Esterase-Sensitive Nitric Oxide Donors of the Diazeniumdiolate Family: In Vitro Antileukemic Activity

Joseph E. Saavedra,[†,‡] Paul J. Shami,[†,§] Lai Yi Wang,[§] Keith M. Davies,[∥] Melissa N. Booth,[⊥] Michael L. Citro,[‡] and Larry K. Keefer*[,⊥]

Intramural Research Support Program, SAIC Frederick, National Cancer Institute, Frederick Cancer Research and Development Center, Frederick, Maryland 21702, Salt Lake City Veterans Administration Medical Center, Salt Lake City, Utah 84148, Department of Chemistry, George Mason University, Fairfax, Virginia 22030, and Chemistry Section, Laboratory of Comparative Carcinogenesis, National Cancer Institute, Frederick Cancer Research and Development Center, Frederick, Maryland 21702

Received July 27, 1999

We have designed a novel prodrug class that is stable in neutral aqueous media but releases bioactive nitric oxide (NO) on metabolism by esterase. Diazeniumdiolates of structure $R_2N-N(O)=N-OR'$, in which $R' = Na$, were reacted with $BrCH_2OAc$ to convert the spontaneously NO-releasing salts **1a** (R_2N = diethylamino) and **1b** (R_2N = pyrrolidino) to prodrugs **2a** (AcOM-DEA/NO) and **2b** (AcOM-PYRRO/NO), respectively, where $R' = CH_2OAc$. In contrast to anions **1a** and **1b** (half-lives in pH 7.4 phosphate at 37 °C of 2 min and 3 s, respectively), **2a** and **2b** showed only minimal decomposition after 16 h under these conditions. Very rapid hydrolysis occurred in the presence of porcine liver esterase, however, with free anion **1a** being observed as an intermediate in the esterase-induced generation of NO from **2a**. The potential utility of this prodrug class is illustrated with a comparison of **1** and **2** as antiproliferative agents in NO-sensitive human leukemia cell lines HL-60 and U937. While the 72-h IC_{50}'s for **1a** and **1b** (which generate NO throughout the medium) in HL-60 cell cultures were >600 μM, those of **2a** and **2b** were 8.3 and 6.4 μM, respectively. This result is consistent with our hypothesis that **2** is selectively hydrolyzed to **1** and thence to NO intracellularly. For U937 cells, the 72-h IC_{50} for both **2a** and **2b** was 53 μM. By contrast, relatively high antiproliferative IC_{50}'s (>100 μM in U937 cells) were observed for analogues in which $R' = CH_2CH_2SC(O)Me$, from which acetyl and 2-mercaptoethyl groups must be successively cleaved to free the NO-releasing diazeniumdiolate function. Within 24 h at initial concentrations of 50 μM, **2a** and **2b** induced apoptosis in 50% and 57% of the HL-60 cells, respectively (35% and 40% of the U937 cells, respectively). The data reveal significant in vitro antileukemic activity on the part of these novel compounds. Moreover, their substantial ease-of-handling advantages over the anionic diazeniumdiolates from which they are derived suggest their use as convenient agents for probing the biological roles of NO.

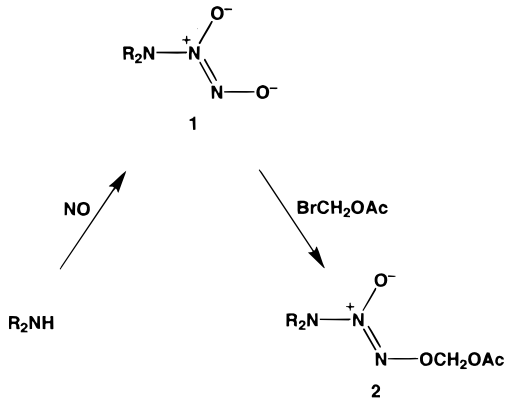
Introduction

A major advantage of the anionic diazeniumdiolates **1** (Scheme 1) as tools for investigating the bioregulatory roles of nitric oxide (NO) is their ability to generate NO spontaneously in aqueous media at rates that depend only upon pH, temperature, and the identity of the R_2N moiety.[1] Half-lives for commercially available diazeniumdiolates in pH 7.4 phosphate buffer range from 2 s to 20 h at 37 °C, with their potencies and durations of action in a variety of in vivo applications correlating well with predictions based on their solution chemistry.[2]

But this inexorability of NO release can be a major disadvantage in many biomedical applications, particularly in the design of drugs for remediating disorders resulting from local deficiencies of endogenous NO. Accordingly, the ultimate goal of our drug discovery effort is to devise strategies for selectively delivering NO to the sites of need while avoiding exposing other NO-sensitive parts of the body to its multipotent effects.[2]

One means of doing this is to convert these spontaneous NO generators to stable prodrug form by attaching

Scheme 1. Synthesis of O^2-Acetoxymethylated Diazeniumdiolates **2**



* To whom correspondence should be addressed. Tel: 301-846-1467. Fax: 301-846-5946. E-mail: keefer@mail.ncifcrf.gov.
† These authors contributed equally to this work.
‡ SAIC Frederick.
§ Veterans Administration.
∥ George Mason University.
⊥ National Cancer Institute.

10.1021/jm9903850 CCC: $19.00
Published on Web 12/30/1999

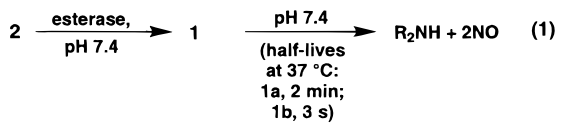
a protecting group (R′) to the terminal oxygen to produce agents of structure $R_2N-N(O)=NOR'$.[3] If one could choose a protecting group that would be selectively removed in the target organ or cell type of interest, dosage with NO could be concentrated at that site even if the prodrug were systemically distributed.

The purpose of this report is to introduce a prodrug class that can be activated to NO-generating form by the action of esterases.

Results

Synthesis and Hydrolysis of O^2-Acetoxymethylated Diazeniumdiolates. The nucleophilic character of the terminal oxygen[3] in anions of structure **1** allowed for their straightforward conversion to acetals **2** by reacting them with bromomethyl acetate in tetrahydrofuran, as shown in Scheme 1. Attachment of the acetoxymethyl group to the terminal oxygen of the anionic diazeniumdiolate resulted in great stabilization. For example, unlike sodium salt **1a** (DEA/NO), whose half-life at 37 °C in pH 7.4 phosphate buffer is 2 min,[1] acetoxymethylated DEA/NO **2a** (AcOM-DEA/NO) was found to be stable in neutral aqueous media. Its characteristic ultraviolet absorption (λ_{max} = 230 nm) showed less than a 2% decrease after 16 h in phosphate-buffered saline (pH 7.4) at 37 °C. Comparable stability was also noted in phosphate buffers at pH 5.5, 6.5, and 8.0. The stability of **2a** decreased markedly, however, at both higher and lower pH. In 1 mM sodium hydroxide, the 230-nm maximum of **2a** decayed exponentially with a half-life of 1.4 min, producing the free diazeniumdiolate **1a** (λ_{max} = 250 nm). In 1 mM hydrochloric acid, the measured half-life of **2a** was 19 min.

