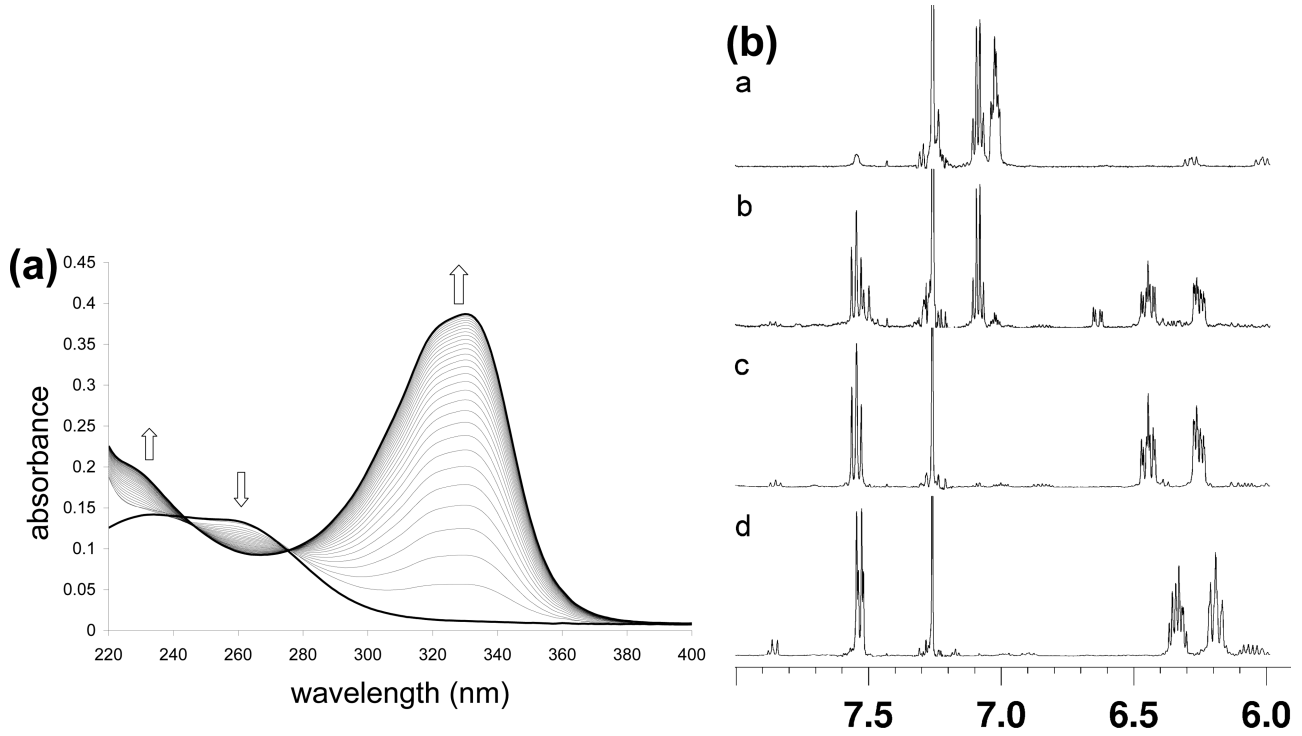


Figure 4. (a) UV−vis spectra (methanol) for base-catalyzed conjugation of nonspecific NO_2-LA. A 20 μM solution of NO_2-LA (**4**−**7**, λ_{max} 257 nm) in MeOH (1 mL) treated with 5.0 μL of 1 M NaOH monitored over 3 min with measurements every 10 s over the region 220−400 nm until equilibrated (λ_{max} 330 nm). (b) ^{1}H NMR (600 MHz, $CDCl_3$, δ 6.0−8.0 ppm shown) of (a) NO_2-LA (**4**−**7**); (b) NO_2-LA after base-catalyzed rearrangement; (c) purified **9a** + **14**; and (d) NO_2-cLA **1** + **2** for comparison.

