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Synthesis, nitric oxide release, and anti-leukemic activity of glutathione-activated nitric oxide prodrugs: Structural analogues of PABA/NO, an anti-cancer lead compound

Harinath Chakrapani,[a,*] Thomas C. Wilde,[a] Michael L. Citro,[b] Michael M. Goodblatt,[a] Larry K. Keefer[a] and Joseph E. Saavedra[b,*]

[a]*Chemistry Section, Laboratory of Comparative Carcinogenesis, National Cancer Institute at Frederick, Frederick, MD 21702, USA*
[b]*Basic Research Program, SAIC-Frederick, National Cancer Institute at Frederick, Frederick, MD 21702, USA*

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Abstract—Diazeniumdiolate anions and their prodrug forms are reliable sources of nitric oxide (NO) that have generated interest as promising therapeutic agents. A number of structural analogues of O^2-(2,4-dinitro-5-(4-(*N*-methylamino)benzoyloxy)phenyl) 1-(*N*,*N*-dimethylamino)diazen-1-ium-1,2-diolate (PABA/NO), an anti-cancer lead compound that is designed to release NO upon activation by glutathione, were prepared. The nitric oxide release patterns of these O^2-(2,4-dinitrophenyl) diazeniumdiolates in the presence of glutathione were tested and it was found that in the absence of competing pathways, these compounds release nearly quantitative amounts of NO. The ability of PABA/NO and its structural analogues to inhibit human leukemia cell proliferation was determined and it was found that compounds releasing elevated amounts of NO displayed superior cytotoxic effects.
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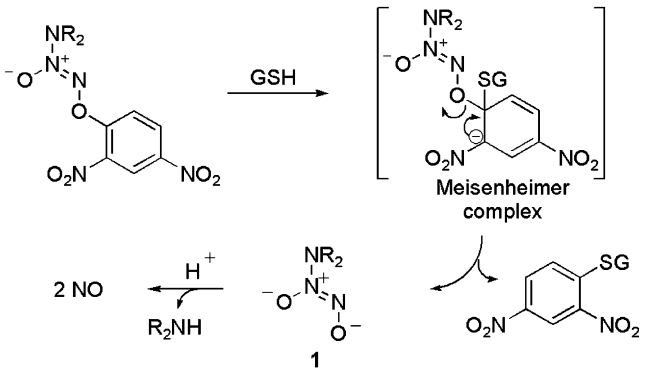
1. Introduction

Nitric oxide (NO) prodrugs of the ionic diazeniumdiolate class (**1**, Scheme 1) that spontaneously dissociate at definitive rates to form NO are not only widely used in biological studies but are also of interest as possible therapeutic agents in the treatment of several disease states, notably cancer.[1–6] Due to the involvement of NO in diverse physiological processes, site-directed delivery of therapeutic nitric oxide is essential to avoid any undesirable side-effects. Derivatization of the O^2-position of the diazeniumdiolate ion with protective groups, converting them into substrates for specific enzymes in a metabolic pathway, is one frequently used strategy.[7–11] The inactive prodrug circulates freely until it encounters the specific protein, which then acts as a trigger, cleaving the protective group, resulting in selective accumulation of NO in that tissue. A successful example of such an approach is the development of



Scheme 1. Mechanism of nitric oxide release from O^2-arylated diazeniumdiolates that are metabolized by glutathione *S*-transferase.

O^2-arylated diazeniumdiolates targeting the detoxification enzyme glutathione *S*-transferase (GST), often over-expressed in cancer tissue.[12–14] The general mechanism of action of such prodrugs involves nucleophilic aromatic substitution[15] by glutathione (GSH), generating the nitric oxide-releasing diazeniumdiolate ion **1** (Scheme 1).[16]

Of the several isoforms of GST, GSTπ is over-expressed in many tumors, which makes it an attractive molecular

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* Corresponding authors. Tel.: +1 301 846 1601; fax: +1 301 846 5946 (H.C.); tel.: +1 301 846 1602 (J.E.S.); e-mail addresses: chakrah@ncifcrf.gov; saavj@ncifcrf.gov

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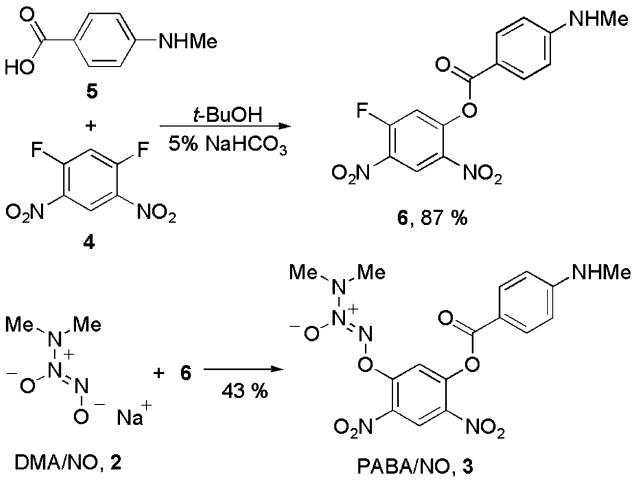


Scheme 2. Original synthesis of PABA/NO (**3**).

target for site-directed delivery of tumoristatic agents.[17–20] Molecular modeling studies of the Meisenheimer complex (Scheme 1) were used as a guide in the design of GSTπ-selective 2,4-dinitroaryl derivatives of DMA/NO (structure **2**, Scheme 2).[12–14]

One such compound, PABA/NO (structure **3**, Scheme 2), has shown tumoristatic activity against A2780 human ovarian cancer xenografts in female SCID mice with a potency comparable to that of cisplatin.[14] This and other in vitro and in vivo activities established PABA/NO to be a promising anti-cancer lead compound.[13] In an effort to identify drug candidates with superior anti-cancer activity, we attempted to synthesize and study structural analogues of PABA/NO.