Exposure of **2a** as a stable aqueous solution to esterase resulted in its rapid disappearance. As illustrated in Figure 1A, when a 0.19 mM solution of **2a** at pH 8 was treated at 37 °C with porcine liver esterase, its characteristic chromophore at 230 nm completely disappeared within 1 min and the spectral characteristics of **1a** were the only ones detectable. In accord with its known first-order dissociation to diethylamine and NO, the spectrum of **1a** had also disappeared after the mixture was allowed to stand overnight (not shown). Comparable results were seen at pH 7.4 (Figure 1B), except that the isosbestic behavior of Figure 1A was compromised because subsequent dissociation of the initially formed **1a** to diethylamine and NO at this lower pH is more rapid than at pH 8. The overall course of esterase-catalyzed hydrolysis of **2a** and **2b** is shown in eq 1.



a series: R_2N = diethylamino
b series: R_2N = pyrrolidino

To confirm that molecular NO is produced in the esterase-mediated hydrolysis of compounds of structure **2**, the reaction was conducted in a flask being continuously swept with inert gas to purge NO as it formed in the dissociation into an NO-specific chemiluminescence analyzer. Both **2a** and **2b** began to generate copious NO after addition of the esterase (chemiluminescence traces shown as Figure A in Supporting Information). Both NO release profiles integrated for the near-theoretical yield of this gas (1.83 and 1.85 mol of NO/mol of **2**, respectively). The time course of NO release from **2a** was significantly more prolonged than that obtained for **2b**. This is consistent with the known half-lives (eq 1) of anions **1a** and **1b**, which are the intermediates in the mechanism of NO release.

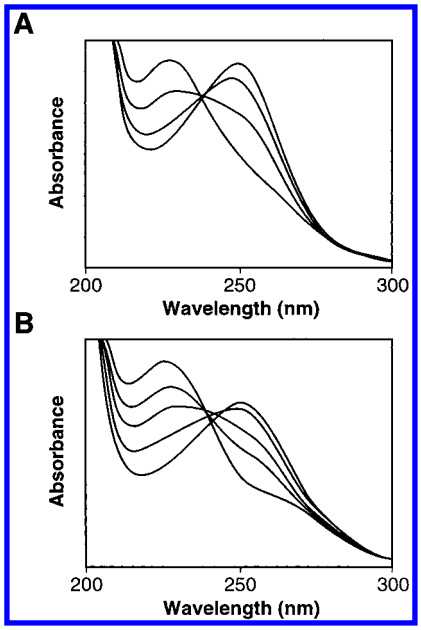
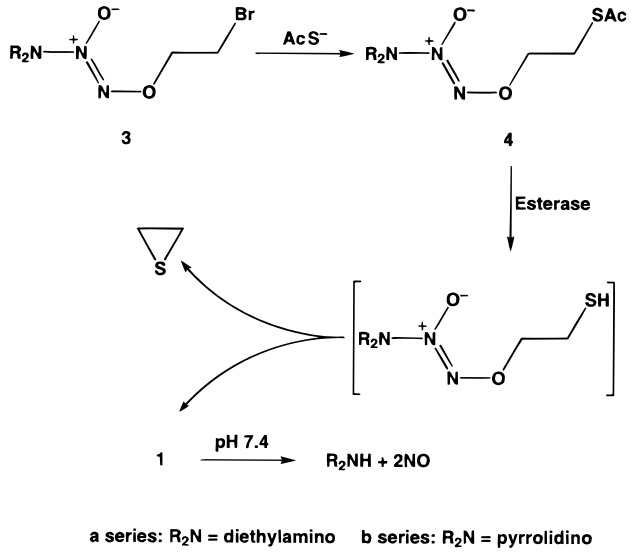


Figure 1. Ultraviolet spectral changes observed after addition of a 0.19 mM solution of **2a** in phosphate buffer at 37 °C to porcine liver esterase at a pH of 8.0 (A) or 7.4 (B). Panel A shows spectra recorded 5, 10, 15, and 30 s after the addition, while panel 1B shows corresponding changes after 10, 25, 35, 60, and 90 s. The peak at 230 nm is characteristic of the starting ester, while that at 250 nm is due to anionic diazeniumdiolate **1a**. The loss of the isosbestic point in panel B is due to the more rapid decomposition of **1a** at pH 7.4 ($t_{1/2}$ = 2 min).

To determine whether nonhepatic esterases could also effect the hydrolysis, mixtures of **2a** with rat blood were studied. We found that 6.7 mM **2a** in whole blood rapidly decomposed even at 4 °C. The apparent half-life under these conditions as measured by gas chromatography was 32 min. Correspondingly faster disappearance was noted as temperature was increased, half-life estimates of 8 and 2 min being recorded at 25 and 37 °C, respectively. Both plasma and the cell fraction were catalytically active; a half-life of 8 min was observed when 12.3 mM **2a** was dissolved at 25 °C in either rat plasma or a suspension of blood cells in phosphate-buffered saline, pH 7.4.

Synthesis and Hydrolysis of Thioacetate Esters 4. Capitalizing on an alternate strategy of esterase-sensitive prodrug design employed previously by Lefebvre et al.,[4] we also performed the stepwise synthesis of thioacetates **4** as outlined in Scheme 2. The 2-bromoethyl derivatives (**3**) of **1** were prepared as previously described[3,5] and then reacted with $MeC(O)S^-$ to give stable products (**4**). According to this strategy, the thioester bond should hydrolyze rapidly under the influence of esterase, freeing the mercaptoethyl derivative as a discrete intermediate. This in turn could hypothetically suffer intramolecular attack by sulfur on

Scheme 2. Synthesis of, and Proposed Mechanism of Esterase-Induced NO Release from, Thioacetate Esters **4**



its β-carbon,[4] displacing diazeniumdiolate anion **1** with formation of thiirane as a byproduct.

That this was actually the course followed was indicated by studying the ultraviolet spectra of DEA/NO derivative **4a** at 37 °C in the absence and presence of porcine liver esterase. The characteristic chromophore of **4a** at 236 nm did not change in intensity in pH 10 buffer over 30 min but shifted to 250 nm within 5 min when the esterase was added, consistent with the rapid esterase-induced regeneration of free anion **1a** from **4a**.

This process was considerably slower at pH 7.4, presumably because the initial hydrolysis produced the thiol rather than the thiolate anion at this pH,[6] decreasing the nucleophilicity of the sulfur atom and slowing the rate of thiirane departure. Nevertheless, formation and dissociation of **1a** did occur in physiological buffer, a total of 1.6 mol of NO/mol of starting material eventually being detected by chemiluminescence when an otherwise stable solution of **4a** was treated with esterase.

