SUPPORTING INFORMATION

Dual Mechanisms of HNO Generation by a Nitroxyl Prodrug of the Diazeniumdiolate (NONOate) Class

Daniela Andrei, Debra J. Salmon, Sonia Donzelli, Azadeh Wahab, John R. Klose, Michael L. Citro, Joseph E. Saavedra, David A. Wink, Katrina M. Miranda, and Larry K. Keefer

Table of Contents	**Page**
Full Authorship for Reference 34	**2**
Materials and Methods	**2**
General	**2**
Preparation of 2	**2**
Kinetic Studies	**3**
Reductive Nitrosylation of Metmyoglobin	**4**
Product Analyses	**5**
Figure S1. Expansion of Figure 6 Showing Formaldehyde NMR Signal	**6**
Esterase-Mediated Hydrolysis of 2, Including Figure S2	**7**
Effects of Prodrug 2 on Ventricular Myocytes	**8**
References	**9**

Full Authorship for Reference 34: Miranda, K. M.; Katori, T.; Torres de Holding, C. L.; Thomas, L.; Ridnour, L. A.; McLendon, W. J.; Cologna, S. M.; Dutton, A. S.; Champion, H. C.; Mancardi, D.; Tocchetti, C. G.; Saavedra, J. E.; Keefer, L. K.; Houk, K. N.; Fukuto, J. M.; Kass, D. A.; Paolocci, N.; Wink, D. A. *J. Med. Chem.* **2005**, *48* , 8220-8228.

Materials and Methods

General. IPA/NO ($Na[(CH_3)_2CHNH(N(O)NO]$, sodium 1-(*N*-isopropylamino)diazen-1-ium-1,2-diolate, **1**) was synthesized and utilized as previously described.[1,2] Concentrations of stock solutions (>10 mM), prepared in 10 mM NaOH and stored at -20 °C, were determined directly prior to use from the absorbance at 250 nm (ε 10 mM^{-1} cm^{-1}).[3] Angeli's salt ($Na_2N_2O_3$)[4,5] and DEA/NO ($Na[Et_2NN(O)NO]$)[1,2] were similarly obtained and handled, except that their extinction coefficients at 250 nm in 10 mM NaOH were taken as 8 mM^{-1} cm^{-1} as reported previously.

Caution*: Primary amine diazeniumdiolate salts such as* ***1*** *have been known to decompose explosively and without warning or obvious provocation on isolation or storage.*

***O*²-(Acetoxymethyl) 1-(Isopropylamino)diazen-1-ium-1,2-diolate (2).** A solution of bromomethyl acetate (867 mg, 5.67 mmol) in 3 mL of tetrahydrofuran was reacted with a slurry of IPA/NO (800 mg, 5.67 mmol, in 10 mL of dimethyl sulfoxide) at room temperature. The reaction mixture was stirred overnight, whereupon 15 mL of water was added, and stirring was continued for another 10 min. The residue was extracted with dichloromethane and the organic layer was washed with 5% sodium bicarbonate, dried over anhydrous sodium sulfate, filtered, and evaporated in vacuo to give a colorless oil. Column chromatography was performed using

hexane:acetone (4:1) on silica gel to give the desired product **2** (880 mg, 81%): UV (ethanol) λ_{max} (ε) 240 nm (8.7 mM^{-1} cm^{-1}); ^{1}H NMR ($CDCl_3$) δ 1.19 (d, J = 6.4 Hz, 6H), 2.12 (s, 3H), 4.00 (doublet of septets, J = 6.4 Hz and 9.1 Hz, 1H), 5.75 (s, 2H), 6.25 (d, J = 9.1 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 20.34, 20.81, 49.17, 87.07, 169.42. Anal. Calcd for $C_6H_{13}N_3O_4$: C, 37.69; H, 6.85; N, 21.98. Found: C, 37.77; H, 6.98; N, 21.82.

