Reactions of Hydro(pero)xy Derivatives of Polyunsaturated Fatty Acids/Esters with Nitrite Ions under Acidic Conditions. Unusual Nitrosative Breakdown of Methyl 13-Hydro(pero)xyoctadeca-9,11-dienoate to a Novel 4-Nitro-2-oximinoalk-3-enal Product

Alessandra Napolitano,[†] Emanuela Camera,[‡] Mauro Picardo,[‡] and Marco d'Ischia*[,†]

Department of Organic Chemistry and Biochemistry, University of Naples "Federico II", Via Cinthia 4, I-80126 Naples, Italy, and Istituto Dermatologico S. Gallicano, Via S. Gallicano 25a, 00153 Rome, Italy

dischia@cds.unina.it

Received July 27, 2001

13(*S*)-Hydroperoxy- and 13(*S*)-hydroxyoctadeca-9,11-dienoic acids (**1a/b**), 15(*S*)-hydroperoxy- and 15(*S*)-hydroxyeicosa-5,8,11,13-tetraenoic acids (**2a/b**), and their methyl esters reacted smoothly with NO_2^- in phosphate buffer at pH 3−5.5 and at 37 °C to afford mixtures of products. **1b** methyl ester gave mainly the 9-nitro derivative **3b** methyl ester (11% yield) and a peculiar breakdown product identified as the novel 4-nitro-2-oximinoalk-3-enal derivative **4** methyl ester (15% yield). By GC−MS hexanal was also detected among the products. Structures **3b** and **4** methyl esters were secured by ^{15}N NMR analysis of the products prepared from **1b** methyl ester upon reaction with $Na^{15}NO_2$. **4** methyl ester (14% yield) was also obtained from **1a** methyl ester along with the nitrated hydroperoxy derivative **3a** methyl ester (10% yield). Under the same conditions, **2a/b** methyl esters gave mainly the corresponding nitrated derivatives **5a/b**, with no detectable breakdown products, whereas the model compound (*E*,*E*)-2,4-hexadienol (**6**) afforded two main nitrated derivatives identified as **7** and **8**. A reaction pathway for **1a/b** methyl esters was proposed involving conversion of nitronitrosooxyhydro(pero)xy intermediates which would partition between two competing routes, viz., loss of HNO_2, to give **3a/b** methyl esters, and a remarkably facile fission leading to **4** methyl ester and hexanal.

Introduction

Reactions of hydroperoxy and hydroxy derivatives of polyunsaturated fatty acids with nitrite (NO_2^-) and related nitrosating species may be implicated in the pathophysiological sequelae of a number of conditions associated with enhanced lipid peroxidation and excessive production of nitric oxide (NO) and other inflammatory modulators.[1] During artherosclerosis, ischemia/reperfusion, neurodegeneration, skin allergy, cancer, and inflammatory disease states, elevated levels of 13(*S*)-hydroperoxy- and 13(*S*)-hydroxyoctadeca-9,11-dienoic acids (**1a/b**) as well as 15(*S*)-hydroperoxy- and 15(*S*)-hydroxyeicosa-5,8,11,13-tetraenoic acids (**2a/b**), lipoxins, leukotrienes, isoprostanes, and other bioactive eicosanoids[2] are generated by both enzymatic and nonenzymatic processes.[3] Because of their peculiar reactive moiety comprising an oxygenated functionality adjacent to a conjugated diene moiety, these compounds are expectedly susceptible to structural modification by reactive nitrogen species and chiefly NO_2^-, the main metabolic end product of NO and an important mediator of nitrosative damaging and mutagenic reactions.[4]

Relatively high levels of NO_2^- are found in physiological fluids (0.5−3.6 μM in plasma, 15 μM in respiratory tract lining fluids, and up to 210 μM in saliva).[5] These levels increase dramatically during pathological conditions, e.g., following ischemia/reperfusion, acute inflammation, or allergy.[5b,6] NO_2^- is also a ubiquitous environmental pollutant, raising increasing concern as a contaminant of drinking waters,[7] and is commonly used as an additive in preserved/pickled food.[8]

At physiological pH, e.g., 7.4, NO_2^- is stable. However, in acidic media it equilibrates with nitrous acid (HNO_2,

* To whom correspondence should be addressed. Phone: +39-081-674132. Fax: +39-081-674393.

[†] University of Naples "Federico II".

[‡] Istituto Dermatologico S. Gallicano.

(1) Schmitt, D.; Shen, Z.; Zhang, R.; Colles, S. M.; Wu, W.; Salomon, R. G.; Chen, Y.; Chisolm, G. M.; Hazen, S. L. *Biochemistry* **1999**, *38*, 16904−16915.

(2) (a) Cao, Y.; Pearman, A. T.; Zimmerman, G. A.; McIntyre, T. M. Prescott, S. M. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 11280−11285. (b) Balazy, M. *J. Physiol. Pharmacol.* **2000**, *51*, 597−607. (c) Ferrante, J. V.; Huang, Z. H.; Nandoskar, M.; Hii, C. S. T.; Robinson, B. S.; Rathjen, D. A.; Poulos, A.; Morris, C. P.; Ferrante, A. *J. Clin. Invest.* **1997**, *99*, 1445−1452. (d) Ziboh, V. A.; Miller, C. C.; Cho, Y. *Am. J. Clin. Nutr.* **2000**, *71*, 361S−366S.

(3) (a) Kuehn, H. *Prog. Lipid Res.* **1997**, *35*, 203−226. (b) Nicolau, K. C.; Ramphal, J. Y.; Petasis, N. A.; Serhan, C. N. *Angew. Chem., Int. Ed. Engl.* **1991**, *30*, 1100−1116.

(4) (a) van der Vliet, A.; Eiserich, J. P.; Halliwell, B.; Cross, C. E. *J. Biol. Chem.* **1997**, *272*, 7617−7625. (b) O'Donnell, V. B.; ChumLey, P. H.; Hogg., N.; Bloodsworth, A.; Darley-Usmar, V. M.; Freeman, B. A. *Biochemistry* **1997**, *36*, 15216−15223.

(5) (a) Leone, A. M.; Francis, P. L.; Rhodes, P.; Moncada, S. *Biochem. Biophys. Res. Commun.* **1994**, *200*, 951−957. (b) Gaston, B.; Reilly, J.; Drazen, J. M.; Fackler, J.; Ramdey, P.; Arnelle, D.; Mullins, M. E.; Sugarbaker, D. J.; Chee, C.; Singel, D. J.; Loscalzo, J.; Stamler, J. S. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 10957−10961. (c) Green, L. C.; Wagner, D. A.; Glogowski, J.; Skipper, P. L.; Wishnok, J. S.; Tannenbaum, S. R. *Anal. Biochem.* **1982**, *126*, 131−138.

(6) (a) Wakita, T.; Shintani, F.; Yagi, G.; Asai, M.; Nozawa, S. *Brain Res.* **2001**, *905*, 12−20. (b) Dweik, R. A.; Comhair, S. A. A.; Gaston, B.; Thunnissen, F. B. J. M.; Farver, C.; Thomassen, M. J.; Kavuru, M.; Hammel, J.; Abu-Soud, H. M.; Erzurum, S. C. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, 98, 2622−2627. (c) O'Donnell, V. B.; Eiserich, J. P.; Bloodsworth, A.; Chumley, P. H.; Kirk, M.; Barnes, S.; Darley-Usmar, V. M.; Freeman, B. A. *Methods Enzymol.* **1999**, *301*, 454−470.

