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Diazeniumdiolated carbamates: A novel class of nitric oxide donors

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ABSTRACT

We report an indirect method for synthesis of previously inaccessible diazeniumdiolated carbamates. Synthesis involves use of previously reported triisopropylsilyloxymethylated isopropylamine diazeniumdiolate (TOM-ylated IPA/NO). These novel diazeniumdiolated carbamate prodrugs upon activation release nitric oxide (NO) similar to their secondary amine counterparts. They are also efficient sources of intracellular NO. These prodrugs may have potential applications as therapeutic NO-donors.

1. Introduction

Diazeniumdiolate prodrugs[1] (**1**, Fig. 1) are considered as reliable sources of the potent bioregulatory agent, nitric oxide[2] (NO). These prodrugs (**1**), on metabolic activation or hydrolysis, form the parent anion (**2**), which further decomposes to release up to two moles of NO and the parent amine (Fig. 1a). The therapeutic applications of these prodrugs are diverse, and largely depend on the *O*-2 protecting group ('R' in structure **1**, Fig. 1) and its mechanism of activation. For example, vinyl protected prodrug V-PYRRO/NO (**3**) is activated by cytochrome P450 to release NO, and shows hepatoprotective properties against a variety of toxins.[3] Glutathione (GSH)-activated arylated prodrug JS-K (**4**) is a lead anticancer agent.[4] Recently, primary amine diazeniumdiolate prodrug AcOM-IPA/NO (**5**)[5] was reported to release nitroxyl (HNO), another potent bioeffector molecule with possible applications in treating heart failure and alcohol abuse.[6]

Secondary amine diazeniumdiolate ions are protonated at *N*-3 (see Fig. 1 for numbering) to release NO,[1] whereas, primary amine diazeniumdiolates release nitroxyl[5] (HNO) on protonation at *N*-2. Given the difference in product distribution between primary and secondary amine diazeniumdiolate ions seen on hydrolyzing them (mixed NO/HNO donors versus pure NO donors, respectively), we speculated that diazeniumdiolated carbamates (**6a**) and amides (**6b**) might hydrolyze with different and potentially advantageous product distributions and/or kinetics (Fig. 1c). We have until now had no success in forming diazeniumdiolate ions by reacting amides or other acylated amines directly with NO under basic conditions, so we have resorted to an indirect method for accessing a relevant prototype. Here we describe the synthesis of prodrugs that can be used to probe the chemistry of anionic

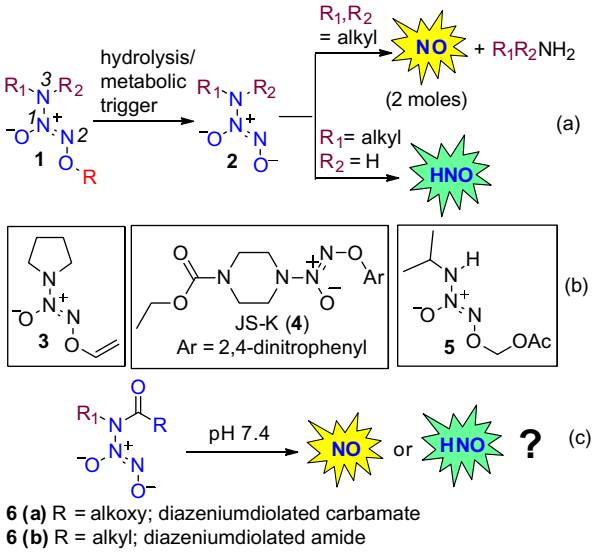

Figure 1. (a) Activation of diazeniumdiolate prodrugs to release NO or HNO (b) Structures of, V-PYRRO/NO (**3**), JS-K (**4**) and AcOM-IPA/NO (**5**). (c) Hydrolysis of diazeniumdiolated amides and carbamates (**6**).

