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Cell-Permeable Esters of Diazeniumdiolate-Based Nitric Oxide Prodrugs

Harinath Chakrapani,*,† Anna E. Maciag,‡ Michael L. Citro,‡ Larry K. Keefer,† and Joseph E. Saavedra*,‡

Chemistry Section, Laboratory of Comparative Carcinogenesis, National Cancer Institute at Frederick, Frederick, Maryland 21702, and Basic Research Program, SAIC-Frederick, National Cancer Institute at Frederick, Frederick, Maryland 21702

chakrah@ncifcrf.gov; saavj@ncifcrf.gov

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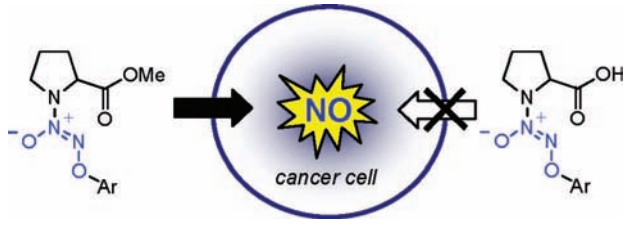
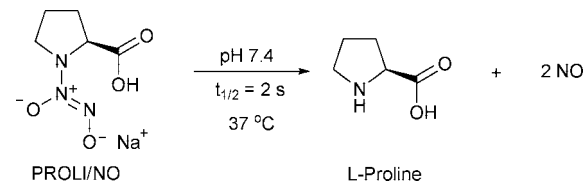
ABSTRACT



Although *O*²-(2,4-dinitrophenyl) derivatives of diazeniumdiolate-based nitric oxide (NO) prodrugs bearing a free carboxylic acid group were activated by glutathione to release NO, these compounds were poor sources of intracellular NO and showed diminished antiproliferative activity against human leukemia HL-60 cells. The carboxylic acid esters of these prodrugs, however, were found to be superior sources of intracellular NO and potent inhibitors of HL-60 cell proliferation.

Nitric oxide (NO) donors of the diazeniumdiolate class are routinely used as sources of nitric oxide for chemical and biological applications.[1] For example, the 1-[2-(carboxylato)pyrrolidin-1-yl]diazen-1-ium-1,2-diolate (PROLI/NO) anion dissociates in pH 7.4 phosphate buffer to form nitric oxide with a half-life of 2 s (Scheme 1).[2]

Scheme 1. Dissociation of PROLI/NO to Form NO



Under aerobic conditions, one of the possible byproducts of PROLI/NO decomposition is *N*-nitrosoproline (NPRO, Scheme 2).[2e]

In contrast to most other *N*-nitrosamines,[3] which are potent carcinogens, NPRO showed no carcinogenic activity at a

† Laboratory of Comparative Carcinogenesis.
‡ SAIC-Frederick.

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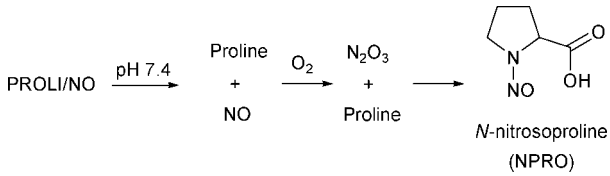
Scheme 2. Proposed Mechanism for the Formation of NPRO from the Decomposition of PROLI/NO under Aerobic Conditions



range of dosages in numerous animal models.[2e,4] For example, *N*-nitrosodiethylamine was shown to induce tumors in a rat model in different organs and sites (Table 1, entry 2).[3b]

Table 1. *N*-Nitrosamine-Induced Carcinogenesis in a Rat Model[a]

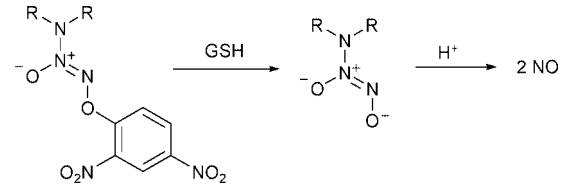
entry	*N*-nitroso	% of treated animals with tumors (organ developing tumors)
1	proline (NPRO)[b]	0
2	diethylamine[c]	95 (esophagus), 65 (liver)
3	pyrrolidine[d]	100 (liver)
4	piperidine[d]	100 (nasal), 67 (esophagus), 27 (liver)
5	pipecolic acid[d]	0
6	isonipecotic acid[d]	0

[a] Administrated through drinking water; ref 3b. [b] Total dose was 100 mmol/animal. [c] Total dose was 1.0 mmol/animal. [d] Total dose was 3.9−4.6 mmol/animal.

However, even at 100-fold higher doses than those of *N*-nitrosodiethylamine, NPRO showed no evidence of tumor formation (Table 1, entry 1).[3b] Not just NPRO but other *N*-nitrosamines with a carboxylic acid group were reported to show no carcinogenic activity in a rodent model (Table 1, entries 5 and 6).[3b] Thus, the use of diazeniumdiolate anions with a carboxylic acid functionality (such as PROLI/NO) would be preferable in clinical settings due to the formation of relatively innocuous byproducts.