(7) Khan, A. R.; Ullah, S.; Hussain, F.; Khan, M.; Riaz, M. *J. Chem. Soc. Pak.* **1999**, *21*, 106−114.

(8) Sen, N. P.; Seaman, S. W.; Baddoo, P. A.; Burgess, C.; Weber, D. *J. Agric. Food Chem.* **2001**, *49*, 2096−2103.

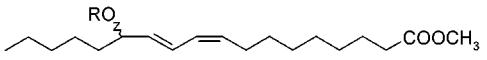
10.1021/jo015973b CCC: $22.00
Published on Web 01/30/2002

pK_a = 3.25),[9] which, in turn, is readily converted to a range of potent nitrosating/nitrating species, such as NO_2 and N_2O_3, capable of inducing profound modifications of polyunsaturated fatty acids.[10]

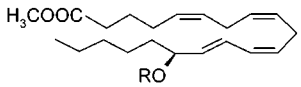
Although several circumstances can be envisioned in which NO_2^- may react with bioactive lipid peroxidation products in an acidic environment, the basic chemistry of this interaction has been investigated only sporadically. In a previous exploratory paper[11] surveying the nitration of unsaturated fatty acids by NO-derived species, **1a** was reported to react with NO_2^- (or $^{15}NO_2^-$) at pH 3 to give mainly nitroepoxide derivatives identified by MS/MS and ^{15}N NMR analysis. However, the nature of the remainder of the reaction products and the detailed reaction pathways of **1a** remained largely uncharted. In fact, a survey of the literature failed to provide more than scattered or indirect information even on the basic reactivity of simple 2,4-dienols or structurally related hydroperoxides toward nitrosating agents.[12]

Yet, the prospects that this reaction might return a practical synthetic access to an array of novel derivatives of **1a/b** and **2a/b** was especially attractive, given the potent physiological effects displayed by the parent compounds. Additional incentives for studies of the interactions of NO_2^- with hydro(pero)xy polyunsaturated fatty acids stemmed from a report showing that **1a** inhibited the formation of *N*-nitrosodimethylamine by reaction of dimethylamine with NO_2^- in citrate buffer (pH 3−5).[13] In that study, however, no insight was gained into the nature of the main products and the reaction mechanisms.

In the present paper we report the isolation, the spectral characterization, and a preliminary biological screening of single nitration products formed by reaction of **1a/b** and **2a/b** and their esters with NO_2^- in acidic media.



1a methyl ester: R=OH
1b methyl ester: R=H



2a methyl ester: R=OH
2b methyl ester: R=H

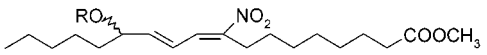
Results and Discussion

Reactions of 1a and 1b and Their Methyl Esters with NO_2^-. Reactions of **1a** and **1b** methyl esters with varying concentrations of NO_2^- were typically conducted in a biphasic system consisting of 0.05 M phosphate buffer, pH 3/ethyl acetate. In both cases, the reactions afforded closely similar patterns of products (TLC), most of which proved positive to the Griess reagent. The main products formed in the reaction mixtures from **1b** methyl ester could be obtained in pure form by preparative TLC and were subjected to extensive spectral analysis.

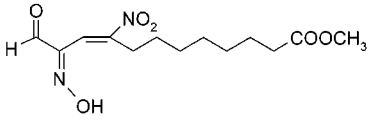
The less polar product (R_f 0.30, about 11% isolated yield) displayed strong bands in the FT-IR spectrum at 1514 and 1327 cm^{-1} for a conjugated nitro group, and was straightforwardly formulated as **3b** methyl ester on the basis of $^1H-^1H$ COSY, $^1H-^{13}C$ HMQC, and HMBC experiments as well as GC−MS analysis in the PICI and NICI modalities. The product was optically active, suggesting that the stereogenic center was not affected under the reaction conditions. This was also confirmed by the similar $[\alpha]_D$ values determined on freshly prepared **1b** methyl ester and on a sample of the same compound recovered by TLC fractionation from a typical reaction mixture.

The more polar product (R_f 0.16, about 15% yield) exhibited a distinct chromophore (λ_{max} = 274 nm) and strong bands in the FT-IR spectrum for a hydroxyl group (3547 cm^{-1}), a conjugated nitro group (1541 and 1337 cm^{-1}), and, apparently, more than one C=O functionality (broad band at 1650 cm^{-1} with inflections).

Besides the resonances of the carbomethoxyl functionality, the ^1H NMR spectrum featured in the low-field region a 1H singlet at δ 9.61 correlating with a CH carbon signal at δ 189.1, diagnostic of an aldehyde group, and a 1H singlet at δ 7.28 correlating with a carbon signal at δ 119.2. These data, and the lack of resonances ascribable to the CH−OH group, revealed a profound modification of the original dienol moiety. The $^1H-^{13}C$ HMBC spectrum showed cross-peaks between the aldehyde proton signal and carbon resonances at δ 119.2 and 153.6, whereas the proton singlet at δ 7.28 correlated only with a carbon signal at δ 157.9. An additional feature was a 2H triplet for a methylene group at δ 2.48 giving cross-peaks with the carbon signals at δ 119.2 and 157.9. Overall these data were suggestive of a fragmentation product of **1b** methyl ester featuring an aldehyde group, an oxime group, and a nitro-substituted double bond in that order. On this basis, the compound was formulated as **4** methyl ester.



3a methyl ester: R=OH
3b methyl ester: R=H



4 methyl ester

In this structure, the geometry of the oxime group was inferred to be *anti* with respect to the aldehyde from the relatively upfield shift of the alkene carbon β to the nitro group.[10] The *E* configuration of the alkene double bond was deduced from the chemical shift of the proton experiencing the magnetic anisotropy effect of the nitro group.

GC−MS analysis of **4** methyl ester in the EI modality gave a spectrum in which the molecular ion peak was below detection limits, though a small M − 2 peak and intense fragmentation peaks at *m*/*z* 157, 171, and 185, due to losses of $(CH_2)_6COOCH_3$, $(CH_2)_5COOCH_3$, and

(9) Lide, D. R. *CRC Handbook of Chemistry and Physics*, 75th ed.; CRC Press: Boca Raton, FL, 1995.
(10) Napolitano, A.; Camera, E.; Picardo, M.; d'Ischia, M. *J. Org. Chem.* **2000**, *65*, 4853−4860.
(11) O'Donnel, 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.* **1999**, *12*, 83−92.
(12) Kelley, D. R.; Jones, S.; Adigun, J. O.; Koh, K. S. V.; Hibbs, D. E.; Hursthouse, M. B.; Jackson, S. K. *Tetrahedron* **1997**, *53*, 17221−17234.
(13) Kikugawa, K.; Kato, T.; Konoe, Y.; Sawamura, A. *Food. Chem. Toxicol.* **1985**, *23*, 339−342.

$(CH_2)_4COOCH_3$ in that order, were clearly discernible. However, the *O*-TMS derivative of **4** methyl ester afforded the expected molecular ion peak at *m*/*z* 372.

To further substantiate the structural formulations for products **3b** methyl ester and **4** methyl ester, and to probe possible relationships with the previously described products,[11] **1b** methyl ester was allowed to react with $Na^{15}NO_2$ at pH 3, and the resulting **3b** methyl ester (10% yield) and **4** methyl ester (14% yield) were isolated and subjected to ^{15}N NMR analysis.

