Hydrolytic Reactivity Trends among Potential Prodrugs of the O^2-Glycosylated Diazeniumdiolate Family. Targeting Nitric Oxide to Macrophages for Antileishmanial Activity

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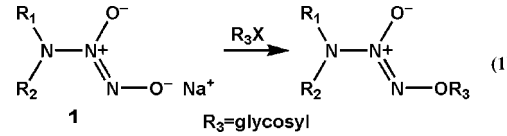
Glycosylated diazeniumdiolates of structure $R_2NN(O)=NO-R'$ (R' = a saccharide residue) are potential prodrugs of the nitric oxide (NO)-releasing but acid-sensitive $R_2NN(O)=NO^-$ ion. Moreover, cleaving the acid-stable glycosides under alkaline conditions provides a convenient protecting group strategy for diazeniumdiolate ions. Here, we report comparative hydrolysis rate data for five representative glycosylated diazeniumdiolates at pH 14, 7.4, and 3.8−4.6 as background for further developing both the protecting group application and the ability to target NO pharmacologically to macrophages harboring intracellular pathogens. Confirming the potential in the latter application, adding $R_2NN(O)=NO-GlcNAc$ (where R_2N = diethylamino or pyrrolidin-l-yl and GlcNAc = *N*-acetylglucosamin-l-yl) to cultures of infected mouse macrophages that were deficient in inducible NO synthase caused rapid death of the intracellular protozoan parasite *Leishmania major* with no host cell toxicity.

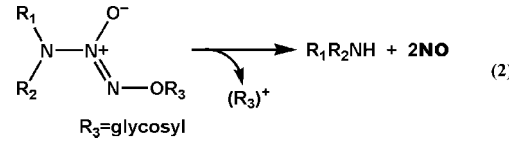
Introduction

Macrophages and the nitric oxide (NO[a]) they produce on stimulation by appropriate cytokine combinations are key players in a mammalian host's defense against microbial pathogens[1] and cancer cells,[2–4] but they can fail in this role, allowing disease to progress.

With the hypothesis that pharmacologic supplementation of the immune cells' NO output might restore their host-protective effects, we are exploring the possibility that prodrugs exploiting the mannose receptor (MR) of macrophages[5] might be actively imported and then selectively activated in the intracellular milieu for NO release and consequent therapeutic benefit.

As a test system, we have chosen *Leishmania major* (*L. major*), a protozoan parasite that is known to be susceptible to NO's toxic effects but that is able to survive and proliferate in highly lytic phagolysosomes of macrophages that have not yet been induced to express inducible NO synthase (iNOS).[6] As prospective prodrugs of NO, we have derivatized a series of monosaccharides reportedly recognized and transported by the MR (including *N*-acetylglucosamine as well as mannose)[7] by substituting them at the anomeric position with diazeniumdiolate ions **1** that in their free form spontaneously release molecular NO at physiological pH levels. Glycosylating ions of structure **1** as in eq 1 has been proposed by Wu et al. as a route to prodrugs capable of delivering bioactive NO for pharmacological benefit.[8] For this strategy to be successful in the present connection, the glycosylated diazeniumdiolate must be hydrolytically stable enough in the systemic circulation to reach the physiological site of need but sufficiently labile under conditions unique to the target site (e.g., the acidic [pH 4−5] interior of a macrophage's phagolysosome) to free the aglycone for proton-induced generation of NO, as depicted in eq 2.





Glycosylation as in eq 1 has also been introduced as a convenient protecting group strategy for ions of structure **1**.[9] Here too, success in a given application depends on a knowledge of the glycoside's hydrolytic reactivity. Best results will be obtained if the glycoside is adequately stable through all the needed transformations at neutral or acidic pH but easily cleaved at high pH to generate the base-stable **1** ion.

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[a] Abbreviations: NO, nitric oxide; *L. major*, *Leishmania major*; MR, mannose receptor; PBS, phosphate buffered saline; iNOS, inducible nitric oxide synthase; IFN, interferon; TNF, tumor necrosis factor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; GlcNAc, *N*-acetylglucosamine; DMF, *N*,*N*-dimethylformamide; NAGase, *β*-*N*-acetylglucosaminidase.

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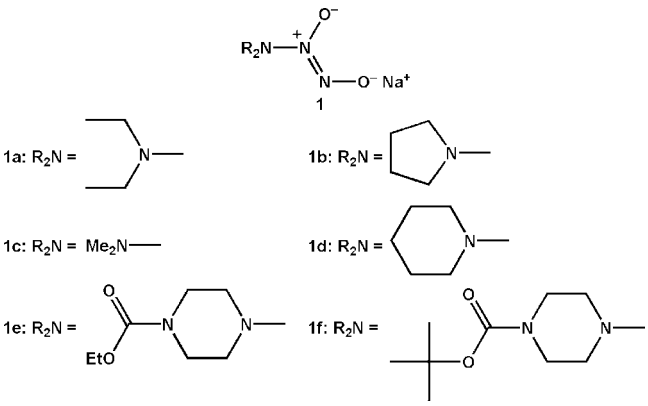
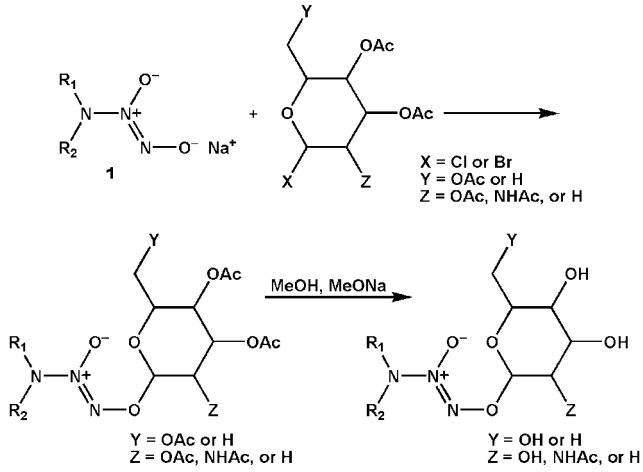
Scheme 1. Ionic Diazeniumdiolates Used as Starting Materials for Preparations Described in This Work



Scheme 2. General Synthesis of Glycosylated Diazeniumdiolates Described in This Work

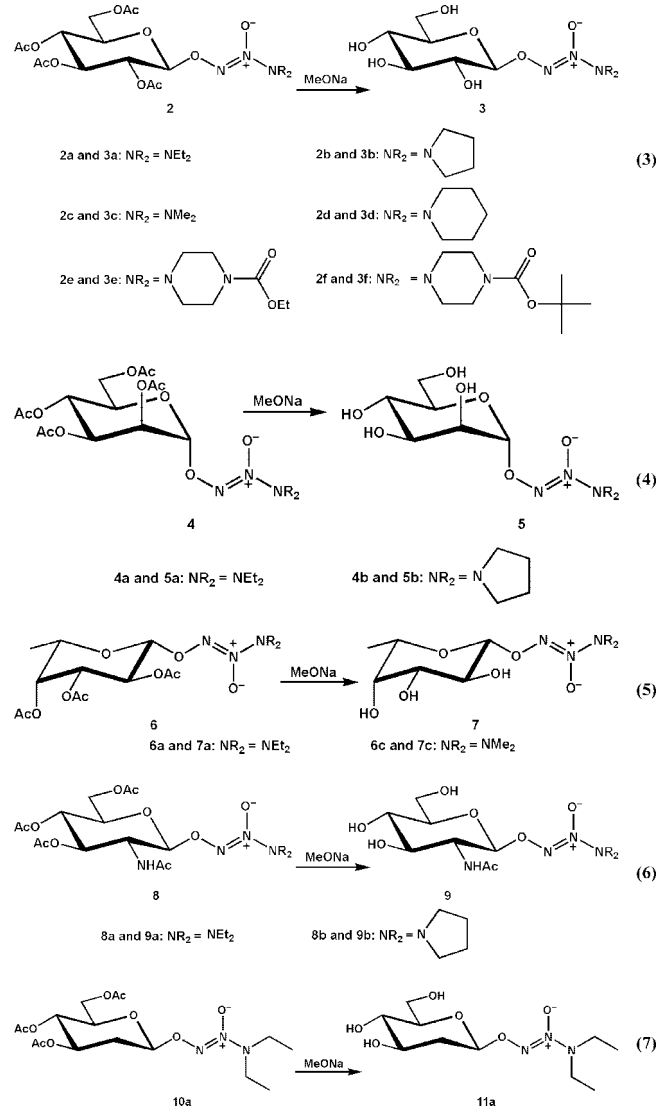


Here we report the physicochemical characterization of a small library of these O^2-glycosylated diazeniumdiolates as protected starting materials for future syntheses as well as for pharmacological testing, focusing especially on the rates of their hydrolytic cleavage at three different pH ranges. We then provide preliminary data suggesting their potential utility for delivering therapeutic levels of NO to intracellular pathogens resident in infected macrophages.

Results

Synthesis. A variety of ions **1** (Scheme 1) were converted to the corresponding peracetylated glycosides by reacting them with the appropriate anomerically activated monosaccharide (i.e., glycosyl halide) (Scheme 2). Although initially silver acetate was commonly added to reaction mixtures comprising peracetylated 1-halopyranoses and sodium salts **1**, it was eventually discovered that this was in most cases unnecessary. Following installation of the diazeniumdiolate group at C-1, the resulting peracetylated, O^2-glycosylated products were treated with catalytic amounts of sodium methoxide in methanol to cleave the ester groupings, as detailed in eqs 3–7. The α stereochemistry of mannosyl derivative **4a** was confirmed by X-ray crystallography, as shown in Figure 1. The α vs β configurations of all other compounds were shown by proton NMR to be as assigned in eqs 3–7.