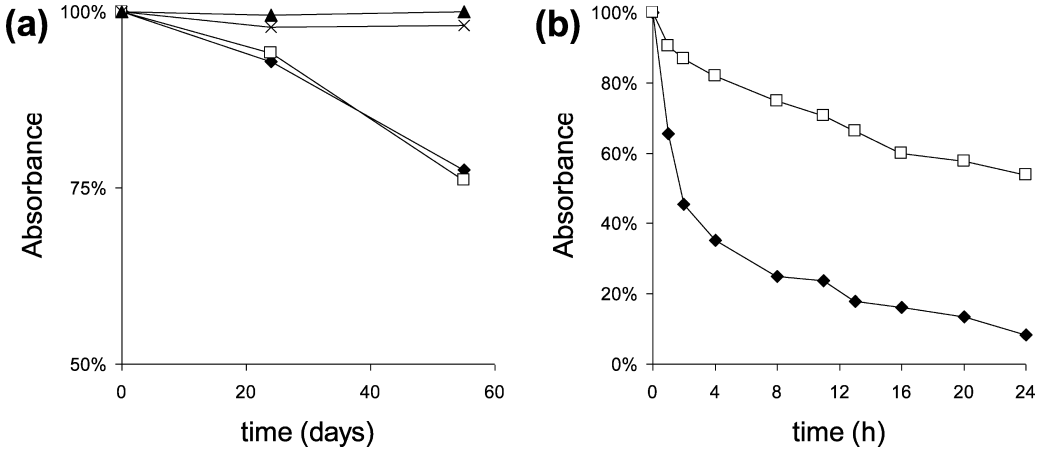


Figure 5. (a) UV−vis absorbance of NO_2-cLA compared to initial absorbance. NO_2-cLA solution (2 mM) stability over 2 months: methanol at rt (□) or −80 °C (▲), DMSO at rt (♦) or −80 °C (×). (b) NO_2-cLA stability over 24 h in aqueous buffer (□) or as neat liquid (♦), both at rt.

and similar environments available when applied to cells or tissue provide both stabilization of the nitroalkene as well as a variety of alternative potential reaction options.

CONCLUSION

In summary, both natural isotopic abundance and ^{15}N-labeled NO_2-cLA **1** and **2**, representative isomers of nitro-conjugated linoleic acids found in vivo, were characterized and synthesized. The structures of the nitrodiene motif, the mechanism of formation and the relationship with methylene-skipped diene nitrolinoleic acids were discussed in the context of previous descriptions of nitrated bis allylic linoleic acid. The structural identification, facile synthetic methods, and optimized storage conditions presented herein provide the investigative tools to further develop the field of electrophilic nitro-lipid signaling. Investigations to dissect the mechanism of diene nitration, synthesize individual regioisomers, and develop the biological and pharmacological applications of these lipids are ongoing.

EXPERIMENTAL SECTION

General Methods. All glassware was oven-dried before use, and reactions were performed under an atmosphere of dry N_2. Specific 9*Z*,11*E*-conjugated linoleic acids (approximately 90% purity) and reagents were purchased and used as received without further purification. Solvents were purchased dry with molecular sieves or HPLC-grade and were used as received. Analytical thin-layer chromatography (TLC) was performed on precoated silica gel F-254 plates and visualized by 254 nm UV light, Greiss reagent (1% sulfanilamide, 0.1% *N*-(1-naphthyl)ethylenediamine in 5% phosphoric