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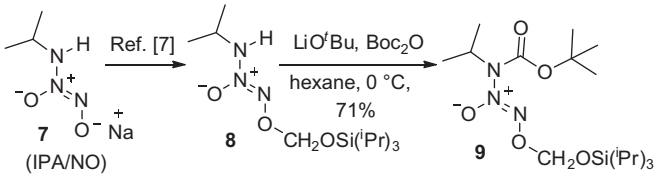
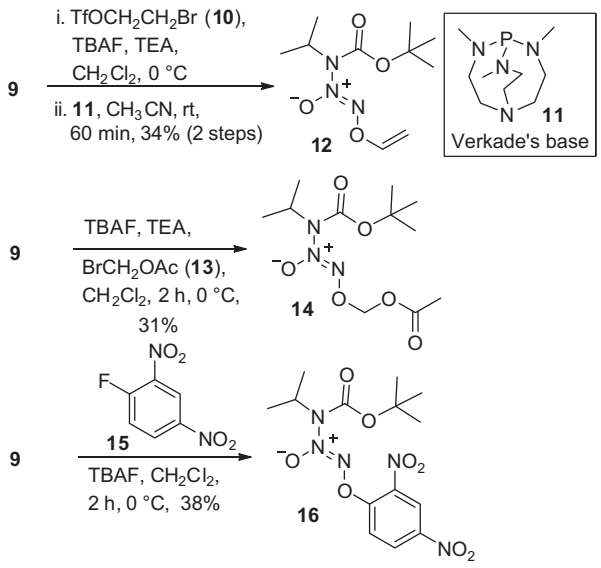


Scheme 1. Synthesis of carbamate diazeniumdiolate **9**.



Scheme 2. Synthesis of carbamate diazeniumdiolate prodrugs **12**, **14** and **16**.

diazeniumdiolated carbamates as well as to generate analogues that can be used for studies of their biological properties.

2. Results and discussion

2.1. Synthesis

We envisaged that the triisopropylsilyloxymethyl (TOM) protecting group strategy[7] developed for *O*-2 substituted diazeniumdiolate prodrugs might be useful for the synthesis of such carbamate or amide derivatives. Thus, IPA/NO (**7**, Scheme 1) was converted to TOM-ylated IPA/NO (**8**, Scheme 1) following the reported procedure.[7] This compound **8** on treatment with a base and Boc-anhydride (Boc_2O) gave carbamate **9**. After experimentation with several bases and reaction conditions for this transformation, it was observed that compound **9** is efficiently formed by using lithium *tert*-butoxide in hexane at 0 °C.

With compound **9** in hand, it was important to remove the TOM protecting group and introduce a biologically significant protecting group helpful for site-directed delivery of NO. We treated compound **9** with tetra-*n*-butylammonium fluoride (TBAF) in the presence of a 4-fold excess of 2-bromo-l- [((trifluoromethane)sulfonyl)oxy]ethane (**10**) and triethylamine (TEA) to obtain the required bromide (Scheme 2). This crude bromide on treatment with Verkade's superbase (**11**) gave the vinyl carbamate prodrug **12** in 34% yield (2 steps). Removal of silyl protection in the presence of bromomethyl acetate (**13**) and TEA gave esterase-labile acetoxymethylated diazeniumdiolate prodrug **14**. Similar deprotection in the presence of 2,4-dinitrofluorobenzene (**15**) gave arylated prodrug **16** in 38% yield. To the best of our knowledge, these are the first examples of diazeniumdiolates with a carbamate functional group at *N*-3 (for atom numbering system, see structure **1**, Fig. 1).

2.2. NO-release

It was important to identify and quantitate the NO and/or HNO released on activation of these prodrugs. Chemiluminescence assay was used to determine NO. After NO evolution had ceased, the residual solution was further used for Griess's assay in the presence of air (oxygen). The Griess's assay was used to determine amount of nitrite (NO_2^-) present in the solution. Nitrite is the oxidation product of NO in aerobic aqueous solution. Quantitation of HNO was carried out using quantitation of nitrous oxide (N_2O), a dimerization product of HNO, by gas chromatography. The results are summarized in Table 1. Compound **14** on activation by porcine liver esterase under physiological conditions for 5 days gave 1.25 mol (63%) of NO, 0.06 mol (3%) of nitrite and an undetectable amount of N_2O. The decreased amount of NO measured on hydrolysis of **14** may be attributed to one or more effects. Nitrosative inactivation of the enzyme could occur, ceasing hydrolysis and resulting in protein nitrosation products not detectable by Griess assay. Formation of *N*-nitrosocarbamate might also be expected. This product could further rearrange to the *O*-isopropyl carbamate with expulsion of N_2, a difficult product to measure. Similarly, compound **16** on activation by GSH in PBS under physiological conditions gave about 1.35 mol of NO, 0.32 mol of nitrite and trace amount of N_2O. Prodrug **16** also shows less than the calculated amount of NO, probably due to similar reasons as speculated for compound **14**. Thus, these *N*-diazeniumdiolated carbamates release NO via a pathway similar to that of their secondary amine counterparts as shown in Figure 2.