In the case of **3b** methyl ester, a single resonance was apparent at δ 376.7 showing cross-peaks in the $^1H-^{15}N$ HMBC spectrum with the proton signals at δ 7.55 and 2.69. Two distinct signals at δ 374.5 and 424.2 substantiated the nitro and oxime[14] functionalities in **4** methyl ester. Whereas the former signal exhibited a well-discernible cross-peak in the $^1H-^{15}N$ HMBC spectrum with the singlet at δ 7.28, no detectable corrrelation was observed between the ^{15}N signal at δ 424.2 and the aldehyde proton, due probably to the exceedingly low long-range coupling constants of the oxime nitrogen. This interpretation was corroborated by the ^1H NMR spectrum of **4** methyl ester, in which the proton resonance at δ 7.28 was split into a double doublet by $^3J_{H-N}$ coupling with the nitro and the oxime group, whereas the aldehyde proton signal at δ 9.61 still appeared as a singlet.

NMR and GC−MS analysis of a crude reaction mixture from **1b** methyl ester confirmed the presence of **3b** and **4** methyl esters, ruling out the possibility that they were artifactually produced during chromatographic separation on silica. Notably, GC−MS analysis revealed a major reaction byproduct which was identified as hexanal by comparison of its chromatographic and mass spectrometric behavior with that of an authentic sample. No products derived from intermolecular cross-linking processes (e.g., dimerization) were apparently present.

Reaction of **1a** methyl ester under the same conditions used for **1b** methyl ester afforded comparable yields of **4** as well as a product closely related to **3b** methyl ester which was identified as **3a** methyl ester by straightforward NMR analysis. Diagnostic for the CH−OOH group was a proton resonance at δ 4.50 correlating with a carbon signal at δ 86.2. These values differed significantly from those of the resonances of the CH−OH group of **3b** methyl ester at δ 4.32 and 72.7. Product **3a** methyl ester, unlike **3b** methyl ester, proved positive to standard tests for peroxides, viz., the iodide/starch and the xylenol orange assays.[15]

Under the typical reaction conditions described above, **1b** gave two main products which proved to be the expected **3b** and **4** methyl esters after methylation of the mixture with diazomethane.

Products **3b** and **4** could also be detected as methyl esters by GC−MS analysis in mixtures obtained by reaction of peroxidized linoleic acid with NO_2^- at pH 3 for 5 h, after ethyl acetate extraction and treatment with diazomethane. In these experiments peroxidized linoleic acid refers to the mixture of products obtained by Fe^{2+}-EDTA-induced autoxidation of linoleic acid at pH 7.4 and at 37 °C following reduction with $NaBH_4$, to limit breakdown of peroxide products and increase formation of hydroxylated derivatives.

(14) Allen, M.; Roberts, J. D. *J. Org. Chem.* **1980**, *45*, 130−135.
(15) Jiang, Z.-Y.; Hunt, J., V.; Wolff, S., P. *Anal. Biochem.* **1992**, *202*, 384−389.

In another series of experiments the reaction of **1b** methyl ester with NO_2^- was investigated under different experimental conditions to gain information about possible early intermediates, changes in product distribution, and effects of acids, oxygen, and NO_2^- concentration.

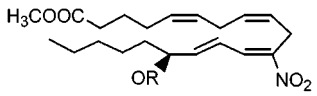
With substoichiometric amounts of NO_2^- (0.5 molar equiv), at pH 3 and with all other parameters unchanged, the reaction proceeded to a lesser extent, as expected; however, the product distribution remained virtually unaffected over prolonged periods of time (up to 10 h) and was substantially similar to the product distribution obtained with higher NO_2^- concentrations (e.g., 10 molar equiv). When incubated at pH 3 under the typical reaction conditions but in the absence of NO_2^-, products **3b** and **4** methyl esters did not suffer appreciable structural modifications or decomposition.

Reaction of **1b** methyl ester with 2 molar equiv of NO_2^- proceeded to a considerable extent also at pH 5.5 in a biphasic system (ethyl acetate/phosphate buffer) to give comparable yields of formation of **3b** and **4** methyl esters. A similar degree of substrate conversion and product pattern was observed in 1% sulfuric acid, whereas the reaction was substantially inhibited at pH above 6.

To assess the effect of oxygen on the reaction course, **1b** methyl ester was incubated at pH 3 with 2 molar equiv of NO_2^- under an Ar atmosphere. Careful analysis of the reaction mixture indicated a modest decrease in the extent of substrate conversion but no change in product distribution. Similar patterns of products were formed when the typical reaction of **1b** methyl ester at pH 3 with 2 molar equiv of NO_2^- was conducted at 0 or 40 °C.

Acid-Promoted Reactions of 2a and 2b and Their Methyl Esters with NO_2^-. Reactions of **2a** and **2b** methyl esters with NO_2^- under the same conditions adopted for **1a** and **1b** methyl esters proceeded similarly to give a main component positive to the Griess assay. The products were isolated by preparative TLC and could be identified by straightforward spectral analysis as **5a** and **5b** methyl esters. The configuration of the stereogenic center at C-15 was apparently unaffected, as judged from the $[\alpha]_D$ value.

As expected, **2a** and **2b** gave **5a** and **5b**, respectively, as the main reaction products, obtained as methyl esters after treatment of the reaction mixture with diazomethane.

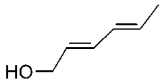


5a methyl ester: R=OH
5b methyl ester: R=H

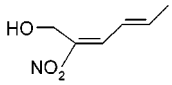
Despite careful analysis, no aldehyde compound with the structural features of **4** was found to be formed to any appreciable extent by the reaction of **2a** or **2b** methyl ester with NO_2^-.

Acid-Promoted Reactions of *(E,E)*-2,4-Hexadienol with NO_2^-. Before the mechanistic issues raised by the formation of nitration products of types **3**−**5** from the corresponding hydro(pero)xide precursors were addressed, a brief investigation into the reaction behavior of a model 2,4-dienol system with NO_2^- seemed desirable. To this end, the commercially available (*E*,*E*)-2,4-hexadienol (**6**) was judged to fulfill the requirements of a suitable model. Consistent with this expectation, the compound reacted

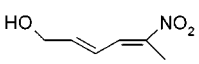
smoothly with 2 molar equiv of NO_2^- at pH 3 to afford two main products in comparable yields, which could be isolated and identified as **7** and **8**.



6



7

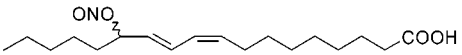


8

Mechanistic Issues. The pH conditions adopted in the present study were compatible with the generation of HNO_2, which would then decompose to give either gaseous species, such as N_2O_3, NO, and NO_2, or ionic species, e.g., NO^+ and NO_2^+. To determine which of these species was actually involved in nitration of **1a/b** and **2a/b**, NO_2^- was allowed to decompose separately in 1% sulfuric acid, and the gases that evolved were conveyed by a stream of Ar through a solution of **1b** methyl ester in ethyl acetate. TLC analysis showed formation of a complex pattern of products virtually identical to that obtained from the NO_2^--dependent reaction in the biphasic system.

