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Evaluation of nitroalkenes as nitric oxide donors

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Abstract—Chemiluminescence experiments demonstrate that simple nitroalkenes release low levels of nitric oxide. UV and EPR measurements suggest but cannot confirm direct NO release from nitroalkenes. Given the biological activity of nitrated unsaturated fatty acids, these results suggest the possible metabolic conversion of nitroalkenes to NO.

In 1998, Furchgott, Ignarro, and Murad received the Nobel Prize for the identification of nitric oxide (NO) as the endothelium-derived relaxing factor. In vivo generation of NO, a free radical signaling mediator, occurs via the nitric oxide synthase (NOS)-catalyzed, five-electron oxidation of L-arginine.[1] The biological activity of NO results from its ability to activate soluble guanylate cyclase, which converts guanosine triphosphate into cyclic guanosine monophosphate, followed by kinase-mediated signal transduction.[2] NO regulates a number of biological processes including smooth muscle relaxation,[3] immune stimulation,[4] neurotransmission,[5] and platelet aggregation.[6]

The medicinal use of gaseous or aqueous solutions of NO remains difficult due to the instability and inconvenient handling of the solutions. These drawbacks and an increased interest in the importance of NO in biology and medicine have led to the widespread development of compounds that release NO in situ, i.e., NO donors.[7] Currently, a number of different classes of NO-related therapeutic agents (*N*-nitro, *N*-nitroso, *O*-nitro, *O*-nitroso, *S*-nitroso, metal-nitrosyl, *C*-nitroso, and *C*-nitro) are being evaluated in preclinical and clinical studies.

C-nitro compounds, namely nitrated unsaturated fatty acids, have recently emerged as a unique class of signaling agents (Fig. 1). Initial studies revealed the presence of nitrated derivatives of linoleic acid (18:2) and oleic acid (18:1) in high concentrations ($\geqslant$500 nM) in human red blood cells, urine, and plasma, making these compounds the single largest pool of bioactive nitrogen oxides in the vasculature.[8] Additional studies showed that nitrated unsaturated fatty acids act as ligands for the peroxisome proliferator-activated receptor γ (PPARγ),[9] activate endothelial heme oxygenase 1 expression,[10] and covalently modify proteins, i.e., glyceraldehyde-3-phosphate dehydrogenase.[11]

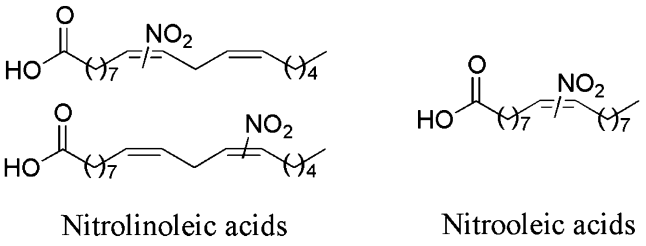

Figure 1. Structures of naturally occurring unsaturated nitrated fatty acids.

Nitrated unsaturated fatty acids also relaxed rat aortic rings with an increase in cyclic guanosine mono-phosphate and inhibition of guanylate cyclase blocked this relaxation suggesting a potential role for NO.[12] Chemiluminescence analysis by three different research groups of the reaction headspace from the incubation of various nitrated unsaturated fatty acids in buffer or buffer/alcohol mixtures revealed the generation of small amounts (<1%) of NO.[12b,13] Electron paramagnetic resonance (EPR) trapping experiments provided evidence of NO formation, but could not be quantified.[12b,13a] Treatment of oxyhemoglobin ($HbFe^{II}$-O_2) with nitrolinoleic acid formed methemoglobin ($HbFe^{III}$) suggesting NO formation.[13a] This rapid conversion does not reconcile with

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the low levels of NO measured by chemiluminescence and may reflect heme oxidation by the organic nitro group.[14] To date, the NO release properties and mechanisms of nitrated unsaturated fatty acids and nitroalkenes remain to be completely described (including our previous report).[13b] Given the intense interest in the biology of these unique molecules, we wished to further characterize the NO donor properties of nitroalkenes with our hypothesis being that simple nitroalkenes, which contain the same functional group as the nitrated unsaturated fatty acids, release NO under similar conditions. We describe the synthesis and NO donor properties of organic nitroalkenes.

