ORGANIC LETTERS

2007
Vol. 9, No. 22
4551−4554

# Nitric Oxide Prodrugs: Diazeniumdiolate Anions of Hindered Secondary Amines

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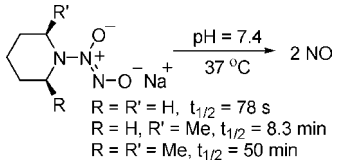
**Received August 15, 2007**

## ABSTRACT




**Nitric oxide prodrugs derived from hindered secondary amines were prepared. The decomposition patterns of these prodrugs indicate that α-methyl groups around the nitrogen bearing the diazeniumdiolate group prolong their half-life in aqueous buffer.**

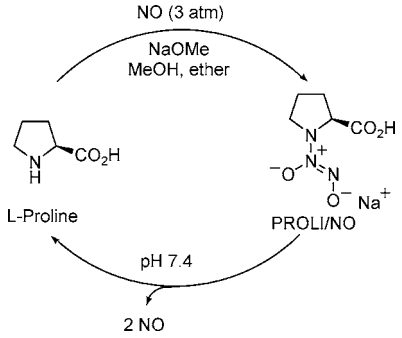
Nitric oxide (NO) participates in a plethora of physiological processes such as regulation of blood pressure, platelet aggregation, neurotransmission, and immune response.[1] Due to the difficulty of performing meaningful biological studies with nitric oxide gas, its progenitors (NO donors) are typically utilized in studies investigating such diverse effects.[2] Several classes of NO donors are known, most with shortcomings.[2] For example, the decomposition of thionitrites to nitric oxide is not spontaneous.[3] Organic nitrates require complex metabolic activation to display NO-like activity, which is poorly understood.[4]

Diazeniumdiolate-based nitric oxide donors are highly effective at delivering nitric oxide at definitive rates with a known mechanism.[4,5] For example, PROLI/NO spontaneously dissociates in buffer to generate nitric oxide with a half-life of 2 s (Scheme 1).[6] Furthermore, diazeniumdiolates can also be easily derivatized into prodrug forms to facilitate localization and site-directed delivery of NO for biomedical applications.[5]

The nature of byproducts formed during the delivery of the desired pharmacological agent is a critical determinant of the scope and utility of any prodrug. Under physiological conditions, in addition to NO and L-proline, PROLI/NO forms *N*-nitrosoproline (NPRO) as a minor byproduct (Scheme 2).[7]

Nitrosamines such as NPRO have been extensively studied for their carcinogenic activity.[8] Using a rodent model, by comparison of the extent of tumor growth and the time taken

**Scheme 1.** PROLI/NO, a Nitric Oxide Donor




† Chemistry Section, Laboratory of Comparative Carcinogenesis.
‡ Basic Research Program, SAIC Frederick.

(1) (a) Ignarro, L. J. *Angew. Chem., Int. Ed.* **1999**, *38*, 1882−1892. (b) Furchgott, R. F. *Angew. Chem., Int. Ed.* **1999**, *38*, 1870−1880. (c) Moncada, S.; Palmer, R. M. J.; Higgs, E. A. *Pharmacol. Rev.* **1991**, *43*, 109−142.
(2) (a) Wang, P. G.; Xian, M.; Tang, X.; Wu, X.; Wen, Z.; Cai, T.; Janczuk, A. J. *Chem. Rev.* **2002**, *102*, 1091−1134. (b) Megson, I. L.; Webb, D. J. *Expert Opin. Invest. Drugs* **2002**, *11*, 587−601.
(3) Stamler, J. S.; Toone, E. J. *Curr. Opin. Chem. Biol.* **2002**, *6*, 779−785.
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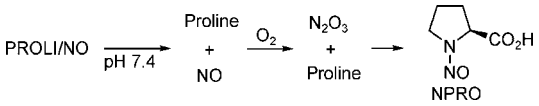
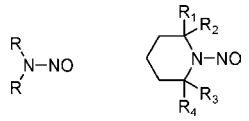
10.1021/ol7019636 CCC: $37.00 
**Published on Web 10/05/2007**

**Scheme 2.** Proposed Mechanism of *N*-Nitrosoproline (NPRO) Formation from PROLI/NO




for such an effect to be observed after chronic treatment with comparable doses of nitrosamine, a relative carcinogenicity scale was derived by Lijinsky (Table 1).[9] For example,