2.3. Intracellular NO-release

Next, we studied the intracellular NO release and cell permeability of these new compounds. The intracellular NO was estimated by using the nitric oxide-sensitive and commercially available dye, 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM diacetate).[8] Normal human skin fibroblast BJ-5ta cells and U937 human leukemia cells were selected, and JS-K (**4**) was taken as a positive control for the experiments. Both the cell lines were pre-loaded with DAF-FM diacetate, followed by treatment with DMSO solutions of prodrugs **14**, **16** and JS-K. The fluorescence measurements after 90 min provided estimates of the levels of intracellular NO. It was observed that at equal concentrations, prodrug **16** showed significantly higher levels of NO as

Table 1
Quantitation of NO, NO_2^- and N_2O for prodrugs **14** and **16**.

Prodrug	Activation conditions	NO mol/mol of prodrug[a] (% yield)	NO_2^- mol/mol of prodrug[a] (% yield)	N_2O mol/mol of prodrug
14	Porcine liver esterase in PBS at 37 °C	1.25 (63%) over 5 days	0.06 (3%)	N.D.[b]
16	GSH (4 mM) in PBS at 37 °C	1.35 (68%) over 400 min	0.32 (16%)	0.02

[a] 2 mol/mol prodrug = 100% yield.
[b] N.D. = not detected.

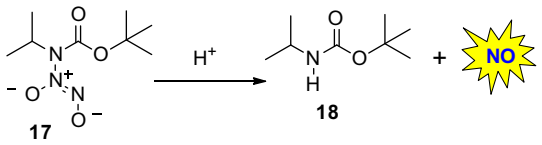


Figure 2. Proposed path for activation of diazeniumdiolated carbamate **17** to release NO, the predominant hydrolysis product.

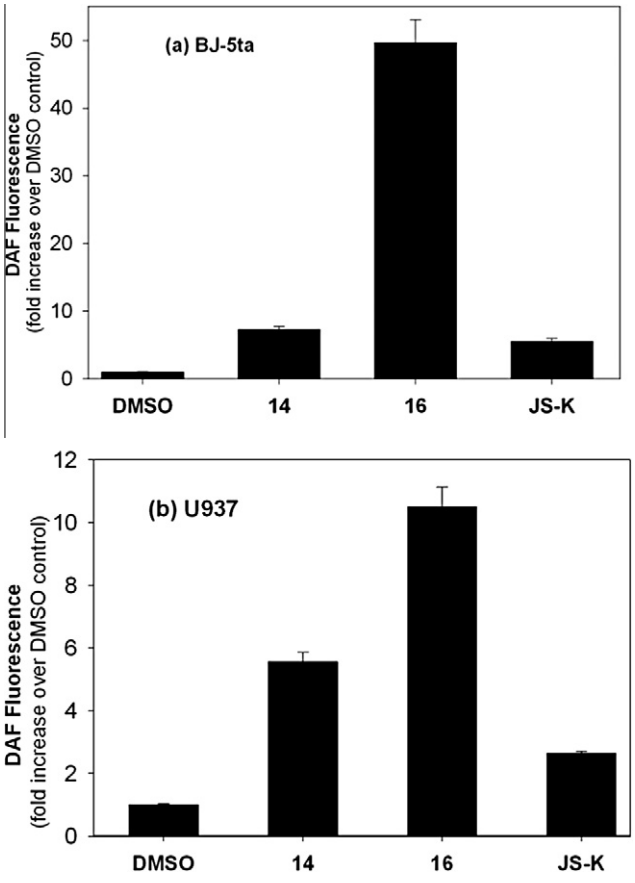


Figure 3. Levels of intracellular NO formation upon treatment of (a) BJ-5ta and (b) U-937 cells with compounds (10 μM final concentration) and DMSO (control) as determined by DAF-FM diacetate fluorescence study.

compared to JS-K, while **14** showed a comparable fluorescence level as that of JS-K for BJ-5ta and U937 cells (Fig. 3). A reviewer has pointed out that the DAF-FM assay is subject to false positive readings under certain experimental settings. Nevertheless, we conclude that the present results are consistent with the view that the prodrugs **14** and **16** are taken up by the cells and are activated therein to release NO.