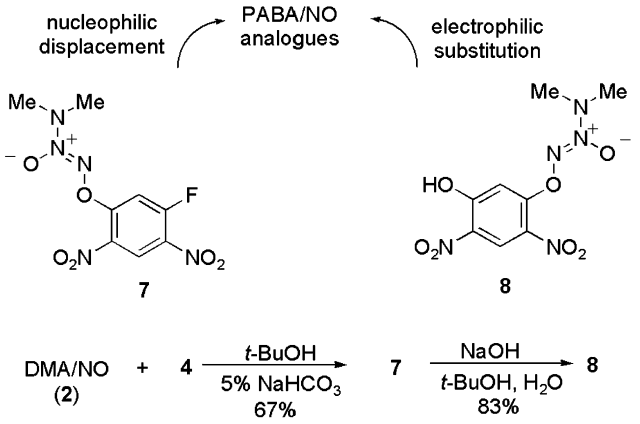
Initially, PABA/NO was prepared in 38% overall yield by the reaction of 1,5-difluoro-2,4-dinitrobenzene (**4**) with (*N*-methyl)-4-aminobenzoic acid (**5**) to form the fluoride **6**, which was then reacted with DMA/NO (Scheme 2).[13] The second step of this synthesis produced a mixture of byproducts that rendered isolation of PABA/NO cumbersome. Hence, devising an efficient synthetic route to PABA/NO was desired.

2. Results and discussion

In order to prepare a variety of PABA/NO analogues, two synthetic intermediates, structures **7** and **8**, of divergent reactivity were envisaged (Scheme 3).

The fluoride **7**, which was prepared by treatment of **4** with DMA/NO, is a candidate for reaction with nucleophiles (Scheme 3).[12] The phenol **8**, which can react with electrophiles, was obtained by hydrolysis of **7** in 83% yield (Scheme 3).[13] This design exploits the opposing organic reactivity of compounds **7** and **8** and should permit facile access to diverse PABA/NO analogues.

As predicted, the two compounds **7** and **8** served as convenient divergence points in the synthesis of numerous PABA/NO analogues. PABA/NO and several ester



Scheme 3. Proposed synthetic intermediates **7** and **8** and their syntheses.

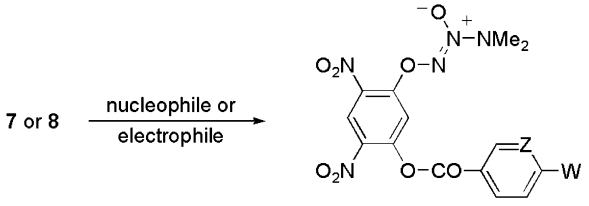
derivatives (**9**–**14**) differing in the substitution pattern in the aroate ester functionality were synthesized in moderate to good yields from **7** or **8** (Table 1).

Nitric oxide donors coupled with non-steroidal anti-inflammatory drugs (NSAIDs) are assuming importance as versatile drug candidates.[21–23] These compounds have shown to minimize the deleterious effects of traditional NSAIDs, while retaining the potent anti-inflammatory activity of the parent NSAIDs.[21–23] PABA/NO analogues (**15**–**17**) of non-sterodial anti-inflammatory drugs (NSAIDs) such as aspirin, ibuprofen, and naproxen were prepared by treating **8** with the corresponding acid chloride (Scheme 4).

In addition, several structurally similar compounds with varying functional groups connecting the two aromatic rings (**18**–**22**) were synthesized starting from **4**, **7** or **8** (Scheme 5).

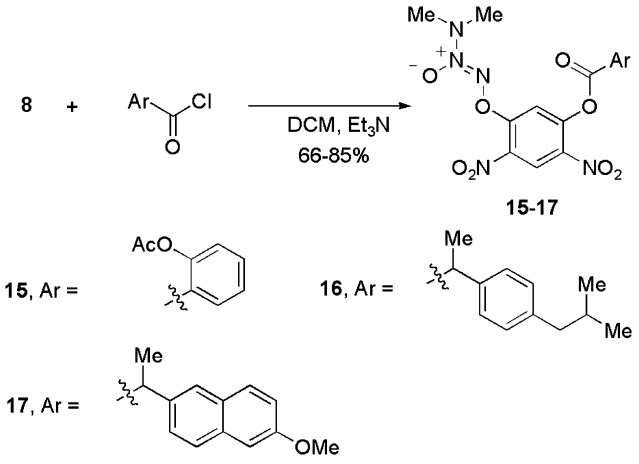
The ambident nucleophilic character of 4-aminobenzoic acid is noteworthy. In acetone, it was reported that 4-aminobenzoic acid reacted through the amino group