dx.doi.org/10.1021/jo4021562 | *J. Org. Chem.* 2014, 79, 25−33

acid), or phosphomolybdic acid (10% in ethanol) dip. Flash chromatography was performed on 40−63 μm silica gel. Preparatory thin-layer chromatography (PTLC) was performed on silica gel plates containing gypsum binder and fluorescent indicator. ^{1}H and ^{13}C NMR spectra were performed at 600 or 400 MHz and 150 or 100 MHz, respectively, at ambient temperature, and spectra are referenced to residual nondeuterated solvent ($CHCl_3$: 7.26 ppm, 77.00 ppm). ^{15}N NMR spectra were performed at 50 MHz. ^{1}H,^{1}H COSY and ^{1}H,^{13}C HMBC experiments were performed using standard pulse programs. FTIR was performed on an FTIR-ATR instrument. Purity of NO_2-FA was assessed by HPLC−UV using a C18 reversed-phase column (2 × 100 mm, 5 μm) at 220 and 312 nm. LC/MS conditions: C18 reversed-phase column (2 × 150 mm, 3 μm) using a water/acetonitrile solvent system containing 0.1% acetic acid as described.[5] Structural analysis of NO_2-FA was conducted by HPLC−ESI−MS/MS using a triple quadrupole mass spectrometer in negative-ion mode in tandem with a high-resolution hybrid mass spectrometer.

Separation of individual isomers was performed after synthesis of NO_2-cLA by method B (Figure 3). Crude reaction mixtures were purified by PTLC and the nitrodiene-containing bands identified by UV. Subsequent preparative reversed-phase HPLC was used to separate this mixture of regioisomers into two fractions. For preparative-scale separation, an LC system with a 5 μm C18 column (250 mm ×20 mm) and a dual-wavelength UV detector (210 and 312 nm) was used. The mobile phases were water and 0.1% trifluoroacetic acid (A) and acetonitrile and 0.1% trifluoroacetic acid (B) with a flow rate of 20 mL/min. A typical reaction mixture (0.5 mmol scale) was divided into two 48 mg portions each containing approximately 50% product by ^{1}H NMR. A single portion was redissolved in methanol/water or acetonitrile/water up to 5 mL and injected. After injection, separation began at 65% A, 35% B for one minute, then increasing to 35% A, 65% B over 20 min, then increasing to 17% A, 83% B over 80 min. NO_2-cLA fractions eluting at 73% B over 45−50 min were collected and analyzed by LC/MS to identify the regioisomers present (12-NO_2-cLA eluting first, Figure 3b). After fractions were pooled and extracted into ethyl acetate, a second PTLC purification was performed to remove hydrolysis products, after which the compounds were evaporated or solvent-switched to chloroform and then deuterated chloroform for analysis by NMR. From an estimated 24 mg crude product, 6.9 mg 12-NO_2-cLA (28.8% recovery of injected material, 8.2% yield based on starting material), 10.0 mg 9-NO_2-cLA (41.7% recovery, 12.3% yield), and 1.8 mg mixed-isomer material (7.5% recovery) were collected.

Method A:[5,25] Acidic Nitration of CLA. A 15 mL culture tube containing a stirbar was charged with 1.0 mL of 1% sulfuric acid (v/v) and 2.5 mL of cyclohexane. To the organic layer was added 100 μL of 9*Z*,11*E*-conjugated linoleic acid (90 mg, 0.3 mmol) in one portion, after which the tube was covered with a septum and sparged with nitrogen with stirring for 15−20 min or until 1 mL of the hexanes had evaporated. An Eppendorf tube was charged with sodium nitrite ($NaNO_2$ or $Na^{15}NO_2$, 140 mg, 2 mmol) in 1 mL of deionized water for a 2 M solution, which was then sparged with N_2 for 5−10 min. A portion (0.75 mL) of the degassed nitrite solution was added to the biphasic lipid solution upon completion of degassing for a final concentration of 500 mM nitrite and 100 mM lipid. The solution was sealed with a septum without inlet or outlet, covered with foil, and stirred vigorously for 3 h.

Afterward, the biphasic solution was transferred to a separatory funnel and extracted with three 5 mL portions of EtOAc. The organic layers were combined and washed once with water and once with brine, and the solution was dried over sodium sulfate. The solution was filtered through a Celite/silica gel plug and the solvent removed by rotary evaporation with minimum heating, and then the residual crude product was applied to a PTLC plate prewashed with methanol/dichloromethane (1:1 v/v). The plate was eluted (1% HOAc, 25% EtOAc/hexanes), and the UV-active band (R_f = 0.70) was removed and extracted with EtOAc. The extracted product was then applied to a flash chromatography column for further purification (0.5% HOAc, 0−10% EtOAc/hexanes) to yield NO_2-cLA as a yellow oil (12 mg, 8%).