Nitroalkenes are readily available and versatile synthetic intermediates in organic chemistry.[15] 1-Nitro-cyclohexene (**1**) was purchased from Sigma–Aldrich and tested as received (Fig. 2). Aromatic nitroalkenes **2** and **3** were synthesized using a two-step method consisting of the condensation (Henry reaction) of benzaldehyde with nitromethane or nitroethane, respectively, followed by dehydration using acetic anhydride and dimethylaminopyridine.[16] Compounds **4** and **5** were synthesized by a similar condensation/dehydration sequence using trifluoroacetic anhydride and triethylamine.[16,17] The nitrated fatty acids (**6**–**7**) were prepared as previously described.[13b]

Chemiluminescence experiments show the ability of simple nitroalkenes to release nitric oxide and/or nitrite, the stable oxidative decomposition product of NO in aqueous solution (Table 1).[18] Solutions of nitroalkenes (50 mM) were incubated in 1:1 EtOH:H_2O at room temperature for 24 h and aliquots of these mixtures were injected into the reaction chamber of a commercial chemiluminescence nitric oxide analyzer (NOA) containing a KI/HOAc solution, which reduces nitrite to NO for detection. The high concentration of nitroalkene, extended incubation time, and organic solvent (to improve substrate solubility) should reveal the presence of either NO or nitrite and these conditions have been utilized in determining the NO releasing properties of nitrated oleic acids.[13b] Under these conditions, nitroalkenes release less than 1% of the possible amount of NO/nitrite (0.016–0.52%, Table 1). Alkyl and aryl nitro compounds (nitro-methane, -ethane, -cyclohexane, and -benzene) do not release NO/nitrite under these conditions after 24 h (data not shown). The amount of observed NO/nitrite varies between compounds and no predictable structural trends emerge. Under these conditions, the nitrated oleic acid derivatives (**6** and **7**) generated significantly less NO/nitrite than the simple nitroalkenes (**1** and **2**, Table 1). Solvent and hydrophobic effects may influence the release of NO/nitrite from these fatty acids as previously noted.[12a]

Table 1. Chemiluminescence detection of NO and nitrite from **1**–**7**

Compound	$[NO + NO_2^-]$[a] (μM)
1	245.34 ± 2.28
2	262.57 ± 7.28
3	8.15 ± 1.23
4	79.59 ± 6.12
5	8.57 ± 1.43
6	15.96 ± 6.12
7	12.30 ± 0.49

[a] Five microliter injection of 50 mM solution (1:1 EtOH:H_2O) after 24-h incubation into KI/HOAc solution in NOA.

Based on these results and its commercial availability, 1-nitro-cyclohexene (**1**) was selected as a model nitroalkene NO donor. Table 2 shows the results of nitrogen oxide analysis under a variety of detection conditions from the room temperature incubation of **1** in 1:1 EtOH:H_2O for 24 h. Direct analysis of the headspace above **1** showed the formation of small amounts of NO. The contents of the chemiluminescence NO detector's reaction chamber dramatically influenced the amount of NO or its oxidation products observed (Table 2). Injection of the incubation solution into phosphate buffer or KI/phosphate buffer failed to produce measurable amounts of NO. However, injection of the incubation solution into KI/HOAc solution or HOAc alone resulted in NO and/or nitrite formation, similar to the above results (Table 2). Addition of a reaction aliquot to a mixture of VCl_3/HCl, used to reduce nitrate to NO, further increased the amount of NO formed (still <1% of the possible amount). In general, increasing the acid and reducing strength of the reaction chamber solution increased the amount of nitrogen oxides formed.

Another group of chemiluminescence experiments gave the time and concentration dependence of NO and/or nitrite release from the incubation of 1-nitro-cyclohexene in buffer (Table 3). Similar to the above results, less than 1% of the overall expected amount of NO/nitrite formed during these reactions (Table 3). Analysis of the reaction headspace again clearly showed the formation of NO within 5 min (15 min for 1 mM, Table 3), but the amounts formed did not linearly increase with either time or concentration. The amount of NO present in solution was smaller for each condition and also did not follow a linear trend for time or concentration. Injection of these solutions into KI/HOAc solution for nitrite analysis resulted in significant (but not linear) increases in NO/nitrite (Table 3). Similar to a previous

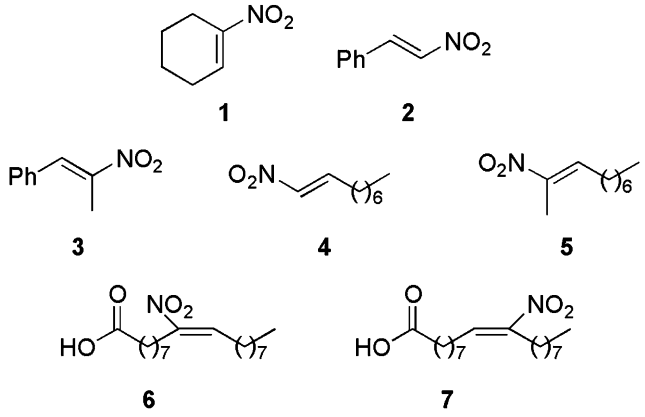

Figure 2. Structures of nitroalkenes used in this study.