Table 1. Synthesis of PABA/NO and its structural analogues

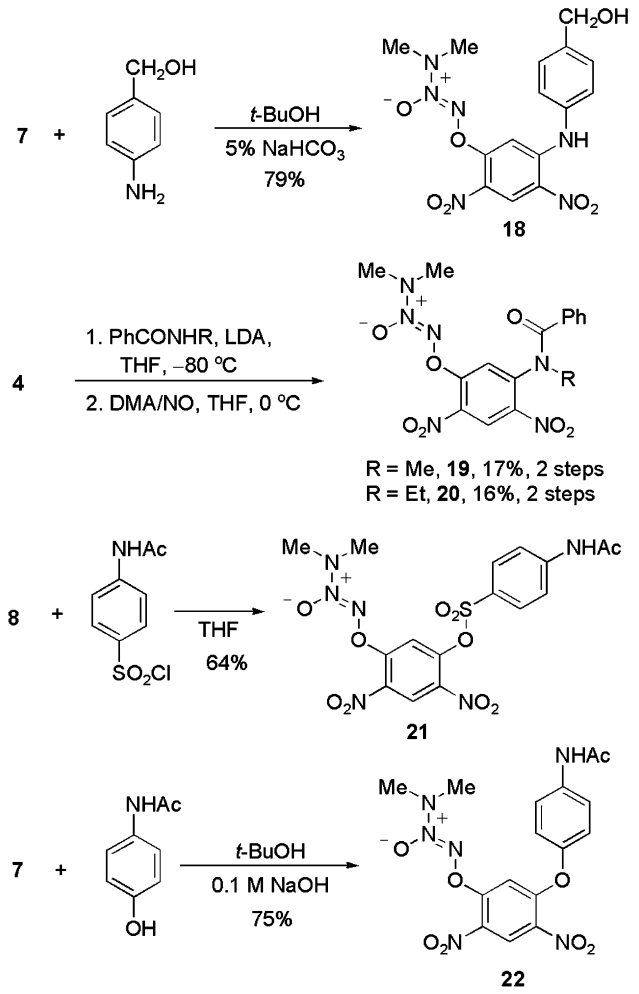


Reactant	Product no	W	Z	Yield[a]
7	**3**	NHMe	CH	79%[b]
7	**9**	NMe_2	CH	66%[b]
7	**10**	NH_2	CH	64%[b]
7	**11**	NHAc	CH	75%[b]
8	**12**	H	CH	34%[c]
8	**13**	H	N	44%[c]
8	**14**	NO_2	CH	71%[c]

[a] Isolated yield.
[b] Prepared using the corresponding benzoic acid.
[c] Prepared using the corresponding aroyl chloride.



Scheme 4. Synthesis of NSAID-based PABA/NO analogues.



Scheme 5. Synthesis of some PABA/NO structural analogues.

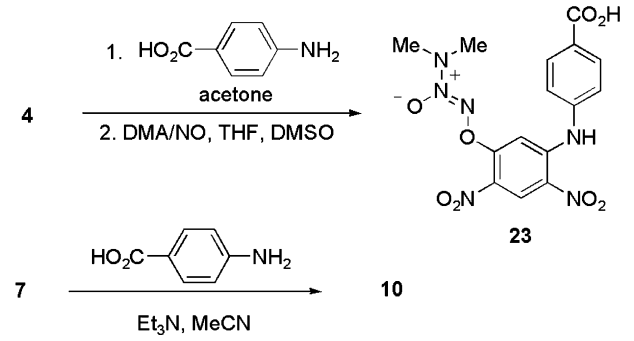
to form **23** ($\upsilon_{C=O}$ = 1685 cm^{-1}), in two steps from **4** (Scheme 6).[13] However, in the presence of triethylamine, the major product of the reaction of **7** and 4-aminobenzoic acid (Scheme 6) was through the carboxylic acid functionality to form the ester **10** ($\upsilon_{C=O}$ = 1742 cm^{-1}).

In a neutral organic solvent such as acetone, presumably the carboxylic acid does not ionize, leaving the nitrogen lone pair as the most nucleophilic species. However, in the presence of a weak base such as triethylamine, it is expected that ionization to the anionic form of 4-aminobenzoic acid occurs. In such a situation, the carboxylate ion is the dominant nucleophile, rationalizing the observed outcome of these reactions of 4-aminobenzoic acid.

PABA/NO and its analogues were independently treated with GSH in 0.1 M, pH 7.4, phosphate buffer and NO release was measured (Table 2). The yields of NO from the ester analogues **9**–**17** were lower than that of PABA/NO (Table 2). All other analogues, **18**–**22**, were found to release higher amounts of NO (⩾93%).

Under similar conditions but in the absence of GSH, negligible amounts of NO were detected from **9**–**22**. In an earlier study, we reported that upon treatment with a range of nucleophiles, 2,4-dinitroarylated diazeniumdiolates released NO, suggesting that arylated diazeniumdiolates are susceptible to activation with other nucleophiles to release nitric oxide.[15]

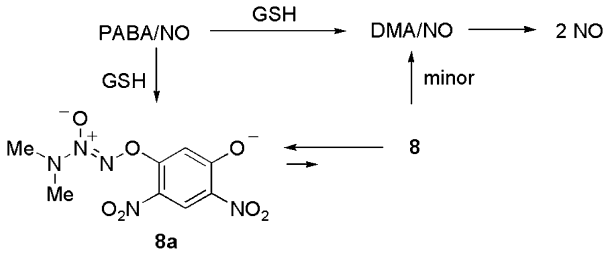
The sub-stoichiometric yield of NO suggests that a pathway other than just nucleophilic aromatic substitution by GSH to release DMA/NO ion is operational. In addi-



Scheme 6. Reactivity of 4-aminobenzoic acid under different reaction conditions with aryl fluorides.

Table 2. Nitric oxide release and anti-leukemic activity for PABA/NO and its structural analogues

Compound	% NO released	IC_{50} (μM)
PABA/NO, **3**	73	3.9
9	30	>25
10	63	13
11	20	22
12	15	>25
13	4	>25
14	0	>25
15	7	>25
16	0	>25
17	5	>25
18	100	6.8
19	99	1.6
20	100	1.4
21	96	3.9
22	93	2.3
8	0	>25



Scheme 7. Mechanism of nitric oxide release from PABA/NO.

tion to nitric oxide release, PABA/NO, in the presence of GSH, is reported to undergo ester hydrolysis to form **8** as a byproduct (Scheme 7).[13] Such a competitive pathway appears to be pronounced in **9**–**17**, leading to lower yields of NO from these compounds in comparison with that of PABA/NO.

The compound **8** itself does not release appreciable amounts of nitric oxide (Table 2), probably due to equilibration to its phenolate ion **8a** (Scheme 7) that is expected to resist reaction with nucleophilic species such as GSH (pK_a of 2,4-dinitrophenol is 4.09).[24]

From the higher amounts of NO released from **18**–**22**, it is inferred that an amine, amide, sulfonate, or ether linker is less labile to cleavage under these conditions. Future work will focus on preparing analogues with such linkages that are resistant to hydrolysis in the presence of GSH.