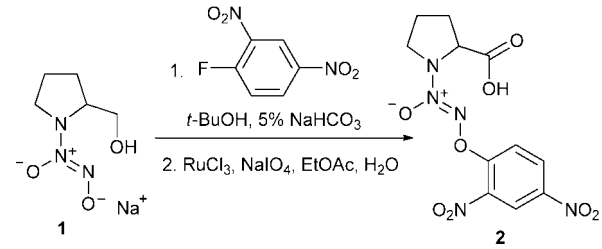
O^2-Derivatization of diazeniumdiolate anions using a suitable protective group facilitates site-directed delivery of nitric oxide.[5] For example, O^2-(2,4-dinitrophenyl) diazeniumdiolates are reported to be activated by glutathione (GSH) to form NO (Scheme 3).[6] Glutathione is an essential component of the biochemical machinery, and its intracellular distribution ranges from 0.1−10 mM.[7]

Scheme 3. Glutathione-Activated Nitric Oxide Prodrugs



Earlier, we prepared the O^2-(2,4-dinitrophenyl) derivative of PROLI/NO, **2**, from diazeniumdiolate salt **1** in two steps (Scheme 4).[8]

Scheme 4. Synthesis of **2** from **1**



Using a similar procedure, carboxylic acids **3**[9] and **4** were prepared (Figure 1).

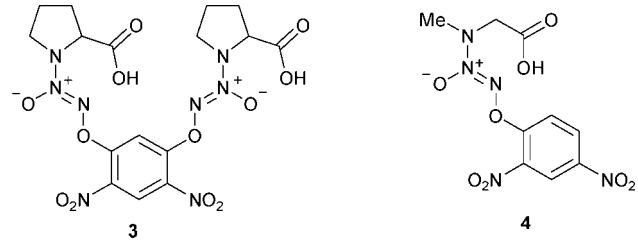


Figure 1. Compounds **3** and **4**.

A chemiluminescence assay was used to study glutathione-activated nitric oxide formation in aqueous buffer (Table 2). Next, the intracellular NO release by these compounds was determined using the nitric oxide-sensitive fluorophore, 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM diacetate);[10] briefly, human leukemia HL-60 cells were preloaded with DAF-FM diacetate, followed by treatment with DMSO solutions of **2**, **3**, and **4**, and fluorescence

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measurements after 40 min provided estimates of levels of intracellular NO (Table 2).

Table 2. Nitric Oxide Release, Fluorescence Measurements, and in Vitro Anti-Proliferative Activity

compd	nitric oxide yield (%)[a]	fluorescence[c] ± S. D.[d] (a.u.)	relative fluorescence (%)	IC_{50} (μM)[e]
DMSO	0	7.7 ± 0.25	100	–
2	100	9.5 ± 0.13	124	>20[f]
3	87[b]	7.9 ± 0.09	103	9.6[g]
4	96	7.4 ± 0.18	96	>20
5	98	17.0 ± 0.49	221	5.4
6	100[b]	28.9 ± 1.26	375	2.7
7	100	17.0 ± 0.49	221	6.0
8	100	24.3 ± 0.85	315	3.4
9	100	26.7 ± 0.85	346	4.8
10	99	24.9 ± 0.70	324	1.4

[a] Nitric oxide yields from the decomposition of the compound (50−100 μL of a 0.1 mM DMSO solution) in the presence of GSH (3.6 mM) in 0.1 M phosphate buffer (3.5 mL) containing 50 μM diethylenetriamine pentaacetic acid (DTPA) at pH 7.4 and 37 °C as measured by chemiluminescence. [b] Calculated based on 4 moles of NO per mole of compound. [c] Mean intracellular NO release measured using the NO-sensitive DAF-FM diacetate dye in HL-60 cells measured in arbitrary units (a.u.). [d] Standard deviations of fluorescence measurements (three independent experiments). [e] The 50% inhibitory concentrations are reported for activity against proliferation of HL-60 cells. [f] Ref 11. [g] Ref 9.

While they were excellent sources of nitric oxide in the presence of glutathione in aqueous phosphate buffer, the carboxylic acids **2**, **3**,[9] and **4** did not form significantly higher levels of intracellular NO than the DMSO control (Table 2). These intracellular NO release observations are consistent with their diminished ability to inhibit in vitro proliferation of human leukemia HL-60 cells (Table 2).[9,11]

To improve cell permeability, it was envisaged that a free carboxylic acid group be masked as an ester. As a neutral, nonionizable species, an ester should be able to cross the cell membrane.[12] Subsequent intracellular ester hydrolysis and glutathione activation should generate the spontaneously nitric oxide-forming diazeniumdiolate anion, which upon decomposition would generate a secondary amine linked to a carboxylic acid such as L-proline (Figure 2).

