Organic Nitrites and NO: Inhibition of Lipid Peroxidation and Radical Reactions

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Organic nitrites, such as *i*-amyl nitrite (IAN), are nitrovasodilator drugs used both clinically and recreationally. Nitrites are also chemically reasonable biological products of NO metabolism, in particular in both inhibition of lipid peroxidation by NO and induction of lipid peroxidation by peroxynitrite and NO_2. Nitrites are also potential products of biomolecule nitrosation and intermediates in biotransformation of nitrate vasodilators. Although mechanisms can be drawn for both prooxidant and antioxidant activity, IAN has been observed to inhibit lipid peroxidation in a variety of systems. To test if the antioxidant activity of nitrites results from NO release alone, inhibition of lipid peroxidation was studied for four organic nitrites and four NO donor NONOates. Iron-induced lipid peroxidation in synaptosomal tissue homogenates and azo compound-initiated lipid peroxidation in liposomes and linoleic acid SDS comicelles were examined. Lipid peroxidation was quantified by TBARS and oxygen uptake analysis. A good correlation of rate of NO release with IC_{50} for inhibition of lipid peroxidation was observed for the NONOates, compatible with lipid radical chain termination by NO, for which a chain termination stoichiometry of 0.4−0.5 mol of lipid peroxyl radicals per mole of NO was determined. In neutral aqueous solution, nitrites also spontaneously released NO as measured by chemiluminescence; however, no correlation was observed between the rate constants of NO release for the nitrites and their inhibitor potency toward lipid peroxidation. Long chain nitrites were seen to be relatively good inhibitors of lipid peroxidation by mechanisms that must involve factors in addition to simple homolysis to release NO. Evidence for direct α-hydrogen atom abstraction from the nitrite by peroxyl radicals was obtained by analysis of aldehyde products and supported by MO calculations. The data suggest that lipid nitrites formed as NO chain termination products have the capacity to further inhibit lipid peroxidation and to release NO.

Introduction

Organic nitrites (RONO) are nitrovasodilator drugs used both clinically and recreationally. The history of nitrites is intertwined with that of organic nitrates ($RONO_2$), since the vasodilatory action of IAN[1] and its potential for use in angina were recognized before that of nitroglycerin (GTN) (*1*). Organic nitrates are important therapeutic agents, GTN, the most famous of which, has been in use in clinical treatment of angina pectoris for more than 125 years. Nitrate vasodilators are widely believed to act via biotransformation to release NO in vivo, an overall $3e^-$ reduction (*2*−*5*). The exact pathway is unknown, but chemical mechanisms may be drawn, including one via an initial $2e^-$ reduction to an organic nitrite, followed by more facile $1e^-$ reduction to NO (*6*). Nitrites, including IAN, are used as medicinal and recreational drugs, although contemporary clinical trials on the use of ethyl nitrite in cardiovascular therapy suggest future new applications of nitrites as therapeutic agents (*7*). Several clinical trials on a family of nitrates, termed cyclooxygenase inhibitory NO donors (CINODs or NO/NSAIDS), and animal model studies on novel nitrates illustrate that the use of nitrates in human health will continue, increase, and in the future broaden considerably beyond cardiovascular therapy (*7*−*10*).

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[1] Abbreviations: ABAP, 2,2′-azobis(2-methylamidinopropane) dihydrochloride; AMVN, 2,2′azobis(2,4-dimethylvaleronitrile); BDE, bond dissociation energy; BODIPY, 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3*a*, 4*a*-diaza-*s*-indacene-3-undecanoic acid (BODIPY 581/591-C_{11}); DCN, 1-decylnitrite; DDN, 1-dodecylnitrite; DEA/NO, diethylamine NONOate diethylammonium salt; DETA/NO, diethylene amino NONOate; DLPC, 1,2-dilinoleoyl-*sn*-glycero-3-phosphocholine; DNPH, 2,4-dinitrophenylhydrazine; DTPA, diethylenetriaminepentaacetic acid; IAN, $C_5H_{11}ONO$, *i*-amyl nitrite; NONOate diazeniumdiolate salt; NOC-9/NO, MAHMA NONOate; PEN, C_7H_9ONO, $PhO(CH_2)_2ONO$, 2-phenoxy-1-ethylnitrite; PUFA, polyunsaturated fatty acids; SDS, sodium dodecyl sulfate; SPE/NO, spermine NONOate, $H_2N(CH_2)_3NH(CH_2)_4-N(NONO)-(CH_2)_3NH_3$), (*Z*)-1-(*N*-[3-aminopropyl]-*N*-[4-(3-aminopropylammonio)butyl]amino) diazen-1-ium-1,2-diolate; Trolox, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid; TBA, 4,6-dihydroxy-2-mercaptopyrimidine (thiobarbituric acid); TBARS, thiobarbituric acid reactive substances.

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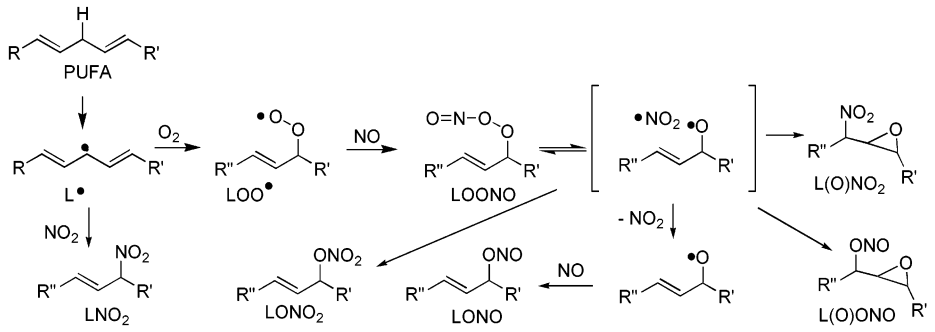
Scheme 1



Nitric oxide is a crucially important biomolecule with multiple physiological functions as evidenced by the ubiquitous distribution of the enzymes responsible for in vivo production of NO: neuronal, inducible, and endothelial nitric oxide synthases (NOS) (*2*−*5*, *11*, *12*). However, there has also been substantial research into the toxicology of NO and its metabolites, reactive nitrogen (oxygen) species (variously RNS or RNOS), which has led to many suggestions of a role for NO in cytotoxicity and genotoxicity (*13*−*16*). The aberrant reactions of reactive oxygen species (ROS), produced from O_2, contribute to oxidative stress, whereas reactions of RNS, produced from reaction of NO, are proposed to contribute to nitrosative stress (*14*). The NO-derived chemical species most routinely implicated in toxicity have been peroxynitrite ($ONOO^-$), nitroxyl (NO^-), nitrosyl chloride (NOCl), and nitrogen dioxide (NO_2). The reaction of peroxynitrite and NO_2 with phenols, including tyrosine, yields nitrophenols, in particular nitrotyrosine (*17*−*19*). Both peroxynitrite and NO_2 have been proposed to initiate lipid peroxidation and degradation of other biomolecules (*16*, *20*−*23*). Whereas peroxynitrite, derived from NO, initiates lipid peroxidation, there is sound evidence that NO itself inhibits lipid peroxidation, even that induced by peroxynitrite (*24*). The apparent paradox that NO can appear to be a causative factor in both toxicity and cytoprotection, with both prooxidant and antioxidant properties, is well-known and requires much further research to determine which mechanisms and outcomes are of physiological relevance (*14*, *25*).

NO inhibits lipid peroxidation; the available data strongly suggest that this apparent antioxidant activity results from radical chain termination, rather than from attenuation of the radical initiator. The radical chain termination products have been proposed to include nitrite ion (NO_2^-) and organic nitrates and nitrites (eq 1) (*21*, *26*−*29*).

$$4NO + 2ROO\bullet + H_2O \rightarrow 2HNO_2 + RONO_2 + RONO \quad (1)$$

Both nitrates and nitrites have been reported as products of the reaction of NO_2 with PUFA, such as linoleic acid, and in these reactions, nitroalkane products were also reported (*22*, *23*, *30*, *31*). These data raise the interesting possibility that lipid nitrates, nitrites, and nitroalkanes are natural membrane components (Scheme 1; the generic homoallylic structure represents *cis* and *trans* PUFAs) (*21*, *28*). Importantly, nitrolinoleate has recently been reported to be biologically active and of possible physiological relevance (*32*).

In previous research, we have compared the effects of NO, nitrites, and nitrates on lipid peroxidation in synaptosomes and liposomes using induction by ferrous ion and by azo initiators (*33*). Whereas classical nitrates had no effect on lipid peroxidation, novel nitrates designed to release NO, SPE/NO, and IAN inhibited lipid peroxidation in all systems studied (*33*). The objective of this present paper is to expand and extend the data obtained on lipid peroxidation by study and comparison of a series of NONOates and a series of nitrite esters, to answer the question as to whether nitrites are simply acting as NO donors in displaying apparent antioxidant activity. These data would assist in understanding the biological activity of exogenous and endogenous nitrites, whether xenobiotics or natural metabolites.

Four nitrites were chosen for this study: IAN, a clinically relevant nitrovasodilator; PEN, as a more hydrolytically labile nitrite; and DCN and DDN, as models of the putative lipid nitrites produced from reactions of NO and NO_2 with lipid chain propagating radicals in biological membranes. For comparison and to further define the antioxidant mechanism of NO itself, four well-characterized NONOates (diazenium diolates) were chosen, spanning a range of reactivity and providing fast to slow release NO donors: NOC-9/NO, DEA/NO, SPE/NO, and DETA/NO (*34*). The rate of NO release from the NO donor NONOates was seen to correlate well with the IC_{50} values measured for inhibition of Fe-induced lipid peroxidation in synaptosomes. Furthermore, the antioxidant stoichiometry of NO was quantified for the first time using the benchmark oxygen pressure transduction method (*35*, *36*). In contrast to the NONOates, the rate of NO release of the nitrites did not correlate with the relative potency of inhibition of lipid peroxidation. To account for this discrepancy, the reaction of IAN with peroxyl radicals was studied, suggesting that alkyl nitrite α-hydrogen abstraction by peroxyl radical may contribute to radical chain termination.

Experimental Procedures

Diazeniumdiolate salts were obtained from RBI (Natick, MA) or Calbiochem (La Jolla, CA). All other chemicals were obtained from Sigma (St. Louis, MO), Aldrich Chemicals (Milwaukee, WI), or BDH (Toronto, Canada), unless otherwise stated. IAN was freshly distilled before use in lipid peroxidation assays, and the purity was 98.3% as assessed by ^{1}H NMR, the impurity being *i*-amyl alcohol.