Reaction of **1b** methyl ester with NO_2^- at pH 3 was also conducted in the presence of a large excess (10 molar equiv) of 1,3-dinitrobenzene, to eliminate radical reactions and provide an opportunity for ionic or other nonradical reactions (e.g., via N_2O_3) to occur. Under such conditions, product formation was not affected, but this could be due to a substantial lack of reactivity of 1,3-dinitrobenzene with NO_2^--derived species.[10] Reaction of **1b** methyl ester with NO_2BF_4 (an established source of NO_2^+ contaminated by some NO^+) in chloroform afforded a quite different pattern of products which was not investigated further.

Attempts to isolate, detect, or intercept intermediate species in the reactions of **1a/b** or **2a/b** with NO_2^- were unsuccessful. To determine whether initial nitrosation of **1b** at the OH group was a requisite in the formation of **3b** and/or **4**, the nitrite ester **9** was prepared by careful

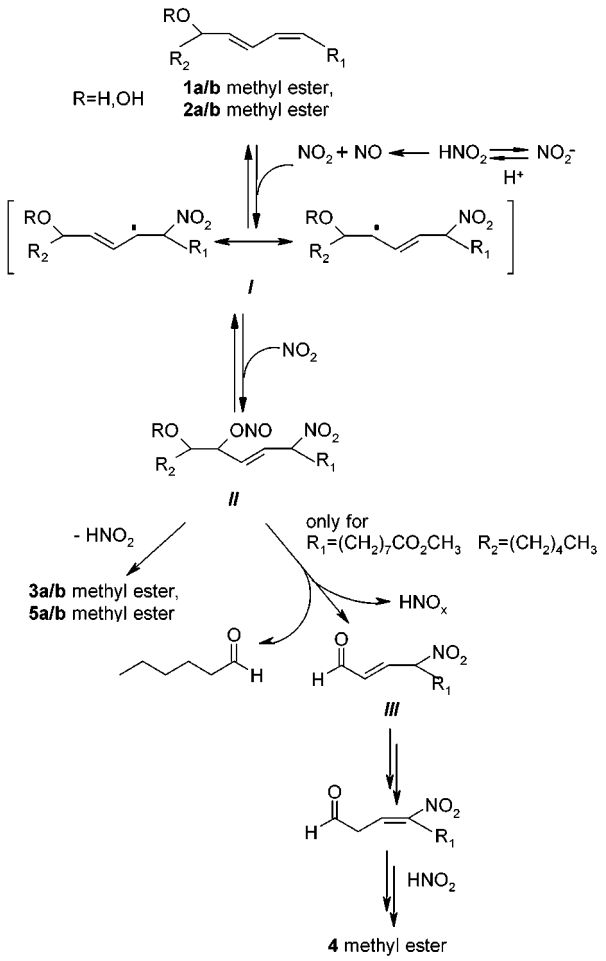


9

nitrosation of **1b** with *tert*-butyl nitrite in chloroform. ^{1}H NMR analysis of the crude reaction mixture indicated a main component (ca. 80%) exhibiting the resonances expected for **9**, in particular a deshielded proton at δ 5.77, correlating with an sp^3 carbon signal at δ 80.1 denoting a CH−ONO functionality. The compound proved too unstable to permit isolation by conventional chromatographic conditions. On being allowed to stand in chloroform at room temperature, crude **9** underwent slow decomposition without appreciable formation of **3b** or **4**. On the other hand, on exposure to aqueous buffer at pH 3, it was rapidly converted to **1b** along with very small amounts of **3b** and **4**, in line with the known facility of allylic nitrites to hydrolysis at acidic pH to give alcohols and HNO_2.[11]

Products **3a/b** and **4** methyl esters shared a nitro group on the 9-position of the fatty acid chain, which suggested a possible mechanistic relationship. This hypothesis

Scheme 1



however was ruled out by the lack of appreciable conversion of **3b** methyl ester to **4** methyl ester upon reaction with various amounts of NO_2^- under the usual conditions. Overall, these observations concurred to delineate a possible mechanistic frame for reaction of **1a/b** and their methyl esters with NO_2^-, which is concisely illustrated in Scheme 1.

In this scheme, NO_2 derived from the acid-induced decomposition of NO_2^- [10] would bring free radical attack to the terminal position of the conjugated diene moiety distal to the hydro(pero)xyl group to generate a resonance-stabilized nitroalkyl radical of type **I**. This regioselectivity, which was completely lost in the case of **6**, is conceivably dictated by the *Z* geometry of the distal double bond in the diene moieties of **1a/b** and **2a/b**. Combination with another molecule of NO_2 via one of the oxygen centers would eventually afford unstable nitronitrite intermediates **II** which would readily eliminate HNO_2[10] to give **3a/b** or **5a/b**.

Allowance for the above mechanistic scheme would provide an attractive rationale for the origin of **4**. The latter may be envisioned as arising from facile cleavage of the postulated β-hydro(pero)xynitrooxy intermediates **II**, possibly through cyclic transition states or intermediates akin to those formed in the early stages of alkene ozonolysis. The final outcome would be formation of hexanal, in accord with GC−MS evidence, and a 4-nitro-3-alkenal intermediate (**III**), which might eventually undergo nitrosation at the reactive methylene group[16] to give **4** methyl ester. Unfortunately, attempts to detect

or intercept the postulated species involved in the cleavage process proved unsuccessful, and its exact nature remains unsubstantiated. The proposed reaction pathway would also account for the similar product distribution and yields obtained from **1a** methyl ester and **1b** methyl ester.

Why breakdown products of type **4** do not appear to form in the case of **2a/b** is an open issue, since all lines of evidence point to early mechanistic steps in common for **1a/b** and **2a/b**. It is possible that peculiar structural factors in **2a/b**, such as the presence of additional double bonds with doubly allylic methylene groups, divert or hinder reaction intermediates from breakdown routes by, e.g., intramolecular H-atom transfer.

In any case, the lack of appreciable changes in product distributions with time and NO_2^- concentration and the stability of reaction products suggested competing nitration pathways, which may reflect kinetic control at some stage.

Recently,[11] a mechanism for the reaction of **1a** with NO_2^- under acidic conditions was proposed, which envisaged homolytic decomposition of a transient peroxynitrite ester leading to caged alkoxyl and NO_2 radicals. Rearrangement of the former via an epoxyallylic carbon-centered radical and radical recombination was then suggested to yield nitroepoxy products. Close inspection (TLC/NMR and GC−MS) of the reaction mixture of **1a** obtained exactly under the reported conditions (viz., 10 min of incubation at pH 3 with NO_2^-) revealed the formation of **3a** and **4** methyl esters after treatment with diazomethane, but no detectable nitroepoxy compound. Of course, the limited range of products identified hampered a complete analysis of the reaction mixture, and the possibility exists that nitroepoxy derivatives are formed as minor components, since nitrosation of organic hydroperoxides is well documented in the literature.[17] In any case, the evidence reported in the present study, though strongly suggestive of the reaction pathways in Scheme 1, does not rule out alternate mechanistic options, e.g., ionic routes or decomposition of peroxynitrite esters. These pathways are not mutually exclusive and may contribute at least in part to the product mixture.

Preliminary Biological Experiments.

Products **3b** methyl ester and **4** methyl ester were briefly evaluated for their toxicity and apoptotic properties against a human keratinocyte cell line (HaCat). While **3b** methyl ester decreased cell viability to 47% of the control, **4** methyl ester proved much less toxic, the cell viability being ca. 80% of the control and similar to that observed in the case of **1b** methyl ester. As a positive control, ethanol decreased the cell viability to 55%.