Hydrolysis of 2 and 4 by Leukemia Cells. To determine whether NO-sensitive leukemia cell lines HL-60 and U937[7] are capable of converting diazeniumdiolates **2** and **4** to NO intracellularly, we conducted a series of experiments in serum-free phosphate-buffered saline (PBS) with and without the cells. Nitrate and nitrite, the oxidative end products of NO release, were measured in the supernatants. Figure 2 shows the quantities of these indicators of total NO release detected under various circumstances. In cell-free PBS, there was minimal spontaneous release of NO by either acetoxymethyl derivative (**2a** or **2b**) when added at concentrations as high as 100 μM. By contrast, when added at the same concentrations in cell-containing PBS, there was significant and concentration-dependent NO release from both compounds. HL-60 cells appeared to induce the release of slightly more NO than U937 cells. When the NO donors were added to HL-60 cells at a concentration of 100 μM, the concentrations of nitrate/nitrite at 24 h were 83 ± 6.1 and 102 ± 0.2 μM for **2a** and **2b**, respectively. The same measurements with U937 cells were 74 ± 12.5 and 95 ± 0.3 μM for **2a** and **2b**, respectively. By comparison, when **4a** was

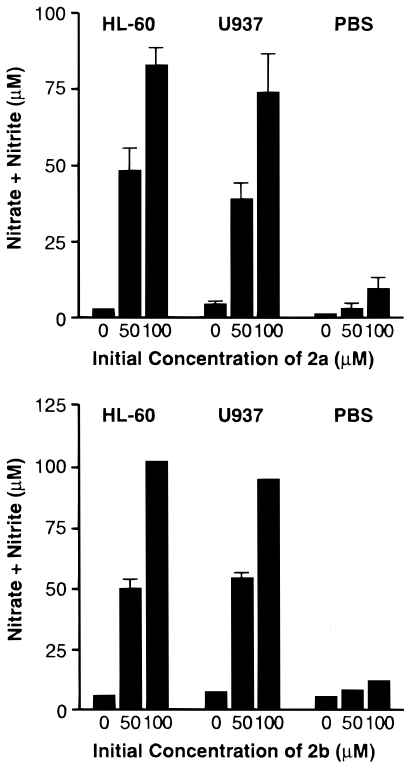


Figure 2. NO release by **2a** and **2b**. HL-60 or U937 cells were cultured at a density of 150 000/mL in serum-free PBS. At the time of culture initiation, we added **2a** or **2b** to the cells or to cell-free PBS. NO release was determined by measuring nitrate/nitrite levels in the supernatants at 24 h. When added to leukemia cells, **2a** and **2b** released a significant amount of NO in a concentration-dependent fashion. There was very little spontaneous release by either compound. Results are the means ± SEM of at least three separate experiments for each data point.

added to HL-60 or U937 cells at 100 μM, the measured nitrate/nitrite concentrations at 24 h were elevated over background by 12.8 ± 0.75 and 18.4 ± 4.8 μM, respectively (not shown).

To determine the time course of intracellular activation of the compounds, **2a**, **2b**, and **4a** were added to HL-60 or U937 cells at a concentration of 100 μM and nitrate/nitrite levels in the supernatants were measured at time points of 2, 4, 6, 8, and 24 h. Acetoxymethyl compounds **2a** and **2b** achieved maximal NO release 6−8 h after addition (Figure 3). No significant NO release was observed from thioacetate **4a** until 24 h after addition. In a separate set of experiments, **2a** and **2b** were added at concentrations of 100 μM to HL-60 or U937 cells cultured at different densities in serum-free PBS. In these experiments, the amount of NO released after 24 h increased with increasing numbers of leukemia cells in the cultures (Figure B in Supporting Information).

To determine whether activation of compounds **2a** and **2b** occurred intracellularly, as hypothesized, and not by release of esterases into the medium, HL-60 and U937 cells were incubated in serum-free PBS for a period of 8 h before **2a** or **2b** was added at a concentration of 100 μM. Supernatants were then collected at 2, 4, 6, 8, and 24 h for measurement of nitrate + nitrite levels using the Griess assay. The results, summarized in Figure 4, reveal an accumulation of NO oxidation products over the first 8 h after adding the drugs; this

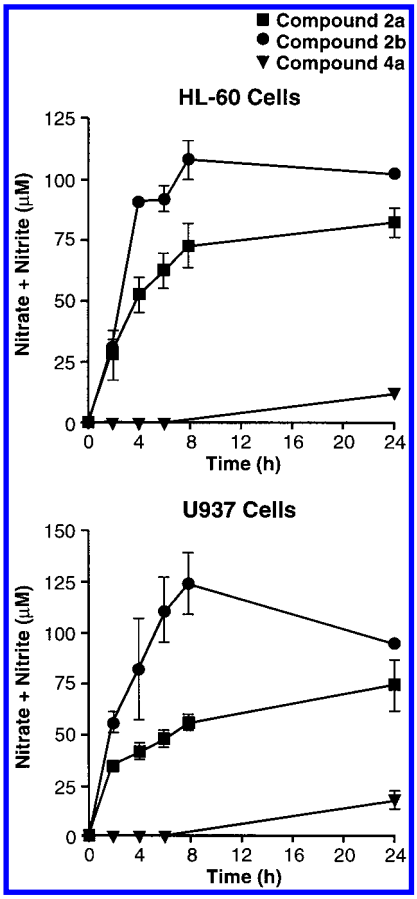


Figure 3. Time course of NO release by **2a**, **2b**, and **4a**. HL-60 or U937 cells were cultured at a density of 150 000/mL in serum-free PBS. At the time of culture initiation, **2a**, **2b**, or **4a** was added to the cells at a concentration of 100 μM. NO release was determined by measuring nitrate/nitrite levels in the supernatants at the indicated time points. NO release from **2a** and **2b** peaked at 6−8 h. NO release from **4a** was very slow and minimal at 24 h. Results are the means ± SEM of at least three separate experiments for each data point.