Synthesis. Alkyl nitrites were prepared by the nitrosation of the corresponding alcohols (*37*). For DCN and DDN, 1-decanol and 1-dodecanol, respectively (0.033 mol), were dissolved in ice-cold ether or dichloromethane together with concentrated sulfuric acid (0.016 mol). To the organic phase, ice-cold sodium nitrite (0.036 mol) in double-distilled water was added dropwise over 1.5 h. The reaction was performed at 5−10 °C under

vigorous stirring. After the entire solution of sodium nitrite was added, the reaction mixture was further stirred for 30 min. The organic phase was extracted, washed twice with a solution of sodium carbonate (1 g) and sodium chloride (12.5 g) in water (50 mL), and dried over anhydrous magnesium sulfate. The crude nitrites were purified by fractional distillation. The distilled yellowish product had a purity of 93% as determined by ^{1}H NMR. The contaminant was the starting alcohol. Further purification was performed by flash chromatography under argon using a short silica gel column and a mixture of 3:2 hexane:dichloromethane as eluant. The purity of the product after column chromatography was >98% as determined by ^{1}H NMR ($CDCl_3$ δ (ppm): 4.70, t, 2 H; 1.75 q, 2 H; 1.30 m, 14−18 H; 0.89 t, 3 H), the impurity being the starting alcohols.

For PEN, sodium nitrite (0.145 mol) was added to 2-phenoxy-1-ethanol (0.084 mol) and the reaction mixture was processed in the same manner as above. The crude nitrite was purified by flash column chromatography, since fractional distillation resulted in the decomposition of PEN. The product yellow oil had a purity of 83%, as assessed by ^{1}H NMR ($CDCl_3$ δ (ppm): 7.30 t, 2 H; 6.98 t, 1 H; 6.90 d, 2 H; 5.12 t, 2 H; 4.28 t, 2 H). The impurity was the starting 2-phenoxy-1-ethanol.

Kinetics. Rate constants for the hydrolytic degradation of NONOates at 37 °C and pH 7.4 were determined spectrophotometrically as previously described (*38*). Rate constants for NO release from NONOates, under the same reaction conditions, were determined using a Clark type NO selective electrode (ISO-NO-II and ISO-NOMC, WPI Inc., Sarasota, FL) as previously described (*38*). The rate of NO release from alkyl nitrites was measured by headspace chemiluminescence detection (Sievers Research Inc., Boulder, CO, model 207B) (*39*). *S*-Nitroso-*N*-acetylpenicillamine of high purity (>98%, Molecular Probes, Eugene, OR) was used to calibrate the system. Stock solutions of alkyl nitrites were prepared in anhydrous HPLC grade acetonitrile. Aliquots (2 mL) of 0.1 M phosphate buffer, pH 7.4, prepared with HPLC grade water, were added to latex septa-capped vials and deoxygenated with pure nitrogen for at least 10 min. Vials were preincubated for approximately 10 min at 37 °C, and 10 μL of alkyl nitrite stock solution was injected through the septum into the phosphate buffer. The initial concentration of alkyl nitrites in the reaction volume was 1 mM. The headspace (0.2 mL) was sampled every 3 min for a total time of 30 min cumulative measurements. All measurements were performed in triplicate.

Synaptosome Assay. The methodology for measurement of TBARS from synaptosomes has been fully described previously (*33*). Briefly, synaptosomes were prepared from cerebral cortex homogenates of adult Sprague−Dawley rats and suspended in Locke's buffer (NaCl, 154 mM; KCl, 5.6 mM; $CaCl_2{\cdot}2H_2O$, 2.3 mM; $MgCl_2{\cdot}6H_2O$, 1.0 mM; $NaHCO_3$, 3.6 mM; glucose, 5 mM; HEPES, 5 mM; pH 7.2) for assay. The protein content, determined by Bradford protein assay using bovine serum albumin as standard, was found to be 1.6 ± 0.3 mg/mL. All experiments were performed in triplicate and on three separate synaptosome preparations from different animals. Potential antioxidants and prooxidants were freshly prepared in Locke's buffer ($FeSO_4$), in NaOH (10 mM) (NONOates), or in organic solvent—DMSO (nitrites; final dilution ≤ 2.5% (v/v) organic component). Pro/antioxidants (or solvent vehicle in control experiments) were added to the synaptosome preparation, followed immediately by induction of lipid peroxidation by freshly prepared $FeSO_4$ (or buffer in control experiments) and incubation for 30 min at 37 °C (air equilibrated). After incubation, TBARS were measured, as previously described, at 530 nm on a Dynex MRX microplate reader. The TBAR solution was freshly made and calibrated using solutions of authentic malondialdehyde (MDA). The choice of a 30 min incubation period was made after preliminary lipid peroxidation experiments exploring the time course of lipid peroxidation, incubating synaptosomes with $FeSO_4$ (10−150 μM) in Locke's buffer, for time intervals from 15 to 180 min. Under our experimental conditions, the level of peroxidation, as measured by TBARS, was seen to be below saturation at 30 min using 50 μM $FeSO_4$. The exact species responsible for initiating Fe-induced lipid peroxidation has been the subject of debate, but there is general agreement that this is a useful system, which may mimic aspects of disrupted metal ion homeostasis in vivo (*40*−*42*).

All appropriate control experiments were performed. Goss et al. noted a slight inhibitory effect for high concentrations of SPE/NO on TBARS formation from MDA (*43*). We observed a 2% reduction in TBARS from MDA (5.25 μM) on incubation with SPE/NO (1 mM) and workup as above. We observed no significant effect in similar experiments with MDA and IAN (1 mM), which we would tentatively interpret as reflecting a minor reaction of spermine in trapping MDA. We conclude that nitrites do not influence MDA−TBA adduct formation (TBARS) and that an effect of NONOates is only seen at high concentrations. The advantages and disadvantages of the TBARS methodology have previously been discussed, and while TBARS may reflect reactive substances in addition to MDA, the TBARS measure allows highly reproducible determination of Fe-induced lipid peroxidation products in tissue homogenates (*44*−*46*). The TBARS technique is ineffective for measurements of azo initiator-induced lipid peroxidation, since the formation of MDA and other TBA reactive aldehydes is strongly amplified by iron-catalyzed degradation of the primary lipid hydroperoxide products of lipid peroxidation. Pure chain-breaking antioxidant activity describes quenching of lipid chain peroxyl radicals; however, a reduction in TBARS will also be caused by other antioxidant mechanisms such as removal of lipid peroxides, which can be estimated by preincubation of initiator with substrate followed by addition of antioxidant. At higher concentrations (1 mM), both NONOates and nitrites, when added after the 30 min incubation of synaptosome substrate with $FeSO_4$ initiator, gave approximately 20% inhibition of TBARS formation. Taken together with data from other antioxidant assays, this indicates that inhibition of TBARS by NONOates and nitrites occurs primarily through quenching of peroxyl radicals (*33*).

Pressure Transducer Experiments. DLPC multilamellar liposomes, with known amounts of lipid soluble initiator (AMVN), were prepared by coevaporation from dichloromethane, followed by vortex mixing in 10 mM phosphate buffer saline, pH 7.4, and 10 freeze−thaw cycles (*47*). Peroxidation experiments were performed at 37 °C under atmospheric pressure of oxygen using a dual channel high sensitivity pressure transducer, the details of which have been fully described elsewhere (*48*). Stock solutions of Trolox and SPE/NO were prepared in phosphate buffer saline and added in small volumes (10 μL) to the liposomes (2 mL) after the rate of oxygen uptake was constant. Micelles of linoleic acid were made in 0.5 M SDS containing 0.1 mM DTPA. Volumes (2 mL) of linoleic acid micelles were added to the sample cell, and pure oxygen was blown over the solution for about 30 s. The peroxidation of linoleic acid was initiated by addition of 50 μL of ABAP stock solution in phosphate buffer saline. When the rate of oxygen uptake became constant, a small volume (10 μL) of Trolox in 0.5 M SDS solution was added. After the rate of oxygen uptake returned to that before the addition of Trolox, the same volume of the SPE/NO stock solution in phosphate buffer saline was added and the rate of oxygen uptake was monitored. Stock solutions of SPE/NO were prepared with ice-cold phosphate buffer saline immediately before injection into the reaction mixture, and the concentration of stock solutions was determined spectrophotometrically immediately after their addition to the reaction cell. Concentrations of substrate, initiator, and inhibitors in micelles were calculated using the micellar reaction volume of 2.54×10^{-4} L for 2.0 mL of 0.5 M SDS (*49*). The oxygen uptake concentration was corrected for nitrogen evolution from the azo initiator and oxygen evolution during peroxyl radical recombination, as well as for the oxygen uptake by the initiator (*36*, *48*).

HPLC Analysis. IAN and ABAP were reacted at 37 °C in 40% acetonitrile/10 mM phosphate buffer saline (50 mM NaCl), pH 7.4. Small aliquots (0.3 mL) were removed from the reaction

Table 1. Calculated Relative Energies for Isodesmic Reaction Scheme Shown in Figure 1[a]

		dE[c]			ΔH		
structure	components[b]	6-31G** (kcal/mol)	6-311+G** (kcal/mol)	cc-pVTZ (kcal/mol)	6-31G** (kcal/mol)	6-311+G** (kcal/mol)	cc-pVTZ (kcal/mol)
allene **1**	$NO_2/O_2/NO$	0.00	0.00	0.00	0.00	0.00	0.00
allyl radical **2**	$HNO_2/O_2/NO$	10.60	10.11	9.92	10.49	10.00	9.81
allyl peroxide **3**	NO_2/NO	−7.66	−8.90	−8.90	−7.62	−8.85	−8.85
allyl peroxyl rad **4**	HNO_2/NO	−3.31	−2.30	−2.58	−3.46	−2.44	−2.73
allyl peroxynitrite **5**	HNO_2	−23.60	−19.19	−21.01	−23.91	−19.50	−21.32
allyl nitrate **6**	HNO_2	−52.80	−49.73	−51.65	−53.40	−50.32	−52.24
nitro oxirane **7**	HNO_2	−63.52	−60.50	−60.87	−64.37	−61.35	−61.73
allyl alkoxy rad **8**	NO_2/HNO_2	−17.20	−18.06	−17.83	−17.73	−18.58	−18.36
oxirane nitrite **9**	HNO_2	−63.90	−58.30	−59.71	−64.64	−59.04	−60.45
aldehyde **10**	$2HNO_2$	−74.98	−75.81	−76.34	−75.49	−76.32	−76.85
oxirane rad **11**	HNO_2/NO_2	−13.25	−12.23	−2.96	−13.84	−12.81	−13.60
oxirane alkoxy rad**12**	HNO_2/NO	−26.61	−25.52	−24.58	−27.21	−26.11	−25.17
oxirane nitrate **13**	HNO	−33.15	−29.41	−29.90	−33.70	−29.95	−30.45
oxirane alcohol **14**	NO_2/NO	−49.93	−50.99	−49.87	−50.38	−51.44	−50.32
allyl peroxynitrate **15**	HNO	6.13	9.93	8.41	5.95	9.75	8.22
allyl alkoxy rad **16**	NO_3/HNO	34.20	32.77	34.23	34.05	32.63	34.09
allyl nitrite **17**	HNO_3	−52.84	−50.01	−51.95	−53.59	−50.76	−52.70
allyl alkoxy rad **16**	HNO_3/NO	−14.78	−16.71	−16.44	−15.37	−17.30	−17.03
epoxy nitroso **18**	HNO_3	−42.39	−40.20	−40.66	−43.35	−41.16	−41.62
endoperoxide **19**	NO_2/NO	−21.91	−17.67	−18.67	−22.81	−18.57	−19.56