Note: All nitrodienes were maintained in (organic) solution whenever possible and fully evaporated only to weigh or switch solvents. Even slightly elevated temperatures during rotary evaporation led to rapid formation of impurities. Methanol or DMSO solutions were preferred for long-term storage of samples for biological experimentation.

Method B:[41,42] Trifluoroacetate Nitration. A 10 mL round-bottom flask containing a stirbar was charged with dichloromethane (5 mL), 9*Z*,11*E*-conjugated linoleic acid (140 mg, 0.5 mmol), trifluoroacetic anhydride (0.13 mL, 0.9 mmol), and ammonium nitrate (40 mg NH_4NO_3 or $NH_4{}^{15}NO_3$; or 152 mg NBu_4NO_3, 0.5 mmol). The RBF was stirred for 10 min, and then 10.0 μL hydrofluoroboric acid (48% aq) was added to the flask. The solution was stirred at room temperature under nitrogen overnight. The next day, the darkened solution was quenched with 5 mL of water, stirred 5 min, and then transferred to a separatory funnel. The organic layer was removed and the aqueous layer extracted with 3 × 5 mL portions of EtOAc. The organic layers were then combined, washed once with water and once with brine, dried over sodium sulfate, and eluted through a small plug of silica gel. The solvent was removed by rotary evaporation and the crude oil transferred to a 25 mL RBF and redissolved in 10 mL of anhydrous diethyl ether. To the stirred flask was added 60 mg of potassium acetate or proprionate (0.6 mmol, 1.2 equiv) and the suspension stirred under N_2 at rt overnight monitored by TLC. After 24 h (or disappearance of starting material by TLC), the solution was partitioned with 5 mL 1 M aqueous hydrochloric acid and stirred 20 min. The aqueous layer was extracted twice with 5 mL portions of Et_2O. The organic layers were combined, washed once with water and once with brine, and dried over sodium sulfate. The solution was filtered through a silica gel plug, and the solvents were removed by rotary evaporation. The residual oil was purified as in method A to yield NO_2-cLA as a yellow oil (40 mg, 25%; 66 mg, 40%) or by preparative HPLC to obtain single isomers as described.

*(9E,11E)-9-Nitrooctadeca-9,11-dienoic Acid (**1**).* ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 10.1 (br s, 1H); 7.54 (−C*H*=CNO_2, d, *J* = 11.4 Hz, 1H); 6.34 (CH_2C*H*=CH, dt, *J* = 14.9, 7.4, 1H); 6.19 (CH=C*H*CH=, dd, *J* = 14.9, 11.4 Hz, 1H); 2.65 (CNO_2CH_2, t, *J* = 7.6 Hz, 2H); 2.34 (CH_2CO_2, t, *J* = 7.4 Hz, 2H); 2.24 (CH_2CH=CH, q, *J* = 7.3 Hz, 2H); 1.63 (m, 2H); 1.51 (m, 2H); 1.45 (m, 2H); 1.33−1.29 (broad m, 12H); 0.89 (−CH_3, t, *J* = 6.7 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 179.8, 149.25, 149.1, 133.9, 123.5, 33.9, 33.7, 31.6, 28.95, 28.88, 28.81, 28.56, 28.01, 26.5, 24.6, 22.5, 14.0.