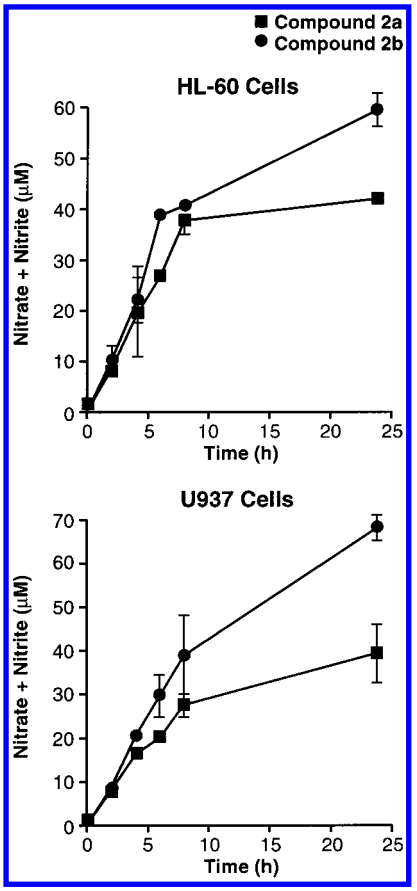


Figure 4. Time course of NO release by **2a** and **2b** after delayed addition to leukemia cells. HL-60 or U937 cells were incubated in serum-free PBS at a density of 150 000/mL for 8 h whereupon **2a** or **2b** was added at a concentration of 100 μM. Nitrate + nitrite levels were measured by the Griess reaction at the indicated time points after the addition of **2a** and **2b**. The kinetics of NO release was similar to those in experiments in which **2a** and **2b** were added at the time of culture initiation (Figure 3). The higher levels of NO metabolites at the 24-h time point (32 h after culture initiation) likely reflect spontaneous cell death rather than secretion of esterases into the medium by viable leukemia cells. Results are the means ± SEM of two separate experiments at each time point.

result was kinetically reminiscent of that seen in Figure 3, except that buildup of metabolites was slower and did not plateau at 8 h but rather was highest at the 24-h time point. The differences can be explained by the fact that in these serum-free media (i.e., containing no growth factors and inadequate nutrients) cellular well-being declines with time, with death occurring roughly 1 day after initiating the culture. Thus at the 24-h time point in Figure 4, which is actually 32 h after culture initiation, a significant portion of the cells can be expected to have lysed, releasing their intracellular esterases into the medium for continued activity.

To confirm that **2a** and **2b** are hydrolyzed intracellularly in these leukemia cell cultures, rather than through the activity of esterases secreted into the extracellular medium, HL-60 or U937 cells were incubated in serum-free PBS for 16 h and centrifuged to remove the cells. Compounds **2a** and **2b** were added to the resulting cell-free supernatants at a concentration of 100 μM, and nitrite/nitrate levels were measured by the Griess assay 24 h later. As shown in Figure 5, concentrations of NO metabolites in the range of 4−5 μM were observed. This is far less than the 70−100 μM concentrations seen at the same time point when the compounds were added to cell-containing media (Figure 3). These data provide further evidence for the intracellular activation of compounds **2**.

Antiproliferative Effects of 2 and 4 on Leukemia Cells in Culture. We next determined the effect of esterase-sensitive diazeniumdiolates **2** and **4** on leukemia cell growth in serum-containing medium using Trypan blue exclusion. When measured at 24, 48, and 72 h, there was a concentration-dependent growth inhibition by both **2a** and **2b**, but even after 3 days of exposure and at a concentration as high as 100 μM, **4b** inhibited cellular growth minimally (Figure 6A). While not very effective at lower concentrations, **4a** at 100 μM inhibited HL-60 and U937 cell growth at 72 h by 90% and 48%, respectively. The most potent growth inhibitors were **2a** and **2b**, with significant growth inhibition in the low micromolar range. For HL-60 cells, the calculated 50% inhibitory concentrations (IC_{50}) at 72 h were 8.3 and 6.4 μM for **2a** and **2b**, respectively. For U937 cells, the 72-h IC_{50} for both **2a** and **2b** was 53 μM.

To determine whether growth inhibitory potency is influenced by the presence of the protecting groups on the terminal oxygen, HL-60 and U937 cells were treated

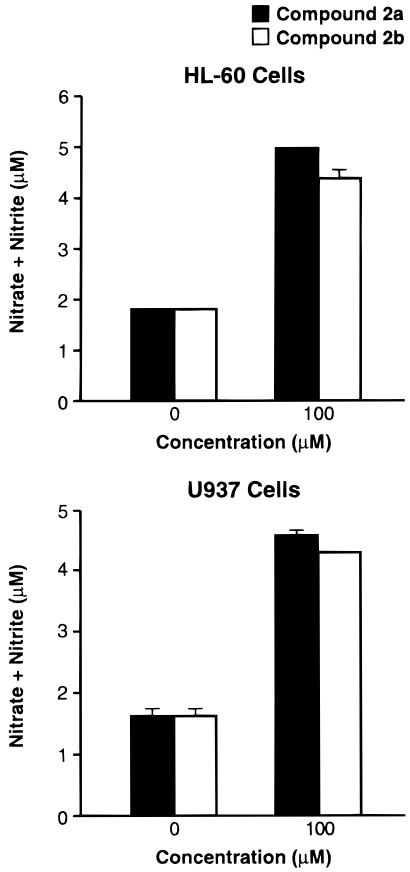
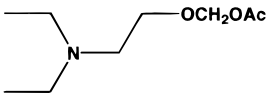


Figure 5. Activation of **2a** and **2b** in cell supernatants. HL-60 or U937 cells were incubated at a density of 150 000/mL in serum-free PBS for 16 h whereupon supernatants were collected. Compounds **2a** and **2b** were added to the cell-free supernatants at a concentration of 100 μM and incubated for 24 h, at which point nitrate + nitrite levels were measured using the Griess assay. There was minimal activation of **2a** and **2b** in cell-free supernatants, further supporting the intracellular mechanism for hydrolysis of these compounds. Results are the means ± SEM of three separate experiments.

with **1a** and **1b**. Even after 72 h of exposure at initial concentrations up to 100 μM, underivatized diazeniumdiolate ions **1a** and **1b** did not substantially affect leukemia cell growth (Figure 6B). We did not observe any significant growth inhibition by either salt until concentrations of 1000 μM were reached. The observed IC_{50} values for **1a** were 620 and 500 μM for HL-60 and U937 cells, respectively. Even at a concentration of 1000 μM, the IC_{50} for **1b** was not reached.

Induction of Leukemia Cell Apoptosis by 2a and 2b. We then determined the effect of the two most potent growth inhibitors (**2a** and **2b**) on HL-60 and U937 cell apoptosis. To ascertain that the observed effects were due to NO release and not to generation of formaldehyde, acetate, and/or the amine byproducts of dissociation of **2**, we used compound **5** [prepared by reacting 2-(*N*,*N*-diethylamino)ethanol first with sodium hydride then with bromomethyl acetate] as a negative



5

control since its esterase-induced hydrolysis would produce formaldehyde, acetate, and an amine but not NO. When assayed 24, 48, and 72 h after culture initiation, **5** did not induce significant apoptosis in either HL-60 or U937 cells (Figure 7). By contrast, when added at the same concentrations, **2a** and **2b** induced extensive apoptosis in both cell lines (Figure 7). These flow cytometric apoptosis assays were confirmed using DNA laddering assays (Figure 8).