[a] All minimum energy structures and thermochemical parameters were obtained at B3LYP/6-31G**. [b] Reaction components for which energies are summed to yield combined energy relative to allene + NO_2 + O_2 + NO. [c] dE + E +ZPE.

mixture and mixed with an equal volume of 1 mM DNPH solution. The DNPH solution (100 mL) was prepared by dissolving the required amount of DNPH (20 mg, recrystallized three times from acetonitrile) into acidulated (20 μL concentrated H_2SO_4) HPLC grade acetonitrile. The purity of the DNPH reagent was verified by HPLC and found to be >99.5%. HPLC analyses were conducted on a Shimadzu LC-10A, using detection at 360 nm and analytical separation on a C-18 column (4.6 mm × 150 mm, Supelco), using gradient elution: 0−15 min a linear gradient of 50−80% acetonitrile−water followed by a 2 min isocratic elution at 80% acetonitrile−water and reequilibration. 2,4-Dinitrophenylhydrazones of different carbonyl compounds were either purchased as HPLC standards from Supelco (formaldehyde, acetaldehyde, acrolein, *n*-butyraldehyde, *i*-butyraldehyde, and *i*-valeraldehyde) or prepared from DNPH reagent and the carbonyl compounds of highest purity (acetone from Sigma and 4-hydroxybutanone from ICI America). Solutions of 2,4-dinitrophenylhydrazone standards and known carbonyl compounds were prepared in the same solvent used for the reaction of IAN with ABAP. Standard curves for *i*-valeraldehyde quantification were constructed with both *i*-valeraldehyde and standard *i*-valeraldehyde hydrazone. With *i*-valeraldehyde, standard curves were performed in both acetonitrile and 40% acetonitrile in phosphate buffer. The amount of *i*-valeraldehyde in aqueous solutions was underestimated using calibration in straight acetonitrile due to hydration of the aldehyde in aqueous solution. Therefore, the standard curve was constructed using known amounts of *i*-valeraldehyde incubated in the same aqueous solution used for the reaction of IAN with ABAP. Control experiments for ABAP alone (no IAN) and IAN alone (no ABAP) were performed under the same conditions. Control experiments for the reaction of *i*-amyl alcohol (the product of IAN hydrolysis) with ABAP were also performed in order to address the question of aldehyde formation from IAN hydrolysis products.

MO Calculations. DFT calculations were carried out at three levels, B3LYP/6-31G**//B3LYP/6-31G**, B3LYP/6-311+G**//B3LYP/6-31G**, and B3LYP/cc-pVTZ//B3LYP/6-31G**, using Spartan 02 Windows (Wavefunction, Irvine, CA). The conformational space was searched at the AM1 level, and thermochemical parameters were obtained for appropriate conformers after geometry optimization at the B3LYP/6-31G** level and confirmation of zero negative eigenvalues. BDE (ΔH_{homo}), ΔH_r, and dE (E + ZPE) were calculated at 298 K using standard thermochemical methods, with ZPE scaled by 0.95. The reliability of similar DFT methods for BDE calculations on nitrosothiols has been reported (*50*, *51*). To study the thermochemistry of various reactions, relative heats of reaction (ΔH_r) were calculated using series of isodesmic transformations, which included O_2, NO, NO_2, HNO, HNO_2, HNO_3, and NO_3, where appropriate. The data shown in Table 1 were calculated using a series of reactions beginning with the homoallylic H atom abstraction from propene by NO_2. Thus, the relative energy of $H_2C{=}CHCH_3 + NO + NO_2 + O_2$ was set at zero and the relative energy was calculated for subsequent isodesmic transformations (Figure 1).

Results

NONOates were observed to inhibit Fe-induced lipid peroxidation in a concentration-dependent manner (Figure 2). The inhibitor potency, expressed as the concentration required to produce 50% inhibition of the iron control (IC_{50}), increased in the order DETA/NO < SPE/NO < DEA/NO < NOC-9/NO. The IC_{50} values experimentally determined in Locke's buffer at pH 7.4 for DETA/NO, SPE/NO, DEA/NO, and NOC-9/NO were 5.89×10^{-4}, 1.98×10^{-4}, 5.90×10^{-5}, and 4.05×10^{-5} M, respectively. This ordering mirrored the trend in the rate constants (k_{NO}) measured for NO release. In phosphate buffer (0.1 M, pH 7.4), k_{NO} for NO release from NONOates at 37 °C, calculated from the measured rate of hydrolysis and the number of equivalents of nitric oxide released per equivalent of NONOate, were 1.92×10^{-5}, 5.62×10^{-4}, 8.10×10^{-3}, and 1.37×10^{-2} s^{-1} for DETA/NO, SPE/NO, DEA/NO, and NOC-9/NO, respectively (*34*, *39*, *52*). No significant difference was observed (data not shown) for the rate constant of NO release in Locke's buffer (used for Fe-induced lipid peroxidation experiments) and phosphate buffer at the same pH 7.4 and 37 °C.

The four organic nitrites studied also inhibited Fe-induced lipid peroxidation in a concentration-dependent manner (Figure 3). The rank order of inhibitor potency of alkyl nitrites (IC_{50}) increased in the order PEN < IAN < DCN ≤ DDN, based upon the experimentally determined IC_{50} values of 4.67×10^{-4}, 1.41×10^{-4}, 3.40×10^{-5}, and 3.17×10^{-5} M, respectively. In contrast to the NONOates, the rank ordering for nitrites did not reflect the trend in the rate constants for NO release. In

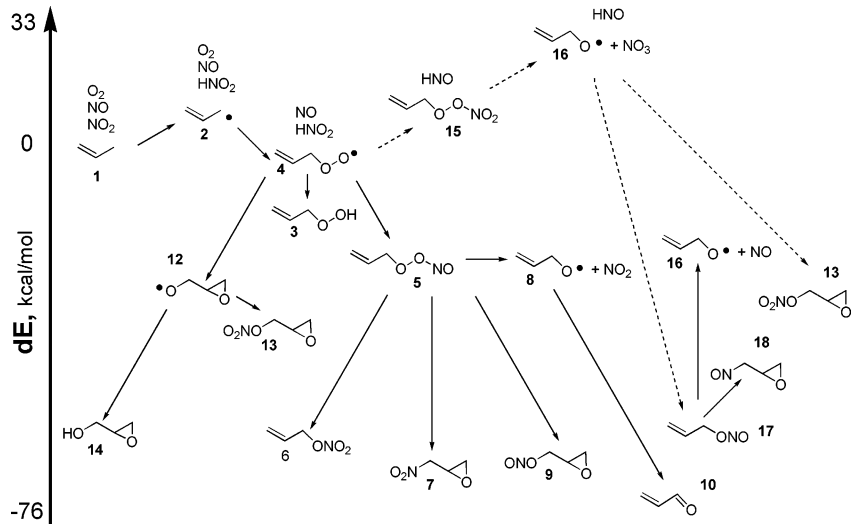


Figure 1. Relative energies of chemical species potentially involved in lipid peroxidation in the presence of NO_x species, calculated at B3LYP/6-311 +G**//B3LYP/6-31G**. The energy (dE = E + ZPE) of $CH_2CHCH_3 + O_2 + NO + NO_2$ was set at zero. All reactions are isodesmic, but for clarity, not all NO compounds have been included in the scheme (see Table 1 for full details of reaction components and calculated energies).

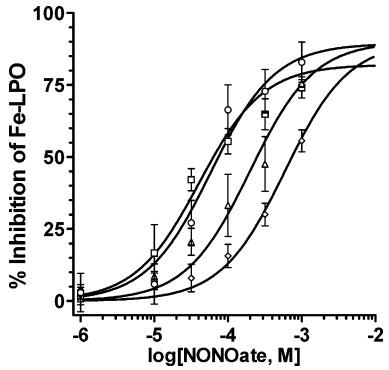


Figure 2. Effect of NONOates. Inhibition of lipid peroxidation induced by $FeSO_4$ (50 μM), as assessed by TBARS determination, on incubation of rat brain synaptosomes with NOC-9/NO (□), DEA/NO (○), SPE/NO (△), and DETA/NO (◇). Percentage inhibition relative to controls: TBARS in the presence of vehicle and absence of $FeSO_4$ (100%); TBARS in the presence of vehicle and $FeSO_4$ (0%). Errors bars show SEM ($n = 3$). Data are fitted to a sigmoidal curve.

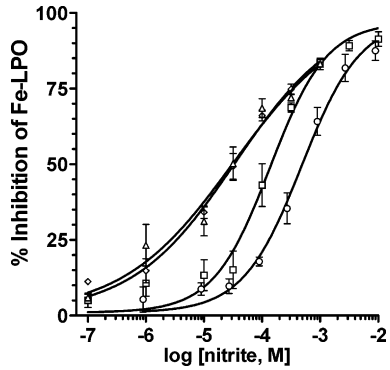


Figure 3. Effect of alkyl nitrates. Inhibition of lipid peroxidation induced by $FeSO_4$ (50 μM), as assessed by TBARS determination, on incubation of rat brain synaptosomes with IAN (□), PEN (○), DCN (△), and DDN (◇). Percentage inhibition relative to controls: TBARS in the presence of vehicle and absence of $FeSO_4$ (100%); TBARS in the presence of vehicle and $FeSO_4$ (0%). Errors bars show SEM ($n = 3$). Data are fitted to a sigmoidal curve.

phosphate buffer (0.1 M, pH 7.4, 37 °C), k_{NO} for the organic nitrites, PEN, IAN, DCN, and DDN, measured by headspace gas chemiluminescence detection, was 7.68 $\times 10^{-5}$, 1.04 $\times 10^{-4}$, 4.95 $\times 10^{-5}$, and 2.77 $\times 10^{-5}$ s^{-1}, respectively.

SPE/NO inhibited oxygen uptake in linoleic acid micelles and DLPC liposome peroxidation initiated by the azo initiators ABAP and AMVN, respectively, showing definite induction periods (Figure 4A,B). These induction periods allowed the calculation of the number of molar equivalents of peroxyl radicals (n) quenched per molar equivalent of NO using the expression $R_i = n$[inhibitor]/τ, where R_i is the rate of chain initiation determined experimentally using Trolox as the antioxidant control and τ is the inhibition period. The experimental stoichiometric factor found for SPE/NO inhibition of ABAP-induced peroxidation of linoleic acid in SDS comicelles was 0.32 ± 0.05. In DLPC liposomes, with peroxidation induced by AMVN, the stoichiometric factor for SPE/NO was 0.43 ± 0.06, which compares with the reported value of 0.52 ± 0.03 from O'Donnell et al. for inhibition of ABAP-induced peroxidation of linoleic acid in sodium cholate micelles by solutions of NO (*28*). In O'Donnell's work, various corrections were applied to the experimentally derived stoichiometric factor (0.31), yielding the stoichiometry of eq 1.