3. Conclusions

In summary, we have synthesized diazeniumdiolated carbamate prodrugs with established, biologically significant protecting groups starting from TOM-ylated diazeniumdiolate. To the best of our knowledge, this is the first report of diazeniumdiolated carbamate prodrugs. We have studied their activation mechanism and measured the amount of NO released. These new prodrugs release NO by a mechanism similar to their secondary amine counterparts. We have also demonstrated their ability to release NO inside cells. The compounds synthesized have led to a new class of NO-donors with potential biological applications.

4. Experimental

4.1. Synthesis

4.1.1. General

Starting materials were purchased from Aldrich Chemical Co. (Milwaukee, WI) unless otherwise indicated. NMR spectra were recorded on a 400 MHz Varian UNITY INOVA spectrometer; chemical shifts (δ) are reported in parts per million (ppm) downfield from tetramethylsilane. Ultraviolet (UV) spectra were recorded on an Agilent Model 8453 or a Hewlett-Packard model 8451A diode array spectrophotometer. High resolution mass spectra (HRMS) were recorded on Agilent 6250 series Accurate-Mass Q-TOF LC/MS by electrospray ionization (ESI). Elemental analyses were performed by Midwest Microlab (Indianapolis, IN). Chromatography was performed on a Biotage SP1 Flash Purification System. Prepacked silica gel flash chromatography columns were purchased from Yamazen Science Inc. (San Bruno, CA). Compounds **8**[7] and **10**[9] were prepared by using the reported procedures. Diethyl ether was used to dissolve and transfer all the liquid compounds from rotary evaporator flask to smaller containers and sample vials.

4.1.2. Procedures and analytical data

Compound **9**. Under N_2, a 1 M solution of LiO^{t}Bu in hexane (3.8 mL, 3.84 mmol) was added to an ice-cold solution of compound **8** (1.06 g, 3.49 mmol) in hexane (10 mL). After 20 min, a solution of Boc_2O in hexane was added to this ice-cold reaction mixture. After 20 min of stirring, the reaction was diluted with distilled water (10 mL) and hexane (10 mL). The organic layer was washed with brine, dried over anhydrous Na_2SO_4 and evaporated. The crude product was purified by flash column chromatography (9:1 hexane/ethyl acetate) to give compound **9** as an oil (1.0 g, 71%). UV (ethanol) λ_{max} (ε) 235 nm (5.9 mM^{-1} cm^{-1}); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.05–1.17 (m, 3H), 1.08 (d, J = 6.1 Hz, 18H), 1.32 (d, J = 6.7 Hz, 6H), 1.49 (s, 9H), 4.36 (septet, J = 6.7 Hz, 1H), 5.53 (s, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 151.42, 91.58, 83.53, 51.44, 27.96, 19.86, 17.64, 11.81. HRMS (ESI) m/z calculated for $C_{18}H_{43}N_4O_5Si$ $[M+NH_4]^+$ 423.2997, found 423.2993. Anal. Calcd for $C_{18}H_{39}N_3O_5Si{\cdot}0.5Et_2O$: C, 54.27; H, 10.02; N, 9.49, Found: C, 54.36; H, 9.79; N, 9.66.

Compound **12**. To a solution of **9** (140 mg, 0.35 mmol), triethylamine (TEA) (0.2 mL, 1.4 mmol) and 2-bromo-l-[((trifluoromethane)sulfonyl)oxy]ethane (**10**) (360 mg, 1.4 mmol) in CH_2Cl_2 (3 mL) was added tetrabutylammonium fluoride trihydrate (TBAF) (133 mg, 0.42 mmol) in CH_2Cl_2 (2 mL) at 0 °C. Reaction was allowed to attain rt over 2 h, then the reaction was diluted with 5% sodium bicarbonate, and extracted with CH_2Cl_2. The combined organic layer was washed with brine, dried over anhydrous Na_2SO_4 and evaporated. The crude product was used for the next step without further purification. This crude product was dissolved in acetonitrile (2 mL) and a solution of Verkade's superbase **11** (151 mg, 1.4 mmol) in acetonitrile (1 mL) was added at rt. After 60 min at rt, volatiles were evaporated and the crude product was purified by flash column chromatography (9:1 hexane/ethyl acetate) to give compound **12** as a colorless oil (29 mg, 34% for 2 steps). UV (ethanol) λ_{max} (ε) 258 nm (5.73 mM^{-1} cm^{-1}); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.33 (d, J = 6.7 Hz, 6H), 1.50 (s, 9H), 4.37 (septet, J = 6.7 Hz, 1H), 4.53 (dd, J = 6.6, 2.7 Hz, 1H), 4.97 (dd, J = 14.0, 2.7 Hz, 1H), 6.90 (dd, J = 14.0, 6.6 Hz, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 151.25, 148.27, 94.08, 83.98, 51.66, 27.94, 19.85.