Discussion

The family of NO donors introduced in this work constitutes a novel class of compounds with a unique mechanism of activation requiring the enzymatic cleavage of an ester bond. In enzyme-free aqueous media, they showed little tendency to release NO spontaneously. When added to leukemia cells, however, they released large amounts of NO as evidenced by the high levels of NO oxidation products in the supernatants of these cultures (Figures 2 and 3). The suggestion in these results that the NO donors were activated intracellularly is supported by the fact that the amount of NO released by the individual compounds in a given time interval was proportional to the number of cells in the cultures. The conclusion that these esters are activated for NO release intracellularly was further supported by the similarity of nitrite/nitrate accumulation rates when substrate was added to the medium at 8 versus 0 h after initiating the cultures (Figure 4). Additional confirmation was found in the fact that cell-free supernatants prepared from cell cultures incubated for 16 h were essentially devoid of esterase activity (Figure 5).

The compounds we screened showed differences in potency as far as leukemia cell growth inhibition is concerned (Figure 6). Indeed, only acetoxymethyl derivatives **2a** and **2b** were potent growth inhibitors in the concentration range studied. Furthermore, this growth inhibition seemed to be induced by triggering apoptosis (Figures 7 and 8). Since control compound **5** did not induce leukemia cell apoptosis, we conclude that growth inhibition was due to NO release rather than to toxicity of formaldehyde, acetate, or the amine carrier moiety.

Since compounds **1**, **2**, and **4** are all expected to release the same amount of NO per molecule on hydrolysis, their differences in growth inhibitory potency are likely due to differences in their rates and location of NO release. Indeed, when added to leukemia cells, **2a** and **2b** (the two most potent growth inhibitors) generated more NO at faster rates over a period of 24 h than **4a** (which was much less potent). HL-60 cells seemed to be somewhat more sensitive than U937 cells to **2a** and **2b** even though both compounds released roughly equal amounts of NO when added to each cell line. Consequently, the observed difference in susceptibility is more likely due to greater inherent resistance to NO by U937 cells than to differing rates of activation of the compounds by the two cell lines.

Once cleaved by esterases, **2a** and **2b** are converted to **1a** and **1b**, respectively. While these anions have very short half-lives of NO release (2 min and 3 s, respectively), they proved to be far less potent growth inhibitors than either **2a** or **2b** at identical concentrations. This discrepancy can be explained by the fact that **1a**

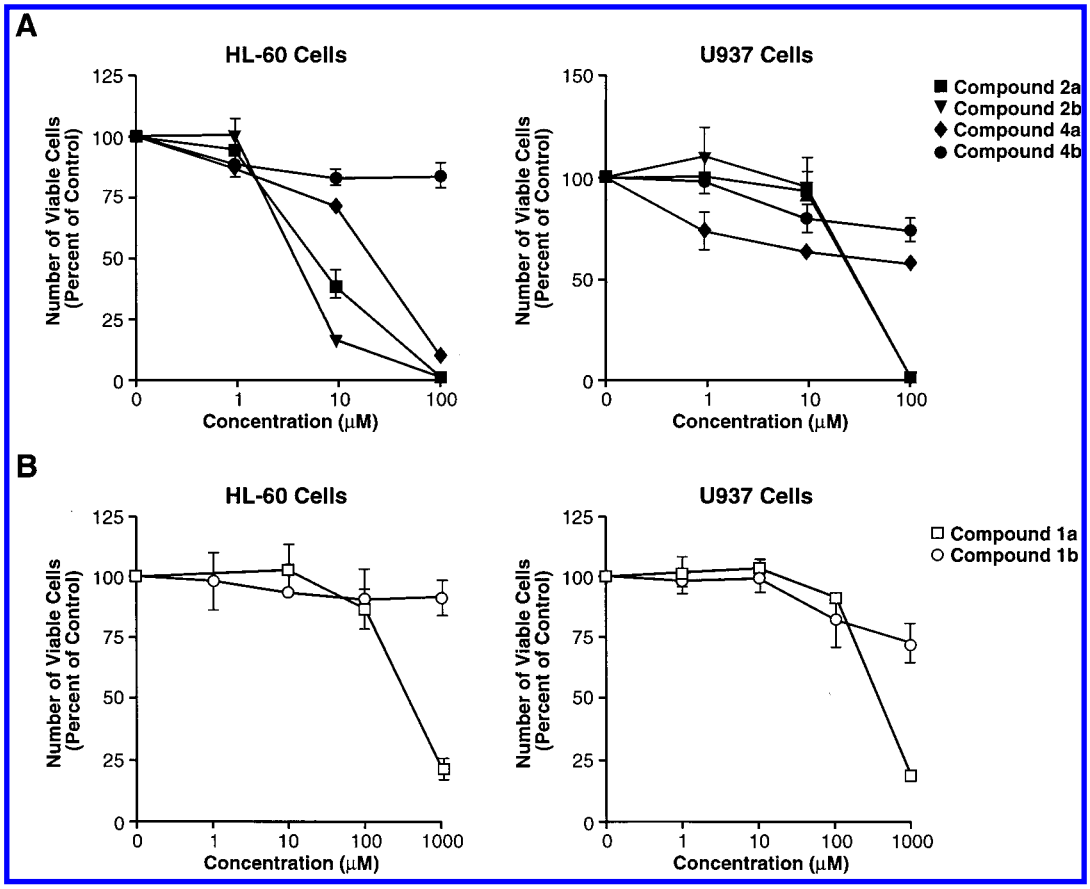


Figure 6. Effect of different NO donors on leukemia cell growth. HL-60 or U937 cells were cultured with the NO donors at the indicated concentrations. Cell growth was assayed by Trypan blue exclusion 72 h after culture initiation. There was a concentration-dependent growth inhibition by the NO donors, with **2a** and **2b** being the most potent agents (A). Similar trends were seen at 24 and 48 h (not shown). By contrast, anions **1a** and **1b** were about 2 orders of magnitude less potent when introduced directly into the culture medium (B). Results are the means ± SEM of at least three separate experiments for each data point.

and **1b** release NO spontaneously and probably do not penetrate leukemia cells significantly before releasing NO. Consequently, they generate NO extracellularly such that a substantial proportion is oxidized before it can enter the cell, wasting its potential antileukemic activity. Additionally, we have previously shown that in order to be effective growth inhibitors, compounds of this class must have a long half-life for NO release (up to several hours).[7] On the other hand, **2a** and **2b** do not release NO spontaneously but rather require activation through esterase cleavage. Since the esterase activity in these experiments was found to be concentrated in the cytoplasm, the amount of NO continuously delivered to its molecular targets was maximized with **2a** and **2b**.