**Table 1.** Relevant Reported Chemical and Biological Data for Selected Nitrosamines (from References 9 and 11)




| R | $R_1$ | $R_2$ | $R_3$ | $R_4$ | relative carcinogenticity[a] | relative rate of nitrosation[b] |
|---|---|---|---|---|---|---|
| Et | – | – | – | – | ++++ | – |
| $^i$Pr | – | – | – | – | + | – |
| – | H | H | H | H | +++ | 1 |
| – | H | Me | H | H | ++ | 0.16 |
| – | H | Me | H | Me | 0 | 0.062 |
| – | Me | Me | Me | Me | 0 | 0.0068 |
| NPRO | | | | | 0 | – |

[a] Chronic administration of comparable doses of nitrosamine in the drinking water of rats was used to derive this scale. [b] Rate constants for nitrosation of the corresponding amine were determined for equimolar (0.2 M) amine/nitrite mixtures at pH 4.1 (acetate buffer) at 27 °C.

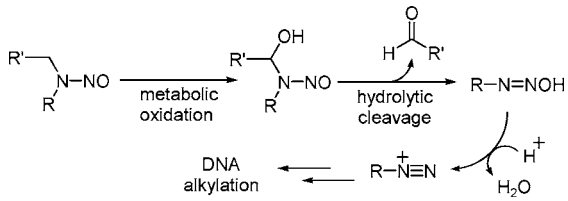
NPRO was found to display no carcinogenic activity in numerous animal models[10] and is assigned a value of 0 in this scale.[9]

Due to its short half-life under physiological conditions, PROLI/NO is not a suitable source of nitric oxide for every experiment. Hence, we sought to prepare diazeniumdiolate ions that upon decomposition release quantitative amounts of nitric oxide with a range of half-lives with concomitant formation of innocuous byproducts.

A notable trend in the structure−carcinogenicity relationship of nitrosamines is that increasing substitution around the *N*-nitroso group decreases its potency (Table 1).[8,9] For example, among piperidine derivatives, the carcinogenic potential diminishes significantly when substituted with methyl groups in the 2- and 6-positions.

DNA alkylation, one of the recognized pathways for nitrosamine carcinogenicity, is proposed to occur through metabolic oxidation of the α-position of nitrosamines (Scheme 3).[8] The introduction of substituents α to the

**Scheme 3.** A Mechanism for DNA Alkylation by Nitrosamines




*N*-nitroso group appears to hinder such oxidation, contributing to diminished carcinogenic potential of such compounds.

The formation of nitrosamines during the decomposition of diazeniumdiolate ions is proposed to occur through *N*-nitrosation of the amine byproduct (Scheme 2). It is reported that the presence of α-methyl substituents retarded *N*-nitrosation of substituted piperidines, presumably by hindering electrophilic addition to the amine (Table 1).[11] For example, under similar conditions, the rate of *N*-nitrosation of piperidine is 16-fold faster than that of 2,6-dimethylpiperidine.[11] Hence, the propensity to form a nitrosamine as a byproduct of diazeniumdiolate decomposition could decrease with increasing substitution around the nitrogen bearing the diazeniumdiolate group. Thus, the synergistic effects of retarded *N*-nitrosation rates of the parent amine and diminished carcinogenic potential of the ensuing nitrosamine should lead to NO donor candidates with a potentially favorable toxicological profile.

Starting from *cis*-2,6-dimethylpiperidine, the desired diazeniumdiolate ion **1** was prepared by using standard diazeniumdiolation conditions (Scheme 4). But these conditions failed to produce the diazeniumdiolate of bis(isopropyl)amine.[12] A stainless steel high-pressure reactor was employed to obtain **2** by exposing bis(isopropyl)amine to 27 atm of NO over 5 days (Scheme 4). 2,2,6,6-Tetramethylpiperidine, however, failed to react even after prolonged exposure to such elevated pressures of NO.

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(8) (a) Lijinsky, W. *Chemistry and Biology of N-Nitroso Compounds*; Cambridge University Press: Cambridge, UK, 1992. (b) Lijinsky, W. *Cancer Metastasis Rev.* **1987**, *6*, 301−356. (c) Lijinsky, W. In *Chemical Carcinogenesis*; Plenum Publishing Corp.: New York, 1988; pp 639−647.

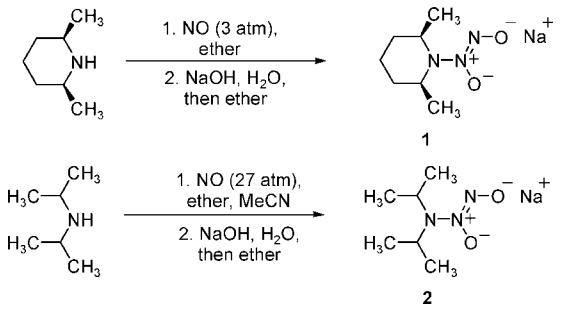
(9) Lijinsky, W. In *Genotoxicology of N-Nitroso Compounds*; Rao, T. K., Lijinsky, W., Epler, J. L., Eds.; Plenum Publishing Corp.: New York, 1984; pp 192−195.