Kinetic Studies. The rate constants of decomposition were measured spectrophotometrically by monitoring the decrease in absorbance of peaks at ~240-250 nm characteristic of the diazeniumdiolate functionality. The hydrolysis medium consisted of the metal chelator DTPA (50 μM) in calcium- and magnesium-free Dulbecco's phosphate-buffered saline (PBS, pH initially 7.4, adjusted by adding NaOH or HCl as needed). Although PBS is a relatively weak buffer, this triprotic system was sufficient to maintain the desired pH values from 3-13 at the low concentrations of **1** or **2** used for spectroscopic analysis. Additionally, since buffer composition can significantly impact measurable signals from HNO, we choose to use phosphate buffer whenever possible. UV-visible spectroscopy was performed with a Hewlett-Packard 8453 diode-array spectrophotometer equipped with an Agilent 89090A thermostat set to 37 °C. The spectrophotometer was blanked after warming the cuvette containing buffer at the appropriate pH in the instrument heat block for 5 min. For esterase-containing reactions, porcine liver esterase (20 μL, 1.4 U/mL; Sigma; suspension in 3.2 M $(NH_4)_2SO_4$, pH 8) was added to the reaction buffer before blanking. Upon addition of **1** or **2** (≤10 μL of stock, **1** in 10 mM NaOH, **2** in methanol, kept on ice), the cuvette was capped, mixing was accomplished either by inversion or by pipette, and spectra were collected at 0.5- to 60-s intervals for time periods of 2-600 min or until $A_\infty < 0.05$. Kinetic analysis was performed by fitting the data to an exponential decay ($A = \Delta A e^{-kt} + A_\infty$) using KaleidaGraph v.3.1.

Reductive Nitrosylation of Metmyoglobin.[6,7] Reductive nitrosylation of ferric myoglobin (metMb) to nitrosyl myoglobin (MbNO) by **1** or **2** was monitored in a quartz cuvette in assay buffer at 37 °C. Formation of HNO was further examined by quenching with GSH, which does not interact directly with low concentrations of NO. To maintain deaerated conditions, all solutions were transferred using gas-tight Hamilton syringes, and the reaction buffer was sparged with ultra-high purity argon at the rate of ≥1 min for each mL of buffer. Aliquots (2-3 mL) were removed by Hamilton syringe and transferred to an argon-flushed, graded seal quartz cuvette (Spectrocell; Oreland, PA) stoppered with a Suba-Seal septum (Sigma-Aldrich). The buffer within the cuvette was again gently bubbled with argon for 5 min. In a separate stoppered vial, a small amount of horse heart myoglobin was purged with argon for 15 min while in an ice bath and then dissolved with deaerated buffer. An aliquot of metMb (>1 mM stock; ε_{502} = 10.2 mM^{-1} cm^{-1})[8] was added by syringe, as were aliquots of GSH (approximately 100×; 250 μM final) as appropriate. For reactions containing porcine liver esterase (PLE; 1.4 U/mL), the viscosity of the suspension required rigorous degassing by three freeze-pump-thaw cycles using a dry ice bath for freezing. Given the varied results from the esterase-free reaction, the enzyme remained active after freezing. The reaction was initiated by introducing a small volume of stock diazeniumdiolate solution (**1** in 10 mM NaOH, **2** in methanol, kept on ice; 100 μM final) to the metMb (50 μM final) solution. Spectra were collected at 30- to 60-s intervals for 60-90 min or until A_{543} and A_{575} reached stable values. The concentration of MbNO was determined spectrophotometrically (ε_{543} = 11.6 mM^{-1} cm^{-1}; ε_{575} = 10.5 mM^{-1} cm^{-1})[8] or, in case of incomplete conversion, using absorbance values from the final spectra and the following equations to solve for the concentrations of metMb and MbNO.

$$A_{543} = \varepsilon(metMb_{543})[metMb] + \varepsilon(MbNO_{543})[MbNO]$$

$$A_{502} = \varepsilon(metMb_{502})[metMb] + \varepsilon(MbNO_{502})[MbNO]$$

The absorbance at 543 nm of a solution of metMb, $\varepsilon(metMb_{543})$ was used to determine an $\varepsilon(metMb_{543})$ of 6300 ± 120 $M^{-1}cm^{-1}$. The maximum absorbance at 502 nm of a solution of metMb (20 or 50 μM) exposed to an excess of HNO donor (100 μM), where it was assumed that $[metMb]_{initial} = [MbNO]_{final}$, was used to determine an $\varepsilon(MbNO_{502})$ of 7400 ± 570 $M^{-1}cm^{-1}$. A simpler calculation using $\Delta\varepsilon_{543} = 5.2\ mM^{-1}\ cm^{-1}$, $\Delta\varepsilon_{575} = 6.7\ mM^{-1}\ cm^{-1}$) results in comparable product concentrations (for Figure 1, 25 μM and 47 μM, respectively).