Significance

One of the main obstacles to the use of NO in the treatment of neoplastic diseases is its effects on other biological targets, particularly in the cardiovascular system.[8,9] Accordingly, we are seeking ways of maximizing NO exposure to the tumor cells while minimizing undesired collateral effects elsewhere in the body. The present work describes a selection of esterase-activated prodrugs that appear to offer substantial cell-targeting advantages over the anions from which they were derived. The observed increases in leukemia cell antiproliferative potency (up to 2 orders of magnitude or more relative to the corresponding anions) suggest considerable potential utility for intracellular targeting of NO in a variety of in vitro applications. Rapid hydrolysis in rat blood of the acetyl esters described here obviously limits the suitability of these specific compounds for in vivo applications. Nevertheless, analogues that are less susceptible to the action of such nonspecific esterases can be conceived. An important goal of this work will be to design agents that are tailored for selective activation by esterases unique to a given target cell type. Noteworthy too are the demonstrable ease-of-handling advantages of the esters reported herein relative to the hydrolytically unstable parent anions. Further work should considerably extend the biomedical utility of this novel class of NO donor.

Experimental Section

NO was purchased from Matheson Gas Products (Montgomeryville, PA). Ultraviolet (UV) spectra were recorded on a Hewlett-Packard model 8451A diode array spectrophotometer. Nuclear magnetic resonance spectra were collected with a 300-MHz Varian Unity Plus or a Varian XL-200 NMR spectrometer; spectra were obtained in deuteriochloroform. Chemical shifts are reported in parts per million (ppm) downfield from tetramethylsilane. Starting materials **1** and **3** were prepared as previously described.[3,5,10] Elemental analyses were done by Atlantic Microlab, Inc. (Norcross, GA).

***O*²-(Acetoxymethyl) 1-(*N*,*N*-Diethylamino)diazen-1-ium-1,2-diolate (2a).** A slurry of 1.1 g (7.1 mmol) of sodium 1-(*N*,*N*-diethylamino)diazen-1-ium-1,2-diolate (**1a**)[10] and 200

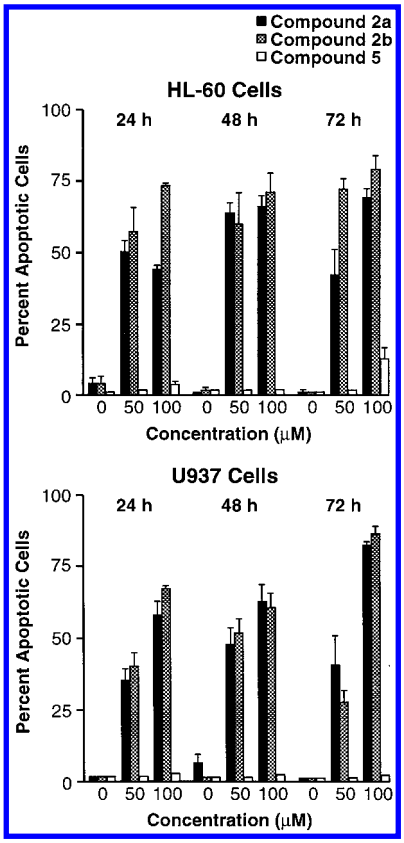


Figure 7. Induction of leukemia cell apoptosis by **2a** and **2b**. HL-60 or U937 cells were cultured with **2a**, **2b**, or **5** at the indicated concentrations. At 24, 48, and 72 h, the percent of apoptotic cells was determined by flow cytometry. Control compound **5**, whose esterase-induced hydrolysis would generate formaldehyde but not NO, did not induce significant leukemia cell apoptosis. At the same concentrations, **2a** and **2b** induced a substantial degree of apoptosis in both cell lines. Results are the means ± SEM of at least three separate experiments for each data point.

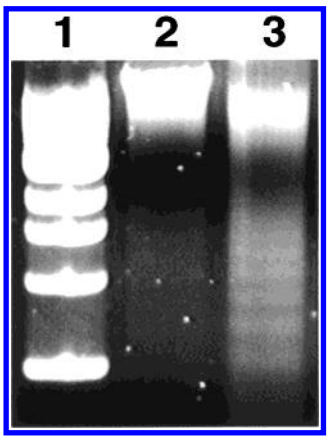


Figure 8. Induction of apoptosis by NO donors in HL-60 cells. Cells were treated with compound **2a** for a period of 24 h at a concentration of 100 μM. Cellular DNA was then isolated and electrophoresed in a 1.2% agarose gel: lane 1 = molecular weight marker; lane 2 = untreated control; lane 3 = 100 μM **2a**. The NO donor induced apoptosis (as evidenced by DNA laddering). The figure shows the results of one experiment that is representative of three performed. Similar results were obtained with U937 cells and with compound **2b**.

mg of anhydrous sodium carbonate in 20 mL of tetrahydrofuran was cooled to 0 °C under a stream of dry nitrogen. To this was added a solution of 1 g (6.5 mmol) of bromomethyl acetate in 5 mL of tetrahydrofuran. The reaction mixture was allowed to warm to room temperature and stirred for 72 h. The mixture was evaporated in vacuo and the residue was extracted with dichloromethane, dried over sodium sulfate, filtered, and evaporated to give 611 mg of an amber oil. The crude mixture was chromatographed on silica gel and eluted with dichloromethane to give 321 mg (22%) of product, **2a**: NMR δ 1.12 (t, 6 H), 2.11 (s, 3 H), 3.21 (q, 4 H), 5.81 (s, 2 H); UV (H_2O) λ_{max} (ϵ) 230 nm (7.6 mM^{-1} cm^{-1}). Anal. ($C_7H_{15}N_3O_4$) C, H, N.

Decomposition of 2a in Rat Blood. Samples of **2a** (20 mM in pH 7.4 phosphate buffer) were mixed with whole blood at various temperatures to provide a final concentration in the blood of 6.7 mM. Aliquots of 200 μL each were quenched at 0 °C with 100 μL of 10% trichloroacetic acid, filtered through glass wool at various times after mixing, and injected into a Shimadzu model 4BM gas chromatograph equipped with a Hewlett-Packard 18652A A/D converter coupled to the recorder of a flame ionization detector. A 3% OV-17 on 80/100 Supelcoport glass column (Supelco, Bellefonte, PA) was used. Oven temperature was kept constant at 150 °C, with a helium flow rate of 40 mL/min. The retention time for **2a** was 5.0 min under these conditions. The decrease in the integral of the peak appearing at the retention time of **2a** was monitored and used to estimate the half-lives under the indicated conditions. Later experiments tracking disappearance of **2a** in plasma and the blood cell fraction were similarly conducted, except that the initial substrate concentration was 12.3 mM, aliquots were quenched with ethanol, and chromatography was performed on a Varian 3700 gas chromatograph equipped with a 30-foot × 0.3-mm DB-1 capillary column (J&W Scientific, Folsom, CA) with a helium flow rate of 19 mL/min; oven temperature was kept at 75 °C for 5 min, then programmed at 10 °C/min to 250 °C. The retention time of **2a** was 14.5 min.