Rates of Base-Induced Deglycosylation: Dependence on Identity of the Saccharide. It was previously shown that attaching acid- and heat-sensitive diazeniumdiolate ions to the anomeric carbon of glucose protected them under a variety of conditions that would otherwise lead to their decomposition but that brief exposure to concentrated aqueous alkali could free the aglycone, regenerating the intact diazeniumdiolate ion under basic conditions that prevented the ion's acid-catalyzed dissociation.[9] The reaction was first order in hydroxide ion with a rate constant k_{OH^-} of 5.3×10^{-3} M^{-1} s^{-1} for the prototypical glycoside **3a**. Indeed, **3a** has proven to be the most sensitive to base-induced hydrolysis of the five glycosides studied here. The half-lives, determined by following the first order decrease of the diazeniumdiolate chromophore's ultraviolet absorbance as a function of time and summarized in Table 1, show that fucosyl derivative **7a** hydrolyzed half as fast as **3a** in 1 M sodium hydroxide, with GlcNAc analogue **9a** being slower than 2-deoxy derivative **11a** by a similar factor under these conditions (half-life of 51 min versus half-life of 23 min, respectively). Mannose derivative **5a** was most resistant of the five with a half-life of 298 min.



The results suggest that β-D-glucosyl is an advantageous saccharide to use as a protecting group for diazeniumdiolate ions. The glucose derivatives could generally be prepared with ease (not always the case in our experience with the other glycosides), and they hydrolyzed in base to regenerate the free diazeniumdiolate ions more quickly than the others as well.

Differing Order of Hydrolytic Reactivity at pH 7.4 and Below. In contrast to their relatively sluggish rates of cleavage in 1 M base, **9a** and **11a** were the most easily hydrolyzed of

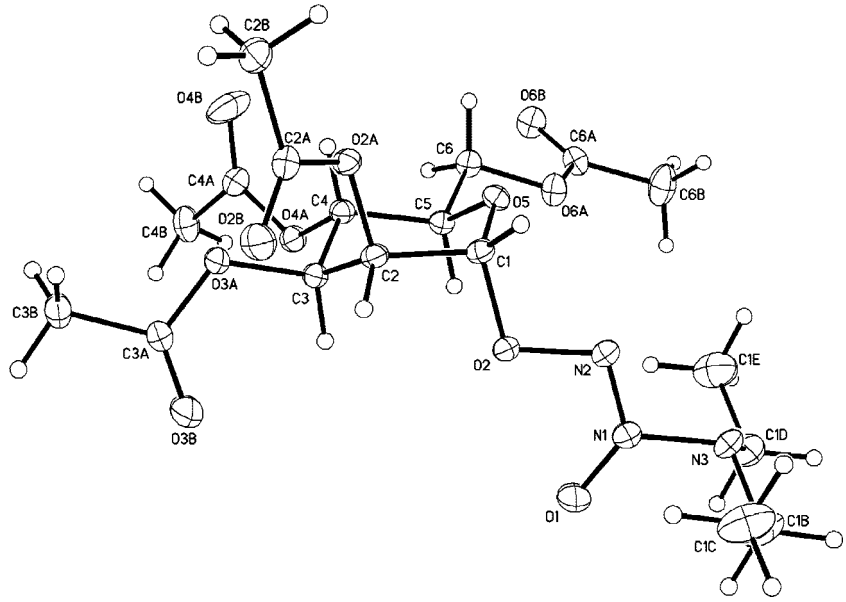


Figure 1. Structure of **4a** as determined by X-ray diffraction analysis. Displacement ellipsoids are at the 40% probability level. Some H-atoms are omitted for clarity.

Table 1. Half-Lives of Hydrolysis at 37 °C for Glycosides of Structure $Et_2NN(O)=NOR$ in 1.0 M NaOH, in 0.1 M Phosphate at pH 7.4, or in 0.05 M Citrate at pH 3.8−4.6

	half-life		
R	pH 14 (min)	pH 7.4 (day)	pH 4.6 (day)
β-D-glucosyl (**3a**)	2.1	>10	>10[a]
β-L-fucosyl (**7a**)	4.0	>10	>10[a]
β-(2-deoxy)-D-glucosyl (**11a**)	23	0.5	1.2[b]
β-(*N*-acetyl-D-glucosaminyl) (**9a**)	51	0.5	0.7[a]
α-D-mannosyl (**5a**)	298	>10	>10[a]

[a] pH 4.6. [b] pH 3.8.

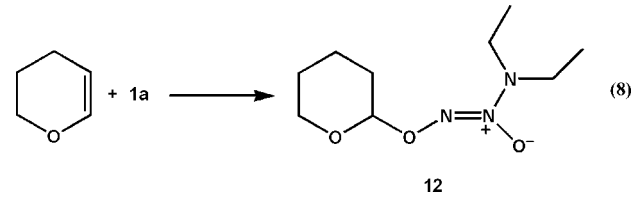
Table 2. Half-Lives of Tetrahydropyranyl Derivative **12** at Various pH Levels at 37 °C

pH	$t_{1/2}$ (min)
14.0 (1.0 M NaOH)	2.5
13.0 (0.10 M NaOH)	2.7
12.1 (0.0125 M NaOH)	3.5
10.0 (0.05 M glycine/NaOH)	2.9
8.1 (0.10 M phosphate)	3.5
7.4 (0.10 M phosphate)	3.1
4.0 (0.05 M citrate)	3.2

the five **1a** derivatives in pH 7.4 phosphate buffer, with half-lives estimated as 0.5 days (Table 1). The other three glycosides included in this study hydrolyzed so slowly that their rates could not be monitored with confidence by following the changes in their absorbance maxima with time.

Rates of hydrolysis at pH 3.8−4.6 were little different from those at pH 7.4, with **9a** and **11a** displaying half-lives of 0.7 and 1.2 days and the other three showing values of >10 days (Table 1). Interestingly, tetrahydropyranyl derivative **12**, produced by reacting **1a** with dihydropyran as in eq 8, hydrolyzed within minutes at all pH values studied (Table 2).

The relatively high susceptibility of GlcNAc derivative **9a** to hydrolysis at pH 7.4 was confirmed by following the accumulation of the nitric oxide auto-oxidation product, nitrite ion, in both phosphate-buffered saline (PBS) and cell culture medium containing 2.5% fetal calf serum. As shown in Figure 2, there was little difference between the PBS and culture medium results, with each one engendering cleavage of **9a** to



(8)

12

NO and thence to nitrite in 10−20% recovery during a 24 h incubation. By contrast, mannose derivative **5a** generated little or no nitrite under the same conditions.

Surprisingly, hydrolysis of pyrrolidine derivative **9b** in PBS was more than an order of magnitude slower than that of **9a** (Figure 2). Differing only in the fact that the two CH_3 groups of **9a** are joined to form the five-membered ring of **9b**, we had anticipated that they would be very similar in rate. However, the half-lives of **9b** in 0.1 M phosphate at pH 7.4 or in 0.05 M citrate at pH 4.6 were both >10 days at 37 °C.

Hydrolysis of 0.25−1 mM **9b** was considerably faster when incubated in RPMI cell culture medium containing 2.5% fetal calf serum instead of PBS, as shown in Figure 2. Importantly, the rates of nitrite release from **9b** were increased even further when the serum-containing medium was used to culture mouse macrophages deficient in inducible NO synthase ($iNOS^{-/-}$). In the experiments summarized in Figure 2, the cells increased the nitrite yield 4-fold in medium initially containing 0.25 mM **9b**. Similarly, the macrophages approximately doubled the rate of nitrite accumulation in a parallel incubation of 0.25 mM **9a**.

These serum- and macrophage-induced catalytic effects were further explored in additional experiments with **9b**. As shown in Figure 3, the rate of nitrite formation in cell culture medium proved quite dependent on serum concentration, with the macrophage-induced acceleration being clearly evident in medium containing 0%, 0.1%, or 1% fetal bovine serum but not in the presence of 5% or, with some batches of serum, 2.5%. Infecting the macrophages with *L. major* did not significantly affect the rate of nitrite formation (data not shown).

Catalysis of Hydrolysis by Glycosidases. The above results suggested that the macrophages may harbor factors capable of accelerating the conversion of glycosides **9a** and especially **9b** to NO. As one possibility, Wu, et al. have reported on the rapid

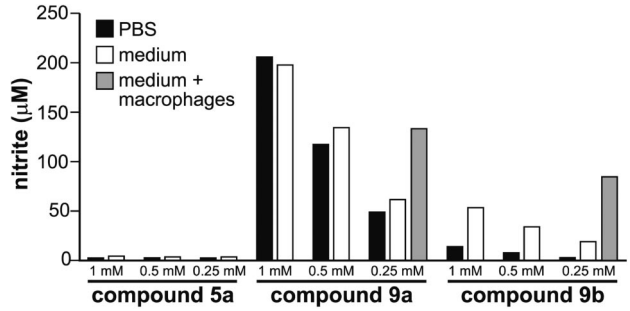


Figure 2. Nitrite yields as a reflection of NO generation on incubating **5a**, **9a**, and **9b** in PBS, in cell culture medium containing 2.5% fetal calf serum, or in the serum-containing medium while being used to culture macrophages for 24 h at 37 °C. When the glycoside solutions were assayed immediately after they were dissolved, no nitrite was detected using the Griess test. Data shown are mean values of triplicates. Standard deviations were <5%. Shown is one of two comparable experiments.