*(9E,11E)-12-Nitrooctadeca-9,11-dienoic acid (**2**).* ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 10.1 (br s, 1H); 7.53 (CNO_2=C*H*−, d, *J* = 11.3 Hz, 1H); 6.32 (CH=C*H*CH_2, dt, *J* = 15.0, 7.4, 1H); 6.19 (=CHC*H*=CH, dd, *J* = 15.0, 11.3 Hz, 1H); 2.65 (CH_2CNO_2, t, *J* = 7.5 Hz, 2H); 2.35 (CH_2CO_2, t, *J* = 7.4 Hz, 2H); 2.24 (CH=CHCH_2, q, *J* = 7.4 Hz, 2H); 1.63 (m, 2H); 1.51 (m, 2H); 1.45 (m, 2H); 1.33−1.29 (broad m, 12H); 0.88 (−CH_3, t, *J* = 6.7 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 180.0, 149.30, 148.9, 133.8, 123.6, 34.0, 33.6, 31.4, 28.97, 28.91, 28.88, 28.82, 28.51, 28.04, 26.6, 24.6, 22.5, 14.0. UV−vis: λ_{max} (MeOH) 312 nm, ε = 11,200 M^{-1} cm^{-1}. FT-IR: 2927, 2856, 1706, 1644, 1559, 1510, 1459, 1434, 1317, 1220, 1169, 971, 862, 727 cm^{-1}. ESI(−)-HRMS: for $C_{18}H_{30}O_4N$ calcd 324.2180 $[M - 1]^-$, found 324.2183. TLC (EtOAc/hexanes, 1:3): R_f = 0.33.

*[^{15}N]-(9E,11E)-9-Nitrooctadeca-9,11-dienoic Acid ([^{15}N]-**1**).* ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 10.1 (br s, 1H); 7.54 (−C*H*=CNO_2, d, *J* = 11.4 Hz, 1H); 6.34 (CH_2C*H*=CH, dt, *J* = 14.9, 7.4, 1H); 6.19 (CH=C*H*CH=, dd, *J* = 14.9, 11.4 Hz, 1H); 2.65 (CNO_2CH_2, t, *J* = 7.6 Hz, 2H); 2.34 (CH_2CO_2, t, *J* = 7.4 Hz, 2H); 2.24 (CH_2CH=CH, q, *J* = 7.3 Hz, 2H); 1.63 (m, 2H); 1.51 (m, 2H); 1.45 (m, 2H); 1.33−1.29 (broad m, 12H); 0.89 (−CH_3, t, *J* = 6.7 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 179.5, 149.25, 149.06 (J_{C-N} = 11.0 Hz), 134.0, 123.50 (J_{C-N} = 3.4 Hz), 33.9, 33.7, 31.6, 28.96, 28.89, 28.82, 28.57, 28.02, 26.5, 24.6, 22.5, 14.0.

*[^{15}N]-(9E,11E)-12-Nitrooctadeca-9,11-dienoic Acid ([^{15}N]-**2**).* ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 10.1 (br s, 1H); 7.53 (CNO_2=C*H*−, d, *J* = 11.3 Hz, 1H); 6.32 (CH=C*H*CH_2, dt, *J* = 15.0, 7.4, 1H); 6.19 (=CHC*H*=CH, dd, *J* = 15.0, 11.3 Hz, 1H); 2.65 (CH_2CNO_2, t, *J* = 7.5 Hz, 2H); 2.35 (CH_2CO_2, t, *J* = 7.4 Hz, 2H); 2.24 (CH=

dx.doi.org/10.1021/jo4021562 | J. Org. Chem. 2014, 79, 25−33

$CHCH_2$, q, J = 7.4 Hz, 2H); 1.63 (m, 2H); 1.51 (m, 2H); 1.45 (m, 2H); 1.33−1.29 (broad m, 12H); 0.88 ($-CH_3$, t, J = 6.7 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 179.2, 149.33 (J_{C-N} = 11.2 Hz), 148.8, 133.77 (J_{C-N} = 1.1 Hz), 123.66 (J_{C-N} = 3.4 Hz), 33.8, 33.6, 31.5, 28.99, 28.93, 28.90, 28.83, 28.54, 28.06, 26.6, 24.6, 22.5, 14.0. ^{15}N NMR (50.6 MHz, $CDCl_3$) δ (ppm): 377.8, 377.9 (combination of positional isomers). FT-IR: 2927, 2857, 1708, 1643, 1521, 1481, 1294, 973, 726 cm^{-1}. ESI(−)-HRMS: for $C_{18}H_{30}O_4{}^{15}N$ calcd 325.2151 $[M - 1]^-$, found 325.2153.