***O*²-(Acetoxymethyl) 1-(Pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (2b).** A slurry of 3.07 g (0.02 mol) of sodium 1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (**1b**),[5] 1 g of anhydrous sodium carbonate, and 20 mg (0.12 mmol) of silver acetate (needed because **1b** seemed to react with bromomethyl acetate more sluggishly than **1a**, requiring silver ion assistance) in 25 mL of dimethyl sulfoxide was stirred with 1.4 mL (0.014 mol) of bromomethyl acetate for 72 h at room temperature. The resulting solution was poured over 100 g of ice−water, extracted with ether, and dried over anhydrous sodium sulfate. The solution was filtered through magnesium sulfate and evaporated to give 1.67 g of an oil. Preliminary purification was carried out on a 4-cm × 15-cm KP-Sil column connected to a Biotage Flash 40 system and eluted with pure dichloromethane at a rate of 25 mL/min. The fractions containing the pure product were combined and evaporated in vacuo to give 1.33 g of a solid that was recrystallized from petroleum ether to give white plates: mp 62−63 °C; NMR δ 1.97 (m, 4 H), 2.12 (s, 3 H), 3.60 (m, 4 H), 5.76 (s, 2 H); UV (H_2O) λ_{max} (ϵ) 218 nm (5.2 mM^{-1} cm^{-1}) and 256 nm (7.8 mM^{-1} cm^{-1}). Anal. ($C_7H_{13}N_3O_4$) C, H, N.

Hydrolysis of Esters with Porcine Liver Esterase. To 1 mL of a 189 μM solution of **2a** in pH 8 buffer was added 1 μL (1 unit) of porcine liver esterase (suspension in 3.2 M ammonium sulfate; Sigma) (1.05 mg of protein in a 0.5-mL solution). The course of the reaction was monitored by ultraviolet spectrophotometry at 37 °C by observing the disappearance of the chromophore at 230 nm and the ensuing formation of **1a** (250 nm). To obtain the early data points for Figure 1, the reaction was also initiated by adding 1.0 mL of a 189 μM **2a** solution in pH 8 buffer at 37 °C to 1 μL of the esterase contained in a 1-mL cuvette prepositioned in the spectrophotometer. Esterase-induced hydrolysis of esters **2b** and **4** was effected under similar conditions.

General Method for the Preparation of *O*²-(2-*S*-Acetylmercaptoethyl) 1-Substituted Diazen-1-ium-1,2-diolates. To a stirring solution of the appropriate *O*²-bromoethyl 1-substituted diazen-1-ium-1,2-diolate **3** in dichloromethane at 0 °C under N_2 was added first triethylamine and then thiolacetic acid (Aldrich). The reaction mixture was allowed to warm gradually to 25 °C over the course of several

hours. The solution resulting from filtration of the reaction mixture under vacuum was washed first with water and then with cold dilute hydrochloric acid. The washed dichloromethane solution was dried with sodium sulfate and filtered under vacuum through magnesium sulfate. Removal of solvent yielded the crude product, which was purified by flash chromatography on a Flash 40 system using a 90-g KP-Sil column eluted with 10:1 dichloromethane:ethyl acetate to give the final product.

***O*²-(2-*S*-Acetylmercaptoethyl) 1-(*N*,*N*-Diethylamino)-diazen-1-ium-1,2-diolate (4a).** *O*²-(2-Bromoethyl) 1-(*N*,*N*-diethylamino)diazen-1-ium-1,2-diolate **(3a)**[3] (2.3 g, 9.6 mmol), dichloromethane (35 mL), triethylamine (4.0 mL, 2.9 g, 29 mmol), and thiolacetic acid (2.0 mL, 2.1 g, 28 mmol) were mixed and stirred for 3 h as described above. The crude product was flash chromatographed to yield a pale yellow oil (775 mg, 3.3 mmol, 34%): NMR δ 1.10 (t, 6 H), 2.35 (s, 3 H), 3.12 (q, 4 H), 3.24 (t, 2 H), 4.36 (t, 2 H); UV (H_2O) λ_{max} (ϵ) 236 nm (12 mM^{-1} cm^{-1}). Anal. ($C_8H_{17}N_3SO_3$) C, H, N, S.

***O*²-(2-*S*-Acetylmercaptoethyl) 1-(Pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (4b).** *O*²-(2-Bromoethyl) 1-(pyrrolidin-l-yl)diazen-1-ium-1,2-diolate **(3b)**[5] (1.7 g, 7.0 mmol) in dichloromethane (15 mL), triethylamine (3.2 mL, 2.3 g, 23 mmol), and thiolacetic acid (1.4 mL, 1.49 g, 19.6 mmol) were used as described in the general procedure. The reaction mixture was allowed to stir at 25 °C for 18 h. The crude product, a yellow oil, was chromatographed to yield a pale yellow oil, which crystallized on standing to yield 690 mg (43%) of pale yellow crystals: mp 38−40 °C; NMR δ 1.95 (m, 4 H), 2.35 (s, 3 H), 3.23 (t, 2 H), 3.56 (m, 4 H), 4.26 (t, 2 H); UV (H_2O) λ_{max} (ϵ) 236 nm (10.9 mM^{-1} cm^{-1}) and 250 nm (9.75 mM^{-1} cm^{-1}). Anal. ($C_8H_{15}N_3SO_3$) C, H, N, S.

2-(*N*,*N*-Diethylamino)ethyl Acetoxymethyl Ether (5). A solution of 711 mL (5.4 mmol) of *N*,*N*-diethylethanolamine in 10 mL of tetrahydrofuran was flushed with dry nitrogen. To this solution was added 198 mg (8.25 mmol) of sodium hydride and the resulting reaction mixture was heated at reflux for 1 h. The mixture was cooled to 0 °C under a stream of nitrogen, whereupon 833 mg (5.4 mmol) of bromomethyl acetate was introduced slowly through a syringe and stirred overnight. Methanol (500 mL) was added and the mixture was stirred for 15 min. The reaction mixture was concentrated on a rotary evaporator; the residue was extracted with dichloromethane and the solution was dried over sodium sulfate, filtered through a layer of magnesium sulfate, and evaporated under vacuum to give 633 mg of a colorless oil. The crude mixture containing roughly 44% of the desired product and 56% of 2-(*N*,*N*-diethylamino)ethyl acetate was fractionally distilled under vacuum to give 120 mg of **5**: bp 80 °C at 5 mmHg; NMR δ 1.05 (t, 6 H), 2.10 (s, 3 H), 2.63 (q, 4 H), 2.73 (t, 2 H), 3.75 (t, 2 H), 5.29 (s, 2 H). Anal. ($C_9H_{19}NO_3$) C, H, N.

Measurement of NO Generation Rates. Generation of molecular NO was quantified as previously described[1] to obtain the solution chemistry data of Figure A (Supporting Information) and associated text.