Anal. Calcd for $C_{10}H_{19}N_3O_4$: C, 48.97; H, 7.81; N, 17.13, Found: C, 48.92; H, 7.73; N, 17.16.

Compound **14**. To a solution of **9** (400 mg, 0.98 mmol), triethylamine (TEA) (0.6 mL, 3.92 mmol) and bromomethyl acetate (**13**) (0.4 mL, 3.92 mmol) in CH_2Cl_2 (9 mL) was added tetrabutylammonium fluoride trihydrate (TBAF) (372 mg, 1.18 mmol) in CH_2Cl_2 (4 mL) at 0 °C. Reaction was allowed to attain rt over 2 h, then the reaction was diluted with 5% sodium bicarbonate and extracted with CH_2Cl_2. The combined organic layer was washed with brine, dried over anhydrous Na_2SO_4 and evaporated. The crude product was purified by flash column chromatography (9:1 hexane/ethyl acetate) to give compound **14** as colorless oil (89 mg, 31%). UV (ethanol) λ_{max} (ε) 247 nm (5.52 mM^{-1} cm^{-1}); ^1H NMR ($CDCl_3$, 400 MHz) δ 1.32 (d, J = 6.7 Hz, 6H), 1.50 (s, 9H), 2.12 (s, 3H), 4.35 (septet, J = 6.7 Hz, 1H), 5.86 (s, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 168.99, 151.14, 87.24, 83.86, 51.61, 27.93, 20.64, 19.78. HRMS (ESI) m/z calculated for $C_{11}H_{22}N_3O_6$ $[M+H]^+$ 292.1503, found 292.1500.

Compound **16**. To a solution of **9** (360 mg, 0.9 mmol) and 2,4-dinitrofluorobenzene (**15**) (170 mg, 0.9 mmol) in CH_2Cl_2 (9 mL) was added tetrabutylammonium fluoride trihydrate (TBAF) (347 mg, 1.10 mmol) in CH_2Cl_2 (4 mL) at 0 °C. Reaction was allowed to attain rt over 2 h, then the volatiles were evaporated and the crude product was purified by flash column chromatography (5:1 hexane/ethyl acetate) to give compound **16** as a yellow solid (130 mg, 38%). UV (ethanol) λ_{max} (ε) 248 nm (6.01 mM^{-1} cm^{-1}) and 286 nm (7.29 mM^{-1} cm^{-1}); ^1H NMR ($CDCl_3$, 400 MHz) δ 1.39 (d, J = 6.8 Hz, 6H), 1.53 (s, 9H), 4.43 (septet, J = 6.8 Hz, 1H), 7.73 (d, J = 9.2 Hz, 1H), 8.49 (ddd, J = 9.2, 2.7, 0.6 Hz, 1H), 8.90 (d, J = 2.7 Hz, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 152.94, 150.94, 143.15, 129.00, 121.98, 118.79, 84.95, 52.50, 27.95, 19.86. HRMS (ESI) m/z calculated for $C_{14}H_{23}N_6O_8$ $[M+NH_4]^+$ 403.1572, found 403.1578.

4.2. NO-release

4.2.1. Chemiluminescence

Calibration of the Sievers Nitric Oxide Analyzer (NOA), model 280i (Instruments Business Group, Boulder, CO) was performed by injecting various volumes of known concentrations of NO in helium (50 ppm, 500 ppm and 5%) certified standards into the reaction chamber and recording the peak areas. Samples and reaction chambers were incubated at 37 °C. The gas was sparged with argon and swept into the chemiluminescence detector. Data were recorded using Agilent Chemstation software and processed using Microsoft Excel.