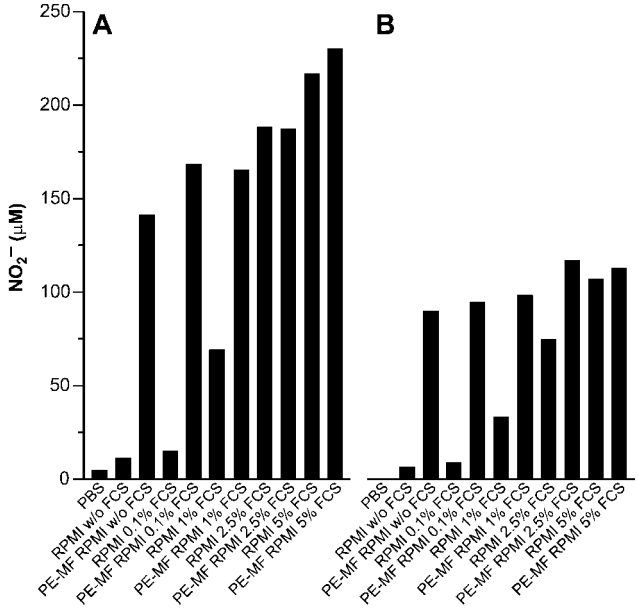


Figure 3. Generation of NO after parallel incubations of **9b** at concentrations of (A) 250 and (B) 125 μM under different conditions. Substance **9b** was incubated in phosphate-buffered saline (PBS) alone; RPMI cell culture medium without (w/o) or with addition of 0.1%, 1%, 2.5%, and 5% fetal calf serum (FCS) in the absence or presence of peritoneal exudate macrophages from iNOS-deficient mice (PE-MF); cultured in serum-free or serum-containing (0.1%, 1%, 2.5%, or 5% FCS) RPMI. Supernatants were harvested after 24 h, and cumulative NO release was estimated by determining its auto-oxidation product, nitrite ion, using the Griess assay. Data shown are mean values of triplicates. Standard deviations were <5%. The results confirm that the macrophages are capable of metabolizing **9b** to NO and that the accelerating effect of serum on the hydrolysis of **9b** is concentration-dependent. Shown are results from one of two identically designed experiments, which yielded comparable results.

enzymatic cleavage of **5b** and certain other diazeniumdiolated carbohydrates by the corresponding glycosidases.[8] To determine whether *N*-acetylglucosamine derivatives **9a** and **9b** are similarly susceptible, we incubated them with *N*-acetylglucosaminidases isolated from jack bean and from human placenta. As summarized in Table 3, both enzymes hydrolyzed both substrates with rates and efficiencies that were comparable to those of the reference compound, 1-(*p*-nitrophenyl)-2-deoxy-2-(*N*-acetyl)-β-D-glucosamine. The time course of NO release in a typical hydrolysis is illustrated in Figure 4. Negligible NO generation was seen in a parallel incubation of mannose derivative **5a**, which was not expected to be hydrolyzed by an *N*-acetylglucosaminidase.

Table 3. Rates and Extents of NO Generation on Hydrolysis of **9a** and **9b** under Catalysis by *N*-Acetylglucosaminidases from Both Jack Bean (JB) and Human Placenta (HP)[a]

		moles produced per mole of glycoside			
compd	enzyme	[NO]	$[NO_2^-]$	$[NO + NO_2^-]$	$k(s^{-1})$
9a	JB	1.26	0.05	1.31	6.2×10^{-4}
9a	HP	1.73	0.24	1.97	4.1×10^{-4}
9b	JB	1.72	0.12	1.84	3.1×10^{-4}
9b	HP	1.69	0.36	2.05	3.6×10^{-4}

[a] All reactions were run at 37 °C in 0.1 M phosphate, pH 7.4, containing 0.05 mM diethylenetriaminepentaacetic acid. Jack bean (JB) enzyme was present at 0.11 units/mL, while its human placenta (HP) counterpart was added at 0.044 units/mL. Substrates were added at the following initial concentrations: **9a**, 0.10 mM; **9b**, 0.16 mM; **5a**, 0.09 mM. NO production was followed by chemiluminescence detection until no more was evolved. Nitrite formed during hydrolysis was measured in the remaining solution by the colorimetric Griess assay. Rate constants were cleanly first order, as determined by ultraviolet spectrophotometry; wavelengths followed were 227 nm for **9a** and **5a** but 256 nm for **9b**. Absorbance changes in the **5a** incubations were negligible under the conditions employed. The enzymes used were confirmed to be fully active by means of control incubations with the manufacturer's recommended substrate, 1-(*p*-nitrophenyl)-2-deoxy-2-(*N*-acetyl)-β-D-glucosamine.

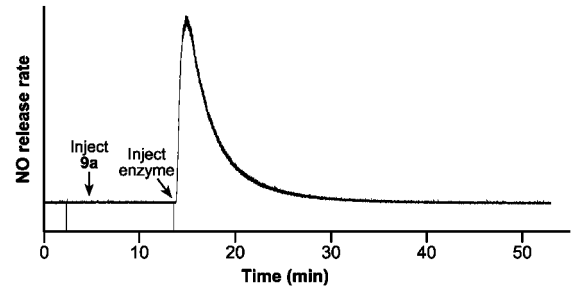


Figure 4. Chemiluminescence trace showing the time course of NO release from 0.39 μM **9a** at 37 °C in 50 mM citrate buffer (pH 5.0) with 1.6 μg/mL of jack bean β-*N*-acetylglucosaminidase.

Antileishmanial Activity of 9a and 9b. We next tested the hypothesis that the infected macrophages' ability to accelerate the hydrolysis of **9a** and **9b** to NO might help them defeat their intracellular *L. major* amastigotes. Previous studies have shown that intracellular *L. major* amastigotes are killed by cytokine-activated macrophages in an iNOS-dependent manner.[10–13] For the analysis of the NO donors we utilized macrophages from iNOS-deficient mice, which allowed us to exclude any contribution by iNOS-derived endogenous NO. As shown in Figure 5, unstimulated macrophages allowed replication of the intracellular parasites during the 72 h observation period. Because of the absence of iNOS, stimulation of the macrophages with interferon (IFN)-γ plus tumor necrosis factor (TNF) had only a very weak antileishmanial effect, as seen before.[14] In contrast, addition of **9a** or **9b** led to a highly significant decrease in the number of intracellular parasites, with **9a** being more potent than **9b**. Non-NO-releasing *N*-acetyl-β-D-glucosaminyl derivative **13** did not have any effect on the number of intracellular *Leishmania* (data not shown).

Importantly, in the concentrations tested, neither of the NO donors caused any toxicity to the macrophage host cells as determined microscopically by trypan blue staining of dead cells or by measuring the mitochondrial activity of the total cell monolayer using the MTT assay (see Experimental Section).

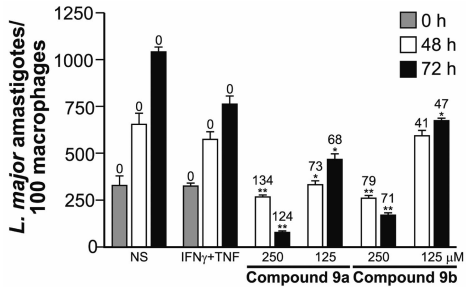
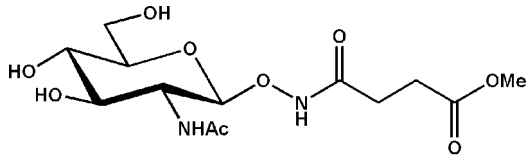


Figure 5. Effect of two glycosylated diazeniumdiolates on the intracellular proliferation of *L. major* in primary mouse macrophages. Peritoneal exudate macrophages from iNOS-deficient mice were cultured in medium alone or stimulated with IFN-γ plus TNF for 16 h prior to a 4 h infection period with *L. major* amastigotes (parasite/macrophage ratio of 5:1). Thereafter, the macrophages remained unstimulated (NS), were stimulated with IFN-γ plus TNF, or were exposed to glycosylated diazeniumdiolates (**9a** or **9b**) at the indicated concentrations. The mean ($\pm$SD) number of intracellular parasites per 100 macrophages (immediately after infection [0 h], after 48 and 72 h), and the amount of nitrite in the culture medium (accumulated between 24 and 48 h and between 48 and 72 h) are given: (*) $p < 0.01$, (**) $p < 0.001$, significantly different compared to unstimulated macrophages. The numbers above the bars represent the mean value of nitrite (units of micromolar) detected in the culture supernatants after the final accumulation period (48−72 h of stimulation). Nitrite ion was devoid of antileishmanial activity under these conditions.



13

Thus, these glycosylated diazeniumdiolates exert potent antiparasitic effects against intracellular *L. major* at concentrations that do not cause overt damage to the host cells.