Next, PABA/NO and its analogues were tested for their ability to inhibit leukemia cell proliferation and IC_{50} values were determined (Table 2). It was found that in addition to PABA/NO, the compounds **18**–**22** were potent inhibitors of cell proliferation. When the % NO released upon treatment with GSH and IC_{50} values of PABA/NO and its analogues were plotted, a noteworthy trend emerged (Fig. 1). Compounds that released elevated amounts of nitric oxide (>70%) were found to display potent anti-proliferative activity (<10 μM). Furthermore, all compounds in this study that released negligible amounts of NO were found to be moderately

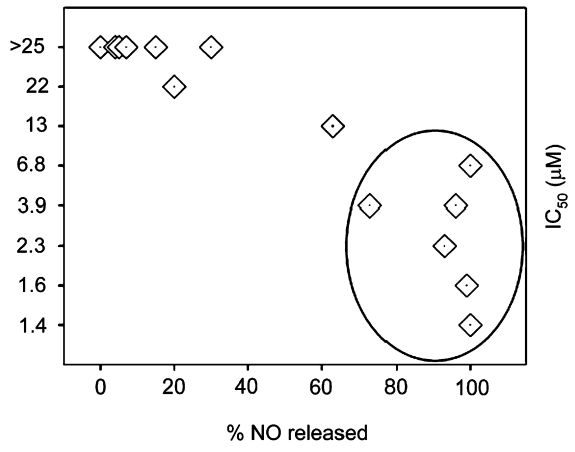


Figure 1. Variation of observed cytotoxic effects with % NO released upon activation by GSH by PABA/NO and its analogues.

active or inactive in the cell proliferation bioassay. Taken together, these observations indicate a role for nitric oxide in the cytotoxic activity of these compounds.

In conclusion, we report the synthesis of a number of O^2-(2,4-dinitrophenyl) diazeniumdiolates that are designed to be activated by glutathione to release nitric oxide using several approaches. The nitric oxide release patterns of these prodrugs in the presence of glutathione were determined; in the absence of competing pathways, nearly quantitative amounts of NO release were observed. Their in vitro anti-leukemic activity indicates that nitric oxide is an important component of cytotoxicity. Finally, this study reveals structural patterns that may assist in future design of improved nitric oxide-based anti-cancer agents.

3. Experimental

3.1. General

NO was purchased from Matheson Gas Products (Montgomeryville, PA). Starting materials were purchased from Aldrich Chemical Co. (Milwaukee, WI) unless otherwise indicated. NMR spectra were obtained in chloroform-*d*, DMSO-d_6, or acetone-d_6 on a Varian UNITY INOVA spectrometer; chemical shifts (δ) are reported in parts per million (ppm) downfield from tetramethylsilane. NMR spectra for publication were prepared using the software MestRe Nova (Mestrelab Research, Spain). Quantification of NO after treatment of compounds with glutathione (GSH) was carried out by chemiluminescence with a Thermal Energy Analyzer Model 502A instrument (Thermedics, Inc., Woburn, MA) or a Sievers 280i Nitric Oxide Analyzer (Boulder, CO).[12] Ultraviolet (UV) spectra were recorded on an Agilent Model 8453 or a Hewlett-Packard model 8451A diode array spectrophotometer. Infrared spectra were recorded on M500 IR spectrometer (Buck Scientific, East Norwalk, CT). Elemental analyses were performed by Midwest Microlab (Indianapolis, IN). Sodium 1-(*N*,*N*-dimethylamino)diazen-1-ium-1,2-diolate (DMA/NO, 2) was prepared as described earlier.[4] O^2-(2,4-Dinitro-5-fluorophenyl) 1-(*N*,*N*-dimethylamino)-diazen-1-ium-1,2-diolate **7** and O^2-(2,4-dinitro-5-hydroxyphenyl) 1-(*N*,*N*-dimethylamino)diazen-1-ium-1,2-diolate **8** were prepared by reported methods.[12]

3.2. General synthetic procedures

Unless otherwise specified, these were the general methods used for the synthesis of PABA/NO analogues.

Method A: Ester formation. A solution or a partial solution of 1.5 mmol of the aryl carboxylic acid in dichloromethane (65 mL) was treated with triethylamine (2 molar excess) giving a homogeneous solution. To this was added a solution of **7** (420 mg, 1.45 mmol) in dichloromethane (65 mL) and the mixture was stirred at room temperature. The progress of the reaction was monitored by TLC and after 72 h, the solution was washed with 5% sodium bicarbonate, then with 1 M HCl, dried

(sodium sulfate), filtered and evaporated to give a solid that was recrystallized from ethanol or from dichloromethane:ether. Unless otherwise indicated, no chromatography was required.

Method B: Alternate method for ester formation. To a solution of **7** (151 mg, 0.52 mmol) in acetonitrile (6 mL), a solution of 0.53 mmol of the aryl carboxylic acid and a slight molar excess of triethylamine in acetonitrile (4 mL) were added and the resulting solution was stirred at room temperature. A precipitate formed within 30 min of reaction and the resulting slurry was stirred overnight. The reaction mixture was diluted with water, and the yellow precipitate was collected by filtration and recrystallized from ethanol afforded pure material.

Method C: Acylation reaction. To a solution of **8** (96 mg, 0.33 mmol) in dichloromethane (5 mL), triethylamine (56 μL, 0.44 mmol) was added. To this an equimolar amount of the acid chloride in dichloromethane (5 mL) was added and the mixture was stirred at room temperature for 20 min to 2 h while being monitored by TLC. The reaction mixture was diluted with dichloromethane (20 mL), then washed with 1 M HCl and subsequently with 5% sodium bicarbonate. The organic layer was dried over sodium sulfate, filtered through magnesium sulfate and evaporated to give either a powder or a glass. The powder was normally recrystallized from mixtures of ethanol and dichloromethane, while the glassy products were dissolved in ether and allowed to crystallize slowly on standing.