Product Analyses. In addition to HNO measurements as described above by reductive nitrosylation of metmyoglobin, NO was quantified by chemiluminescence assay, as previously described.[9] Organic products of hydrolysis of **1** and **2** were quantified by integrating their NMR spectra; for pH 7.4, 3.9 mM solutions in 0.1 M phosphate buffer were employed, while for pH 11, 4.9 mM solutions of **1** and **2** in 50 mM sodium carbonate were studied. Samples were run on a Varian Inova 400 MHz NMR with a Dell Precision 390 workstation. The samples were run at 37 ^{o}C and water suppression was achieved by using the preset pulse sequence. Peaks were identified by comparison with authentic standards. Authentic propene was produced as a reference for the identification of this gas as a product of the reactions by the following procedure. Isopropylamino methyl carbamate was dissolved in dichloromethane and mixed with an equivalent volume of 1 M hydrochloric acid. A solution of sodium nitrite in water was added gradually. The bottom yellow-green layer was separated, dried over sodium sulfate, and concentrated under vacuum. A portion of the resulting oil (~5 μL) was dissolved in 10% sodium deuterioxide in D_2O and allowed to decompose for 1 h. The NMR spectrum exhibited signals corresponding to methanol, isopropanol, starting carbamate, and a small (presumably saturating)

concentration of propene. Its methyl group signals appeared as a doublet of triplets (δ 1.70 and 1.72, J = 1.5 and 6.5 Hz) identical to those observed in the hydrolysis of **1** and **2**.

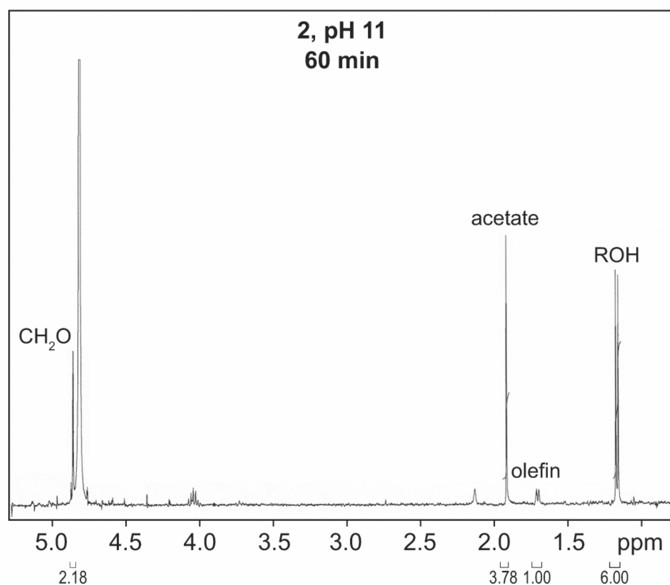

Figure S1. Expansion of the NMR spectrum in the lower right panel of Figure 6 to include the formaldehyde signal at 4.82 ppm, which was identical in chemical shift to that of aqueous 1,3,5-trioxane (a cyclic trimer of formaldehyde). The small numbers underneath the ppm axis are the observed integrals for the four organic products identified in the hydrolysis of **2**. The observed integral for acetate corresponded to a yield (taken as 100%) essentially matching that expected for the sum of propene plus isopropanol (26% + 79%, respectively) suggesting that analysis of the organic products in these reactions can provide reliable quantitative insight into the partition between the NO- and HNO-forming pathways.

Esterase-Mediated Hydrolysis of 2. Figures S2A and B show that addition of porcine liver esterase does not impact reductive nitrosylation of metMb by IPA/NO (**1**), in neither the presence nor absence of GSH (compare to Figures 1A and 2A; product yield in the presence of GSH is somewhat variable between batches of myoglobin). Similar spectral changes are apparent for AcOM-IPA/NO (**2**) under the same conditions (Figure S2C and D). Comparison to Figures 1B, which has a higher product yield, and 2C, in which GSH inhibits product formation, supports that esterase hydrolyzes **2** to produce **1** as an intermediate in the production of both NO and HNO.