For antioxidants that give clear inhibition of oxygen uptake and definite breaks in the induction period, absolute rate constants for inhibition (k_{inh}) can be determined. These values are determined from measurements of oxygen uptake during the inhibition periods by using the integrated expression for kinetics during inhibition: $\Delta[O_2] = -k_p/k_{inh}[RH]\ln(1 - t/\tau)$, where $\Delta[O_2]$ is the oxygen uptake, [RH] is the substrate concentration, and k_p is the rate constant for chain propagation, which for linoleate is known (36.1 M^{-1} s^{-1}) under these experimental conditions (*53*). These plots are shown in Figure 4C,D. The absolute inhibition rate constants for Trolox and SPE/NO for the ABAP-induced peroxidation of linoleic acid in SDS comicelles were 1.51 $\times 10^4$ and 1.73 $\times 10^4$ M^{-1} s^{-1}, respectively. In DLPC liposomes, with peroxidation induced by AMVN, the rate constants for inhibition by

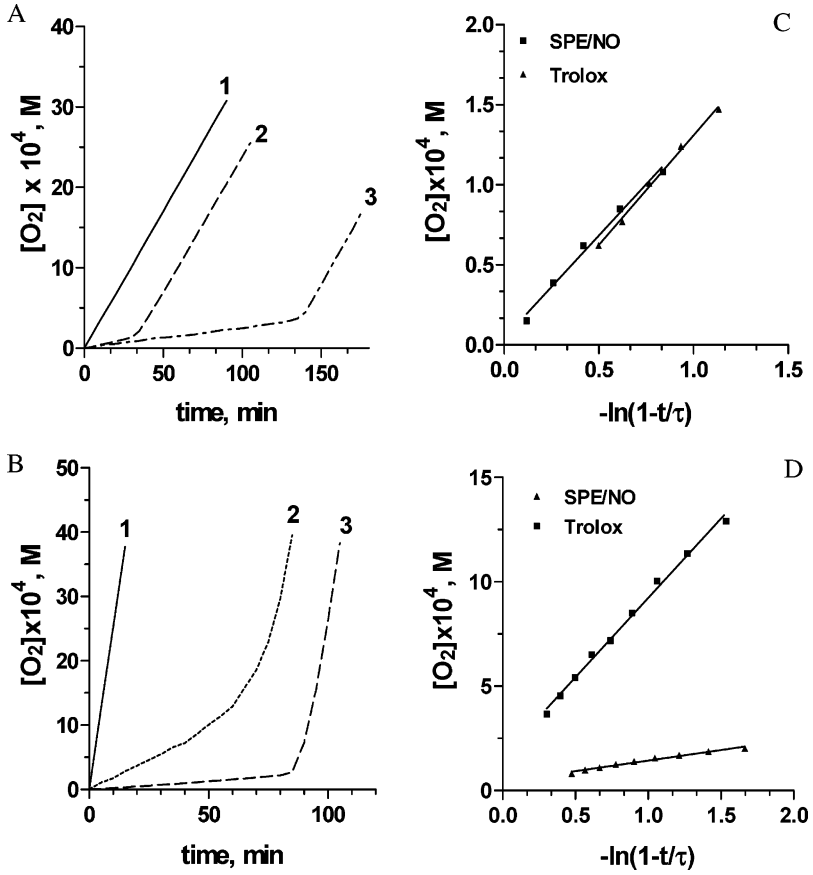


Figure 4. (A) Effect of SPE/NO on ABAP-initiated (4.01×10^{-2} M) peroxidation of linoleic acid (6.1×10^{-2} M) in 0.5 M SDS (pH 7.4) at 37 °C; 1, uninhibited peroxidation; 2, 1.02×10^{-4} M SPE/NO, $\tau = 44$ min; 3, 4.96×10^{-5} M Trolox, $\tau = 139.5$ min, $R_i = 1.18 \times 10^{-8}$ M s^{-1}. (B) Effect of SPE/NO on AMVN-initiated (8.59×10^{-6} mol) peroxidation of DLPC liposomes (9.23×10^{-5} mol) in 10 mM phosphate buffer saline at 37 °C; 1, uninhibited peroxidation; 2, 1.11×10^{-8} mol Trolox, $\tau = 76.5$ min, $R_i = 4.84 \times 10^{-12}$ mol s^{-1}; 3, 4.33×10^{-8} mol SPE/NO, $\tau = 92.5$ min. (C) Plots for oxygen uptake during inhibition period in linoleic acid SDS micelles for inhibitors in B: Trolox $k_{inh} = 1.51 \times 10^4$ M^{-1} s^{-1}, SPE/NO $k_{inh} = 1.73 \times 10^4$ M^{-1} s^{-1}. (D) Plots of oxygen uptake during inhibition period in DLPC liposomes for inhibitors in A: Trolox $k_{inh} = 4.94 \times 10^4$ M^{-1} s^{-1}, SPE/NO $k_{inh} = 3.67 \times 10^5$ M^{-1} s^{-1}.

Trolox and SPE/NO were 4.94×10^4 and 3.67×10^5 M^{-1} s^{-1}, respectively. Interestingly, experiments carried out at either lower concentrations of NONOates or higher concentrations of azo initiator and at a greater level of peroxyl radical flux both yielded only a reduction of the rate of oxygen consumption and not a true lag phase (data not shown). It is important to note that this observation demonstrates the requirement for a flux of NO greater than that of lipid peroxyl radicals for chain-breaking antioxidant activity; otherwise, only chain retardation is observed.

The HPLC analysis of the product mixture for the reaction of IAN with ABAP in 40% acetonitrile in phosphate buffer saline after 24 h of incubation at 37 °C revealed the presence of *i*-valeraldehyde, formaldehyde, acetone, and 4-hydroxybutanone (Figure 5a). The identity and amount of aldehyde formation were determined by formation of the hydrazone derivatives and construction of appropriate standard curves using authentic hydrazones. Acetone and formaldehyde were observed on degradation of ABAP in the absence of IAN and likely result from the fragmentation of the ABAP-derived peroxyl radical $R(CH_3)_2COO\bullet$ (Scheme 2) (*54*). Fragmentation pathways to formaldehyde and 4-hydroxybutanone can also be drawn from IAN through intermediate radical species (Scheme 2). The percentage yield of *i*-valeraldehyde in the reaction mixture, when substrate had been entirely consumed, was found to be 5% based upon the initial concentration of IAN. In a control reaction of ABAP with *i*-amyl alcohol, *i*-valeraldehyde was also observed in the product mixture but at only half the yield observed in the reaction of ABAP with IAN (Figure 5b).

DFT MO calculations have proven useful for comparison with experimental data related to NO release and antioxidant activity in solution (*50*, *51*, *55*, *56*). Because of the importance of NO and NO_2 in atmospheric chemistry, a larger body of computational data has been generated to model this chemistry; for example, a recent study on trapping of isoprene-derived peroxyl radicals by NO reported a stabilization of 21 kcal/mol in formation of the peroxynitrite and a further 23−27 kcal/mol on isomerization to the nitrate (*57*). Furthermore, the transformations of the peroxynitrite were shown to be barrierless. In this work, calculations were performed on a variety of model compounds of relevance to formation and degradation of nitrites in lipid peroxidation in the presence of NO or NO_2, including nitro, nitroso, and nitrooxy derivatives and those alkoxyl and peroxyl species normally seen in lipid peroxidation (Table 1).

Allyl peroxyl radical (**4**) was used as a model for a lipid peroxyl radical, and a series of isodesmic reactions were set up (Figures 1 and 6). The transformations shown in Figure 6 yielded a stabilization of 17 kcal/mol on trapping of allyl peroxyl radical by NO and a further stabilization of 30 kcal/mol on isomerization to allyl nitrate. However, a variety of exothermic rearrangements were illustrated

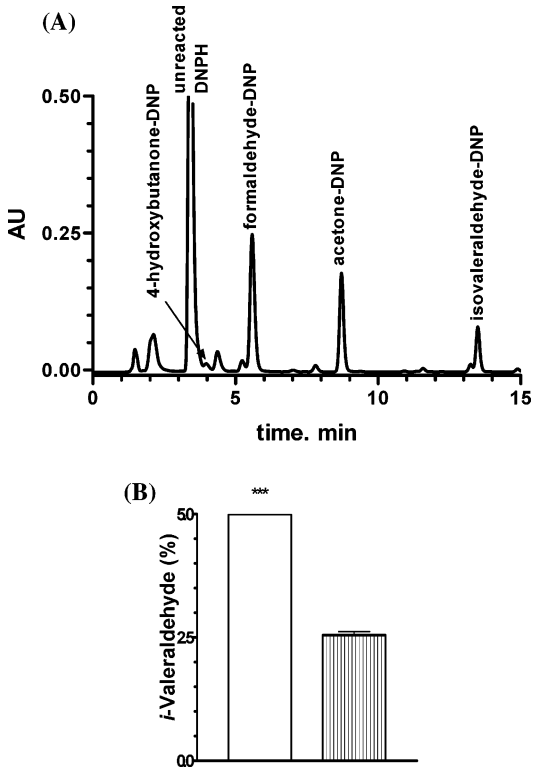


Figure 5. (A) DNPH adducts identified in the IAN 1 mM with ABAP 30 mM reaction mixture after 24 h of incubation at 37 °C in 40% acetonitrile/10 mM phosphate buffer saline (50 mM aCl) at pH 7.4. (B) Percent yield of *i*-valeraldehyde formed after 24 h of incubation at 37 °C of 30 mM ABAP with IAN (empty bar) and *i*-amyl alcohol (stripped bar), respectively. The percent yield was calculated relative to the initial concentration of substrate. Results represent means ± SD; $p < 0.01$.

to be theoretically possible, in particular formation of the nitrooxirane and oxirane nitrite (Figure 6). Formation of allyl nitrite and oxirane nitrate is also thermodynamically favorable in transformations in reactions of NO and NO_2 with oxygen radicals derived from lipid peroxidation (Figure 6). However, the concentrations of NO_2 and peroxyl radical are unlikely to be sufficient for this combination to be of significance in the antioxidant activity of NO. No attempt was made to explore the detailed reaction pathways either via a concerted transition state or via a geminate radical pair. An alternative set of isodesmic reactions was constructed to provide a reaction energy profile incorporating most species of potential relevance in the antioxidant action of NO (Figure 1; this profile should be used as a general guide only, since up to four separate chemical species must be included to balance all transformations. Also note that other species may be involved as shown in Scheme 3).