■ ASSOCIATED CONTENT

Supporting Information

Copies of ^1H and ^{13}C NMR for compounds **1** and **2** (NO_2-cLA). Copies of ^1H, ^{13}C, $^1H-^1H$ COSY, $^1H-^{13}C$ HMBC, ^{15}N, and $^1H-^{15}N$ HMBC NMR for compounds [^{15}N]-NO_2-cLA (**1** and **2**). UV−vis spectra and graphs. LC/MS analysis of products including fragmentation. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: 412-648-9319. Fax: 412-648-2229. E-mail: srw22@pitt.edu.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This study was supported by NIH Grant Nos. R01-HL058115, R01-HL64937, and P01-HL103455 (B.A.F.) and R01-AT006822 (F.J.S.). F.J.S. and B.A.F. acknowledge interest in Complexa, Inc. We thank Dr. Damodaran Krishnan Achary (University of Pittsburgh) for assistance with heteronuclear NMR, Dr. Alessandro Bisello (University of Pittsburgh) for assistance with purification, and Dr. Andrew Robak (Keuka College) for helpful discussions.

■ REFERENCES

(1) O'Donnell, V. B.; Eiserich, J. P.; Chumley, P. H.; Jablonsky, M. J.; Krishna, N. R.; Kirk, M.; Barnes, S.; Darley-Usmar, V. M.; Freeman, B. A. *Chem. Res. Toxicol.* **1998**, *12*, 83−92.

(2) Rudolph, V.; Rudolph, T. K.; Schopfer, F. J.; Bonacci, G.; Woodcock, S. R.; Cole, M. P.; Baker, P. R. S.; Ramani, R.; Freeman, B. A. *Cardiovasc. Res.* **2009**, *85*, 155−66.

(3) Nadtochiy, S. M.; Baker, P. R. S.; Freeman, B. A.; Brookes, P. S. *Cardiovasc. Res.* **2009**, *82*, 333−340.

(4) Baker, P. R. S.; Schopfer, F. J.; O'Donnell, V. B.; Freeman, B. A. *Free Radical Biol. Med.* **2009**, *46*, 989−1003.

(5) Bonacci, G.; Baker, P. R. S.; Salvatore, S. R.; Shores, D.; Khoo, N. K. H.; Koenitzer, J. R.; Vitturi, D. A.; Woodcock, S. R.; Golin-Bisello, F.; Watkins, S.; St Croix, C.; Batthyany, C. I.; Freeman, B. A.; Schopfer, F. J. *J. Biol. Chem.* **2012**, *287*, 44071−82.

(6) Freeman, B. A.; Baker, P. R. S.; Schopfer, F. J.; Woodcock, S. R.; Napolitano, A.; d'Ischia, M. *J. Biol. Chem.* **2008**, *283*, 15515−15519.

(7) Schopfer, F. J.; Cipollina, C.; Freeman, B. A. *Chem. Rev.* **2011**, *111*, 5997−6021.

(8) Baker, P. R. S.; Lin, Y.; Schopfer, F. J.; Woodcock, S. R.; Groeger, A. L.; Batthyany, C. I.; Sweeney, S.; Long, M. H.; Iles, K. E.; Baker, L. M. S.; Branchaud, B. P.; Chen, Y. E.; Freeman, B. A. *J. Biol. Chem.* **2005**, *280*, 42464−75.

(9) Baker, L. M. S.; Baker, P. R. S.; Golin-Bisello, F.; Schopfer, F. J.; Fink, M.; Woodcock, S. R.; Branchaud, B. P.; Radi, R.; Freeman, B. A. *J. Biol. Chem.* **2007**, *282*, 31085−31093.

(10) Batthyany, C. I.; Schopfer, F. J.; Baker, P. R. S.; Duran, R.; Baker, L. M. S.; Huang, Y.; Cervenansky, C.; Branchaud, B. P.; Freeman, B. A. *J. Biol. Chem.* **2006**, *281*, 20450−20463.