Method D: Arylether formation. To a partial solution of **7** (50 mg, 0.17 mmol) in *tert*-butanol (2 mL), an equivalent of the phenol in 0.1 M NaOH (2 mL) was added. The slurry was stirred at room temperature overnight and diluted with water; the resulting yellow precipitate was collected by filtration and recrystallized from ethanol.

Method E: Amide formation. To a solution of 1.6 g (11 mmol) of *N*-substituted benzamide in anhydrous THF, cooled to −80 °C, lithium diisopropyl amide (LDA, 1.5 M in hexane, 8.5 mL, 12.8 mmol) was added. After 1 h, this solution, by syringe, was added to **7** (2.1 g, 10.1 mmol), also dissolved in anhydrous THF, and cooled to −80 °C. After an additional hour, solvents were removed and the remaining solids were extracted with dichloromethane and washed with dilute HCl and brine, then dried (Na_2SO_4 and $MgSO_4$), filtered, and flash chromatographed (1:1 hexanes:ethyl acetate) to obtain pure material.

3.2.1. O^2-(2,4-Dinitro-5-(4-(*N*-methylamino)benzoyloxy)-phenyl) 1-(*N,N*-dimethylamino)diazen-1-ium-1,2-diolate (PABA/NO, 3). Prepared using method A in 79% yield. Its physicochemical properties matched those described previously.

3.2.2. O^2-(2,4-Dinitro-5-(4-(*N,N*-dimethylamino)benzoyloxy)phenyl) 1-(*N,N*-dimethylamino)diazen-1-ium-1,2-diolate (9). A solution of **7** in acetonitrile was treated with 4-dimethylaminobenzoic acid and triethylamine according to Method B. The crude product was recrystallized from ethanol to give a 66% yield of **9**: mp 160–161 °C; UV λ_{max} (ε) 229 nm (29.1 mM^{-1} cm^{-1}), 320 nm (47.5 mM^{-1} cm^{-1}); ^1H NMR ($CDCl_3$) δ 3.11 (s, 6 H), 3.27 (s, 6H), 6.71 (d, 2H, J = 9.2 Hz), 7.55 (s, 1H), 8.03 (d, 2H, J = 9.2 Hz), 8.90 (s, 1H); ^{13}C NMR δ 40.1, 41.8, 110.9, 113.4, 124.6, 132.7, 133.4, 135.9, 150.0, 153.9, 154.3, 163.4. Anal. Calcd for $C_{17}H_{18}N_6O_8 \cdot 0.05$ EtOH: C, 47.02; H, 4.23; N, 19.24. Found: C, 46.59; H, 4.17; N, 18.85.

3.2.3. O^2-(2,4-Dinitro-5-(4-(amino)benzoyloxy)phenyl) 1-(*N,N*-dimethylamino)diazen-1-ium-1,2-diolate (10). A solution of **7** in acetonitrile was treated with aminobenzoic acid and triethylamine according to Method B. The crude product was recrystallized from ethanol to give a 64% yield of **10**: mp 160–1601 °C; IR (film) 1742 cm^{-1}; ^1H NMR ($CDCl_3$) δ 3.27 (s, 6H), 4.30 (broad, 2H), 6.70–6.72 (m, 2H), 7.53 (s, 1H), 7.98–8.00 (m, 2H), 8.91 (s, 1H); ^{13}C NMR δ 41.8, 113.4, 113.9, 116.3, 128.6, 133.1, 135.8, 149.8, 152.4, 152.5, 154.0, 163.1. Anal. Calcd for $C_{15}H_{14}N_6O_8$: C, 44.34; H, 3.47; N, 20.68. Found: C, 43.90; H, 3.45; N, 20.97.

3.2.4. O^2-(2,4-Dinitro-5-(4-(*N*-acetylamino)benzoyloxy)-phenyl) 1-(*N,N*-dimethylamino)diazen-1-ium-1,2-diolate (11). This product was obtained in 75% yield from the reaction of 4-acetamidobenzoyl chloride and **8** as described in Method C: mp 151–2 °C; UV (ethanol) λ_{max} (ε) 292 nm (46.1 mM^{-1} cm^{-1}); ^1H NMR (acetone-d_6) δ 2.25 (s, 3H), 3.28 (s, 6H), 7.43 (broad, 1H), 7.56 (s, 1H), 7.73 (d, 2H, J = 8.8 Hz), 8.17–8.19 (m, 2H), 8.96 (s, 1H); ^{13}C NMR δ 24.9, 41.8, 113.3, 119.0, 122.7, 124.9, 132.2, 133.9, 135.5, 143.7, 149.3, 154.1, 162.8, 168.5. Anal. Calcd for $C_{17}H_{16}N_6O_9$: C, 45.54; H, 3.60; N, 18.74. Found: C, 45.41; H, 3.58; N, 18.45.

3.2.5. O^2-(2,4-Dinitro-5-(benzoyloxy)phenyl) 1-(*N,N*-dimethylamino)diazen-1-ium-1,2-diolate (12). A solution of **8** in dichloromethane was treated with benzoyl chloride as described in Method C to give a 34% yield of **12**: mp 130–1 °C; UV (ethanol) λ_{max} (ε) 232 nm (16.9 mM^{-1} cm^{-1}), 293 nm (8.2 mM^{-1} cm^{-1}); ^1H NMR ($CDCl_3$) δ 3.28 (s, 6H), 7.54 (s, 1H), 7.57–7.59 (m, 2H), 7.70–7.74 (m, 1H), 8.19–8.22 (m, 2H), 8.96 (s, 1H); ^{13}C NMR δ 41.8, 113.3, 124.9, 127.6, 128.9, 130.7, 133.9, 134.8, 149.3, 154.1, 163.5. Anal. Calcd for $C_{15}H_{13}N_5O_8$: C, 46.04; H, 3.35; N, 17.90. Found: C, 45.76; H, 3.34; N, 17.59.