For the cell culture experiments, to avoid the confounding effect of nitrate in the medium or serum, cells were cultured at 37 °C in a 5% CO_2 humidified atmosphere in serum-free PBS and at a density of 25 000−150 000 cells/mL as indicated in the individual experiments. The NO donors were added at the indicated concentrations. In parallel, NO donors were added at the same concentrations in cell-free PBS and incubated under the same conditions. At the indicated time points, 100-μL aliquots of the supernatant were removed and used for measurement of nitrite levels after treatment with nitrate reductase using the Griess reaction as previously described.[11]

Cell Lines and Culture Conditions. HL-60 and U937 cells were from ATCC (Rockville, MD). For the cell growth and apoptosis experiments, cells were cultured at a density of 150 000 cells/mL in RPMI-1640 with 10% fetal bovine serum at 37 °C in a 5% CO_2 humidified atmosphere. Cells were cultured in a total volume of 1 mL with one well per variable. The NO donors were added at the indicated concentrations at the time of culture initiation. At the indicated time intervals, cells were harvested and washed twice in PBS, whereupon assays for growth and apoptosis were done.

Cell Growth and Apoptosis Assays. The number of viable cells was determined by Trypan blue exclusion. Apoptosis was assayed by flow cytometry and by determining DNA laddering using gel electrophoresis as previously described.[7] For the flow cytometry assay, we used the method of Nicoletti et al. with slight modification.[12] In brief, after two PBS washes, cells were resuspended in 1 mL of lysis buffer consisting of 0.2% NP40 solution containing 5% bovine serum albumin in PBS. Ten microliters of a 4 mg/mL propidium iodide solution and 10 μL of a 10 mg/mL RNAse A solution were then added. The fraction of cells with low DNA content (which is consistent with the apoptotic fraction)[12,13] was determined using a Becton-Dickenson flow cytometer.

For the DNA laddering assay, cells were cultured in suspension medium with the NO donors as outlined above. They were then centrifuged and washed in PBS. DNA was isolated with the DNA Blood Kit from QIAGEN (Chatsworth, CA) using the manufacturer's protocol. Five micrograms of DNA was electrophoresed in a 1.2% agarose gel and stained with ethidium bromide. NP40, RNAse A, and propidium iodide were from Sigma (St. Louis, MO). Results are expressed as averages of multiple experiments ± the standard error of the mean (SEM). SEM was calculated as the standard deviation of different measurements divided by the square root of the number of measurements.

Acknowledgment. We thank Mary McGuire and John Klose for determining the NMR spectra. P.J.S. is a Translational Research Awardee from the Leukemia Society of America. This project was funded in part by the National Cancer Institute under Contract No. NO1-CO-56000.

Supporting Information Available: Time course of NO generation after adding esterase to aqueous **2** solutions (Figure A) and dependence of NO generation rate on cell density in cultures of HL-60 or U937 cells (Figure B). This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Keefer, L. K.; Nims, R. W.; Davies, K. M.; Wink, D. A. "NONOates" (1-Substituted Diazen-1-ium-1,2-diolates) as Nitric Oxide Donors: Convenient Nitric Oxide Dosage Forms. *Methods Enzymol.* **1996**, *268*, 281−293.

(2) Keefer, L. K. Nitric Oxide-Releasing Compounds: From Basic Research to Promising Drugs. *CHEMTECH* **1998**, *28*, 30−35.

(3) Saavedra, J. E.; Dunams, T. M.; Flippen-Anderson, J. L.; Keefer, L. K. Secondary Amine/Nitric Oxide Complex Ions, R_2N-$[N(O)NO]^-$. O-Functionalization Chemistry. *J. Org. Chem.* **1992**, *57*, 6134−6138.

(4) Lefebvre, I.; Périgaud, C.; Pompon, A.; Aubertin, A.-M.; Girardet, J.-L.; Kirn, A.; Gosselin, G.; Imbach, J.-L. Mononucleoside Phosphotriester Derivatives with *S*-Acyl-2-thioethyl Bioreversible Phosphate-Protecting Groups: Intracellular Delivery of 3′-Azido-2′,3′-dideoxythymidine 5′-monophosphate. *J. Med. Chem.* **1995**, *38*, 3941−3950.

(5) Saavedra, J. E.; Billiar, T. R.; Williams, D. L.; Kim, Y.-M.; Watkins, S. C.; Keefer, L. K. Targeting Nitric Oxide (NO) Delivery in Vivo. Design of a Liver-Selective NO Donor Prodrug That Blocks Tumor Necrosis Factor-α-Induced Apoptosis and Toxicity in the Liver. *J. Med. Chem.* **1997**, *40*, 1947−1954.

(6) The pK_a of the thiol group in cysteine is 8.3. See: Zubay, G. L. *Biochemistry*, 4th ed.; Wm. C. Brown: Dubuque, IA, 1998; p 64.

(7) Shami, P. J.; Sauls, D. L.; Weinberg, J. B. Schedule and Concentration-Dependent Induction of Apoptosis in Leukemia Cells by Nitric Oxide. *Leukemia* **1998**, *12*, 1461−1466.

(8) Griffith, O. W.; Kilbourn, R. G. Design of Nitric Oxide Synthase Inhibitors and Their Use to Reverse Hypotension Associated with Cancer Immunotherapy. *Adv. Enzyme Regul.* **1997**, *37*, 171−194.

(9) Shahidi, H.; Kilbourn, R. G. The Role of Nitric Oxide in Interleukin-2 Therapy Induced Hypotension. *Cancer Metastasis Rev.* **1998**, *17*, 119−126.

(10) Maragos, C. M.; Morley, D.; Wink, D. A.; Dunams, T. M.; Saavedra, J. E.; Hoffman, A.; Bove, A. A.; Isaac, L.; Hrabie, J. A.; Keefer, L. K. Complexes of ·NO with Nucleophiles as Agents for the Controlled Biological Release of Nitric Oxide. Vasorelaxant Effects. *J. Med. Chem.* **1991**, *34*, 3242−3247.

(11) Granger, D. L.; Anstey, N. M.; Miller, W. C.; Weinberg, J. B. Measuring Nitric Oxide Production in Human Clinical Studies. *Methods Enzymol.* **1999**, *301*, 49−61.
(12) Nicoletti, I.; Migliorati, G.; Pagliacci, M. C.; Grignani, F.; Riccardi, C. A Rapid and Simple Method for Measuring Thymocyte Apoptosis by Propidium Iodide Staining and Flow Cytometry. *J. Immunol. Methods* **1991**, *139*, 271−279.
(13) Darzynkiewicz, Z.; Bruno, S.; Del Bino, G.; Gorczyca, W.; Hotz, M. A.; Lassota, P.; Traganos, F. Features of Apoptotic Cells Measured by Flow Cytometry. *Cytometry* **1992**, *13*, 795−808.

JM9903850