Discussion

Parasites of the genus *Leishmania* are among a variety of pathogens that thrive intracellularly in the macrophage despite their demonstrated sensitivity to the cytostatic and microbicidal action of NO, a major component of this cell type's normal immunological armamentarium.[15] It has been suggested that the parasites' ability to survive in the normally hostile interior of phagolysosomes of activated macrophages is due at least in part to impairment of endogenous NO production by the phagocytized parasites.[16–18]

With the hypothesis that pharmacological supplementation of the macrophages' NO reserves might restore the immune system's capacity to contain and eliminate these pathogens, we are exploring ways of delivering exogenously derived NO to the intracellular compartments of infected macrophages. The strategy described here is aimed at exploiting the so-called mannose receptor [MR, formerly also termed mannose/fucose receptor (MFR)] that decorates the surfaces of macrophages and certain other cells of the immune system. If these receptors can be harnessed to import glycosylated, diazeniumdiolated NO prodrugs into the cell, one might rely on intracellular glycosidases and/or the acidic environment in the phagolysosome (pH 4−5.5) to free the sequestered NO and kill the pathogens.

The infectious agents in our test system were *L. major* amastigotes. As host cells, we used primary peritoneal macrophages from iNOS-knockout mice to minimize the possibility that endogenously produced NO might contribute significantly to the screening results; accordingly, stimulation of the cells with cytokines, a procedure that in normal cells resoundingly activates wild type macrophages to produce iNOS-derived antimicrobial NO in abundance, had little effect on the parasites.

Recognizing that the MR serves as a receptor not only for mannose and fucose but also for certain other saccharides such as *N*-acetylglucosamine, we added glycosides **5a**, **5b**, **7a**, **7c**, **9a**, and **9b** at concentrations of 125 or 250 μM to culture medium containing the infected macrophages. Neither of the fucose derivatives had any perceivable effect on the parasite burden of these cells, and only one of the mannosides (**5a**) showed any activity, that being of borderline significance. However, both of the *N*-acetylglucosamine (GlcNAc) derivatives (**9**) were decidedly antileishmanial (Figure 5).

The data are consistent with the interpretation that the cultured macrophages were able to effect cleavage of the glycosidic bonds of prodrugs **9a** and **9b**, with the resulting spontaneous release of NO significantly reducing the parasite burden with no observable toxicity to the host cells.

Significance. The results suggest that *N*-acetylglucosamine derivatives **9a** and **9b** may be promising lead compounds for the development of antileishmanial agents. They were designed with the hypothesis that they might enter the infected macrophages via the cells' mannose receptor and be cleaved therein to concentrate intracellular NO for antileishmanial activity. While none of the analogous fucosides and mannosides was more than marginally active, if at all, the results for **9a** and **9b** were consistent with that hypothesis. The findings for **9b** were particularly illuminating. Its hydrolysis to generate NO in cell culture medium was greatly accelerated in the presence of macrophages, as reflected in an increased rate of accumulation of nitrite (the auto-oxidation product of NO) that correlated suggestively with antileishmanial activity.

Very importantly, the iNOS-knockout macrophages harboring the affected parasites were well spread and quite healthy, more so than their wild type counterparts that were stimulated to kill resident *L. major* by up-regulation of iNOS using interferon-γ/tumor necrosis factor treatment. We conclude that NO donors **9a** and **9b** exert their antileishmanial activity by cell-mediated activation to one or more metabolites (presumably including NO) that directly harm the parasites and not to any nonspecific toxicity toward the host cells.

Future work with these lead compounds will focus on the additional hypothesis that divalent or polyvalent analogues might have exponentially increased potency. We are also examining the mechanistic origins of the often surprising differences in hydrolysis rate among the test agents studied here. Preliminary indications suggest that the >20-fold increase in hydrolysis rate for acyclic **9a** in pH 7.4 phosphate buffer relative to its more constrained heterocyclic analogue **9b** may be a consequence of electronic interaction between the pyrrolidino nitrogen and the −N(O)═NO− group to which it is attached. An X-ray crystallographic study is currently underway aimed at further exploring this hypothesis.

The chemical data reported here may also aid in the synthesis of future prodrug candidates as well as in the design of drugs with NO release profiles needed for specific pharmacological effects. In particular, the results support the utility of glucosylation as a protecting group strategy for diazeniumdiolate ions **1** to provide derivatives that remain stable through synthetic transformations at neutral-to-acidic pH and/or at elevated

temperatures but that can be efficiently removed at high pH levels, conditions that stabilize the newly formed diazeniumdiolate ions.

Experimental Section

NO was purchased from Matheson Gas Products (Montgomeryville, PA). 2,3,4,6-Tetraacetyl-1-α-glucopyranosyl bromide (acetobromoglucose) was purchased from Fluka Chemical Corp. (Milwaukee, WI). Sodium salts **1a**,[19] **1b**,[20] **1c**,[21] **1e**,[22] and **1f**[22] were prepared as previously described, as were **2a**,[9] **3a**,[9] α-D-acetobromomannose,[23] α-D-acetochloro-(*N*-acetylglucosamine),[24] and α-L-acetobromofucose.[25] Unless otherwise indicated, all other chemicals were purchased from Aldrich Chemical Co. (Milwaukee, WI). Nuclear magnetic resonance (NMR) spectra were collected with a 400 MHz NMR spectrometer. Chemical shifts (δ) are reported in parts per million downfield from tetramethylsilane or 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt. Elemental analyses were performed by Atlantic Microlab, Inc. (Norcross, GA) or Midwest Microlab, LLC (Indianapolis, IN).

Sodium 1-(Piperidin-1-y1)diazen-1-ium-1,2-diolate (1d) (JS-11-105). A solution of 100 mL of freshly distilled piperidine in 100 mL of ether was placed in a Parr bottle, purged with nitrogen, and cooled to −80 °C. The cold solution was charged with 60 psi of nitric oxide. A solid mass formed within 30 min, but the mixture was allowed to stand for an additional 3 h. The pressure was released; the cold material was collected by filtration and washed with ether. The piperidinium salt was treated with 20 mL of 10 M NaOH and 10 mL of methanol for cation exchange, and the resulting pasty mass was flooded with 500 mL of ether. The diazeniumdiolate **1d** was collected by filtration and dried under vacuum to give 18 g of product: UV (0.01 M NaOD) λ_{max} (ε) 249 nm (8.15 mM^{-1} cm^{-1}); ^{1}H NMR (10 mM NaOD) δ 1.46−1.52 (m, 2 H), 1.72−1.78 (m, 4 H), 3.06 (t, 4 H, J = 5.6 Hz); ^{13}C NMR δ 25.31, 27.39, 55.80.

General Procedure for the Preparation of O^2-Glycosylated Diazeniumdiolates. A 0.5 M solution of the appropriate peracetylated glycosyl halide in tetrahydrofuran was slurried with solid sodium bicarbonate to remove any traces of acid, filtered, injected dropwise into a 0.5 M solution of the appropriate diazeniumdiolate salt (**1**) in dimethyl sulfoxide, and stirred for 24−78 h at room temperature. The reaction mixture was poured over ice−water (100 mL) and extracted with ether. The ether layer was washed with water, dried over sodium sulfate, and treated with charcoal. The solution was filtered through magnesium sulfate, concentrated on a rotary evaporator, and dried under vacuum. The glucose derivatives were purified by recrystallization, while the glassy mannose, fucose, and GlcNAc adducts required column chromatography. Deacylation was carried out according to established procedures prescribed by Wolfram and Thompson[26] by adding 10 mol % of a 25% solution of sodium methoxide in methanol to 0.1 M solutions of the peracetylated/glycosylated diazeniumdiolates in methanol. Reactions were monitored by thin layer chromatography on 2.5 cm × 7.5 cm silica gel plates developed using various solvents. Spots were visualized by ultraviolet quenching.

***O*^{2}-(2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl) 1-(Pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (2b) (CV-05).** The general procedure above was employed as follows. To 4.48 g (0.029 mol) of **1b** were added catalytic amounts of silver acetate and 12 g (0.029 mol) of acetobromoglucose. A solid (9.2 g, 69%) was isolated: mp 146−147 °C; UV (H_2O) λ_{max} (ε) 258 nm (7.9 mM^{-1} cm^{-1}); ^{1}H NMR ($CDCl_3$) δ 1.94−1.98 (m, 4 H), 2.02 (s, 3 H), 2.04 (s, 6 H), 2.09 (s, 3 H), 3.58−3.62 (m, 4 H), 3.78−3.84 (m, 1 H), 4.14−4.19 (m, 1 H), 4.25−4.30 (m, 1 H), 5.12−5.19 (m, 2 H), 5.26 (t, 1 H, J = 9.2 Hz), 5.33−5.39 (m, 1 H); ^{13}C NMR δ 20.54, 20.60, 20.70, 23.01, 50.60, 61.74, 69.14, 72.50, 72.91, 100.27, 169.05, 169.33, 170.17, 170.63. Anal. ($C_{18}H_{27}N_3O_{11}$) C, H, N.

***O*^{2}-(β-D-Glucopyranosyl) 1-(Pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (3b) (CV-10).** Compound **2b** was deacylated to give a 79% yield of **3b** as a white foam: UV (methanol) λ_{max} (ε) 252 nm (7.2 mM^{-1} cm^{-1}); ^{1}H NMR (DMSO-d_6) δ 1.83−1.92 (m, 4 H), 3.06−3.26 (m, 4 H), 3.41−3.49 (m, 5 H), 3.63−3.69 (m, 1 H), 4.59 (t, 1 H, J = 5.9 Hz), 4.85 (d, 1 H, J = 7.8 H), 5.00 (d, 1 H, J = 5.2 Hz), 5.09 (d, 1 H, J = 4.3), 5.36 (d, 1 H, J = 5.0 Hz); ^{13}C NMR δ 22.32, 50.59, 60.59, 69.34, 71.57, 76.60, 77.33, 103.17. Anal. ($C_{10}H_{19}N_3O_7 \cdot H_2O$) C, H, N.