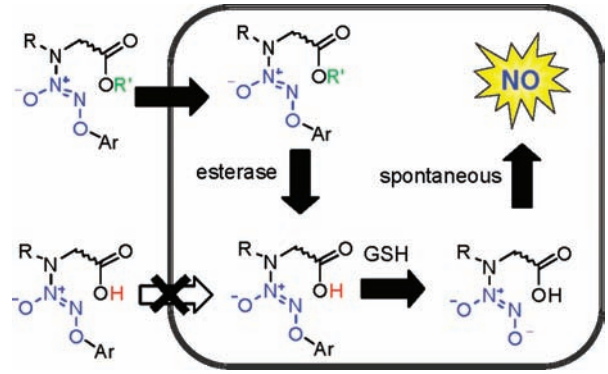
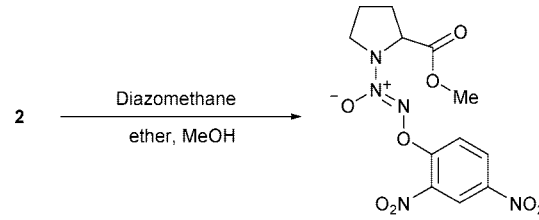


Figure 2. Design of cell-permeable nitric oxide prodrugs.

Accordingly, **5**, the methyl ester of **2**, was prepared by treating the carboxylic acid with diazomethane (Scheme 5).

Scheme 5. Synthesis of **5** by Methylation of **2**



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Using a similar procedure, compounds **6** and **7** were prepared from **3** and **4**, respectively (Figure 3). Next, diazeniumdiolation of the requisite secondary amines, followed by arylation, produced esters of isonipecotic acid, **8** and **9**, and nipecotic acid, **10** (Figure 3).

Glutathione-activated nitric oxide release for these esters was determined. Quantitative nitric oxide yields from a majority of the prodrug esters were observed (Table 2). Then, these esters were tested for their ability to deliver nitric oxide intracellularly using the DAF-FM diacetate assay. All the esters were found to release much higher levels of intracellular NO than their carboxylic acid counterparts (Table 2). Under these conditions, the prodrug **6**, which released 4 moles of NO in the presence of GSH, produced the most intracellular nitric oxide. A plot of the relative levels of intracellular NO shows that esters **8**, **9**, and **10** formed nearly 3-fold higher levels of NO than **2** (Figure 4).

Finally, the ability of these compounds to inhibit proliferation of human leukemia HL-60 cells was determined.

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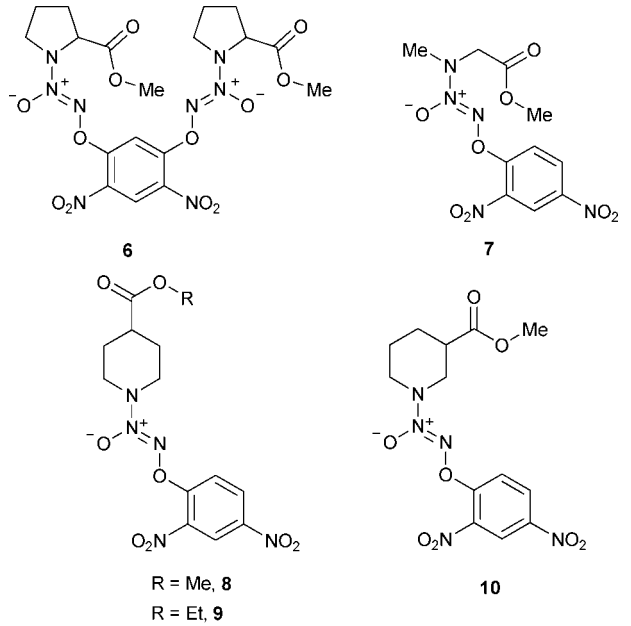

Figure 3. Compounds **6**, **7**, **8**, **9**, and **10**.

Esterification of the carboxylic acids **2**−**4** significantly improved in vitro antiproliferative activity of the resulting esters **5**−**7** against HL-60 human leukemia cells (Table 2). For example, the PROLI/NO ester analogues **5** and **6** were superior inhibitors of HL-60 cell proliferation relative to their carboxylic acid counterparts **2** and **3** (Table 2). All the other esters (**7**−**10**) showed excellent antiproliferative activity that was consistent with elevated levels of intracellular nitric oxide. While other mechanisms might be operational, cell permeability of O^2-(2,4-dinitrophenyl) diazeniumdiolates to release nitric oxide intracellularly appears to be a crucial determinant of inhibitory potential.

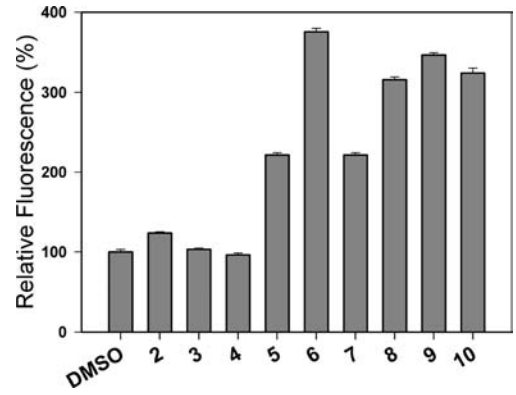

Figure 4. Relative levels of intracellular nitric oxide formation upon treatment of HL-60 cells with compounds **2**−**10** (5 μM DMSO solutions) and DMSO (control) as determined by DAF-FM diacetate fluorescence study.

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

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