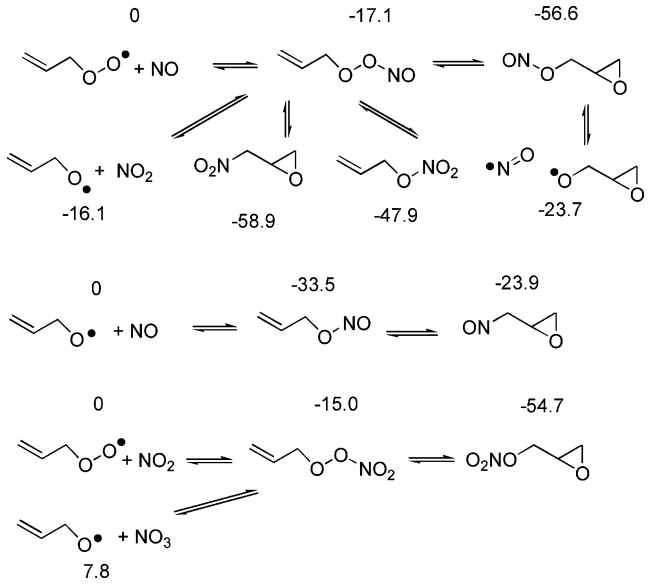


Figure 6. Relative reaction enthalpy (ΔH_r, kcal/mol) for chain termination reactions of NO and NO_2, calculated at B3LYP/6-311 +G**//B3LYP/6-31G**.

Values of BDE for the O−N bond of acyl nitrite esters of 26−27 kcal/mol and for the S−N bond of *t*-alkyl nitrosothiols of 25 kcal/mol have been measured experimentally (*50*, *58*). Other estimates of BDE for the S−N bond of nitrosothiols from experiment and calculations (at the CBS-QB3 level) are approximately 5 kcal/mol higher (*51*). The values calculated herein at the DFT B3LYP/6-311+G**//B3LYP/6-31G** level of calculation for acyl nitrites and nitrosothiols are 27 and 26 kcal/mol, respectively (Table 2). The calculated O−N BDE values for PEN and IAN were higher at 33−34 kcal/mol, and for the putative allyl and oxirane nitrites formed during NO-terminated lipid peroxidation, the calculated BDE was 37 kcal/mol (Table 2 and Figures 1 and 7). Comparison of the thermodynamics of homolysis and α-H abstraction from IAN demonstrated that hydrogen atom abstraction combined with NO release was endothermic by 52.5 kcal/mol (Figure 7). However, when the reaction with allyl peroxyl and alkoxyl radicals was incorporated, this process was calculated to be highly exothermic, with ΔH_r ranging from 27 to 50 kcal/mol (Scheme 4 and Table 3).

Discussion

Nitrites and nitrates are of importance as xenobiotics and potentially as endogenous products of nitrosative metabolism, including as products of the chain-breaking

Scheme 2

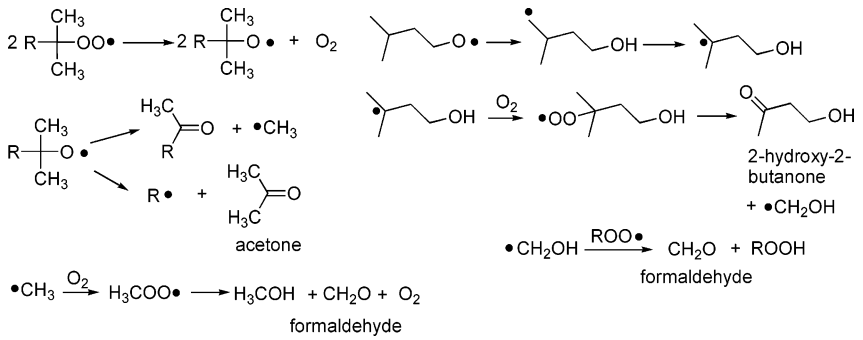


Table 2. Calculated RZ−NO Bond Dissociation Energies (ΔH_r) and dE Values for Homolysis of Nitrites and Nitrosothiols at Three Levels of Calculation

	dE (kcal/mol)			BDE (kcal/mol)		
	6-31G**	6-311+G**	cc-pVTZ	6-31G**	6-311+G**	cc-pVTZ
AcONO	29.7	27.0	28.7	29.5	**26.9**	28.5
BzONO	29.6	27.1	28.7	29.4	**27.0**	28.5
MeONO	38.9	34.0	36.4	39.3	**34.4**	36.8
PEN	36.7	33.6	35.1	36.5	**33.2**	35.0
IAN	37.5	34.2	35.9	37.5	**34.2**	35.9
allyl nitrite **17**	38.1	33.3	35.5	42.0	**37.2**	39.5
oxirane nitrite **9**	37.8	33.2	35.4	41.7	**37.1**	39.3
MeSNO	28.9	27.0	28.6	29.1	**27.2**	28.8
tBuSNO	27.4	25.8	27.1	27.3	**25.7**	27.1

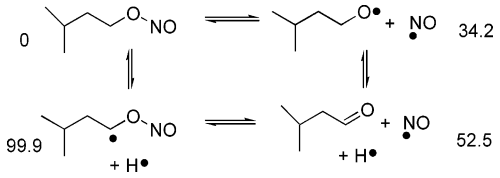
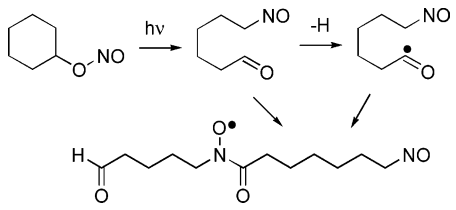


Figure 7. Relative heats of reaction (ΔH_r, kcal/mol) for O−NO bond homolysis and α-H abstraction from IAN, calculated at B3LYP/6-311 +G**//B3LYP/6-31G**.

Scheme 3



Scheme 4

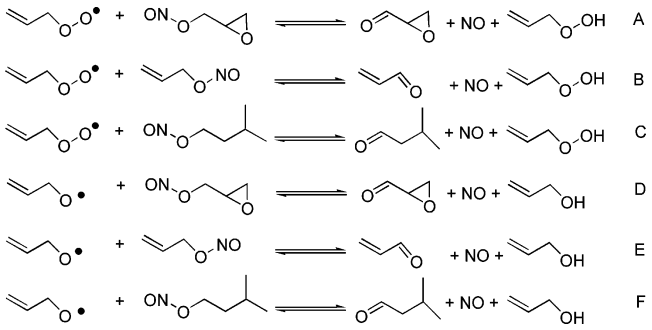


Table 3. Calculated ΔH_r (kcal/mol) for α-H Abstraction Reactions Shown in Scheme 4 at Three Levels of Calculation

	6-31G**	6-311+G**	cc-pVTZ
A	−25.63	**−27.59**	−25.90
B	−23.70	**−30.69**	−28.94
C	−19.01	**−27.30**	−25.93
D	−45.21	**−47.08**	−45.38
E	−43.28	**−50.18**	−48.42
F	−38.59	**−46.79**	−45.42

antioxidant action of NO. Antioxidant and prooxidant mechanisms can be drawn for nitrites and nitrates; therefore, we studied the influence of these agents on Fe-induced lipid peroxidation in synaptosomes and on azo initiator-induced lipid peroxidation in liposomes, wherein classical nitrates had no effect, but novel nitrates that release NO and the nitrite ester, IAN, inhibited lipid peroxidation (*33*). The present study, in which the antioxidant activity of a series of four NONOate NO donors was compared with four nitrite esters, was designed to test whether the observed antioxidant activity of nitrites resulted simply from NO released from nitrite degradation. To confirm that NONOates behave in an entirely analogous manner to solutions of NO, we analyzed oxygen consumption by pressure transduction, for comparison to the seminal work of O'Donnell et al. (*28*).

Organic nitrites and nitrates represent vasodilators of clinical importance. Nitrates and nitrites are widely believed to provide exogenous sources of NO, requiring reductive biotransformation systems for conversion to NO in vivo (*6*, *59*). Nitrites are putative intermediates in the $3e^-$ biotransformation of nitrates, and because reduction of nitrites to NO is only a $1e^-$ reduction, unsurprisingly, differences between the biological activity of nitrates and nitrites have been observed (*60*). Both the chemical mechanism and the biotransformation apparatus responsible for reduction of both nitrates and nitrites are not clearly defined.

Xenobiotic organic nitrates and nitrites are well-known medicinal agents, but they may also constitute endogenous, naturally occurring products of the reactions of NO, peroxynitrite and NO_2 with lipids, and other biomolecules under oxidative and nitrosative stress. The free radical gases, NO and NO_2, have metamorphosed in the eyes of researchers from environmental toxins to important biomolecules requiring much further study. NO is, of course, an important biological messenger molecule with many roles, including in learning, memory, and smooth muscle relaxation. NO reacts very rapidly with superoxide to generate peroxynitrite, which under physiological conditions will, in part, undergo homolysis to yield NO_2. The major metabolite of NO in vivo is inorganic nitrite, NO_2^-, which has been shown to be reactive in the presence of hydrogen peroxide and transition metal catalysts to give NO_2 (*22*, *61*−*63*). Early work, in particular by Pryor and co-workers, demonstrated that the environmental toxin NO_2 would be reactive toward biomolecules and in the case of lipids would undergo addition to unsaturated bonds under anaerobic conditions and in the presence of O_2 to form nitro, nitroso (nitrite), and nitrooxy (nitrate) derivatives after initial H atom abstraction (*22*, *23*, *30*, *31*, *63*).

Recently, the potential for formation of nitro, nitroso, and nitrooxy lipids from reaction of NO with lipid peroxy radicals has been revisited, although many products are yet to be isolated and fully characterized (*21*, *32*, *64*−*66*). DFT MO calculations have proven useful in explaining and predicting experimental data related to NO release and antioxidant activity; therefore, we explored the thermodynamics of formation of these potential nitro, nitroso, and nitrooxy lipid products (*50*, *51*, *55*). The calculated relative energies of species potentially involved in the trapping of the lipid peroxyl radical (modeled by the allyl peroxyl radical **4**) by NO are

outlined in Figure 1. The initially formed peroxynitrite ester (**5**), in analogy with peroxynitrous acid, may yield via the geminate radical pair [NO_2••OR] both cage rearrangement products and the cage escape homolysis products NO_2 plus alkoxyl radical (**8**) (Figure 1 and Table 1). Thermodynamically favorable rearrangement products include nitro oxirane (**7**), oxirane nitrite (**9**), and allyl nitrate (**6**) (Figures 1 and 6). Several other routes to nitrite and nitrate products are also apparent (Figure 1) in addition to those resulting from nitrosation of lipid components by N_2O_3 (not shown).