***O*^{2}-(2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl) 1-(*N*,*N*-Dimethylamino)diazen-1-ium-1,2-diolate (2c) (CV-30).** To 3.81 g (0.03 mol) of **1c** were added catalytic amounts of silver acetate and 12 g (0.029 mol) of acetobromoglucose as described in the general procedure. A foam (1.5 g, 12%) was isolated: UV (methanol) λ_{max} (ε) 228 nm (8.3 mM^{-1} cm^{-1}); ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.02 (s, 3 H), 2.037 (s, 3 H), 2.04 (s, 3 H), 2.09 (s, 3 H), 3.08 (s, 6 H), 3.82−3.88 (m, 1 H), 4.08−4.19 (m, 1 H), 4.23−4.31 (m, 1 H), 5.12−5.37 (m, 1 H); ^{13}C NMR δ 20.34, 20.48, 42.09, 61.51, 67.67, 69.00, 72.35, 72.62, 100.12, 168.82, 169.16, 169.93, 170.41. Anal. ($C_{16}H_{25}N_3O_{11}$) C, H, N.

***O*^{2}-(β-D-Glucopyranosyl) 1-(*N*,*N*-Dimethylamino)diazen-1-ium-1,2-diolate (3c) (CV-36).** Compound **2c** was deacylated to give a 66% yield of **2a** as a white foam: UV (methanol) λ_{max} (ε) 224 nm (4.3 mM^{-1} cm^{-1}); ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.96 (s, 6 H), 3.09−3.30 (m, 4 H), 3.39−3.48 (m, 2 H), 3.67 (d, 1 H, J = 11.2 Hz,), 4.61 (br, 1 H), 4.90 (d, 1 H, J = 7.8 Hz), 5.03−5.11 (br, 2 H), 5.40 (br, 1 H); ^{13}C NMR δ 42.19, 60.63, 69.38, 71.62, 76.62, 77.45, 103.42. Anal. ($C_8H_{17}N_3O_7 \cdot H_2O$) C, H, N.

***O*^{2}-(2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl) 1-(Piperidin-1-yl)diazen-1-ium-1,2-diolate (2d) (CV-16).** To 5.0 g (0.03 mol) of **1d** were added catalytic amounts of silver acetate and 12 g (0.029 mol) of acetobromoglucose as described in the general procedure. A white solid (0.512 g, ∼4%) was isolated: mp 112−113 °C; UV (methanol) λ_{max} (ε) 230 nm (6.9 mM^{-1} cm^{-1}); ^{1}H NMR ($CDCl_3$) δ 1.48−1.56 (m, 2 H), 1.72−1.82 (m, 4 H), 2.02 (s, 3 H), 2.03 (s, 3 H), 2.04 (s, 3 H), 2.09 (s, 3 H), 3.39−3.52 (m, 4 H), 3.78−3.84 (m, 1 H), 4.14−4.19 (m, 1 H), 4.24−4.32 (m, 1 H), 5.12−5.38 (m, 4 H); ^{13}C NMR δ 20.53, 20.69, 23.34, 24.60, 52.24, 61.68, 67.82, 69.21, 72.58, 72.82, 100.34, 168.99, 169.30, 170.15, 170.60. Anal. ($C_{19}H_{29}N_3O_{11}$) C, H, N.

***O*^{2}-(β-D-Glucopyranosyl) 1-(Piperidin-1-yl)diazen-1-ium-1,2-diolate (3d) (CV-21).** Compound **2d** was deacylated to give a quantitative yield of **3d** as a white foam: UV (methanol) λ_{max} (ε) 230 nm (7.7 mM^{-1} cm^{-1}); ^{1}H NMR ($CDCl_3$) δ 1.43−1.55 (m, 2 H), 1.66−1.78, (m, 4 H), 3.30−3.46 (m, 5 H), 3.57−3.78 (m, 3 H), 3.79−4.00 (m 3 H), 4.95−5.14 (m, 2 H), 5.24−5.53 (m, 2 H); ^{13}C NMR δ 23.31, 24.64, 52.28, 61.16, 69.01, 71.40, 76.05, 76.25, 103.32. Anal. ($C_{11}H_{21}N_3O_7 \cdot 0.5H_2O$) C, H, N.

***O*^{2}-(2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl) 1-[(4-Ethoxycarbonyl)piperazin-1-yl]diazen-1-ium-1,2-diolate (2e) (CV-06).** To 1.2 g (0.005 mol) of **1e** were added catalytic amounts of silver acetate and 1.83 g (0.0044 mol) of acetobromoglucose. A colorless syrup (1.33 g, 55%) was isolated: UV (ethanol) λ_{max} (ε) 228 nm (7.2 mM^{-1} cm^{-1}); ^{1}H NMR ($CDCl_3$) δ 1.28 (t, 3 H, J = 7.1), 2.02 (s, 3H), 2.04 (s, 3 H), 2.04 (s, 3 H), 2.09 (s, 3 H), 3.44−3.48 (m, 4 H), 3.65−3.68 (m, 4 H), 3.80−3.86 (m, 1 H), 4.16 (q, 2 H, J = 7.2), 4.11−4.19 (m, 1 H), 4.25−4.30 (m, 1 H), 5.11−5.37 (m, 4 H); ^{13}C NMR δ 14.52, 20.46, 20.49, 20.63, 42.21, 50.71, 61.58, 61.76, 67.71, 69.06, 72.62, 100.33, 154.99, 168.92, 169.24, 170.02, 170.48. Anal. ($C_{21}H_{32}N_4O_{13}$) C, H, N.

***O*^{2}-(β-D-Glucopyranosyl) 1-[(4-Ethoxycarbonyl)piperazin-1-yl]diazen-1-ium-1,2-diolate (3e) (CV-11).** Compound **2e** was deacylated to give an 86% yield of **3e** as a white foam after column chromatography (silica gel, 9:1 CH_2Cl_2/MeOH): UV (methanol) λ_{max} (ε) 232 nm (8.8 mM^{-1} cm^{-1}); ^{1}H NMR (DMSO-d_6) δ 1.19 (t, 3 H, J = 7.1 Hz), 3.08−3.14 (m, 1 H), 3.21−3.25 (m, 3 H), 3.32−3.35 (m, 5 H), 3.42−3.55 (m, 5 H), 3.65−3.68 (m, 1 H), 4.06 (q, 2 H, J = 7.1 Hz), 4.52−4.68 (m, 1 H), 4.90−4.93 (m, 1 H), 5.04 (br, 1 H), 5.42 (br, 1 H); ^{13}C NMR δ 14.52, 42.05, 50.34, 54.92, 60.58, 61.10, 69.31, 71.54, 77.51, 103.53, 154.49. Anal. ($C_{13}H_{24}N_4O_9 \cdot 0.75H_2O$) H, N, C: calcd, 39.19; found, 39.67.

***O*^{2}-(2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl) 1-[(4-*tert*-Butoxycarbonyl)piperazin-1-yl]diazen-1-ium-1,2-diolate (2f) (CV-42).** To 1.0 g (0.0037 mol) of **1f** were added catalytic amounts of silver acetate and 1.73 g (0.0042 mol) of acetobromoglucose. A

white solid (1.17 g, 55%) was isolated: mp 122−125 °C; UV (methanol) λ_{max} (ε) 232 nm (9.3 mM^{-1} cm^{-1}); ^1H NMR ($CDCl_3$) δ 1.47 (s, 9 H), 2.02 (s, 3 H), 2.04 (s, 3 H), 2.04 (s, 3 H), 2.09 (s, 3 H), 3.43−3.46 (m, 4 H), 3.59−3.63 (m, 4 H), 3.79−3.89 (m, 1 H), 4.14−4.19 (m, 1 H), 4.25−4.30 (m, 1 H), 5.11−5.38 (m, 4 H); ^{13}C NMR δ 20.52, 20.56, 20.70, 28.31, 50.84, 61.64, 67.78, 69.12, 72.69, 80.50, 100.40, 154.17, 168.99, 169.29, 170.10, 170.56. Anal. ($C_{23}H_{36}N_4O_{13}$) C, H, N.

***O*^{2}-(*β*-D-Glucopyranosyl) 1-[(4-*tert*-Butoxycarbonyl)piperazin-1-yl]diazen-1-ium-1,2-diolate (3f) (CV-167).** Compound **3e** was deacylated as described above to give a 63% yield of **3f** as a white foam: UV (methanol) λ_{max} (ε) 232 nm (8.2 mM^{-1} cm^{-1}); ^1H NMR ($CDCl_3$) δ 1.46 (s, 9 H), 2.70 (br, 1 H), 3.32−3.51 (m, 4 H), 3.51−3.79 (m, 7 H), 3.86 (br, 3 H), 4.93−5.12 (m, 2 H), 5.25−5.47 (m, 2 H); ^{13}C NMR δ 28.34, 42.19, 42.36, 50.92, 61.01, 68.84, 71.39, 76.00, 76.28, 80.48, 103.42, 154.22. Anal. ($C_{15}H_{28}N_4O_9$) C, H, N.