More interesting data emerged from a preliminary screening of pro-apoptotic effects using the Annexin V assay, which allows detection of apoptotic changes significantly earlier than DNA-based assays. With this method, a marked pro-apoptotic effect of **4** methyl ester was observed (ca. 240% with respect to the control), versus a value of ca. 120% for **1b** methyl ester. A lower, yet significant effect (180%) was exerted by **3b** methyl ester. Finally, neither **3b** methyl ester nor **4** methyl ester was found to affect the intracellular formation of reactive oxygen species, as tested by the dichlorofluorescein diacetate assay.

Conclusions

To the best of our knowledge, this is the first paper dealing with the isolation and structural characterization of single products from nitrosation/nitration of bioactive hydro(pero)xy derivatives of polyunsaturated fatty acids under pH conditions of physiological relevance. The main highlights include the discovery of an intriguing nitrosative breakdown pathway of **1a/b** methyl esters operating even under mildly acidic conditions (pH 5.5), and the high regioselectivity of the nitration reaction of **1a/b** and **2a/b** methyl esters, reflecting the expectedly preferential attack of NO_2 to the *Z* double bond. Elucidation of these pathways integrates and puts on a solid experimental ground the chemistry adumbrated by previous workers.[11]

In addition to the chemical aspects, these results may warrant interest for their potential physiological relevance. Tissue damage, apoptosis, and/or alteration of lipid signaling in inflammatory pathways may well result in vivo via the observed acidic NO_2^- reactions targeted to peroxidized unsaturated lipids, e.g., in the stomach (pH from 2.5 to 4.5 during digestion), in the environment of phagocytic lysosomes (pH 3.0),[11] in severely inflamed skin during chronic allergy, and in ischemic tissues (pH 5.8−6.2).[18] Detailed elucidation of the biochemical properties of the new products may yield valuable leads to the rational design of potential agonists or antagonists of bioactive lipids,[11] and the emergence of **1b**, in addition to **1a**,[13] as a novel efficient scavenger of NO_2^- in acidic media seems relevant in this frame.

Experimental Section

General Procedures. Linoleic acid (99%), arachidonic acid (99%), nitronium tetrafluoborate (95% containing nitrosonium tetrafluoroborate), (*E*,*E*)-2,4-hexadienol (97%), and ethyl linoleate (98%) were used as obtained. Soybean lipoxidase (linoleate:oxygen reductase, EC 1.13.11.12) type IB was used.

The EI spectra were obtained at 70 eV. UV and IR spectra were obtained using a diode array and an FT-IR spectrophotometer, respectively. ^{1}H, ^{13}C, and ^{15}N NMR spectra were recorded at 400.1, 100.6, and 40.5 MHz, in that order. For ^{15}N NMR experiments delay values up to 10 s were used. ^{1}H−^{1}H COSY, ^{1}H−^{13}C HETCOR, ^{1}H−^{13}C HMBC, and ^{1}H−^{15}N HMBC experiments were run at 400.1 MHz using standard pulse programs from the Bruker library. ^{15}N chemical shifts were referenced to [^{15}N]urea in DMSO. Analytical and preparative TLC analyses were performed on F254 0.25 and 0.5 mm silica gel plates and high-performance TLC (HPTLC) analyses using 70:30 cyclohexane−ethyl acetate containing 1% acetic acid (eluant A) or 80:20 cyclohexane−ethyl acetate (eluant B). Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine in 5% phosphoric acid),[19] potassium dichromate in 20% sulfuric acid, and iodine were used for product detection on TLC plates. Flash chromatography was performed using 270−400 mesh silica gel. The purity of products obtained as methyl esters was determined from ^{1}H NMR analysis taking the $-OCH_3$ methyl at δ 3.67 as the internal reference (3H). Diazomethane was prepared by reaction of *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide in ethanolic KOH and collected in peroxide-free ether in a dry ice/acetone bath. *Caution! Diazomethane is explosive and must be kept at −20 °C.* *tert*-Butyl nitrite was prepared from *tert*-butyl alcohol by treatment

(16) Williams, F. J. *Nitrosation*; Cambridge University Press: Cambridge, 1988; pp 1−45.

(17) Pryor, W. A.; Castle, L.; Church, D. F. *J. Am. Chem. Soc.* **1985**, *107*, 211−217.

(18) Waldmann, R.; Champigny, G.; Lingueglia, E.; Coscoy, S.; de Weille, J.-R.; Heurteaux, C.; Lazdunskia, M. *Int. Congr. Ser.* **2000**, *1208*, 141−145.

(19) Klebanoff, S. J. *Free Radical Biol. Med.* **1993**, *14*, 351−360.

with sodium nitrite in 10% sulfuric acid.[20] Xylenol orange15 and the iodide/starch test were used for the hydroperoxide assay. All reported yields were determined by product isolation.

GC−MS Analyses. GC−MS was carried out on a GC instrument coupled with a quadrupole mass spectrometer. Helium was the carrier gas with a 1 mL/min flow rate. CI-MS measurements were carried out using methane as the reagent gas. Data were processed using G1701AA data analysis software. The following analytical conditions were used. Method A: 50 m cross-linked poly(ethylene glycol)−terephthalic acid modified column (0.32 mm i.d., 0.52 μm df). Temperature program: 60 °C, hold time 2 min; from 60 to 220 °C, rate 15 °C/min; from 220 to 240 °C, rate 5 °C/min. The inlet, transfer line, source, and quadrupole were taken at 270, 240, 200, and 100 °C in that order. The acquisition started 6 min after the injection (solvent delay 6 min), and was set in scan mode in the range 44−600 amu. The threshold was fixed at 10, and sampling was 0.6 scan/s. Method B: 30 m cross-bond 5% diphenyl−95% dimethylpolysiloxane column (0.25 mm i.d., 0.25 μm df). Temperature program: 40 °C, hold time 1 min; up to 280 °C, rate 5 °C/min. The inlet and detector were taken at 180 and 250 °C, respectively. The acquisition started 5 min after the injection (solvent delay 5 min), and was set in scan mode in the range 50−550 amu. The threshold was fixed at 50, and sampling was 1.6 scans/s. When required, the samples were treated with bis(trimethylsilyl)trifluoroacetamide.

Iron-Promoted Oxidation of Linoleic Acid. To linoleic acid (0.2 mmol) in 0.05 M phopshate buffer, pH 7.4 (400 mL), was added Fe(II)−EDTA complex (50 μM), obtained by premixing $(NH_4)_2Fe(SO_4)_2$ and EDTA, to start the reaction. The mixture was maintained under vigorous stirring in a thermostated bath at 37 °C for 16 h, and then it was treated with solid sodium borohydride (1 mmol) and carefully acidified to pH 3. Sodium nitrite (2 molar equiv) was then added, and after an additional 5 h of stirring, the mixture was extracted with ethyl acetate. The combined organic layers were dried over sodium sulfate to afford a yellow to brown residue which was treated portionwise with ethereal diazomethane until the yellow coloration persisted in solution. After 15 min, excess diazomethane was destroyed by addition of glacial acetic acid, and after removal of the volatile components, the residue was subjected to TLC (eluant B) and GC−MS (method B) analysis.