(10) (a) Brunnemann, K. D.; Enzmann, H. G.; Perrone, C. E.; Iatropoulos, M. J.; Williams, G. M. *Arch. Toxicol.* **2002**, *76*, 606−612. (b) Negishi, T.; Shiotani, T.; Fujikawa, K.; Hayatsu, H. *Mutat. Res.* **1991**, *252*, 119−128. (c) Mirvish, S. S.; Bulay, O.; Runge, R. G.; Patil, K. *J. Natl. Cancer Inst.* **1980**, *64*, 1435−1442. (d) Nixon, J. E.; Wales, J. H.; Scanlan, R. A.; Bills, D. D.; Sinnhuber, R. O. *Food Cosmetics Toxicol.* **1976**, *14*, 133−135.

(11) Jones, A. R.; Lijinsky, W.; Singer, G. M. *Cancer Res.* **1974**, *34*, 1079−1081.

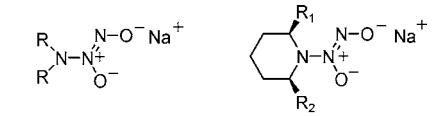
(12) A similar result was reported in: Drago, R. S.; Karstetter, B. R. *J. Am. Chem. Soc.* **1961**, *83*, 1819−1822.

**Scheme 4.** Synthesis of Diazeniumdiolates of Hindered Secondary Amines




In an effort to study decomposition patterns, these compounds were placed in 0.1 M pH 7.4 phosphate buffer at 37 °C (Table 2).[5f,13] Figure 1 illustrates the change in intensity of the absorbance maximum (248 nm) corresponding to the diazeniumdiolate group of **1**. Furthermore, to investigate any neighboring group effects, homologous acyclic amine- and piperidine-based diazeniumdiolate ions **3**−**5** were prepared by reported procedures.[12−14] Their rates of decomposition and calculated half-lives that were determined under similar conditions are listed in Table 2.

**Table 2.** Kinetic Parameters for Diazeniumdiolate Anion Decomposition[a]



DMA/NO, **3**, R = Me
DEA/NO, **4**, R = Et

**5**, $R_1 = R_2 = H$
**6**, $R_1$ = Me, $R_2$ = H

| compd | $k \times 10^4$ ($s^{-1}$)[b] | half-life |
|---|---|---|
| **1** | 2.3 | 50 min |
| **2** | 27 | 4.3 min |
| DMA/NO, **3** | 290 | 24 s |
| DEA/NO, **4** | 59 | 117 s |
| **5** | 89 | 78 s |
| **6** | 14 | 8.3 min |

[a] Based on first-order decomposition in 0.1 M pH 7.4 phosphate buffer at 37 °C. [b] Average value of duplicate experiments.

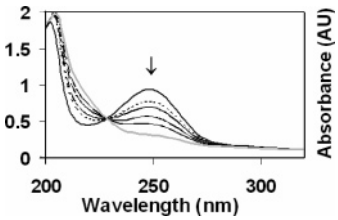



**Figure 1.** Change in UV−visible spectral features during decomposition of **1** in 0.1 M pH 7.4 phosphate buffer at 37 °C.

The half-life of diazeniumdiolate decomposition was prolonged with increasing α-substitution around the nitrogen bearing the diazeniumdiolate group. For example, moving from DMA/NO (**3**) to DEA/NO (**4**) to **2**, the half-life of NO release increased from 24 s to 117 s to 4.3 min (Table 2). The effect of α-methyl substitution is accentuated in substituted piperidines. The first-order rate constants for decomposition of **5**, **6**, and **1** were determined as $8.9 \times 10^{-3}$, $1.4 \times 10^{-3}$, and $2.3 \times 10^{-4}$ $s^{-1}$, respectively, that corresponded to half-lives of 78 s, 8.3 min, and 50 min (Table 2).

In a separate set of experiments, nitric oxide release was studied by using a chemiluminescence assay described earlier.[5f] Nitric oxide release from **1** (Figure 2) was found to be first order with a rate constant of $2.7 \times 10^{-4}$ $s^{-1}$. Under similar conditions, the rate constant of NO release from **2** was determined to be $3.5 \times 10^{-3}$ $s^{-1}$.