***O*^{2}-(2,3,4,6-Tetra-*O*-acetyl-α-D-mannopyranosyl) 1-(*N*,*N*-Diethylamino)diazen-1-ium-1,2-diolate (4a) (JS-37-137).** A solution of 5 g (0.012 mol) of acetobromomannose in tetrahydrofuran (50 mL) was added to 7.75 g (0.05 mol) of **1a** in 30 mL of DMSO and 250 mg of silver acetate. After being stirred for 24 h, the reaction mixture was treated with 100 mL of ether, cooled in an ice bath to avoid possible hydrolysis on adding water, placed in a separatory funnel, and washed with cold water. The ether solution was dried over sodium sulfate, filtered through a layer of magnesium sulfate, and concentrated under vacuum to give 1.7 g of an amber resin. The product was purified on column chromatography (silica gel) using 1:1 hexane/ethyl acetate as the eluant. A fraction containing about 25 mg of product was concentrated, and the isolated resin was crystallized on standing while covered with ether/petroleum ether. The rest of the fractions containing 814 mg of product could be isolated only as a foam. Because of the difficulty in totally purifying the tetraacetylmannose adduct, it is recommended that the deacylation step proceed without thorough purification. However, sufficient material was isolated for characterization: mp 99−100 °C; UV (H_2O) λ_{max} (ε) 228 nm (8.4 mM^{-1} cm^{-1}); ^1H NMR ($CDCl_3$) δ 1.12 (t, 6 H), 2.00 (s, 3 H), 2.05 (s, 3 H), 2.07 (s, 3 H), 2.19 (s, 3 H), 3.15−3.30 (m, 4 H), 4.04−4.08 (m, 1 H), 4.10−4.14 (m, 4 H), 4.26−4.31 (m, 1 H), 5.31−5.36 (m, 1 H), 5.41−5.44 (dd, 1 H, J = 3.51 Hz, J = 9.86 Hz), 5.49−5.51 (dd, 1 H, J = 1.85 Hz, J = 3.51 Hz), 5.58 (d, 1 H, J = 1.75 Hz); ^{13}C NMR δ 11.53, 20.68, 20.70, 20.76, 20.89, 48.49, 62.55, 66.15, 69.94, 68.52, 68.72, 92.21, 169.79, 169.99, 170.16, 170.79. Anal. ($C_{18}H_{29}N_3O_{11}$) C, H, N.

***O*^{2}-(α-D-Mannopyranosyl) 1-(*N*, *N*-Diethylamino)diazen-1-ium-1,2-diolate (5a) (CV-123).** A solution of 624 mg of **4a** in 10 mL of methanol was treated with 0.15 mL of 25% methanolic sodium methoxide and stirred at 25 °C for 30 min. Dowex-50W-H^+ resin (250 mg) was added to the methanolic solution to give 390 mg of a white foam. Purification was carried out on a 4 cm × 7 cm KP-Sil column fitted into a Flash 40 chromatography system and the product was eluted with 9:1 dichloromethane/methanol to give 208 mg of foamy **5a**: UV (methanol) λ_{max} (ε) 228 nm (8.3 mM^{-1} cm^{-1}); ^1H NMR (D_2O) δ 1.06 (t, 6 H), 3.14−3.24 (m, 4 H), 3.60−3.64 (m, 1 H), 3.74−3.91 (m, 2 H), 4.19−4.21 (dd, 1 H, J = 1.76 Hz, J = 3.42 Hz), 5.73 (d, 1 H, J = 1.75 Hz); ^{13}C NMR (D_2O) δ 13.31, 51.70, 63.37, 68.93, 70.95, 73.07, 77.52, 104.86. Anal. ($C_{10}H_{21}N_3O_7 \cdot 0.5\ H_2O$) C, H, N.

***O*^{2}-(α-D-Mannopyranosyl) 1-(Pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (5b) (CV-2-09).** To a slurry of 4.29 (28 mmol) of **1b** in 30 mL of dimethyl sulfoxide and 10 mL of tetrahydrofuran was added 140 mg of silver acetate. A solution of 10 g (24.3 mmol) of acetobromomannose in 10 mL of tetrahydrofuran was injected gradually into the slurry under a blanket of argon. After being stirred at room temperature overnight, the reaction mixture was poured over ice−water and extracted with ether. The organic layer was washed with 5% sodium bicarbonate solution followed by aqueous sodium chloride. The solution was dried over sodium sulfate, filtered, and evaporated to give a brown syrup. This was chromatographed on silica gel using ethyl acetate/hexane in a 7:6 ratio increasing to 3:2 to yield 4.1 g of *O*^{2}-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl) 1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate, **4b**. The product, without further purification, was deacylated as described above to give crude **5b**. The product was chromatographed on silica gel using a gradient of 95%−8% dichloromethane in methanol: UV (H_2O) λ_{max} (ε) 254 nm (7.8 mM^{-1} cm^{-1}); ^1H NMR (D_2O) δ 2.00−2.01 (m, 4 H), 3.46−3.52 (m, 1 H), 3.56−3.63 (m, 4 H), 3.65−3.82 (m, 3 H), 3.93−3.97 (m, 1 H), 4.23 (dd, 1 H, J = 0.9 Hz, J = 2.9 Hz), 5.38 (d, 1 H, J = 0.9 Hz); ^{13}C NMR δ 25.34, 53.84, 63.55, 69.10, 72.23, 75.36, 79.73, 103.02.

***O*^{2}-(2,3,4-Tri-*O*-acetyl-*β*-L-fucopyranosyl) 1-(*N*,*N*-Diethylamino)diazen-1-ium-1,2-diolate (6a) (JS-33-181 Triacetate).** This compound was prepared as described in general methods but without using silver ion. Purification of 1.8 g of crude product was carried out by column chromatography on 80 g of silica gel and the product was eluted with 5:1 dichloromethane/ethyl acetate to give 986 mg of a clear glass (43%): UV (methanol) λ_{max} (ε) 226 nm (7.0 mM^{-1} cm^{-1}); ^1H NMR ($CDCl_3$) δ 1.11 (t, 6 H, J = 7.2 Hz), 1.23 (d, 3 H, J = 6.4 Hz), 1.99 (s, 3 H), 2.03 (s, 3 H), 2.17 (s, 3 H), 3.19 (q, 4 H, J = 7.2 Hz), 4.10−4.14 (m, 1 H), 5.05−5.30 (m, 3 H), 5.44−5.53 (m, 1 H). This compound was used without further purification or characterization in the procedure described in the next paragraph below.

***O*^{2}-(*β*-L-Fucopyranosyl) 1-(*N*,*N*-Diethylamino)diazen-1-ium-1,2-diolate (7a) (JS-33-182).** A solution of 920 mg (2.21 mmol) of the above glass in methanol was deacylated as described for other sugar analogues, yielding 532 mg (86%) of pure **7a** as a white foam: UV (H_2O) λ_{max} (ε) 228 nm (6.3 mM^{-1} cm^{-1}); ^1H NMR (methanol-d_4) δ 1.01 (t, 6 H, J = 7.1 Hz), 1.29 (d, 3 H, J = 6.4 Hz), 3.16 (q, 4 H, J = 7.1 Hz), 3.68−3.76 (m, 3 H), 3.81 (b, 1 H), 3.98−4.08 (m, 1 H), 4.15 (b, 1 H), 5.01 (d, 1 H, J = 8 Hz), 5.08 (b, 1 H); ^{13}C NMR δ 11.45, 16.13, 48.19, 71.35, 71.42, 73.84, 107.71. Anal. ($C_{10}H_{21}N_3O_5 \cdot H_2O$) C, H, N.

***O*^{2}-(2,3,4-Tri-*O*-acetyl-*β*-L-fucopyranosyl) 1-(*N*,*N*-Dimethylamino)diazen-1-ium-1,2-diolate (6c) (JS-33-164).** Acetobromofucose (2.66 g, 0.0075 mol) in tetrahydrofuran was added to 1.52 g (12 mmol) of **1c** without silver acetate to give 2.74 g of crude product. Purification on column chromatography (silica gel) using 5:1 CH_2Cl_2/EtOAc as the eluant provided 2.2 g of a white glass: UV (methanol) λ_{max} (ε) 231 nm (7.9 mM^{-1} cm^{-1}); ^1H NMR ($CDCl_3$) δ 1.25 (d, 3 H, J = 6.5 Hz), 2.02 (s, 3 H), 2.08 (s, 3 H), 2.18 (s, 3 H), 3.07 (s, 6 H), 3.9−4.35 (m, 1 H), 5.07−5.10 (dd, 1 H, J = 3.4, 10.4 Hz), 5.26−5.27 (dd, 1 H, J = 0.9, 8.4 Hz), 5.47−5.52 (dd, 1 H, J = 8.4, 10.4 Hz), 5.15 (d, 1 H, J = 8.4 Hz); ^{13}C NMR δ 16.01, 20.58, 20.61, 20.71, 42.42, 66.94, 69.90, 70.08, 71.46, 100.97, 169.13, 170.14, 170.56. Anal. ($C_{14}H_{23}N_3O_9 \cdot {}^1/_4\ H_2O$) C, H, N.