Synthesis of 1a/b, 2a/b, and Methyl Esters. Linoleic acid or arachidonic acid (0.89 mmol) in 0.1 M sodium borate buffer at pH 9.0 (700 mL) was oxidized with lipoxidase (1580 U/mL) at 25 °C, and the reaction course was monitored spectrophotometrically at 234 nm. Formation yields of **1a** and **2a** were determined taking the extinction coefficients at 234 nm as 23.3 $\times$ 10^3 [21] and 27.0 $\times$ 10^3 [22] M^{-1} cm^{-1}, respectively. The oxidation mixtures were acidified to pH 3 and extracted repeatedly with chloroform (4 $\times$ 250 mL). The combined organic layers were washed with brine and dried over sodium sulfate to give a colorless oil. When **1b** or **2b** was desired, the oxidation mixture was treated with solid sodium borohydride (3.5 mmol) before acidification. The methyl esters were obtained by treatment of the residue with an ethereal solution of diazomethane as above. The purity of each compound was assessed by TLC and/or NMR analysis. Preparations with purity lower than 90% were fractionated by PTLC (eluant A for free acids or B for methyl esters). The hydroperoxy fatty acids were used within 1 h after preparation.

Reaction of 1a/1b, 2a/2b, and Methyl Esters with Nitrite. To a solution of **1a/1b** or **2a**/**2b** (64 mM) in ethyl acetate was added 0.1 M sodium phosphate, pH 3 (2.5:1, v/v, with respect to the organic layer), followed by sodium nitrite (2 molar equiv), and the biphasic system was taken under vigorous stirring in a stoppered round-bottom flask at room temperature. After 3 h, the organic layer was separated, washed with brine, and dried over sodium sulfate to give a yellow residue which was analyzed as described below. When required, the reaction was carried out with purging of the biphasic system with argon for at least 30 min prior to addition of a deareated solution of nitrite in water (1 g/mL). The reaction mixture was neutralized by addition of a solution of 0.5 M sodium acetate in an argon atmosphere before workup as above.

In other experiments the reaction of **1b** methyl ester was run (i) in ethyl acetate/0.1 M phosphate, pH 3, varying the amount of nitrite added in the range 0.5−15 molar equiv with respect to the substrate, (ii) at 37 or 0 °C, (iii) with addition of 1,3-dinitrobenzene in the range 1−10 molar equiv, and (iv) using 1% sulfuric acid or 0.1 M phosphate buffer, pH 4 or 5.5, as the aqueous layer in the biphasic system. The reaction of **1a** with nitrite was carried out under the reported conditions[11] with addition of varying amounts of sodium nitrite up to 15 molar equiv. Product analysis was carried out periodically by TLC (eluant A). For hexanal identification, reaction of **1b** with nitrite (2 molar equiv) was carried out in the ethyl acetate/0.1 M sodium phosphate, pH 3, biphasic system. After 3 h the organic layer was separated and directly analyzed by GC−MS using method B.

Isolation of Compounds 3a/b Methyl Ester and 4 Methyl Ester. For preparative purposes, reaction of **1a** methyl ester with nitrite was carried out in ethyl acetate/0.1 M phosphate buffer, pH 3, as described above, using 175 mg (0.54 mmol) of the starting material. Workup as above gave a residue (190 mg) which was fractionated by PTLC using eluant B to afford pure **3a** methyl ester (R_f 0.34, 20 mg, 10% yield) and **4** methyl ester (R_f 0.16, 25 mg, 15% yield). Under the same conditions **1a** (175 mg, 0.56 mmol) afforded after PTLC fractionation (eluant A) two products, R_f 0.26 (15 mg) and R_f 0.08 (20 mg), which after methylation with diazomethane proved identical to **3a** methyl ester and **4** methyl ester, respectively. Reaction of **1b** methyl ester (175 mg, 0.56 mmol) afforded **3b** methyl ester (R_f 0.30, eluant B, 22 mg, 11% yield) and **4** methyl ester (R_f 0.16, eluant B, 26 mg, 15% yield). When **1b** was used as the substrate, PTLC fractionation (eluant A) gave two main products, R_f 0.17 (18 mg) and R_f 0.08 (22 mg), which after methylation with diazomethane proved identical to **3b** methyl ester and **4** methyl ester, respectively.

Data for methyl (9*E*,11*E*)-13-hydroperoxy-9-nitrooctadeca-9,11-dienoate (3a methyl ester): UV λ_{max} ($CHCl_3$) 304 nm; $[\alpha]_D$ −5.85 (*c* 1.4, $CHCl_3$); FT-IR ($CHCl_3$) ν_{max} 3533, 1732, 1653, 1523, 1333 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ, ppm, 0.89 (m, 3H), 1.30−1.44 (m, 12H), 1.49−1.65 (m, 6H), 2.28 (t, J = 7.6 Hz, 2H), 2.68 (t, J = 7.2 Hz, 2H), 3.65 (s, 3H), 4.50 (m, 1H), 6.26 (dd, J = 15.2, 6.8 Hz, 1H), 6.47 (dd, J = 15.2, 11.2 Hz, 1H), 7.53 (d, J = 11.2 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ, ppm, 14.7 (CH_3), 23.2 (CH_2), 25.5 (CH_2), 25.6 (CH_2), 27.3 (CH_2), 28.6 (CH_2), 29.4 (CH_2), 29.5 (CH_2), 29.6 (CH_2), 32.4 (CH_2), 33.1 (CH_2), 34.8 (CH_2), 52.3 (CH_3), 86.2 (CH), 126.2 (CH), 133.0 (CH), 145.8 (CH), 152.1 (C), 175.4 (C).

Data for methyl (9*E*,11*E*)-13-hydroxy-9-nitrooctadeca-9,11-dienoate (3b methyl ester): UV λ_{max} ($CHCl_3$) 308 nm; $[\alpha]_D$ +25.4 (*c* 0.46, $CHCl_3$); FT-IR ($CHCl_3$) ν_{max} 3518, 1730, 1647, 1514, 1327 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ, ppm, 0.92 (m, 3H), 1.33 (m, 8H), 1.55 (m, 8H), 1.63 (m, 2H), 2.31 (t, J = 7.6 Hz, 2H), 2.69 (t, J = 7.6 Hz, 2H), 3.67 (s, 3H), 4.32 (m, 1H), 6.33 (dd, J = 15.2, 5.2 Hz, 1H), 6.46 (ddd, J = 15.2, 11.6, 0.8 Hz, 2H), 7.55 (d, J = 11.6 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ, ppm, 14.7 (CH_3), 23.3 (CH_2), 25.5 (CH_2), 25.7 (CH_2), 27.2 (CH_2), 28.6 (CH_2), 29.5 (CH_2), 29.6 (CH_2), 30.5 (CH_2), 32.4 (CH_2), 34.8 (CH_2), 37.8 (CH_2), 52.2 (CH_3), 72.7 (CH), 122.8 (CH), 133.4 (CH), 150.0 (CH), 151.6 (C), 176.0 (C); GC−MS (*O*-TMS derivative) t_R 28.5 min (method A), PICI m/z 428 (M + H)/427/425, 411, 354, 309, NICI m/z 426 (M − H)/425, 354/353, 307.

Data for methyl (9*E*,11*E*)-9-nitro-11-oximino-12-oxododec-9-enoate (4 methyl ester): UV λ_{max} ($CHCl_3$) 274 nm; FT-IR ($CHCl_3$) ν_{max} 3547, 1732, 1653, 1563, 1541, 1462, 1337 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ, ppm, 1.2−1.7 (m, 8H), 2.28 (m, 2H), 2.31 (t, J = 7.2 Hz, 2H), 2.48 (t, J = 7.6 Hz, 2H),

(20) Noyes, W. A. *Organic Syntheses*; Wiley: New York, 1943; Collect. Vol. II, p 108.