3.2.6. O^2-(2,4-Dinitro-5-(nicotinoyloxy)phenyl 1-(*N,N*-dimethylamino)diazen-1-ium-1,2-diolate (13). A solution of **8** in dichloromethane was treated with two equivalents of triethylamine followed by acylation with one equivalent of nicotinoyl chloride hydrochloride as described in Method C; recrystallization of the resulting solid from ether afforded **13** (44% yield): mp 123–124 °C; UV (ethanol) λ_{max} (ε) 264 nm (36.5 mM^{-1} cm^{-1}), 299 nm (33.7 mM^{-1} cm^{-1}); ^1H NMR ($CDCl_3$) δ 3.30 (s, 6H), 7.54 (s, 1H), 7.53–7.56 (m, 1H), 8.46–8.49 (m, 1H), 8.93–8.94 (m, 1H), 8.99 (s, 1H), 9.41 (s, 1H); ^{13}C NMR δ 41.8, 110.6, 121.1, 121.3, 122.4, 131.6, 132.6, 148.9, 151.6, 152.1, 159.7,

162.4. Anal. Calcd for $C_{14}H_{12}N_6O_8$: C, 42.86; H, 3.08; N, 21.42. Found: C, 42.64; H, 3.12; N, 21.11.

3.2.7. O^2-(2,4-Dinitro-5-(4-(nitrobenzoyl)oxy)phenyl) 1-(*N,N*-dimethylamino)diazen-1-ium-1,2-diolate (14). This compound was obtained in 71% yield from **8** and *p*-nitrobenzoyl chloride as described in Method C. The product was recrystallized from ether:dichloromethane: mp 133–134 °C; UV (ethanol) λ_{max} (ε) 261 nm (35.7 mM^{-1} cm^{-1}), 299 nm (20.9 mM^{-1} cm^{-1}); ^1H NMR ($CDCl_3$) δ 3.29 (s, 6H), 7.55 (s, 1H), 8.40 (m, 4H), 8.99 (s, 1H); ^{13}C NMR δ 41.7, 113.1, 124.0, 125.1, 131.9, 132.9, 134.3, 135.0, 148.5, 151.5, 154.3, 161.9. Anal. Calcd for $C_{15}H_{12}N_6O_{10}$: C, 41.29; H, 2.77; N, 19.26. Found: C, 41.28; H, 2.80; N, 19.09.

3.2.8. O^2-(2,4-Dinitro-5-(2-acetoxybenzoyl)oxy)phenyl) 1-(*N,N*-dimethylamino)diazen-1-ium-1,2-diolate (15). A solution of **8** in dichloromethane was acylated with the acid chloride of acetylsalicylic acid containing a slight molar excess of triethylamine according to Method C to give a 66% yield of **15**: mp 128–9 °C; UV (ethanol) λ_{max} (ε) 235 nm (21.8 mM^{-1} cm^{-1}), 293 nm (11.9 mM^{-1} cm^{-1}); ^1H NMR ($CDCl_3$) δ 2.30 (s, 3H), 3.29 (s, 6H), 7.21–7.24 (m, 1H), 7.42–7.46 (m, 1H), 7.49 (s, 1H), 7.71–7.75 (m, 1H), 8.25–8.26 (dd, 1H, J = 1.8 Hz, J = 8.0 Hz), 8.95 (s, 1H); ^{13}C NMR δ 21.0, 41.7, 113.5, 120.7, 124.2, 124.9, 126.4, 132.4, 134.1, 135.4, 135.8, 148.8, 151.7, 154.2, 160.9, 169.5. Anal. Calcd for $C_{17}H_{15}N_5O_{10}$: C, 45.44; H, 3.36; N, 15.59. Found: C, 45.28; H, 3.33; N, 15.29.

3.2.9. O^2-(2,4-Dinitro-5-(2-(4-(2-methylpropyl)phenyl) propionoyloxy)phenyl) 1-(*N,N*-dimethylamino)diazen-1-ium-1,2-diolate (16). This adduct was prepared in 85% yield from the reaction of **8** with the acid chloride of 4-isobutyl-α-methyl phenylacetic acid as described in Method C: mp 121–122 °C; UV (ethanol) λ_{max} (ε) 297 nm (24.4 mM^{-1} cm^{-1}); ^1H NMR ($CDCl_3$) δ 0.91 (dd, 6H, J = 0.8, 6.7 Hz), 1.67 (d, 3H, J = 7.2 Hz), 1.87 (septet, 1H, J = 6.9 Hz), 2.48 (d, 2H, J = 7.2 Hz), 3.24 (s, 6H), 4.06 (q, 1H, J = 7.2 Hz), 7.15–7.18 (m, 2H), 7.20 (s, 1H), 7.29–7.31 (m, 2H), 8.87 (s, 1H); ^{13}C NMR δ 18.3, 22.4, 30.2, 41.7, 45.0, 45.2, 113.0, 124.8, 127.5, 129.6, 134.1, 135.7, 141.3, 149.0, 154.4, 171.3. Anal. Calcd for $C_{21}H_{25}N_5O_8$: C, 53.05; H, 5.30; N, 14.23. Found: C, 52.69; H, 5.20; N, 14.37.