O'Donnell et al. estimated a stoichiometric factor of 0.5 for inhibition of ABAP-induced peroxidation of linoleic acid in sodium cholate micelles by solutions of NO, corresponding to a requirement for 2 equiv of NO per equivalent of peroxyl radical (*28*). It was proposed that 1 equiv of NO quenches one lipid peroxyl radical to give a lipid peroxynitrite, with the second equivalent of NO required to quench the alkoxyl radical formed by homolysis of the peroxynitrite. However, these workers did also discuss the complexity of the system with respect to the unknown reactivity of the putative chain termination products, including lipid nitrites and nitrates. Lipid nitrites clearly can act as antioxidants themselves, whereas other products, such as NO_2, can function as chain propagating oxidants.

Available evidence suggests that NONOates act as reliable NO donors, releasing NO in a pH-dependent manner at readily measurable rates (*67*). The NONOate SPE/NO was examined in the classical oxygen uptake antioxidant assay, in comparison with the vitamin E analogue, Trolox, using both hydrosoluble (ABAP) and lipophilic (AMVN) azo initiator-induced lipid peroxidation in linoleic acid micelles and DLPC liposomes, respectively (Figure 4A,B). A kinetic chain length of 6−23 was measured for the inhibition period produced by SPE/NO in SDS micelles, which suggested that the inhibition is mainly due to trapping of lipid peroxyl radicals (LOO•) and not peroxyl radicals derived from the initiator. Under these conditions, the kinetic chain length in the absence of inhibitor was 50−56. The number of equivalents (stoichiometric factor) of peroxyl radicals quenched by NO generated from SPE/NO was found to be 0.32 ± 0.05. These values were calculated from the experimentally determined rate of chain initiation ($R_i = 1.18 \times 10^{-8}$ M^{-1} s^{-1}) using Trolox. Barclay et al. showed that ABAP partitions considerably (up to 91%) into SDS possibly due to electrostatic interactions between the positive amidino groups of ABAP and the anionic headgroups of SDS (*49*); thus, it is reasonable to assume that NO can also quench the peroxyl radicals (ROO•) generated from the initiator. Therefore, a better estimation of the number of equivalents of peroxyl radicals quenched would consider the rate of peroxyl radical generation from ABAP ($R_g = 1.87 \times 10^{-8}$ M^{-1} s^{-1}) calculated using the expression $R_g = 2$ e k_d[ABAP], where e and k_d are the ABAP efficacy (0.43) and decomposition rate constant (5.42×10^{-7} s^{-1}) in SDS micelles. The recalculated value for SPE/NO is 0.51 ± 0.08. The stoichiometric factor found for SPE/NO inhibition of AMVN-induced peroxidation in DLPC liposomes was 0.43 ± 0.06. These numbers are in good agreement with measurements on inhibition of oxygen uptake in NO solutions (*28*).

In our experimental lipid peroxidation model, the four tested NONOates (NOC-9/NO, DEA/NO, SPE/NO, and DETA/NO) inhibited Fe-induced lipid peroxidation in rat brain synaptosomes in a concentration-dependent manner (Figure 2). An excellent linear correlation ($R^2 = 0.99$) between the inhibitory efficiency of NONOates and the rate constant for NO release from each NONOate was found (Figure 8). This correlation emphasizes that NO is mediating inhibition of lipid peroxidation and that NONOates represent excellent experimental NO donors (*68*). Over the relatively short time course of the experiment, the results indicate that the rate of NO release is the important parameter in determining the extent of chain termination. SPE/NO has been reported to inhibit peroxidation and oxidation in other systems (*43*, *69*); in particular, the elegant study of Goss et al. reported the time course of low density lipoprotein oxidation induced by Cu^{II} and ABAP in the presence of NONOates, with reactivity spanning that of SPE/NO (*70*). This paper presented a qualitative relationship between the effectiveness of the NONOates in inhibiting oxidation and rates for NO release, oxidation, and the loss of NO, such that the most reactive NONOate was ineffective as an antioxidant over the long period of oxidation studied.

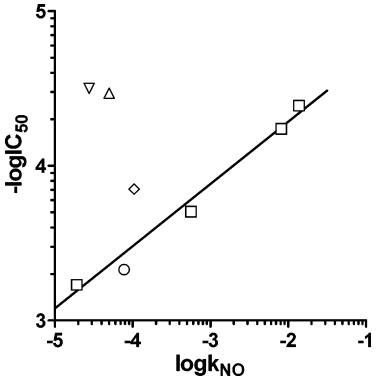


Figure 8. Correlation between inhibitory effect on iron-induced lipid peroxidation in rat brain synaptosomes and rate constants of NO release for NONOates (□). $R^2 = 0.988$. The data points for alkyl nitrites are represented by IAN (◇), PEN (○), DCN (△), and DDN (▽).

The four nitrites used in this study all inhibited Fe-induced lipid peroxidation in a concentration-dependent manner (Figure 3). This apparent antioxidant activity could potentially be explained by NO release, either directly from nitrite homolysis or from the nitrosothiol products of synaptosome nitrosation (*71*). Second, alkyl nitrites and NO might oxidize and/or complex Fe(II) (*72*). The observation of inhibition of lipid peroxidation by IAN using $FeCl_3$/NADPH to induce lipid peroxidation (data not shown) in place of $FeSO_4$ rules out oxidation as a significant contributor. Most importantly, for both NONOates and nitrites, the observation of inhibition of azo initiator-induced lipid peroxidation negates a significant role for interactions with iron ions (*33*). Nevertheless, no correlation was observed between the rate of NO release measured for the four nitrites and the IC_{50} for lipid peroxidation in synaptosomes (Figure 8). Clearly, the long chain esters are the better nitrite inhibitors of lipid peroxidation with the measured IC_{50} values of 30 μM demonstrating greater potency than even the most reactive NONOate, despite the rate of NO release being 3 orders of magnitude less for the nitrites (Figures 2 and 3). Furthermore, long chain nitrites inhibit lipid peroxidation at submicromolar concentrations (Figure 3). By comparison with the IC_{50} value (70 μM) obtained for the vitamin E analogue Trolox under identical conditions,

long chain nitrites are relatively potent antioxidants (*33*). In inhibition of synaptosomal lipid peroxidation by nitrites, other mechanisms must be in operation, in addition to radical chain termination by NO that is produced by nitrite homolysis.

The electron-withdrawing phenoxyl group causes PEN to be more reactive to heterolytic nitrosyl transfer than the simple alkyl nitrites (*37*). Because PEN is a less potent inhibitor than the alkyl nitrites IAN, DCN, and DDN, a mechanism relying on nitrosation is unlikely. Long chain nitrites, such as DCN and DDN, that accumulate in the synaptosome lipid membranes make possible a "concentration effect"; that is, the nitrite local concentration reaches high enough values to compete with chain propagation reactions. However, because the rate of NO release from nitrites is much reduced in organic solvents, an alternative mechanism must be in play. A contribution to antioxidant activity from membrane stabilization is possible but must not be dominant since antioxidant activity is a class effect observed for IAN and PEN.

The chemistry of nitrites includes heterolytic and homolytic reactions, in addition to oxidation of hemoglobin (*73*). In several elegant mechanistic studies on nitrite hydrolysis, the sole N-containing product has reasonably been presumed to be inorganic nitrite; therefore, there has been no attempt to detect other products (*28*, *73*−*75*). Nitrites are excellent nitrosating agents: the nitrosation of different substrates (alcohols, thiols, and amines) by alkyl nitrites (especially tertiary alkyl nitrites) occurs at rates several order of magnitudes higher than competing hydrolysis (*76*). In an aqueous biological compartment, the fast rate of reaction with GSH (approximately 2 M^{-1} s^{-1}) will result in the biological activity of the nitrite being mediated through *S*-nitrosoglutathione (*77*). The homolytic cleavage of the O−NO bond of alkyl nitrites produces NO, with values for the BDE quoted as 35−40 kcal/mol (*78*). A number of synthetically useful photochemical reactions of nitrites have been reported initiated by O−N bond photolysis, but in general, there has not been an attempt to detect and quantify NO as a byproduct (*79*, *80*). In aqueous solution, we observed that alkyl nitrites spontaneously generate NO, as detected electrochemically and by chemiluminescence, which differentiates nitrites from nitrates, which do not spontaneously release NO, and from nitrosothiols, which generally in purified form and in the dark do not spontaneously release NO (*38*). Photolysis of nitrites in organic solvents yields aldehydes and other products, including compounds such as nitrosoalkanes and nitroxyl radicals that are themselves capable of radical scavenging (Scheme 3). Although NO release was not quantified, the papers of Grossi and co-workers are particularly interesting in this respect (*81*−*83*).

Values of BDE for the O−N bond of acyl nitrite esters of 26−27 kcal/mol have been measured experimentally (*58*). These are in excellent comparison with values of 26.9−27.0 kcal/mol calculated herein at the B3LYP/6-311+G**//B3LYP/6-31G** level of calculation (Table 2). The calculated O−N BDE values for PEN and IAN were higher at 33−34 kcal/mol, and for the putative allyl and oxirane nitrites formed during NO-terminated lipid peroxidation, the calculated BDE was 37 kcal/mol (Table 2 and Figures 1 and 7). The simple thermal homolysis of nitrites in biological systems would release NO and alkoxyl radical, the latter having the potential to initiate and propagate radical reactions such as lipid peroxidation. We have previously reported that nitrites are inhibitors of lipid peroxidation, suggesting that nitrites do not inhibit lipid peroxidation via NO release or that a free alkoxyl radical is not released as the homolysis product. One alternative possible mechanism leading to inhibition of lipid peroxidation by nitrites is α-H atom abstraction (Figure 7). The calculated α-H BDE for IAN is almost 100 kcal/mol, but when coupled with O−N bond homolysis and release of NO, the enthalpy of reaction is reduced to 52.5 kcal/mol. Furthermore, the α-H atom abstraction reactions of both allyl peroxyl and alkoxyl radicals with nitrites were calculated to be highly exothermic, with ΔH_r ranging from 27 to 50 kcal/mol (Scheme 4 and Table 3).

The product of α-H abstraction from IAN, *i*-valeraldehyde, was observed in aqueous reaction mixtures containing the water soluble azo initiator, ABAP, and IAN and was not observed in control reactions, including decomposition of IAN in the absence of ABAP. Smaller amounts of *i*-valeraldehyde were observed from reaction of ABAP with *i*-amyl alcohol in the presence of O_2, which is not unexpected, since alcohols can act as antioxidants, although relatively poor ones, via α-H abstraction. Direct reaction of peroxyl radicals with nitrite via α-H abstraction yielding hydroperoxide and NO provides a mechanism for chain termination independent of simple nitrite homolysis. The long chain nitrites would be expected to show an enhanced effect due to higher local concentrations in lipid membranes.