(21) Graff, G.; Anderson, L. A.; Jaques L. W. *Anal. Biochem*. **1990**, *188*, 38−47.

(22) Pryor, W. A.; Cornicelli, J. A.; Devall, L. J., Tait, B.; Trivedi, B. K.; Witiak, D. T., Wu, M. *J. Org. Chem*. **1993**, *58*, 3521−3532.

3.67 (s, 3H), 7.28 (s, 1H), 9.61 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ, ppm, 25.5 (CH_2), 26.9 (CH_2), 28.8 (CH_2), 29.2 (CH_2), 29.8 (CH_2), 30.5 (CH_2), 34.7 (CH_2), 52.3 (CH_3), 119.2 (CH), 153.6 (C), 157.9 (C), 175.2 (C), 189.1 (C); GC−MS t_R 39.8 min (method B), EI *m/z* 298 (10), 252 (47), 185 (100), 171 (15), 157 (18), 87 (61), 74 (93), (*O*-TMS derivative) t_R 45.6 min (method B), EI *m/z* 372 (M^+, 4), 299 (16), 225 (5), 74 (100).

Reaction of 1b with $Na^{15}NO_2$. The reaction was performed under the conditions described above using **1b** methyl ester (175 mg, 0.56 mmol) and $Na^{15}NO_2$ (78 mg, 1.12 mmol). After 3 h, the mixture was worked up as above, and the residue obtained (190 mg) was fractionated on PTLC (eluant B) to give **3b** methyl ester (20 mg, 10% yield) and **4** methyl ester (22 mg, 14% yield).

Data for methyl [^{15}N](9*E*,11*E*)-13-hydroxy-9-nitrooctadeca-9,11-dienoate (3b methyl ester): ^{1}H NMR (400 MHz, $CDCl_3$) δ, ppm, 0.91 (m, 3H), 1.25−1.37 (m, 10H), 1.50−1.62 (m, 8H), 2.31 (t, J = 7.4 Hz, 2H), 2.69 (dt, J = 6.9, 3.4 Hz, 2H), 3.67 (s, 3H), 4.32 (m, 1H), 6.32 (dd, J = 15.0, 5.0 Hz, 1H), 6.41 (dd, J = 15.0, 11.2 Hz, 2H), 7.55 (dd, J = 11.2, 3.6 Hz, 1H); ^{15}N NMR δ, ppm, 376.7. GC−MS (*O*-TMS derivative) t_R 41.7 min (method B), NICI *m/z* 427 (M − H), EI *m/z* 356 (7), 341 (100).

Data for methyl [^{15}N](9*E*,11*E*)-9-nitro-11-oximino-12-oxododec-9-enoate (4 methyl ester): ^{1}H NMR (400 MHz, $CDCl_3$) δ, ppm, 1.25−1.60 (m, 10H), 2.32 (t, J = 7.2 Hz, 2H), 2.48 (dt, J = 7.5, 3.4 Hz, 2H), 3.67 (s, 3H), 7.28 (dd, 4.0, 2.1 Hz, 1H), 9.61 (s, 1H); ^{15}N NMR δ, ppm, 374.5, 424.2; GC−MS (*O*-TMS derivative) t_R 15.8 min (method A), NICI *m/z* 374 (M^+, 100), EI *m/z* 327 (100).

Isolation of 5a Methyl Ester and 5b Methyl Ester. Reaction of **2a** methyl ester (70 mg, 0.20 mmol) with nitrite ions (2 molar equiv) was carried out as above. PTLC fractionation (eluant B) of the residue (80 mg) afforded **5a** methyl ester (R_f 0.38, eluant B, 16 mg, 20% yield). When **2b** methyl ester was used as the substrate (70 mg, 0.21 mmol), PTLC fractionation of the residue (eluant B) afforded **5b** methyl ester (R_f 0.69, 18 mg, 22% yield). Under the same reaction conditions, **2b** gave after fractionation of the residue (eluant A) a main product (R_f 0.38, 17 mg) identified as **5b** methyl ester after methylation as above.

Data for methyl (5*Z*,8*Z*,11*E*,13*E*)-15-hydroperoxy-11-nitroeicosa-5,8,11,13-tetraenoate (5a methyl ester): UV λ_{max} ($CHCl_3$) 301 nm; $[\alpha]_D$ −7.2 (*c* 0.75, $CHCl_3$); FT-IR ($CHCl_3$) ν_{max} 3533, 1735, 1654, 1523, 1337 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ, ppm, 0.90 (m, 3H), 1.25−1.36 (m, 6H), 1.68−1.73 (m, 6H), 2.14 (m, 2H), 2.33 (t, J = 7.6 Hz, 2H), 2.90 (m, 2H), 3.47 (d, J = 6.4 Hz, 2H), 3.67 (s, 3H), 4.50 (m, 1H), 5.33−5.49 (m, 4H), 6.30 (dd, J = 15.2, 6.8 Hz, 1H), 6.52 (dd, J = 15.2, 11.2 Hz, 1H), 7.53 (d, J = 11.2 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ, ppm, 14.7 (CH_3), 23.2 (CH_2), 25.5 (CH_2), 25.6 (CH_2), 26.0 (CH_2), 26.5 (CH_2), 27.4 (CH_2), 32.4 (CH_2), 33.2 (CH_2), 34.2 (CH_2), 52.4 (CH_3), 86.1 (CH), 124.4 (CH), 125.7 (CH), 129.0 (CH), 130.3 (CH), 131.9 (CH), 133.0 (CH), 146.4 (CH), 150.4 (C), 175.5 (C).

Data for methyl (5*Z*,8*Z*,11*E*,13*E*)-15-hydroxy-11-nitroeicosa-5,8,11,13-tetraenoate (5b methyl ester): UV λ_{max} ($CHCl_3$) 302 nm; $[\alpha]_D$ +22.3 (*c* 0.60, $CHCl_3$); FT-IR ($CHCl_3$) ν_{max} 3503, 1713, 1646, 1515, 1375, 1326 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ, ppm, 0.91 (m, 3H), 1.25−1.49 (m, 6H), 1.59 (m, 2H), 1.69 (m, 2H), 2.14 (m, 2H), 2.33 (t, J = 7.6 Hz, 2H), 2.89 (t, J = 5.6 Hz, 2H), 3.48 (d, J = 6.8 Hz, 2H), 3.66 (s, 3H), 4.33 (m, 1H), 5.29−5.48 (m, 4H), 6.35 (dd, J = 15.2, 4.8 Hz, 1H), 6.53 (dd, J = 15.2, 11.6 Hz, 1H), 7.57 (d, J = 11.6 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ, ppm, 14.8 (CH_3), 23.3 (CH_2), 25.5 (CH_2), 25.7 (CH_2), 25.9 (CH_2), 26.5 (CH_2), 27.4 (CH_2), 32.5 (CH_2), 34.2 (CH_2), 37.8 (CH_2), 52.3 (CH_3), 72.6 (CH), 122.5 (CH), 124.6 (CH), 129.0 (CH), 130.2 (CH), 131.8 (CH), 133.6 (CH), 149.9 (C) 150.7 (CH), 176.2 (C); EIMS *m/z* 362 (35), 344 (75), 333 (37), 331 (75), 308 (100).