3.2.10. O^2-(2,4-Dinitro-5-(2-(6-methoxynaphth-2-yl)propionoyloxy)phenyl) 1-(*N,N*-dimethylamino)diazen-1-ium-1,2-diolate (17). Method C was followed in the synthesis of **17**: mp 118–119 °C; UV (ethanol) λ_{max} (ε) 299 nm (24.4 $mM^{-1}cm^{-1}$); ^1H NMR ($CDCl_3$) δ 1.76 (d, 3H, J = 7.2 Hz), 3.17 (s, 6H), 3.93 (s, 3H), 4.22 (q, 1H, J = 7.2 Hz), 7.15–7.18 (m, 3H), 7.47–7.50 (m, 1H), 7.73–7.77 (m, 3H), 8.87 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 18.2, 41.7, 45.5, 55.4, 77.2, 105.6, 113.0, 119.3, 124.8, 126.2, 126.5, 128.9, 129.3, 133.6, 134.0, 135.4, 149.0, 154.0, 158.0, 171.3; Anal. Calcd for $C_{22}H_{21}N_5O_9 \cdot 0.25H_2O$: C, 52.43; H, 4.30; N, 13.90. Found: C, 52.24; H, 4.24; N, 14.06.

3.2.11. O^2-(2,4-Dinitro-5-(4-(hydroxymethyl)phenyl)aminophenyl) 1-(*N,N*-dimethylamino)diazen-1-ium-1,2-diolate (18). To a solution of **7** (1.9 g, 6.5 mmol) in 40 mL of THF/acetone (1:1), 0.81 g (6.6 mmol) of 4-aminobenzyl alcohol in acetone (15 mL) was added. The reaction was stirred at room temperature for 48 h and progress was monitored by TLC. Solvents were evaporated under vacuum and the resulting solids were taken up in dichloromethane. This solution was was washed with 5% sodium bicarbonate, dried over sodium sulfate, filtered and evaporated. Recrystallization from ethanol gave a 79% yield of **18**: mp 137–140 °C; UV (ethanol) λ_{max} (ε) 304 nm (26.1 mM^{-1} cm^{-1}); ^1H NMR (DMSO-d_6) δ 3.02 (s, 6H), 4.54 (d, 2H, J = 5.7 Hz), 5.28 (t, 1H, J = 5.7 Hz), 6.87 (s, 1H), 7.36 (d, 2H, J = 8.4 Hz), 7.45 (d, 2H, J = 8.4 Hz), 8.91 (s, 1H), 10.16 (s, 1H); ^{13}C NMR (DMSO-d_6) δ 40.9, 62.3, 100.2, 125.7, 126.1, 126.4, 127.3, 127.5, 136.0, 141.7, 147.4, 154.1. Anal. Calcd for $C_{15}H_{16}N_6O_7$: C, 45.92; H, 4.11; 21.42. Found: C, 45.95; H, 4.05; N, 21.19.

3.2.12. O^2-(2,4-Dinitro-5-(*N*-benzoyl-*N*-methylamino) phenyl) 1-(*N,N*-dimethylamino)diazen-1-ium-1,2-diolate (19). To a slurry of **2** (1.5 g, 12.0 mmol) in anhydrous THF (30 mL) at 0 °C, **24** (1.3 g, 4.1 mmol) dissolved in anhydrous THF (25 mL) was added. After 1 h, solvents were removed and solids were extracted with dichloromethane and brine. The organic layers were dried and filtered, and solvents were removed. Recrystallization from ethanol gave **19** (640 mg, 1.6 mmol, 39% yield): mp 120–123 °C; UV (ethanol) λ_{max} (ε) 288 nm (20.8 mM^{-1} cm^{-1}); ^1H NMR ($CDCl_3$) δ 3.26 (s, 6H), 3.46 (s, 3H), 7.28–7.32 (m, 2H), 7.37–7.41 (m, 4H), 8.66 (s, 1H); ^{13}C NMR δ 38.6, 41.7, 118.0, 124.6, 128.0, 128.3, 131.1, 138.9, 144.3, 151.2, 170.3. Anal. Calcd for $C_{16}H_{16}N_6O_7$: C, 47.53; H, 3.99; N, 20.78. Found: C, 47.77; H, 4.10; N, 20.41.

3.2.13. O^2-(2,4-Dinitro-5-(*N*-benzoyl-*N*-ethylamino)phenyl) 1-(*N,N*-dimethylamino)diazen-1-ium-1,2-diolate (20). To a slurry of **2** (1.1 g, 8.8 mmol) in anhydrous THF (20 mL) at 0 °C, **25** (0.9 g, 2.8 mmol) dissolved in anhydrous THF (15 mL) was added. After 2 h, solvents were removed and solids were extracted with dichloromethane and brine. The organic layer was dried and filtered, and the solvents were removed. Recrystallization from ethanol gave **20** (670 mg, 1.6 mmol, 57% yield): mp 129–130 °C; UV (ethanol) λ_{max} (ε) 288 nm (20.8 $mM^{-1}cm^{-1}$); ^1H NMR ($CDCl_3$) δ 1.28 (t, 3H, J = 7.1), 3.27 (s, 6H), 3.94 (broad, 2H), 7.29–7.41 (m, 6H), 8.65 (s, 1H); ^{13}C NMR δ 13.3, 41.7, 46.4, 118.1, 124.6, 127.8, 128.5, 134.3, 134.4, 139.5, 142.9, 153.0, 170.0. Anal. Calcd for $C_{17}H_{18}N_6O_7$: C, 48.81; H, 4.34; N, 20.09. Found: C, 48.75; H, 4.35; N, 19.94.

3.2.14. O^2-(2,4-Dinitro-5-(4-(*N*-acetylamino)benzenesulfonyl)oxy)phenyl) 1-(*N,N*-dimethylamino)diazen-1-ium-1,2-diolate (21). To a solution of acetylsulfanilyl chloride (145 mg, 0.62 mmol) in acetone (7 mL), a solution of **8** (178 mg, 0.62 mmol) in 0.1 M sodium hydroxide (7 mL). The resulting homogeneous solution was stirred at room temperature and gradually became cloudy. After stirring overnight, the product was collected by fil-

tration, washed with water followed by ether, and allowed to dry to form a yellow powder that was recrystallized from acetone:dichloromethane to afford **21** (193 mg, 0.4 mmol, 64%): mp 138–139 °C; UV (ethanol) λ_{max} (ε) 280 nm (28.2 $mM^{-1}cm^{-1}$), 338 nm (18.5 $mM^{-1}cm^{-1}$); ^1H NMR (acetone-d_6) δ 2.17 (s, 3H), 3.26 (s, 6H) , 7.36 (s, 1H), 7.86–7.88 (m, 2H), 7.95–7.97 (m, 2H), 8.82 (s, 1H), 9.79 (broad, 1H); ^{13}C NMR (acetone-d_6) δ 24.5, 41.7, 113.7, 119.7, 119.8, 125.5, 127.6, 131.2, 146.6, 146.9, 154.3, 169.9, 170.0. Anal. Calcd for $C_{16}H_{16}N_6O_{10}S$: C, 39.67; H, 3.33; N, 17.35; S, 6.62. Found: C, 39.42; H, 3.24; N, 16.95; S, 6.97.