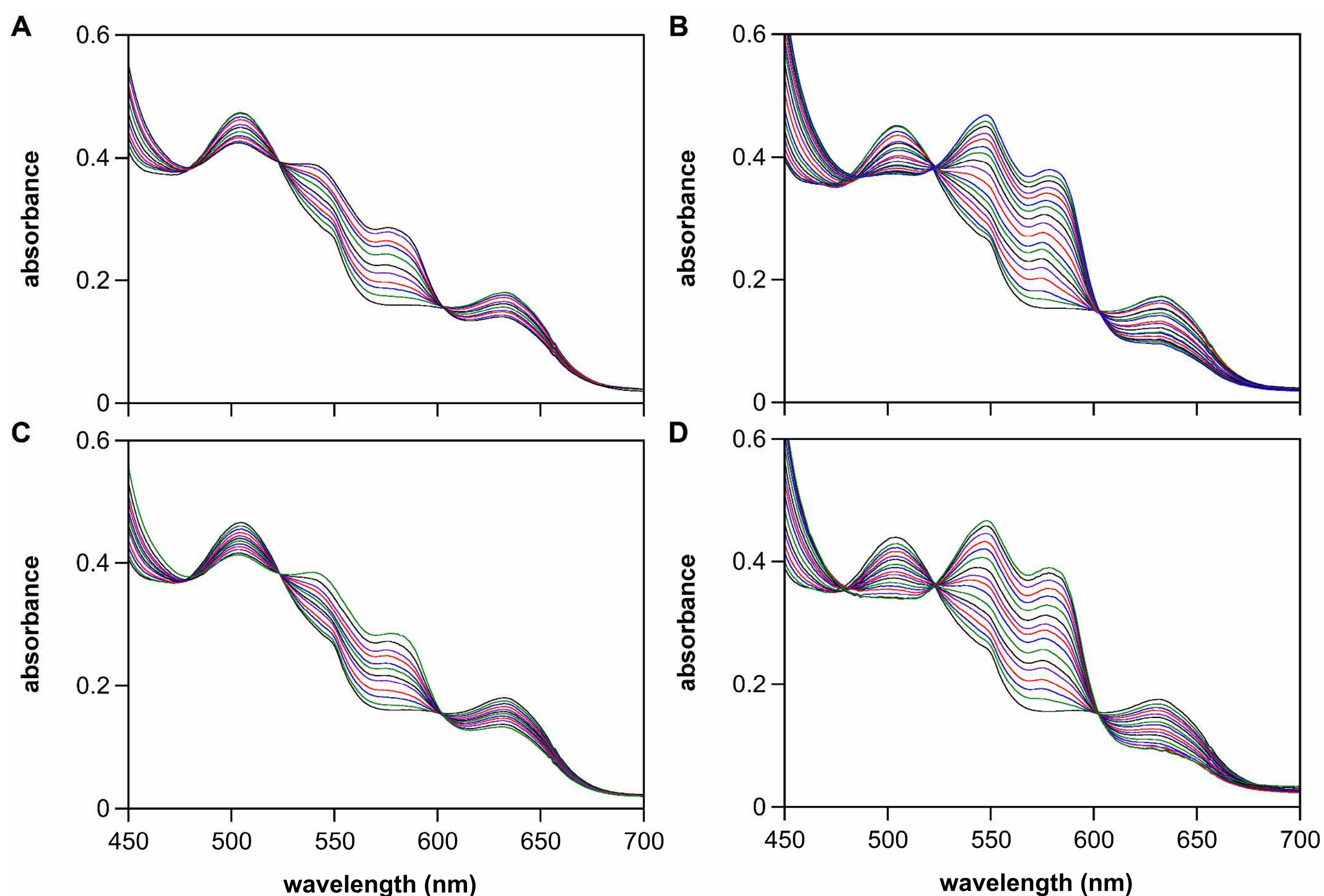

Figure S2. Esterase-mediated hydrolysis of **1** (A and B) and **2** (C and D) in the presence (panels B and D) or absence (panels A and C) of 250 μM GSH. All reaction mixtures contained

100 µM diazeniumdiolate, 50 µM metMb, and 1.4 U/mL of porcine liver esterase, and were incubated at pH 7.4 and 37 °C under deaerated conditions for 1 h.

Effects of Prodrug 2 on Ventricular Myocytes. Stock solutions of **1** and **2** were prepared in 10 mM NaOH and DMSO, respectively, immediately prior to each experiment and stored on ice protected from light. Liberase blendzyme 3 was purchased from Roche Diagnostics (Mannheim) while $CaCl_2$, DMSO, and glucose were purchased from Merck (Darmstadt). Calcium-free perfusion buffer contained 113 mM NaCl, 4.7 mM KCl, 0.6 mM KH_2PO_4, 0.6 mM Na_2HPO_4, 1.2 mM $MgSO_4$, 12 mM $NaHCO_3$, 10 mM $KHCO_3$, 30 mM taurine, 5.5 mM glucose, 10 mM 2,3-butanedione monoxime, and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) at pH 7.4. Digestion buffer contained an additional 0.1 mg/mL of recombinant collagenases/proteases (Liberase blendzyme 3) and 12.5 µM $CaCl_2$. IonOptix solution for the sarcomere shortening measurements contained 135 mM NaCl, 4.7 mM KCl, 0.6 mM KH_2PO_4, 0.6 mM Na_2HPO_4, 1.2 mM $MgSO_4$, 1.25 mM $CaCl_2$, 20 mM glucose, and 10 mM HEPES at pH 7.46.