(11) Cui, T.; Schopfer, F. J.; Zhang, J.; Chen, K.; Ichikawa, T.; Baker, P. R. S.; Batthyany, C. I.; Chacko, B. K.; Feng, X.; Patel, R. P.; Agarwal, A.; Freeman, B. A.; Chen, Y. E. *J. Biol. Chem.* **2006**, *281*, 35686−35698.

(12) Kelley, E. E.; Batthyany, C. I.; Hundley, N. J.; Woodcock, S. R.; Bonacci, G.; Del Rio, J. M.; Schopfer, F. J.; Lancaster, J. R., Jr.; Freeman, B. A.; Tarpey, M. M. *J. Biol. Chem.* **2008**, *283*, 36176−36184.

(13) Kansanen, E.; Bonacci, G.; Schopfer, F. J.; Kuosmanen, S. M.; Tong, K. I.; Leinonen, H.; Woodcock, S. R.; Yamamoto, M.; Carlberg, C.; Yla-Herttuala, S.; Freeman, B. A.; Levonen, A.-L. *J. Biol. Chem.* **2011**, *286*, 14019−14027.

(14) Kansanen, E.; Jyrkkanen, H.-K.; Volger, O. L.; Leinonen, H.; Kivela, A. M.; Hakkinen, S.-K.; Woodcock, S. R.; Schopfer, F. J.; Horrevoets, A. J.; Yla-Herttuala, S.; Freeman, B. A.; Levonen, A.-L. *J. Biol. Chem.* **2009**, *284*, 33233−33241.

(15) Li, Y.; Zhang, J.; Schopfer, F. J.; Martynowski, D.; Garcia-Barrio, M. T.; Kovach, A.; Suino-Powell, K.; Baker, P. R. S.; Freeman, B. A.; Chen, Y. E.; Xu, H. E. *Nat. Struct. Mol. Biol.* **2008**, *15*, 865−867.

(16) Schopfer, F. J.; Cole, M. P.; Groeger, A. L.; Chen, C.-S.; Khoo, N. K. H.; Woodcock, S. R.; Golin-Bisello, F.; Motanya, U. N.; Li, Y.; Zhang, J.; Garcia-Barrio, M. T.; Rudolph, T. K.; Rudolph, V.; Bonacci, G.; Baker, P. R. S.; Xu, H. E.; Batthyany, C. I.; Chen, Y. E.; Hallis, T. M.; Freeman, B. A. *J. Biol. Chem.* **2010**, *285*, 12321−12333.

(17) Schopfer, F. J.; Lin, Y.; Baker, P. R. S.; Cui, T.; Garcia-Barrio, M. T.; Zhang, J.; Chen, K.; Chen, Y. E.; Freeman, B. A. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 2340−2345.

(18) Napolitano, A.; Crescenzi, O.; Camera, E.; Giudicianni, I.; Picardo, M.; d'Ischia, M. *Tetrahedron* **2002**, *58*, 5061−5067.

(19) Napolitano, A.; Camera, E.; Picardo, M.; d'Ischia, M. *J. Org. Chem.* **2000**, *65*, 4853−4860.

(20) Baker, P. R. S.; Schopfer, F. J.; Sweeney, S.; Freeman, B. A. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 11577−82.

(21) Alexander, R. L.; Wright, M. W.; Gorczynski, M. J.; Smitherman, P. K.; Akiyama, T. E.; Wood, H. B.; Berger, J. P.; King, S. B.; Morrow, C. S. *Biochemistry* **2008**, *48*, 492−498.

(22) Dunny, E.; Evans, P. *J. Org. Chem.* **2010**, *75*, 5334−5336.

(23) Zanoni, G.; Valli, M.; Bendjeddou, L.; Porta, A.; Bruno, P.; Vidari, G. *J. Org. Chem.* **2010**, *75*, 8311−8314.

(24) Manini, P.; Capelli, L.; Reale, S.; Arzillo, M.; Crescenzi, O.; Napolitano, A.; Barone, V.; d'Ischia, M. *J. Org. Chem.* **2008**, *73*, 7517−7525.