3.2.15. O^2-(2,4-Dinitro-5-(4-(acetamido)phenoxy)) 1-(*N*,*N*-dimethylamino)diazen-1-ium-1,2-diolate (22). A solution of **7** in *tert*-butanol was reacted with 4-acetamidophenol in 0.1 M NaOH according to Method D. The product was recrystallized from ethanol to give a 75% yield of **22**: mp 159–160 °C; UV (acetonitrile) λ_{max} (ε) 224 nm (26.6 mM^{-1} cm^{-1}), 281 nm (18.9 mM^{-1} cm^{-1}); ^1H NMR (acetone-d_6) δ 2.21 (s, 3H), 3.11 (s, 6H), 7.06 (s, 1H), 7.25–7.28 (m, 2H), 7.81 (d, 2H, J = 8.7 Hz), 8.86 (s, 1H), 9.35 (broad, 1H); ^{13}C NMR (acetone-d_6) δ 23.6, 41.0, 105.7, 121.1, 121.2, 125.6, 131.0, 133.7, 138.3, 149.2, 154.6, 157.2, 168.4. Anal. Calcd for $C_{16}H_{16}N_6O_8 \cdot 0.5\,H_2O$: C, 44.76; H, 3.99; 19.57. Found: C, 44.57; H, 3.81; N, 19.46.

3.2.16. 2,4-Dinitro-1-fluoro-5-(*N*-benzoyl-*N*-methylamino)-benzene (24). *N*-Methylbenzamide was reacted with **4** according to Method E to afford **24** (43% yield): mp 153–156 °C; ^1H NMR (acetone-d_6) δ 3.54 (s, 3H), 7.34–7.50 (m, 5H), 8.02 (d, 1H, J = 11.5 Hz), 8.72 (d, 1H, J = 7.5 Hz); ^{13}C NMR (acetone-d_6) δ 38.9, 120.6, 125.0, 128.9, 129.3, 131.6, 135.3, 142.5, 145.5, 157.0, 159.7, 170.5. Anal. Calcd for $C_{14}H_{10}FN_3O_5$: C, 52.67; H, 3.16; 13.16. Found: C, 52.52; H, 3.18; N, 13.01.

3.2.17. 2,4-Dinitro-1-fluoro-5-(*N*-benzoyl-*N*-ethylamino) benzene (25). *N*-Ethylbenzamide[25] was reacted with **4** according to Method E to afford **25** (29% yield): mp 113–114 °C; ^1H NMR ($CDCl_3$) δ 1.29 (t, 3 H, J = 7.1 Hz), 3.96 (q, 2 H, J = 7.0 Hz), 7.32–7.46 (m, 6H), 8.70 (d, 1H, J = 7.5 Hz); ^{13}C NMR ($CDCl_3$) δ 13.5, 46.6, 119.4, 124.5, 127.7, 128.7, 131.2, 134.0, 141.7, 143.6, 156.0, 158.7, 170.2. Anal. Calcd for $C_{15}H_{12}FN_3O_5$: C, 54.06; H, 3.63; 12.61. Found: C, 53.94; H, 3.62; N, 12.51.

3.3. Nitric oxide release

Chemiluminescence detection and quantification of NO evolving from the reactions of PABA/NO and its analogues were conducted by preparing 10 mM stock solutions of these compounds in pH 7.4 phosphate buffer. A pH 7.4 phosphate buffer containing 1 mM GSH was sparged with inert gas until a steady detector response was established. The stock solution of the diazeniumdiolate prodrug was injected into this GSH solution and the NO release profile was followed at 37 °C. The final concentrations of the NO prodrug in such solutions were 100–150 nM. The resulting curve was integrated to quantify the amount of NO released/mol of compound.

3.4. HL-60 cell culture and growth assay

Human myeloid leukemia HL-60 cells (American Type Culture Collection, Manassas, VA) were cultured in RPMI 1640 with 10% fetal bovine serum at 37 °C in a 5% CO_2-humidified atmosphere. Cells were plated in 96-well plates at a concentration of 10,000 cells in 100 μL. Stock DMSO solutions of each compound were serially diluted in phosphate-buffered saline (PBS) before addition to cultures with a final DMSO concentration in the cultures of 0.5%. Agents were added after 24 h of culture initiation. Three days after agent addition, the number of viable cells was determined using the MTS assay (CellTiter 96, Promega, Madison, WI) according to the manufacturer's protocol. IC_{50} values were calculated by nonlinear fitting of the absorbance/concentration data to the following equation,

$$y = A_{min} + (A_{max} - A_{min})/(1 + 10^{(\mathrm{Log\ IC_{50}} - x) * \mathrm{HS}})$$

where A_{min} is the (background) absorbance observed with no living cells, A_{max} is the (control) absorbance with no compound added, Log IC_{50} is the logarithm of the IC_{50}, HS is the Hill slope, and x is the concentration of the compound in the culture medium. Alternatively, the IC_{50} values were calculated using the fitting program available in Sigma Plot.

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Supplementary data

NMR spectra for compounds **10**–**22**. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2007.11.035.

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