Table 2. Chemiluminescence detection of nitrogen oxide release from **1**

Time (h)	NOA[a]	[NO][b] (μM)	$[NO + NO_2^-]$[c] (μM)	$[NO + NO_2^- + NO_3^-]$[c] (μM)
24	H_2O	1.0 ± 0.1	–	–
24	KI/HOAc	–	279.2 ± 2.2	–
24	HOAc	–	179.5 ± 16.8	–
24	PB	–	0	–
24	KI/PB	–	0	–
24	VCl_3/HCl	–	–	405.3 ± 29.9

[a] Contents of NOA reaction chamber; PB, phosphate buffer (pH 7.4, 50 mM).
[b] Fifty microliter injection of the reaction headspace.
[c] Five microliter injection of 50 mM solution of **1** in 1:1 EtOH:H_2O after 24-h incubation.

Table 3. Time- and concentration-dependent NO and nitrite release from **1**

Time (min)	(mM)[a]	[NO][b] (μM)	[NO][c] (μM)	$[NO + NO_2^-]$[d] (μM)
5	1	0	0	0
15	1	0.26	0	3.42 ± 0.36
30	1	0.25 ± 0.06	0	3.75 ± 0.18
60	1	0.26 ± 0.05	0	4.57 ± 0.19
5	10	0.53 ± 0.15	0	12.00 ± 0.18
15	10	0.72 ± 0.02	0.06 ± 0.02	16.85 ± 0.86
30	10	0.99 ± 0.04	0.07 ± 0.02	18.41 ± 4.30
60	10	0.99 ± 0.05	0.09 ± 0.01	18.95 ± 0.70
5	50	0.69 ± 0.10	0.11 ± 0.01	36.10 ± 2.52
15	50	1.15 ± 0.16	–	41.42 ± 3.59
30	50	1.69 ± 0.14	0.20 ± 0.03	57.96 ± 3.48
60	50	2.02 ± 0.05	0.21 ± 0.04	77.65 ± 1.98

[a] Concentration of **1** in deoxygenated phosphate buffer (PB).
[b] Two-hundred and fifty microliters injection of the reaction headspace into deionized water in the NOA.
[c] Five microliters injection of incubation solution into deionized water in the NOA.
[d] Five microliters injection of incubation solution into KI/HOAc solution in NOA.

report, incubation of **1** with the NO-specific trap carboxy-PTIO resulted in the loss of the EPR signal preventing accurate NO detection or quantification (data not shown).[12b]

Ultraviolet (UV) spectroscopy in the presence of oxy- and deoxyhemoglobin was further employed to explore the NO releasing reactions of **1**. NO reacts with deoxyhemoglobin ($HbFe^{II}$) to cleanly form iron-nitrosyl-hemoglobin ($HbFe^{II}$-NO) and with oxyhemoglobin ($HbFe^{II}$-O_2) to form methemoglobin ($HbFe^{III}$). The absorbance spectrum for the reaction of **1** (50 mM) and $HbFe^{II}$ (70 μM) in phosphate buffer (pH 7.4, 50 mM) over 1 h showed a decrease in the absorbance at 554 nm with an increase in absorbance at 540 and 573 nm characteristic of $HbFe^{II}$-NO (Fig. 3). Careful examination of this spectrum revealed the formation of methemoglobin (increase of absorbance at 630 nm), which indicates separate redox chemistry opposed to simple NO release from **1** (Fig. 3). EPR spectroscopic analysis of this reaction mixture showed the characteristic absorbance of an iron nitrosyl complex (data not shown). These results appear reminiscent of the reaction of nitrite with deoxyhemoglobin where the ferrous iron reduces nitrite producing NO.[19] Reaction of $HbFe^{II}$-O_2 (70 μM) with **1** (50 mM) in phosphate buffer (pH 7.4, 50 mM) showed a decrease in absorbance at 540 and 576 nm with a concomitant increase in absorbance at 630 characteristic of $HbFe^{III}$ formation over 1 h, similar to the reported reaction of nitrolinoleic acid with oxyhemoglobin (Fig. 4).[13a] Similar results were obtained