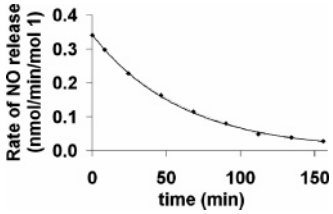



**Figure 2.** Time course of nitric oxide release from **1** in 0.1 M pH 7.4 phosphate buffer at 37 °C.

Excellent yields of nitric oxide from **1** (96%) and **2** (98%) were observed. The predictable release of nitric oxide and the accompanying high yield of NO from **1** and **2** suggest that nitrosamine formation is a minor process. Altogether, the nitric oxide donor profiles of PROLI/NO, **1**, and **2** are amenable to biological studies that require NO release for durations ranging from a few seconds to an hour.

Our laboratory and others have prepared numerous $O^2$-derivatives of diazeniumdiolates to facilitate site-directed delivery of nitric oxide.[15] The conversion of the diazeniumdiolate ions prepared in this study into their corresponding $O^2$-protected forms was accomplished by treatment with dimethyl sulfate or 1-fluoro-2,4-dinitrobenzene (FDNB) (Table 3). The $O^2$-aryl derivatives **8**, **10**, and **11** are designed to target glutathione-*S*-transferase, an important detoxification enzyme that is frequently overexpressed in cancer tissue.[16]

The proposed mechanism of dissociation of diazeniumdiolate ions of secondary amines is initial protonation of the nitrogen bearing the diazeniumdiolate group, followed by

(13) Saavedra, J. E.; Srinivasan, A.; Bonifant, C. L.; Chu, J.; Shanklin, A. P.; Flippen-Anderson, J. L.; Rice, W. G.; Turpin, J. A.; Davies, K. M.; Keefer, L. K *J. Org. Chem.* **2001**, *66*, 3090−3098.
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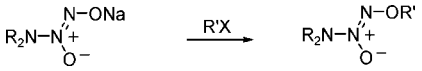
(15) (a) Chakrapani, H.; Showalter, B. M.; Kong, L.; Keefer, L. K.; Saavedra, J. E. *Org. Lett.* **2007**, *9*, 3409−3412. (b) Cai, T. B.; Lu, D.; Landerholm, M.; Wang, P. G. *Org. Lett.* **2004**, *6*, 4203−4205. (c) Saavedra, J. E.; Dunams, T. M.; Flippen-Anderson, J. L.; Keefer, L. K. *J. Org. Chem.* **1992**, *57*, 6134−6138.

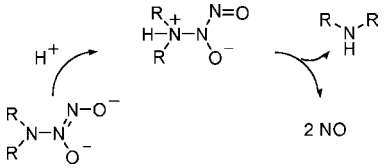
**Table 3.** Preparation of Some Diazeniumdiolate Prodrug Forms




| $R_2N-N_2O_2Na$ | R′X | R′ | product | yield (%) |
|---|---|---|---|---|
| **1** | $Me_2SO_4$ | Me | **7** | 80 |
| **1** | FDNB | 2,4-dinitrophenyl | **8** | 13 |
| **2** | $Me_2SO_4$ | Me | **9** | 33 |
| **2** | FDNB | 2,4-dinitrophenyl | **10** | 19 |
| **6** | FDNB | 2,4-dinitrophenyl | **11** | 31 |

decomposition to produce 2 mol of NO per mole of diazeniumdiolate (Scheme 5).[17]

**Scheme 5.** Proposed Mechanism of Nitric Oxide Release from Diazeniumdiolate Anions




The rate constants for decomposition and NO release are comparable, suggesting that NO release is the major reaction pathway for **1** and **2** in buffer. The nearly quantitative yield of nitric oxide indicates that nitrosamine is a minor byproduct of the decomposition of **1** and **2**. The α-methyl groups could block hydrolytic access to the diazeniumdiolate group of **1**, which would account for the observed diminished rate of decomposition and nitric oxide release. The reported p$K_a$ values of *N*-methyl-*cis*-2,6-dimethylpiperidine (9.35) and *N*-methylpiperidine (10.08) suggest a steric effect on the protonation of *N*-substituted piperidines (Figure 3).[18]

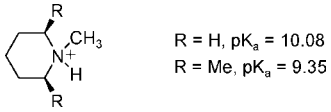



**Figure 3.** p$K_a$ values of some *N*-methylpiperidinium ions (ref 18).

A similar effect, but presumably lower in magnitude, could be operational in the acyclic diazeniumdiolate series that would rationalize the lower rate of decomposition of **2** in comparison with DMA/NO and DEA/NO.

**Acknowledgment.** This research was supported in part by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research, as well as by National Cancer Institute contract N01-CO-12400 to SAIC.

**Supporting Information Available:** Preparative procedures, analytical data for new compounds, and NMR spectra of all compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

OL7019636

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