C57BL/6 male mice (13-15 weeks) were used in the present study. All animal care and experiments in this study were undertaken in accordance with German and European legislation regarding the use of animals for experimental protocols, and all efforts were made to minimize animal suffering and to reduce the number of animals used. Animals were sacrificed by cervical dislocation under light CO_2 anesthesia. Ventricular myocytes were isolated from wild type hearts using a modified protocol of O'Connell.[10] Briefly, the thorax was opened, and the aorta was cut about 2 mm from its entry into the heart. Then the heart was quickly excised, and the aorta was placed onto a cannula with two fine curve-tip forceps and tied with a thread. The cannulated

heart was mounted on a temperature-controlled perfusion system and perfused for 8 min with a calcium-free perfusion buffer before switching to the digestion buffer. After 8-10 min of digestion the ventricles were removed and placed in a digestion buffer, and the tissue was dissociated using forceps. Further enzyme activity was inhibited by addition of 5% fetal bovine serum. Debris was sedimented by gravity and the supernatant, containing the intact cardiac myocytes, was centrifuged. The pellet was resuspended in fresh buffer and $CaCl_2$ was slowly reintroduced to a final concentration of 1 mM. After further centrifugation, IonOptix buffer was added on the pellet and sarcomere shortening was assessed on field stimulation (1 Hz with 4-ms duration, 10 V) using a video-based sarcomere length detection system (IonOptix Corporation) at room temperature.

Reference List

(1) **Maragos, C. M.; Morley, D.; Wink, D. A.; Dunams, T. M.; Saavedra, J. E.; Hoffman, A.; Bove, A. A.; Isaac, L.; Hrabie, J. A.; Keefer, L. K. *J. Med. Chem.* 1991, *34*, 3242-3247.**

(2) **Drago, R. S.; Karstetter, B. R. *J. Am. Chem. Soc.* 1961, *83*, 1819-1822.**

(3) **Miranda, K. M.; Katori, T.; Torres de Holding, C. L.; Thomas, L.; Ridnour, L. A.; McLendon, W. J.; Cologna, S. M.; Dutton, A. S.; Champion, H. C.; Mancardi, D.; Tocchetti, C. G.; Saavedra, J. E.; Keefer, L. K.; Houk, K. N.; Fukuto, J. M.; Kass, D. A.; Paolocci, N.; Wink, D. A. *J. Med. Chem.* 2005, *48* , 8220-8228**.

(4) **Smith, P. A. S.; Hein, G. E. *J. Am. Chem. Soc.* 1960, *82*, 5731-5740.**

(5) **Addison, C. C.; Gamlen, G. A.; Thompson, R. *J. Chem. Soc.* 1952, 338-345.**

(6) **Miranda, K. M.; Paolocci, N.; Katori, T.; Thomas, D. D.; Ford, E.; Bartberger, M. D.; Espey, M. G.; Kass, D. A.; Feelisch, M.; Fukuto, J. M.; Wink, D. A. *Proc. Natl. Acad. Sci. USA* 2003, *100*, 9196-9201.**

(7) **Doyle, M. P.; Mahapatro, S. N.; Broene, R. D.; Guy, J. K. *J. Am. Chem. Soc.* 1988, *110*, 593-599.**

(8) Antonini, E.; Brunori, M. *Frontiers Biol.* 1971, *21*, 19.

(9) Keefer, L. K.; Nims, R. W.; Davies, K. M.; Wink, D. A. *Methods Enzymol.* 1996, *268*, 281-293.

(10) O'Connell, T. D.; Ni, Y. G.; Lin, K.-M.; Han, H.; Yan, Z. *Isolation and culture of adult mouse cardiac myocytes for signaling studies*; AfCS Research Reports 1: No. 5 CM; http://www.signaling-gateway.org/reports/v1/CM0005/CM0005.htm], 03.