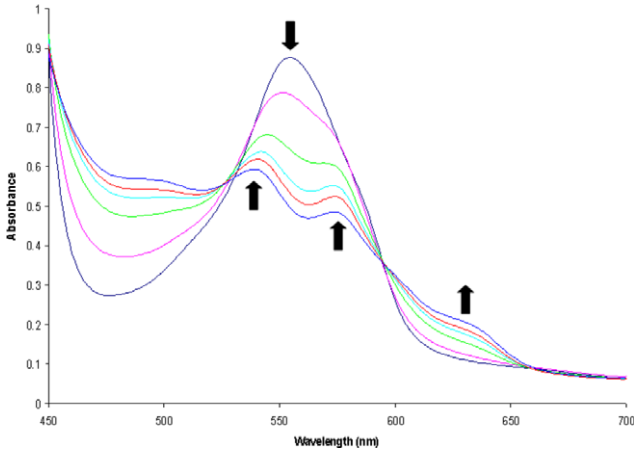

Figure 3. UV spectrum of the reaction of **1** and deoxyhemoglobin in phosphate buffer.

using a lower concentration of **1** (500 μM). While these results may indicate NO release, the rapid oxidation of oxyhemoglobin to methemoglobin does not match with the small amounts of NO measured by chemiluminescence detection (Tables 1–3). Other direct chemistry between the nitroalkene and heme iron cannot be excluded at this time.

Mechanistically, NO release from nitroalkenes remains speculative. Similar to nitrated alcohols (nitroglycerin) and nitrite, the release of NO from nitroalkenes chemically requires reduction. Currently, two mechanisms

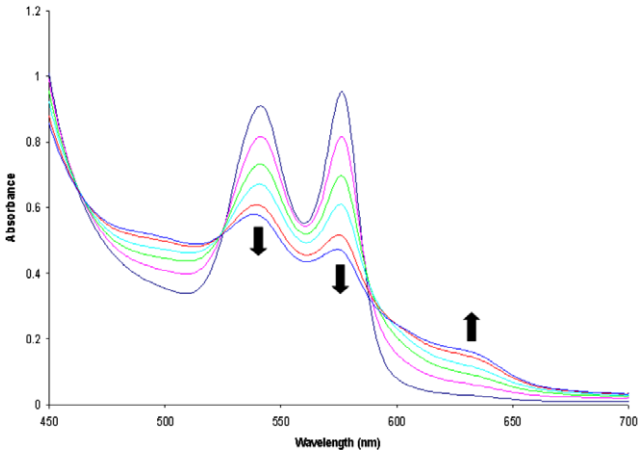

Figure 4. UV spectrum of the reaction of **1** and oxyhemoglobin in phosphate buffer.

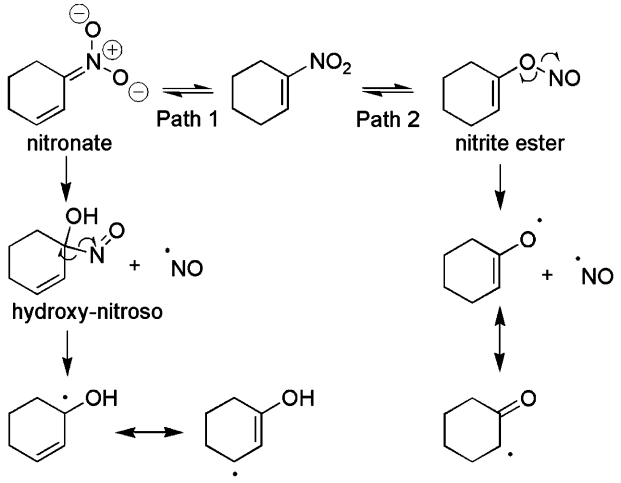

Figure 5. Proposed mechanisms for NO release from nitrated fatty acids—modified Nef reaction (Path 1) and nitroalkene rearrangement (Path 2).