Esterase-activated: 3.0 mL of pH 7.4 buffer containing porcine liver esterase (ammonium sulfate suspension, Sigma, 5KU) (0.55 μL) and diethylenetriaminepentaacetic acid (DTPA, 50 μM) was placed into the reaction chamber of the NOA and then sparged for several minutes with argon. A DMSO solution (10 mM) of the prodrug **14** (60 μL) was injected into the reaction chamber and nitric oxide release was recorded. Total amount of NO released was determined by integrating the area under the curve and applying a calibration curve. The NO release is an average of three independent experiments.

Glutathione (GSH)-activated: 3.0 mL of pH 7.4 buffer containing GSH (3.6–3.9 mM) and DTPA (50 μM) was placed into the reaction chamber of the NOA and then sparged for several minutes with argon. A DMSO solution (10 mM) of the prodrug **16** (100 μL) was injected into the reaction chamber and nitric oxide release was recorded by Sievers NOA. Total amount of NO released was determined by integrating the area under the curve and applying a calibration curve. The NO release is an average of three independent experiments.

4.2.2. Griess assay

Sample volume was measured after the chemiluminescence experiment in the presence of air (oxygen). 100 μL of the Griess reagent, 300 μL of sample and 2.6 mL of deionized water were added in a spectrophotometer cuvette. The mixture was incubated for 30 min at rt. Photometric reference sample was prepared by mixing 100 μL of the Griess reagent and 2.9 mL of deionized water. The absorbance of the nitrite-containing sample at 548 nm was measured relative to the reference sample. Then absorbance readings were converted to the nitrite concentrations according to calibration curve.

4.2.3. Gas Chromatography for N_2O

Instrument: Shimadzu GC-2014
Detector: ECD (Electron capture detector)
Column: Restek ShinCarbon 80/100 Packed Column
Length = 2 m; ID = 2.0 mm;
Test condition: Injector temp = 250 °C, Detector temp = 250 °C; Initial column temp = 90 °C; Final temp = 200 °C; Rate = 20 °C/min; Final temp Hold = 1.1 min; Total program time = 6.7 min; Carrier: He (UHP) = 30 mL/min.

4.3. Cell culture and intracellular NO-release

BJ-5ta: Human hTERT immortalized BJ-5ta skin fibroblasts were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Invitrogen, Carlsbad, CA):199 medium (Sigma) (4:1) supplemented with 10% fetal calf serum (Gemini Bio-Products, Sacramento, CA), 100 U/mL penicillin and 2 mM glutamine. The intracellular level of nitric oxide after diazeniumdiolate prodrug treatment was quantified using the NO-sensitive fluorophore 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM diacetate; Invitrogen, Carlsbad, CA). BJ-5ta cells growing on 96-well plates were loaded with 2.5 μM DAF-FM diacetate in Hanks' balanced salt solution (HBSS) at 37 °C and 5% CO_2. After 30 min of incubation the cells were rinsed with HBSS to remove excess probe, and fresh HBSS was added to the wells. The compounds were added to the cells at 10 μM final concentration. After 90-min incubation the fluorescence of the benzotriazole derivative formed on DAF-FM's reaction with aerobic NO was analyzed using a PerSeptive Biosystems CytoFluor 4000 microplate reader with the excitation source at 485 nm and emission at 525 nm. The mean value of 16 independent experiments is reported (see Table S1 in the Supplementary data).

U937: U937 cell lines were obtained from ATCC, Manassas, VA. Cells were maintained in RPMI 1640 medium (Gibco, Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (Gemini Bio-Products, Sacramento, CA), 100 U/mL penicillin and 2 mM glutamine, at 37 °C and 5% CO_2. U937 cells were loaded with 2.5 μM DAF-FM diacetate in HBSS at 37 °C and 5% CO_2. After 30 min of incubation the cells were collected by centrifugation, rinsed with HBSS to remove excess probe, and resuspended in fresh HBSS. The compounds were added to the cells at 10 μM final concentration. After 90-min incubation the fluorescence of the benzotriazole derivative formed on DAF-FM's reaction with aerobic NO was analyzed using a PerSeptive Biosystems CytoFluor 4000 microplate reader with the excitation source at 485 nm and emission at 525 nm. The mean value of 16 independent experiments is reported (see Table S2 in the Supplementary data).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2012.01.046.

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