***O*^{2}-(*β*-L-Fucopyranosyl) 1-(*N*,*N*-Dimethylamino)diazen-1-ium-1,2-diolate (7c) (JS-33-177).** Deacylation in methanol with catalytic amounts of sodium methoxide was carried out as described above. A white glass was isolated: UV (methanol) λ_{max} (ε) 235 nm (9.6 mM^{-1} cm^{-1}); ^1H NMR ($CDCl_3$) δ 1.36 (d, 3 H, J = 6.4 Hz), 2.99 (s, 6 H), 3.70−3.79 (m, 3 H), 3.96−4.10 (m, 1 H), 4.96 (d, 1 H, J = 8.2 Hz); ^{13}C NMR δ 11.45,16.13, 48.19, 71.35, 71.42, 73.84, 107.71. Anal. ($C_8H_{17}N_3O_6 \cdot 0.67H_2O \cdot 0.33CH_3OH$) C, H, N.

***O*^{2}-(3,4,6-Tri-*O*-acetyl-*β*-D-*N*-acetylglucosaminyl) 1-(*N*,*N*-Diethylamino)diazen-1-ium-1,2-diolate (8a) (CV-144).** To 1.2 g (9.3 mmol) of **1a** in 3:1 dimethylsulfoxide/tetrahydrofuran was added 2-acetamido-2-deoxy-α-D-glucopyranosyl chloride 3,4,6-triacetate (3.3 g, 9.0 mmol) and 47 mg of silver acetate. A white foam was isolated (3.0 g, 70%): UV (methanol) λ_{max} (ε) 226 nm (7.2 mM^{-1} cm^{-1}); ^1H NMR ($CDCl_3$) δ 1.12 (t, 6 H, J = 7.1 Hz), 1.92 (s, 3 H), 2.03 (s, 3 H), 2.04 (s, 3 H), 2.06 (s, 3 H), 3.27 (q, 4 H, J = 7.1 Hz), 3.83−3.89 (m, 1 H), 3.93−4.02 (m, 1 H), 4.12−4.28 (m, 2 H), 5.06−5.12 (m, 1 H), 5.54−5.60 (m, 1 H), 5.68 (d, 1 H, J = 8.8 Hz), 6.22 (d, 1 H, J = 8.2 Hz); ^{13}C NMR 11.35, 20.58, 20.60, 20.64, 23.23, 47.62, 53.42, 61.91, 68.36, 71.65, 72.35, 99.76, 169.40, 170.39, 170.42, 170.58. Anal. ($C_{18}H_{30}N_4O_{10}$) C, H, N.

***O*^{2}-(*N*-Acetyl-*β*-D-glucosaminyl) 1-(*N*,*N*-Diethylamino)diazen-1-ium-1,2-diolate (9a) (CV-159).** A solution of 309 mg (0.67 mmol) of **8a** in methanol was deacylated, yielding 141 mg (63%

yield) of a white foam: UV (H_2O) λ_{max} (ε) 228 nm (7.6 mM^{-1} cm^{-1}); ^1H NMR (D_2O) δ 1.05 (t, 6 H, $J = 7.2$ Hz), 2.02 (s, 3 H), 3.19 (q, 4 H, $J = 7.1$ Hz), 3.46−3.69 (m, 4 H), 3.77−3.96 (m, 3 H), 4.03 (t, 1 H, $J = 9.0$ Hz), 5.37 (d, 1 H, $J = 8.9$ Hz); ^{13}C NMR δ 13.29, 24.91, 51.56, 56.63, 63.19, 72.07, 76.48, 79.70, 104.57, 177.32. Anal. ($C_{12}H_{24}N_4O_7$) C, H, N.

***O*²-(3,4,6-Tri-*O*-acetyl-β-D-*N*-acetylglucosaminyl) 1-(Pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (8b) (CV-154).** This compound was prepared in 73% yield by reacting **1b** with acetochloroglucosamine in the presence of 25 mg of silver acetate. Purification was carried out on a silica gel column by eluting with 7:3 ethyl acetate/hexane increasing to 100% ethyl acetate: mp 159−161 °C; UV (methanol) λ_{max} (ε) 254 nm (7.7 mM^{-1} cm^{-1}); ^1H NMR ($CDCl_3$) δ 1.93 (s, 3 H), 1.96−2.00 (m, 4 H), 2.03 (s, 3 H), 2.04 (s, 3 H), 2.07 (s, 3 H), 3.60−3.65 (m, 4 H), 3.83−3.89 (m, 1 H), 3.96−4.31 (m, 3 H), 5.06 (t, 1 H, $J = 13.3$ Hz), 5.59 (t, 1 H, $J = 9.4$ Hz), 5.68 (d, 1 H, $J = 8.9$ Hz), 6.75 (d, 1 H, $J = 8.4$ Hz); ^{13}C NMR δ 20.58, 20.66, 22.96, 23.29, 53.13, 61.90, 68.35, 71.80, 72.26, 99.63, 169.42, 170.34, 170.59, 170.64. Anal. ($C_{18}H_{28}N_4O_{10}$) C, H, N.

***O*²-(β-D-*N*-Acetylglucosaminyl) 1-(Pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (9b) (CV-166).** A solution of 1 g (2.17 mmol) of **8b** in 13 mL of methanol was treated with 25% sodium methoxide in methanol as described above. The hydrolyzed product was chromatographed on silica gel and eluted with 9:1 dichloromethane/ethyl acetate to give 660 mg (91%) of **9b** as a white foam: UV (methanol) λ_{max} (ε) 254 nm (7.5 mM^{-1} cm^{-1}); ^1H NMR (D_2O) δ 1.96−2.0 (m, 4 H), 2.03 (s, 3 H), 3.54−3.62 (m, 4 H), 3.62−3.96 (m, 4 H), 4.04 (t, 1 H, $J = 9.9$ Hz), 5.25 (d, 1 H, $J = 7$ Hz); ^{13}C NMR δ 24.93, 25.39, 53.75, 56.38, 63.24, 72.17, 76.57, 79.61, 103.91, 177.38. Anal. ($C_{12}H_{22}N_4O_6 \cdot 0.5H_2O$) C, H, N.

***O*²-(3,4,6-Tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl) 1-(*N*,*N*-Diethylamino)diazen-1-ium-1,2-diolate (10a) (JS-45-76).** 1,3,4,6-Tetraacetyl-2-deoxy-β-D-glucose (1.59 g, 4.8 mmol), prepared from 2-deoxyglucose with pyridine and acetic anhydride using the method of Wolfrom and Thompson,[26] was brominated in 25 mL of dichloromethane with 24 mL of 30% HBr/acetic acid according to a literature procedure[27] to give 1.16 g (68%) of 3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl bromide. A solution of 950 mg (2.69 mmol) of this compound in 10 mL of tetrahydrofuran was cooled to 0 °C and stirred under nitrogen. In a separate container were placed 551 mg (3.6 mmol) of **1a**, 10 mL of tetrahydrofuran, and 1 mL of *N*,*N*-dimethylformamide (DMF). The resulting slurry was treated with 792 mg (3.6 mmol) of 15-crown-5, resulting in a near-homogeneous solution that was slowly added to the bromosugar and stirred at 0−4 °C. The reaction was complete within an hour as indicated by TLC on silica gel. The reaction flask was placed on a rotary evaporator and concentrated to an oil (1.6 g) containing the product, impurities, and crown ether as well as DMF. The oil was flash-chromatographed on a 3.3 cm × 29 cm prepacked silica gel column and eluted with 5:1 dichloromethane/ethyl acetate to give 640 mg (59%) of **10a** as a colorless glass that was difficult to separate from the 3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucose. The analytical sample contained the latter compound and **10a** in a 1:3 ratio: UV (methanol) λ_{max} (ε) 227 nm (8.2 mM^{-1} cm^{-1}); ^1H NMR ($CDCl_3$) δ 1.15 (t, 6 H, $J = 7.13$ Hz), 2.05 (s, 3 H), 2.06 (s, 3 H), 2.06 (s, 3 H), 1.99−2.10 (m, 1 H), 2.49 (ddd, 1 H, $J = 2.32$, 4.69, 12.49 Hz), 4.27 (dd, 1 H, $J = 4.9$, 12.2 Hz), 5.01−5.12 (m, 2 H), 5.36 (dd, 1 H, $J = 2.34$, 9.96 Hz); ^{13}C NMR ($CDCl_3$) δ 11.53, 20.69, 20.86, 33.42, 48.51, 62.19, 68.35, 69.90, 72.53, 99.25, 169.63, 170.19, 170.65 (note: only 13 out of 14 carbons are detected). Anal. ($C_{16}H_{27}N_3O_9 \cdot 0.33C_{12}H_{18}O_9$) C, H, N.

***O*²-[2-Deoxy-β-D-glucopyranosyl] 1-(*N*,*N*-Diethylamino)diazen-1-ium-1,2-diolate (11a) (JS-45-112).** To a solution of 208 mg (0.51 mmol) of **10a** in 5 mL of methanol was added 5 μL (0.023 mmol) of 25% methanolic sodium methoxide. The resulting solution was stirred at room temperature for 2 h whereupon 500 mg of prewashed Dowex 50W-H^+ resin was added. After stirring for an additional 5 min, the mixture was filtered and evaporated in vacuo. The residue was taken up in dichloromethane and filtered through a short alumina column to give 130 mg of JS-45-112 as a colorless glass: UV (methanol) λ_{max} (ε) 229 nm (8.2 mM^{-1} cm^{-1}); ^1H NMR ($CDCl_3$) δ1.11 (t, 6 H, $J = 7.13$ Hz), 1.84−1.93 (m, 1 H), 2.39−2.48 (m, 1 H), 3.93−3.95 (m, 5 H), 5.34 (dd, 1 H, $J = 2.2$, 10.2 Hz); ^1H NMR (D_2O) δ 1.07 (t, 6 H, $J = 7.1$ Hz), 1.74−1.82 (m, 1 H), 2.45 (ddd, 1 H, $J = 2.22$, 5.04, 12.3 Hz), 3.20 (q, 4 H, $J = 7.2$ Hz), 3.37−3.43 (m, 1 H), 3.50−3.54 (m, 1 H), 3.76−3.85 (m, 2 H), 3.93 (dd, 1 H, $J = 2.3$, 7.17 Hz), 5.55 (dd, 1 H, $J = 2.22$, 9.94 Hz); ^{13}C NMR ($CDCl_3$) δ 11.49, 36.21, 48.29, 61.64, 71.09, 71.01, 76.18, 100.25: ^{13}C NMR (D_2O) δ 13.33, 38.69, 51.64, 63.39, 72.86, 73.09, 79.60, 103.28. The chemical shifts proved to be solvent-dependent. Anal. ($C_{10}H_{21}N_3O_6 \cdot 0.2H_2O \cdot 0.2C_6H_{12}O_5$) C, H, N.

