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Bioorganic & Medicinal Chemistry Letters 17 (2007) 2013–2017
Bioorganic & Medicinal Chemistry Letters

Evaluation of nitroalkenes as nitric oxide donors

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Received 3 October 2006; revised 4 January 2007; accepted 8 January 2007
Available online 17 January 2007

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.
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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]

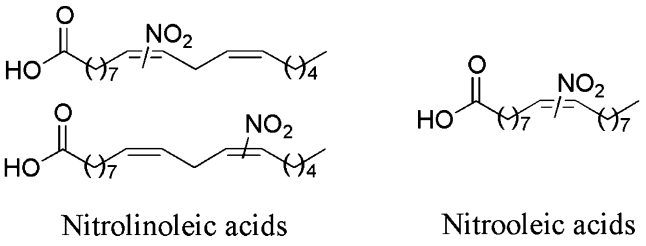

Figure 1. Structures of naturally occurring unsaturated nitrated fatty acids.

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

Keywords: Nitric oxide; Nitric oxide donors; Nitroalkenes; Free radicals.
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0960-894X/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2007.01.016

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

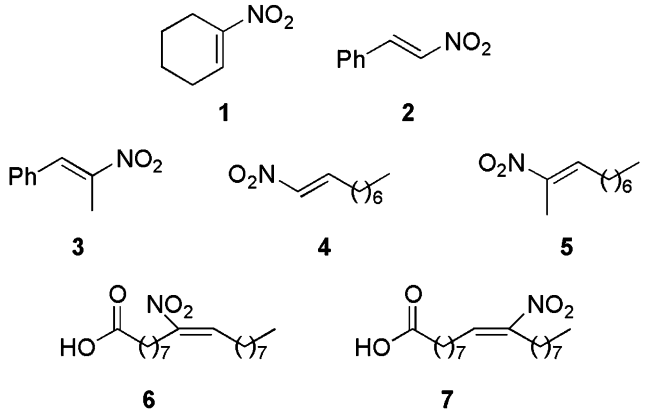

Figure 2. Structures of nitroalkenes used in this study.

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