Summary. The present study further demonstrates that the NO donor NONOates provide reliable and useful fluxes of NO, which may quantitatively be used to assess the biological reactivity and activity of NO. In particular, the rate of NO release from NONOates was quantitatively correlated with the potency for inhibition of Fe-induced lipid peroxidation in rat brain synaptosomes, and an experimentally determined stroichiometric factor of 0.4−0.5 was calculated for inhibition of lipid peroxidation in DLPC liposomes and in linoleic acid SDS comicelles. Lipid nitrites are likely products of lipid peroxyl chain termination by NO, and alkyl nitrites are medicinal agents. It was observed that nitrites are relatively good inhibitors of lipid peroxidation in synaptosomes but that inhibitor potency did not correlate with measured rates of NO release. Theoretical calculations and product distribution analysis supported a novel antioxidant mechanism for nitrites via α-H atom abstraction. There is evidence that nitrolipids and lipid nitrites are products of lipid peroxyl chain termination by NO and that nitrolinoleate is biologically active with properties of an NO donor (*32*). DFT calculations confirm that nitrolipids, lipid nitrates, and lipid nitrites are thermodynamically equally accessible products of lipid peroxyl chain termination by NO. However, lipid nitrites are labile and will function as NO donors and antioxidants. Further work on the biological chemistry of nitrites is warranted to define mechanisms of reaction of biological significance.

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References

(1) Brunton, T. L. (1867) On the use of nitrite of amyl in angina pectoris. *Lancet 2*, 97.

(2) Furchgott, R. E. (1999) Endothelium-derived relaxing factor: discovery, early studies, and identifcation as nitric oxide (Nobel Lecture). *Angew. Chem. Int. Ed. 38*, 1870−1880.
(3) Ignarro, L. J. (1999) Nitric Oxide: A unique endogenous signaling molecule in vascular biology (Nobel Lecture). *Angew. Chem. Int. Ed. 38*, 1882−1892.
(4) Murad, F. (1999) Discovery of some of the biological effects of nitric oxide and Its role in cell signaling (Nobel Lecture). *Angew. Chem. Int. Ed. 38*, 1856−1868.
(5) Moncada, S., Palmer, R. M., and Higgs, E. A. (1991) Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol. Rev. 43*, 109−142.
(6) Thatcher, G. R. J., and Weldon, H. (1998) NO problem for nitroglycerin: organic nitrate chemistry and therapy. *Chem. Soc. Rev. 27*, 331−337.
(7) Moya, M. P., Gow, A. J., Califf, R. M., Goldberg, R. N., and Stamler, J. S. (2002) Inhaled ethyl nitrite gas for persistent pulmonary hypertension of the newborn. *Lancet 360*, 141−143.
(8) Burgaud, J. L., Ongini, E., and Del Soldato, P. (2002) Nitric oxide-releasing drugs: a novel class of effective and safe therapeutic agents. *Ann. N. Y. Acad. Sci. 962*, 360−371.
(9) Smith, S., Dringenberg, H. C., Bennett, B. M., Thatcher, G. R., and Reynolds, J. N. (2000) A novel nitrate ester reverses the cognitive impairment caused by scopolamine in the Morris water maze. *Neuroreport 11*, 3883−3886.
(10) Reynolds, J. N., Bennett, B. M., Boegman, R. J., Jhamandas, K., Ratz, J. D., Zavorin, S. I., Scutaru, D., Dumitrascu, A., and Thatcher, G. R. J. (2002) Neuroprotection against Ischemic brain injury conferred by a novel nitrate ester. *Bioorg. Med. Chem. Lett. 12*, 2863−2866.
(11) Marletta, M. A. (1994) Nitric oxide synthase: aspects concerning structure and catalysis. *Cell 78*, 927−930.
(12) Raman, C. S., Li, H., Martasek, P., Kral, V., Masters, B. S., and Poulos, T. L. (1998) Crystal structure of constitutive endothelial nitric oxide synthase: a paradigm for pterin function involving a novel metal center. *Cell 95*, 939−950.
(13) Graziewicz, M., Wink, D. A., and Laval, F. (1996) Nitric oxide inhibits DNA ligase activity: potential mechanisms for NO-mediated DNA damage. *Carcinogenesis 17*, 2501−2505.
(14) Wink, D. A., and Mitchell, J. B. (1998) Chemical biology of nitric oxide: Insights into regulatory, cytotoxic, and cytoprotective mechanisms of nitric oxide. *Free Radical Biol. Med. 25*, 434−456.
(15) Beckman, J. S., and Koppenol, W. H. (1996) Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *Am. J. Physiol. 271*, C1424−C1437.
(16) Halliwell, B., Hu, M. L., Louie, S., Duvall, T. R., Tarkington, B. K., Motchnik, P., and Cross, C. E. (1992) Interaction of nitrogen dioxide with human plasma. Antioxidant depletion and oxidative damage. *FEBS Lett. 313*, 62−66.
(17) Crow, J. P., and Ischiropoulos, H. (1996) Detection and quantitation of nitrotyrosine residues in proteins: in vivo marker of peroxynitrite. *Methods Enzymol. 269*, 185−194.
(18) Ischiropoulos, H. (1998) Biological tyrosine nitration: a pathophysiological function of nitric oxide and reactive oxygen species. *Arch. Biochem. Biophys. 356*, 1−11.
(19) Reiter, C. D., Teng, R. J., and Beckman, J. S. (2000) Superoxide reacts with nitric oxide to nitrate tyrosine at physiological pH via peroxynitrite. *J. Biol. Chem. 275*, 32460−32466.
(20) Byun, J., Mueller, D. M., Fabjan, J. S., and Heinecke, J. W. (1999) Nitrogen dioxide radical generated by the myeloperoxidase-hydrogen peroxide-nitrite system promotes lipid peroxidation of low-density lipoprotein. *FEBS Lett. 455*, 243−246.
(21) Rubbo, H., Radi, R., Trujillo, M., Telleri, R., Kalyanaraman, B., Barnes, S., Kirk, M., and Freeman, B. A. (1994) Nitric oxide regulation of superoxide and peroxynitrite-dependent lipid peroxidation. Formation of novel nitrogen-containing oxidized lipid derivatives. *J. Biol. Chem. 269*, 26066−26075.
(22) Pryor, W. A., Lightsey, J. W., and Church, D. F. (1982) Reaction of nitrogen dioxide with alkenes and polyunsaturated fatty acids: addition and hydrogen abstraction mechanism. *J. Am. Chem. Soc. 104*, 6685−6692.
(23) Gallon, A. A., and Pryor, W. A. (1994) The reaction of low levels of nitrogen dioxide with methyl linoleate in the presence and absence of oxygen. *Lipids 29*, 171−176.
(24) Laskey, R. E., and Mathews, W. R. (1996) Nitric oxide inhibits peroxynitrite-induced production of hydroxyeicosatetraenoic acids and F2-isoprostanes in phosphatidylcholine liposomes. *Arch. Biochem. Biophys. 330*, 193−198.
(25) Hogg, N., and Kalyanaraman, B. (1999) Nitric oxide and lipid peroxidation. *Biochim. Biophys. Acta 1411*, 378−384.
(26) Rubbo, H. (1998) Nitric oxide and peroxynitrite in lipid peroxidation. *Medicina 58*, 361−366.
(27) Rubbo, H., Parthasarathy, S., Barnes, S., Kirk, M., Kalyanaraman, B., and Freeman, B. A. (1995) Nitric oxide inhibition of lipoxygenase-dependent liposome and low-density lipoprotein oxidation: termination of radical chain propagation reactions and formation of nitrogen-containing oxidized lipid derivatives. *Arch. Biochem. Biophys. 324*, 15−25.
(28) O'Donnell, V. B., Chumley, P. H., Hogg, N., Bloodsworth, A., Darley-Usmar, V. M., and Freeman, B. A. (1997) Nitric oxide inhibition of lipid peroxidation: kinetics of reaction with lipid peroxyl radicals and comparison with alpha-tocopherol. *Biochemistry 36*, 15216−15223.
(29) Rubbo, H., and Freeman, B. A. (1996) Nitric oxide regulation of lipid oxidation reactions: formation and analysis of nitrogen-containing oxidized lipid derivatives. *Methods Enzymol. 269*, 385−394.
(30) Gallon, A. A., and Pryor, W. A. (1993) The identification of the allylic nitrite and nitro derivatives of methyl linoleate and methyl linolenate by negative chemical ionization mass spectroscopy. *Lipids 28*, 125−133.
(31) Pryor, W. A., Castle, L., and Church, D. F. (1985) Nitrosation of organic hydroperoxides by nitrogen dioxide/dinitrogen tetraoxide. *J. Am. Chem. Soc. 107*, 211−217.
(32) Lim, D. G., Sweeney, S., Bloodsworth, A., White, C. R., Chumley, P. H., Krishna, N. R., Schopfer, F., O'Donnell, V. B., Eiserich, J. P., and Freeman, B. A. (2002) Nitrolinoleate, a nitric oxide-derived mediator of cell function: synthesis, characterization, and vasomotor activity. *Proc. Natl. Acad. Sci. U.S.A. 99*, 15941−15946.
(33) Nicolescu, A. C., Zavorin, S. I., Turro, N. J., Reynolds, J. N., and Thatcher, G. R. (2002) Inhibition of lipid peroxidation in synaptosomes and liposomes by nitrates and nitrites. *Chem. Res. Toxicol. 15*, 985−998.
(34) Maragos, C. M., Morley, D., Wink, D. A., Dunams, T. M., Saavedra, J. E., Hoffman, A., Bove, A. A., Isaac, L., Hrabie, J. A., and Keefer, L. K. (1991) Complexes of NO with nucleophiles as agents for the controlled biological release of nitric oxide. Vasorelaxant effects. *J. Med. Chem. 34*, 3242−3247.
(35) Barclay, L. R., Vinqvist, M. R., Mukai, K., Goto, H., Hashimoto, Y., Tokunaga, A., and Uno, H. (2000) On the antioxidant mechanism of curcumin: classical methods are needed to determine antioxidant mechanism and activity. *Org. Lett. 2*, 2841−2843.
(36) Barclay, L. R. C., Locke, S. J., MacNeil, J. M., and VanKessel, J. (1985) Quantitative studies of the autoxidation of linoleate monomers sequestered in phosphatidylcholine bilayer. Absolute rate constants in bilayer. *Can. J. Chem. 63*, 2633−2638.
(37) Buckell, F., Hartry, J. D., Rajalingam, U., Bennett, B. M., Whitney, R. A., and Thatcher, G. R. (1994) Hydrolysis of Nitrite Esters: Putative Intermediates in the Biotransformation of Organic Nitrates. *J. Chem. Soc., Perkin Trans. 2*, 401−403.
(38) Artz, J. D., and Thatcher, G. R. (1998) NO release from NO donors and nitrovasodilators: comparisons between oxyhemoglobin and potentiometric assays. *Chem. Res. Toxicol. 11*, 1393−1397.
(39) Artz, J. D., Toader, V., Zavorin, S. I., Bennett, B. M., and Thatcher, G. R. (2001) In vitro activation of soluble guanylyl cyclase and nitric oxide release: a comparison of NO donors and NO mimetics. *Biochemistry 40*, 9256−9264.
(40) Braughler, J. M., Duncan, L. A., and Chase, R. L. (1986) The involvement of iron in lipid peroxidation. Importance of ferric-to-ferrous ratios in initiation. *J. Biol. Chem. 261*, 10282−10289.
(41) Schafer, F. Q., Qian, S. Y., and Buettner, G. R. (2000) Iron and free radical oxidations in cell membranes. *Cell. Mol. Biol. 46*, 657−662.
(42) Minotti, G., and Aust, S. D. (1992) Redox cycling of iron and lipid peroxidation. *Lipids 27*, 219−226.
(43) Goss, S. P., Hogg, N., and Kalyanaraman, B. (1995) The antioxidant effect of spermine NONOate in human low-density lipoprotein. *Chem. Res. Toxicol. 8*, 800−806.
(44) Rikans, L. E., and Hornbrook, K. R. (1997) Lipid peroxidation, antioxidant protection and aging. *Biochim. Biophys. Acta 1362*, 116−127.
(45) Gutteridge, J. M., and Halliwell, B. (1990) The measurement and mechanism of lipid peroxidation in biological systems. *Trends Biochem. Sci. 15*, 129−135.
(46) Liu, J., Yeo, H. C., Doniger, S. J., and Ames, B. N. (1997) Assay of aldehydes from lipid peroxidation: gas chromatography−mass spectrometry compared to thiobarbituric acid. *Anal. Biochem. 245*, 161−166.
(47) Barclay, L. R., and Vinqvist, M. R. (1994) Membrane peroxidation: inhibiting effects of water-soluble antioxidants on phospholipids of different charge types. *Free Radical Biol. Med. 16*, 779−788.