have been proposed to account for NO release from nitrated unsaturated fatty acids (Fig. 5). The first evokes a modified Nef reaction, a process that normally converts nitroalkanes to the corresponding aldehyde or ketone and nitroxyl (HNO). HNO quickly dimerizes and dehydrates to nitrous oxide (N_2O). In the modified Nef sequence, abstraction of an acidic allylic proton yields the nitronate that subsequently forms a hydroxy-nitroso species that undergoes homolytic bond cleavage to form NO and an allylic radical. The second mechanism relies upon nitroalkene rearrangement to a nitrite ester followed by N–O bond homolysis to form NO and a radical.[12b] Both mechanisms produce radicals as the final carbon product and 3,5-dibromo-4-nitrosobenzene sulfonate (DBNBS), a water soluble spin trap for carbon-centered radicals,[20] has been used to detect a lipid-derived radical during nitrolinoleic acid decomposition.[12b] Time-dependent EPR spectroscopy of the mixture of **1** (10 mM) with DBNBS (10 mM) in phosphate buffer (pH 7.4, 50 mM) at room temperature over 0.5 h showed a six-line EPR signal consistent with the trapping of a secondary carbon-centered radical

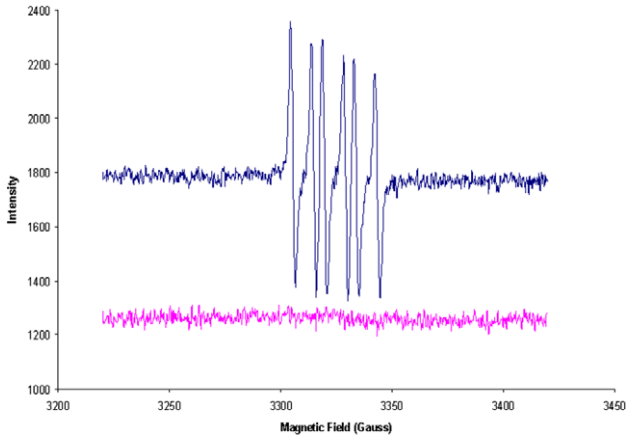

Figure 6. EPR spectrum of the reaction of **1** and DBNBS in phosphate buffer (top line) and the EPR spectrum of DBNBS in phosphate buffer (bottom line).

(Fig. 6). The mixture of DBNBS and NO-saturated phosphate buffer gave a much weaker intensity three-line signal that disappeared over 0.5 h confirming the observed signal was not due to reactions of the trap and NO. These results support the formation of carbon radicals during the decomposition of nitroalkenes to NO but do not distinguish the two proposed pathways. Other experiments provide evidence that disfavors a modified Nef reaction. First, neither cyclohexenone (NMR) nor N_2O (gas chromatography), the normal Nef reaction products, was identified from the incubation of **1** in AcOH or KI/AcOH. Moreover, the styrene derivatives **2** and **3** do not contain acidic allylic hydrogens and cannot undergo the Nef reaction. However, these compounds released NO/nitrite and these results strongly suggest other pathways besides a Nef reaction may account for NO release. The final carbon end products of these reactions (or nitrated unsaturated fatty acids) have not been identified likely due to the low percent conversion and also the formation of reactive radical intermediates.

In summary, nitroalkenes release NO in very low yields as determined by chemiluminescence methods. Other spectroscopic methods do not clearly identify these compounds as spontaneous NO donors, especially compared to known NO donors, such as diazonium diolates.[21] At these levels of NO formation, low-level, analytically difficult to detect impurities (nitrite, organic nitrites) in the samples cannot be eliminated as the ultimate NO source. While these results may indicate that **1** decomposes into NO, nitrite, and/or nitrate, it appears plausible that the highly acidic and reducing analytical conditions trigger NO-releasing chemistry. Such results should be kept in mind during the chemiluminescence analysis of biological samples for nitrated unsaturated fatty acids or any other species. Given the ability of nitrated unsaturated fatty acids to relax pre-constricted rat aortas and the structural similarity of *C*-nitro and *O*-nitro compounds (nitroglycerin), these results may suggest reductive or hydrolytic (to nitrite) metabolic pathways of NO formation in vivo. Studies with compound **1** do not necessarily eliminate the possibility of some unique structural

features of the nitrated unsaturated fatty acids to promote NO formation. Clearly, more work, which we are currently pursuing, must be achieved to further define the ability and mechanism of nitroalkenes (including nitrated unsaturated fatty acids) to act as NO donors.

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Supplementary data

Experimental procedures for the syntheses of **2**–**5** and DBNBS as well as details about chemiluminescence, EPR, and UV experiments are supplied. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.01.016.

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