(25) Napolitano, A.; Camera, E.; Picardo, M.; d'Ischia, M. *J. Org. Chem.* **2002**, *67*, 1125−1132.

(26) Ballini, R.; Araujo, N.; Gil, M. V.; Roman, E.; Serrano, J. A. *Chem. Rev.* **2013**, *113*, 3493−3515.

(27) Mele, M. C.; Cannelli, G.; Carta, G.; Cordeddu, L.; Melis, M. P.; Murru, E.; Stanton, C.; Banni, S. *Prostaglandins Leukot. Essent. Fatty Acids* **2013**, *89*, 115−119.

(28) Pariza, M. W.; Park, Y.; Cook, M. E. *Prog. Lipid Res.* **2001**, *40*, 283−298.

(29) Kramer, J. G.; Parodi, P.; Jensen, R.; Mossoba, M.; Yurawecz, M.; Adlof, R. *Lipids* **1998**, *33*, 835−835.

(30) Salvatore, S. R.; Vitturi, D. A.; Baker, P. R. S.; Bonacci, G.; Koenitzer, J. R.; Woodcock, S. R.; Freeman, B. A.; Schopfer, F. J. *J. Lipid Res.* **2013**, *54*, 1998−2009.

(31) Lim, D. G.; Sweeney, S.; Bloodsworth, A.; White, C. R.; Chumley, P. H.; Krishna, N. R.; Schopfer, F. J.; O'Donnell, V. B.; Eiserich, J. P.; Freeman, B. A. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 15941−15946.

(32) Unpublished data.

(33) Woodcock, S. R.; Marwitz, A. J. V.; Bruno, P.; Branchaud, B. P. *Org. Lett.* **2006**, *8*, 3931−3934.

(34) *CRC Handbook of Chemistry and Physics*, 64th ed.; Weast, R. C., Ed.; CRC Press: Boca Raton, 1983.

(35) Claridge, R. P.; Deeming, A. J.; Paul, N.; Tocher, D. A.; Ridd, J. H. *J. Chem. Soc., Perkin Trans. 1* **1998**, 3523−3528.

(36) d'Ischia, M.; Rega, N.; Barone, V. *Tetrahedron* **1999**, *55*, 9297−9308.

(37) Pryor, W. A.; Lightsey, J. W.; Church, D. F. *J. Am. Chem. Soc.* **1982**, *104*, 6685−6692.
(38) Hata, E.; Yamada, T.; Mukaiyama, T. *Bull. Chem. Soc. Jpn.* **1995**, *68*, 3629−3636.
(39) Golding, P.; Powell, J. L.; Ridd, J. H. *J. Chem. Soc., Perkins Trans. 2* **1996**, 813−819.
(40) Najera, C.; Yus, M.; Karlsson, U.; Gogoll, A.; Baeckvall, J.-E. *Tetrahedron Lett.* **1990**, *31*, 4199−4202.
(41) Bloom, A. J.; Mellor, J. M. *Tetrahedron Lett.* **1986**, *27*, 873−876.
(42) Bloom, A. J.; Mellor, J. M. *J. Chem. Soc., Perkin Trans. 1* **1987**, 2737−2741.
(43) Woodcock, S. R.; Bonacci, G.; Gelhaus, S. L.; Schopfer, F. J. *Free Radical Biol. Med.* **2013**, *59*, 14−26.
(44) Denmark, S. E.; Gomez, L. *J. Org. Chem.* **2003**, *68*, 8015−8024.
(45) Alexander, R. L.; Bates, D. J. P.; Wright, M. W.; King, S. B.; Morrow, C. S. *Biochemistry* **2006**, *45*, 7889−7896.
(46) Creech, G. S.; Kwon, O. *J. Am. Chem. Soc.* **2010**, *132*, 8876−8877.
(47) Barrett, A. G. M.; Graboski, G. G. *Chem. Rev.* **1986**, *86*, 751−762.

dx.doi.org/10.1021/jo4021562 | *J. Org. Chem.* 2014, 79, 25−33