(48) Barclay, L. R. C., Baskin, K. A., Kong, D., and Locke, S. J. (1987) Autoxidation of model membrane. The kinetics and mechanism of autoxidation of mixed phospholipid bilayer. *Can. J. Chem. 65*, 2541−2549.

(49) Barclay, L. R. C., Baskin, K. A., Locke, S. J., and Schaefer, T. D. (1987) Benzophenone-photosensitized autoxidation of linoleate in solution and sodium dodecyl sulfate micelles. *Can. J. Chem. 65*, 2529−2540.

(50) Lu, J. M., Wittbrodt, J. M., Wang, K., Wen, Z., Schlegel, H. B., Wang, P. G., and Cheng, J. P. (2001) NO affinities of s-nitrosothiols: a direct experimental and computational investigation of RS−NO bond dissociation energies. *J. Am. Chem. Soc. 123*, 2903−2904.

(51) Bartberger, M. D., Mannion, J. D., Powell, S. C., Stamler, J. S., Houk, K. N., and Toone, E. J. (2001) S−N dissociation energies of S-nitrosothiols: on the origins of nitrosothiol decomposition rates. *J. Am. Chem. Soc. 123*, 8868−8869.

(52) Mooradian, D. L., Hutsell, T. C., and Keefer, L. K. (1995) Nitric oxide (NO) donor molecules: effect of NO release rate on vascular smooth muscle cell proliferation in vitro. *J. Cardiovasc. Pharmacol. 25*, 674−678.

(53) Barclay, L. R. C., Baskin, K. A., Dakin, K. A., Locke, S. J., and Vinqvist, M. R. (1990) The antioxidant activities of phenolic antioxidants in free radical peroxidation of phospholipid membrane. *Can. J. Chem. 68*, 2258−2269.

(54) Dikalov, S., Kirilyuk, I., and Grigor'ev, I. (1996) Spin trapping of O-, C-, and S-centered radicals and peroxynitrite by 2*H*-imidazole-1-oxides. *Biochem. Biophys. Res. Commun. 218*, 616−622.

(55) Foti, M. C., Johnson, E. R., Vinqvist, M. R., Wright, J. S., Barclay, L. R., and Ingold, K. U. (2002) Naphthalene diols: a new class of antioxidants intramolecular hydrogen bonding in catechols, naphthalene diols, and their aryloxyl radicals. *J. Org. Chem. 67*, 5190−5196.

(56) Cameron, D. R., Borrajo, A. M. P., Bennett, B. M., and Thatcher, G. R. J. (1995) Organic nitrates, thionitrates, peroxynitrites, and nitric oxide: a molecular orbital study of the $RXNO_2 \langle = \rangle RXONO$ (X=O, S) rearrangement, a reaction of potential bilogical significance. *Can. J. Chem. 73*, 1627−1638.

(57) Zhang, D., Zhang, R., Park, J., and North, S. W. (2002) Hydroxy peroxy nitrites and nitrates from OH initiated reactions of isoprene. *J. Am. Chem. Soc. 124*, 9600−9605.

(58) Xian, M., Zhu, X. Q., Lu, J., Wen, Z., and Cheng, J. P. (2000) The first O−NO bond energy scale in solution: heterolytic and homolytic cleavage enthalpies of O-nitrosyl carboxylate compounds. *Org. Lett. 2*, 265−268.

(59) Bennett, B. M., McDonald, B. J., Nigam, R., and Simon, W. C. (1994) Biotransformation of organic nitrates and vascular smooth muscle cell function. *Trends Pharmacol. Sci. 15*, 245−249.

(60) Kowaluk, E. A., and Fung, H. L. (1991) Vascular nitric oxide-generating activities for organic nitrites and organic nitrates are distinct. *J. Pharmacol. Exp. Ther. 259*, 519−525.

(61) van der Vliet, A., Eiserich, J. P., Halliwell, B., and Cross, C. E. (1997) Formation of reactive nitrogen species during peroxidase-catalyzed oxidation of nitrite. A potential additional mechanism of nitric oxide- dependent toxicity. *J. Biol. Chem. 272*, 7617−7625.

(62) Sampson, J. B., Ye, Y., Rosen, H., and Beckman, J. S. (1998) Myeloperoxidase and horseradish peroxidase catalyze tyrosine nitration in proteins from nitrite and hydrogen peroxide. *Arch. Biochem. Biophys. 356*, 207−213.

(63) Giamalva, D. H., Kenion, G. B., Church, D. F., and Pryor, W. A. (1987) Rates and mechanism of reaction of nitrogen dioxide with alkenes in solutions. *J. Am. Chem. Soc. 109*, 7059−7063.

(64) O'Donnell, V. B., Eiserich, J. P., Chumley, P. H., Jablonsky, M. J., Krishna, N. R., Kirk, M., Barnes, S., Darley-Usmar, V. M., and Freeman, B. A. (1999) Nitration of unsaturated fatty acids by nitric oxide-derived reactive nitrogen species peroxynitrite, nitrous acid, nitrogen dioxide, and nitronium ion. *Chem. Res. Toxicol. 12*, 83−92.

(65) O'Donnell, V. B., Eiserich, J. P., Bloodsworth, A., Chumley, P. H., Kirk, M., Barnes, S., Darley-Usmar, V. M., and Freeman, B. A. (1999) Nitration of unsaturated fatty acids by nitric oxide-derived reactive species. *Methods Enzymol. 301*, 454−470.

(66) Goss, S. P., Singh, R. J., Hogg, N., and Kalyanaraman, B. (1999) Reactions of *NO, *NO2 and peroxynitrite in membranes: physiological implications. *Free Radical Res. 31*, 597−606.

(67) Keefer, L. K., Nims, R. W., Davies, K. M., and Wink, D. A. (1996) "NONOates" (1-substituted diazen-1-ium-1,2-diolates) as nitric oxide donors: convenient nitric oxide dosage forms. *Methods Enzymol. 268*, 281−293.

(68) Fitzhugh, A. L., and Keefer, L. K. (2000) Diazeniumdiolates: pro- and antioxidant applications of the "NONOates". *Free Radical Biol. Med. 28*, 1463−1469.

(69) Hogg, N., Struck, A., Goss, S. P., Santanam, N., Joseph, J., Parthasarathy, S., and Kalyanaraman, B. (1995) Inhibition of macrophage-dependent low-density lipoprotein oxidation by nitric-oxide donors. *J. Lipid Res. 36*, 1756−1762.

(70) Goss, S. P., Kalyanaraman, B., and Hogg, N. (1999) Antioxidant effects of nitric oxide and nitric oxide donor compounds on low-density lipoprotein oxidation. *Methods Enzymol. 301*, 444−453.

(71) Williams, D. L. H. (1996) The mechanism of nitric oxide formation from *S*-nitrosothiols (thionitrites). *Chem. Commun.* 1085−1091.

(72) Cooper, C. E. (1999) Nitric oxide and iron proteins. *Biochim. Biophys. Acta 1411*, 290−309.

(73) Doyle, M. P., LePoire, D. M., and Pickering, R. A. (1981) Oxidation of hemoglobin and myoglobin by alkyl nitrites. Inhibition By Oxygen. *J. Biol. Chem. 256*, 12399−12404.

(74) Oh, S. M. N. Y. F., and Williams, D. L. (1991) Nitrosation by alkyl nitrites. Part 7. Comparison with thionitrites: reactions with phenols. *J. Chem. Soc., Perkin Trans. 2*, 685−688.

(75) Iglesias, E., Garcia Rio, L., Leis, J. R., Pena, M. E., and Williams, D. L. H. (1992) Evidence for concerted acid hydrolysis of alkyl nitrites. *J. Chem. Soc. Perkin Trans. 2*, 1673−1679.

(76) Williams, D. L. H. (1988) *Nitrosation*, p 214, Cambridge University Press, Cambridge, New York, Melbourne.

(77) Patel, H. M. S., and Williams, D. L. H. (1989) Nitrosation by alkyl nitrites. Part 3. Reactions with cysteine in water in the pH range 6−13. *J. Chem. Soc., Perkin Trans. 2* 339−341.

(78) Kochi, J. K. (1973) Free radicals. In *Reactive Intermediates in Organic Chemistry* (Olah, G. A., Ed.) p 906, John Wiley & Sons, New York.

(79) Nussbaum, A. L., and Robinson, C. H. (1962) Recent developments in the preparative photolysis of organic nitrites. In *Tetrahedron 17*, 35−39.

(80) Barton, D. H. R., Hesse, R. H., Pechet, M. M., and Smith, L. C. (1979) The mechanism of the Barton reaction. *J. Chem. Soc., Perkin Trans. 1* 1159−1165.

(81) Grossi, L., and Strazzari, S. (1999) A New Synthesis of Alkyl Nitrites: The Reaction of Alkyl Alcohols with Nitric Oxide in Organic Solvents. *J. Org. Chem. 64*, 8076−8079.

(82) Grossi, L. (1997) The photolysis of cycloalkyl nitrite esters: the radical intermediates as studied by EPR. *Tetrahedron 53*, 6401−6410.

(83) Grossi, L. (1997) The photoinduced ring expansion of five membered ring nitrites: a 1,6-exo ring closure process of the intermediate 5-nitrosopentanoyl-type radical. *Tetrahedron 53*, 3205−3214.

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