***O*²-Tetrahydropyran-2-yl 1-(*N*,*N*-Diethylamino)diazen-1-ium-1,2-diolate (12) (JS-45-97).** A solution of 360 mg (3.02 mmol) of 2-chlorotetrahydropyran (prepared by the method of Viola et al.)[28] in 5 mL of tetrahydrofuran was cooled to 0 °C under nitrogen. A solution of 468 mg (3 mmol) of **1a** in 5 mL of THF, 1 mL of DMF, and 660 mg (3 mmol) of 15-crown-5 was added slowly to the cold solution and stirred for 1 h, at which time TLC indicated that the reaction was complete. The volatile solvents were removed on a rotary evaporator, and the residual oil was extracted with ether that was quickly washed with cold, dilute sodium bicarbonate solution, dried over sodium sulfate, filtered, and evaporated to give 662 mg of a brown oil. Flash chromatography on a 13.5 cm × 3.3 cm silica gel column eluted with 5:1 dichloromethane/ethyl acetate gave 358 mg (55%) of JS-45-97 as a colorless liquid: UV (ethanol) λ_{max} (ε) 233 nm (6.2 mM^{-1} cm^{-1}); ^1H NMR ($CDCl_3$) δ 1.11 (t, 6H, $J = 7.12$ Hz), 1.58−1.99 (m, 6H), 3.4 (q, 4H, $J = 7.1$ Hz), 3.56−3.68 (m, 1H), 3.86−3.92 (m, 1H), 5.51 (t, 1H, $J = 2.83$ Hz); ^{13}C NMR ($CDCl_3$) δ 11.53, 18.36, 24.82, 28.03, 48.59, 62.07, 100.15. Anal. ($C_9H_{19}N_3O_3 \cdot 0.1C_5H_{10}O_2$) C, N. H: calcd, 8.85; found, 9.27. (Note: $C_5H_{10}O_2$ is 2-hydroxytetrahydropyran.)

***N*-(β-D-*N*′-Acetylglucosaminyloxy)succinamate *O*-Methyl Ester (13) (BMS-3-80).** This non-NO-releasing control compound was prepared as previously described. The compound's NMR spectrum matched that reported by Cao et al.[29]

Rates of Hydrolysis. Rate constants for hydrolysis of **3a**, **5a**, **7a**, **9a**, and **11a** measured at pH 7.4 and below under protection from light were determined by measuring absorption spectra (200−400 nm) of l mL aliquots taken daily from 40 mL reaction solutions maintained in sealed bottles thermostated at 37 °C for up to 11 days. First-order rate constants were obtained from the slope of plots of $\ln(A_t - A_{inf})$ versus time, where A is absorbance at time t at λ_{max} and where A_{inf} is the constant absorbance value at the end of the reaction. For reactions that had not gone to completion, infinity absorbance values were estimated from the exponential A_t versus time decay curves that were obtained at ≥80% of reaction.

Faster reactions occurring in 1.0 M NaOH solutions were followed directly in the thermostated cell compartment of the HP 8453 diode-array spectrophotometer, and rate constants were obtained through the instrument's kinetics software.

Rates of NO release in cell culture medium containing fetal calf serum in the presence and absence of macrophages were estimated by following the accumulation of its auto-oxidation product, nitrite ion, using the colorimetric Griess reagent assay as described.[30]

Glycosidase-Induced Hydrolysis. The specific activity for hydrolysis of PNP-NAG (*p*-nitrophenyl-*N*-acetyl-D-glucosaminide) by β-*N*-acetylglucosaminidase (NAGase) was confirmed at 25 °C as recommended in the manufacturer's protocol by monitoring UV absorbance changes at 345 nm. Activities for enzymes from both jack bean and human placenta were determined at their pH rate maxima, 5.0 and 4.25, respectively. Chemiluminescence detection and quantification of NO evolving from the reactions were conducted using a Sievers 280i nitric oxide analyzer (Boulder, CO) essentially as described previously.[31] The NO release profile was followed at 37 °C, and the resulting curve was integrated to quantify the amount of NO released per mole of compound. Details of the enzymatic hydrolysis studies on **5a**, **9a**, and **9b** are described in Figure 4 and Table 3.

X-ray Crystallographic Characterization of Mannose Derivative 4a. Single-crystal X-ray diffraction data on compound **4a** were collected at 103 K using Mo Kα radiation and a Bruker SMART 1000 CCD area detector. A 0.38 × 0.21 × 0.20 mm^3 crystal was prepared for data collection coating with high viscosity microscope oil (Paratone-N, Hampton Research). The oil-coated crystal was mounted on a glass rod and transferred immediately to the cold stream (−170 °C) on the diffractometer. The crystal was orthorhombic in space group $P2_12_12_1$ with unit cell dimensions a = 8.919(3) Å, b = 11.086(3) Å, and c = 23.857(7) Å. Corrections were applied for Lorentz, polarization, and absorption effects. Data were 99.7% complete to 28.28° θ (approximately 0.75 Å). The structure was solved by direct methods and refined by full-matrix least-squares on F^2 values using the programs found in the SHELXTL suite (Bruker, SHELXTL version 6.10, 2000, Bruker AXS Inc., Madison, WI). Parameters refined included atomic coordinates and anisotropic thermal parameters for all non-hydrogen atoms. Hydrogen atoms on carbons were included using a riding model [coordinate shifts of C applied to H atoms] with C−H distance set at 0.96 Å. The absolute configuration was set on the basis of the known configuration of the sugar. Atomic coordinates for compound **4a** have been deposited with the Cambridge Crystallographic Data Centre (deposition number 260728). Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge, CB2 1EZ, UK [fax, +44(0)-1223-336033; e-mail, deposit@ccdc.cam.ac.uk].

***Leishmania* Parasites, Macrophages, and Assay for Leishmanicidal Activity.** *L. major* amastigotes were prepared from the skin lesions of BALB/c mice that had been infected subcutaneously with promastigotes of the *L. major* strain MHOM/IL/81/FE/BNI.[14,32,33]

Thioglycolate-elicited peritoneal exudate macrophages were prepared from C57BL/6 iNOS-deficient mice[34] (Jackson Laboratories, Bar Harbor, ME) 4 days after ip injection of 2 mL of 4% Brewer's thioglycolate broth.[14,35] The macrophages were seeded into eight-well LabTek Tissue Culture Permanox Chambers (Nalge Nunc International, Naperville, IL) at 3 × 10^5 macrophages/well and cultured in RPMI-1640 medium (supplemented with 2 mM glutamine, 10 mM HEPES, 13 mM $NaHCO_3$, 50 μM 2-mercaptoethanol, 100 μg/mL penicillin, 100 μg/mL streptomycin, and 2.5% or 5% fetal bovine serum [lot 77H3398; Sigma, Deisenhofen, Germany]). After an adherence phase of 2−3 h, nonadherent cells were washed off and the macrophages were either cultured in medium alone or stimulated with interferon (IFN)-γ (obtained from Dr. G. Adolf at the Ernst Boehringer Institut, Vienna, Austria; 20 ng/mL) and tumor necrosis factor (TNF, 10 ng/mL) for 16 h. Thereafter, the monolayers were infected with amastigotes of *L. major* at a parasite/macrophage ratio of 5:1 for 4 h (with the remaining extracellular *Leishmania* washed off thereafter). For the following 72 h, the macrophages were cultured in medium alone (no stimulus), stimulated with IFN-γ plus TNF, or incubated with glycosylated diazeniumdiolates at the indicated concentrations. After 24 and 48 h, the culture medium was replaced with fresh medium containing the respective stimuli. Immediately after the 4 h pulse infection period (time point 0 h after the infection) as well as 48 and 72 h after infection, the percentage of infected macrophages and the number of intracellular parasites per infected macrophage were determined microscopically using the Diff-Quick staining procedure (Dade Behring, Marburg, Germany). The results were used to calculate the number of parasites per 100 macrophages in culture. All given values are mean values (±SD) and based on the evaluation of at least 4 × 100 macrophages for the infection rate and 3 × 60 macrophages for the number of parasites per cell.

The viability of the macrophage monolayers was verified by microscopic analysis using trypan blue staining of dead cells as well as by the ability of viable macrophages to transform yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into violet formazan (this photometric assay was performed in triplicate using an ELISA reader as described[36,37]).

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Supporting Information Available: Complete crystal structure report for **4a** and results from elemental analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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