Reaction of 1a/1b with Nitrogen Oxides. A solution of nitrite in water (1 M) was added to 10% sulfuric acid over 10 min. The red-orange gas which developed was conveyed with a flux of argon into a solution of **1b** methyl ester in ethyl acetate or cyclohexane (0.06 M). Three hours after the development of the red fumes was complete, the reaction mixture was washed with brine, and the organic layer was dried over sodium sulfate and evaporated to dryness. TLC analysis of the reaction mixture (eluant B) showed a substantial consumption of the starting material. PTLC fractionation of the reaction mixture (eluant B) afforded **3b** methyl ester (9% yield) and **4** methyl ester (12% yield).

Reaction of 1a Methyl Ester with NO_2BF_4. The reaction was carried out and worked up as described.[10]

(9*E*,11*E*)-13-Nitrosooxy-9-nitrooctadeca-9,11-dienoic Acid (9). To a solution of **1b** (15 mg, 0.053 mmol) in chloroform (180 mM) was added *tert*-butyl nitrite (100 μL), and the mixture was kept in an ice−water bath. After 30 min, the volatile components were removed to give an oily residue consisting mainly of **9**, which was stored at −20 °C and subjected to NMR analysis within 1 h after preparation: ^{1}H NMR (400 MHz, $CDCl_3$) δ, ppm, 0.94 (m, 3H), 1.22−1.49 (m, 10H), 1.56 (m, 6H), 1.70 (m, 2H), 2.12 (m, 2H), 2.27 (t, J = 7.6 Hz, 2H), 5.46 (dt, J = 10.8, 10.4 Hz, 1H), 5.65 (dd, J = 14.2, 7.6 Hz, 1H), 5.77 (m, 1H), 5.92 (dd, J = 10.8, 7.6 Hz, 1H), 6.51 (dd, J = 14.2, 10.4, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ, ppm, 13.8 (CH_3), 22.3 (CH_2), 24.6 (CH_2), 24.8 (CH_2), 27.6 (CH_2), 28.9 (CH_2), 29.3 (CH_2), 30.9 (CH_2), 31.4 (CH_2), 33.9 (CH_2), 34.4 (CH_2), 80.1 (CH), 127.3 (CH), 128.0 (CH), 130.8 (CH), 133.9 (C).

To a solution of **9** in ethyl acetate (64 mM) was added 0.1 M phosphate buffer, pH 3 (2.5:1, v/v, with respect to the organic layer), and the mixture was stirred at room temperature. After 40 min, the organic layer was washed with water, dried over sodium sulfate, and taken to dryness. The residue was analyzed by TLC (eluant A). In other experiments, a solution of **9** in dry chloroform (100 mM) was kept in a stoppered round-bottom flask and periodically analyzed by TLC (eluant A).

Reaction of 6 with Nitrite. The reaction of **6** (100 mg, 1.0 mmol) was run under the conditions described for **1a/b** methyl ester and **2a/b** methyl ester using 2 molar equiv of nitrite. After 1 h, the mixture was worked up as above and the residue purified by PTLC (eluant cyclohexane−ethyl acetate, 7:3) to give **7** (R_f 0.30, 13 mg, 9% yield) and **8** (R_f 0.16, 10 mg, 7% yield).

Data for (*E*,*E*)-2-nitro-2,4-hexadienol (7): UV λ_{max} ($CHCl_3$) 306 nm; FT-IR ($CHCl_3$) ν_{max} 3604, 3375, 1653, 1538, 1329 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ, ppm, 1.97 (dd, J = 6.8, 1.2, 3H), 4.61 (s, 2H), 6.39 (ddq, 14.8. 11.2, 1.2 Hz, 1H), 6.53, (dq, J = 14.8, 6.8 Hz, 1H), 7.65 (d, J = 11.2 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ, ppm, 20.3 (CH_3), 56.5 (CH_2), 125.0 (CH), 137.8 (CH), 148.0 (CH); EIMS *m/z* 143 (M^+, 100), 126 (26), 97/96 (28/58).

Data for (*E*,*E*)-5-nitro-2,4-hexadienol (8): UV λ_{max} ($CHCl_3$) 289 nm; FT-IR ($CHCl_3$) ν_{max} 3605, 3410, 1653, 1530, 1327 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ, ppm, 2.27 (s, 3H), 4.38 (d, J = 4.0 Hz, 2H), 6.45 (dt, 15.2, 4.4 Hz, 1H), 6.51, (ddt, J = 15.2, 10.8, 1.6 Hz, 1H), 7.60 (d, J = 10.8 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ, ppm, 13.6 (CH_3), 63.4 (CH_2), 123.3 (CH), 133.2 (CH), 145.8 (CH); EIMS *m/z* 143 (M^+, 9), 141 (84), 126 (3), 95 (100).

Biological Experiments. Immortalized human keratinocytes (cell line HaCat) were grown in Dulbecco modified Eagle's medium and seeded in 24-well plates (1×10^4 per well). **3b** methyl ester, **4** methyl ester, **1a** methyl ester, linoleic acid ethyl ester, and linoleic acid, at a concentration of 1 μg/mL or 10 μg/mL in ethanol, were added to the medium. The control cells were treated only with 95% ethanol. After incubation, the medium was removed, and the cells were washed with phosphate-buffered saline freed of calcium or magnesium ions, and detached by 0.25% trypsin in EDTA containing buffer. The viability of the cultured cells was determined at 24 and 48 h using an adapted 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide eluted stain assay.[23] The effect of the tested substances on the treated cells was expressed as a percentage of the control cell viability. For the Annexin assay, cells in suspension were treated with 2′,7′-dichlorofluorescein diacetate

for 30 ft at 37 °C in a 5% CO_2 atmosphere, stained with Annexin V CyP for 5 min at rt, and then analyzed by flow cytometry. All assays were carried out in duplicate. The flow-cytometric analysis was based on differential light scatter analysis to discriminate viable from apoptotic/necrotic cells, fluorescence intensity as a marker of intracellular reactive oxygen species production, and the percentage of events positive to both 2′,7′-dichlorofluorescein diacetate and Annexin V as a parameter of the early apoptotic process. The setting of the flow cytometer was excitation at 488 nm and emission at 530 and 570 nm for 2′,7′-dichlorofluorescein diacetate and Annexin V, respectively. Trypan Blue exclusion was performed as an independent assay to evaluate cell viability.

Acknowledgment. We acknowledge financial support from the Ministry of Health (Rome). This work was carried out in the frame of the MURST (PRIN 2001) and CNR projects. GC−MS facilities were available at the Istituto Dermatologico S. Gallicano (Rome). We thank the Centro Interdipartimentale di Metodologie Chimico-Fisiche of Naples University for NMR facilities and mass spectra. We thank M. L. Dell' Anna, M.D., for biological experiments.

Supporting Information Available: ^{1}H, ^{13}C, COSY, HMQC, and HMBC NMR spectra of compounds **3b** methyl ester, **5b** methyl ester, and **4** methyl ester, ^{1}H, COSY, and HMQC NMR spectra of compound **9**, and ^{1}H, ^{15}N, and $^{1}H-^{15}N$ HMBC NMR spectra of ^{15}N-enriched **3b** methyl ester and **4** methyl ester. This material is available free of charge via the Internet at http://pubs.acs.org.

JO015973B

(23) Denizot, F.; Lang, R. *J. Immunol. Met.* **1986**, *89*, 271−277.