

## Acyloxy Nitroso Compounds as Nitroxyl (HNO) Donors: Kinetics, Reactions with Thiols, and Vasodilation Properties

Mai E. Shoman,<sup>†</sup> Jenna F. DuMond,<sup>†</sup> T. S. Isbell,<sup>‡</sup> J. H. Crawford,<sup>‡</sup> Angela Brandon,<sup>‡</sup> Jaideep Honovar,<sup>‡</sup> Dario A. Vitturi,<sup>‡</sup> C. R. White,<sup>§</sup> R. P. Patel,<sup>‡</sup> and S. Bruce King<sup>\*,†</sup>

<sup>†</sup>Department of Chemistry, Wake Forest University, Winston-Salem, North Carolina 27109, United States, <sup>‡</sup>Department of Pathology and Center for Free Radical Biology, University of Alabama—Birmingham, Birmingham, Alabama, United States, and <sup>§</sup>Department of Medicine and Center for Free Radical Biology, University of Alabama—Birmingham, Birmingham, Alabama, United States

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Acyloxy nitroso compounds hydrolyze to nitroxyl (HNO), a nitrogen monoxide with distinct chemistry and biology. Ultraviolet–visible spectroscopy and mass spectrometry show hydrolysis rate depends on pH and ester group structure with the observed rate being trifluoroacetate (**3**) > acetate (**1**) > pivalate (**2**). Under all conditions, **3** rapidly hydrolyzes to HNO. A combination of spectroscopic, kinetic, and product studies show that addition of thiols increases the decomposition rate of **1** and **2**, leading to hydrolysis and HNO. Under conditions that favor thiolates, the thiolate directly reacts with the nitroso group, yielding oximes without HNO formation. Biologically, **3** behaves like Angeli's salt, demonstrating thiol-sensitive nitric oxide-mediated soluble guanylate cyclase-dependent vasorelaxation, suggesting HNO-mediated vasorelaxation. The slow HNO-donor **1** demonstrates weak thiol-insensitive vasorelaxation, indicating HNO release kinetics determine HNO bioavailability and activity. These results show that acyloxy nitroso compounds represent new HNO donors capable of vasorelaxation depending on HNO release kinetics.

### Introduction

Nitroxyl (HNO<sup>•</sup>) is a nitrogen-containing compound chemically related to the well-known signaling agent nitric oxide (NO) through one-electron reduction and protonation.<sup>1,2</sup> Nitroxyl demonstrates separate chemistry and biology compared to NO, which has driven studies to better understand its chemical reactions, potential endogenous formation, and the development of HNO-based therapeutics.<sup>1–10</sup> Endogenous mechanisms of HNO formation have been proposed including direct enzymatic production from nitric oxide synthases, heme protein-mediated oxidation of various substrates, and nonenzymatic reactions between thiols and *S*-nitrosothiols.<sup>11–15</sup> Possible specific functions for endogenously produced HNO range from antioxidant<sup>16</sup> to vasorelaxant<sup>17–25</sup> to cytotoxic mediator.<sup>26</sup> Some of these HNO associated activities may occur via oxidation to NO, and subsequent stimulation of NO-signaling and various 1-electron acceptors have been

proposed to mediate this biochemistry including ferric heme proteins, flavins, and quinones.<sup>21,22</sup>

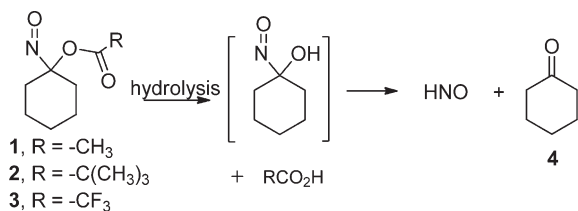
Irrespective of endogenous HNO formation, a parallel line of work entails the development of HNO donors as therapeutics.<sup>1–10</sup> This area stems from previous studies demonstrating that HNO mediates the action of cyanamide,<sup>27,28</sup> a drug used to treat alcoholism. Exciting newer studies show the HNO donor (sodium trioxodinitrate, Angeli's salt, AS) exerts positive inotropic effects in both normal and failing hearts, and decreases venous pressures, therapeutic properties optimal for the treatment of heart failure.<sup>5,29</sup> An increasing understanding of HNO reactivity reveals NO-independent mechanisms of biological action and identifies thiols as critical reaction targets. Recent studies suggest thiols represent the primary reaction target for HNO and likely mediate much of their biological activity.<sup>5</sup> For example, nitroxyl inhibits the thiol containing enzymes aldehyde dehydrogenase (ALDH) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), showing both reversible and irreversible components of inhibition.<sup>27</sup> The majority of the actions of HNO on cardiac tissue (increasing inotropy and calcium sensitivity) have also been attributed to HNO interaction with key thiols.<sup>29–31</sup> Consistent with this concept, we showed HNO selectively modifies mitochondrial thiols.<sup>32</sup>

The chemical behavior of HNO (rapid dimerization and dehydration to nitrous oxide, N<sub>2</sub>O) requires the use of HNO donors in these studies.<sup>6</sup> A current major limitation in HNO-based therapeutic development and our understanding of HNO biology is the reliance on a select few HNO donors.<sup>2,5,10,33</sup> The most widely utilized is Angeli's salt, which also produces nitrite that possesses its own biological activity.<sup>34,35</sup>

\*To whom correspondence should be addressed. Phone: 336 758 5774. Fax: 336 758 4656. E-mail: kingsb@wfu.edu.

<sup>a</sup> Abbreviations: ALDH, aldehyde dehydrogenase; AS, Angeli's salt; C-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxidecarboxy-PTIO; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GSH, reduced glutathione; GSSG, oxidized glutathione; HNO, nitroxyl; KH, Krebs–Henseleit buffer; L-NAME, L-N<sup>G</sup>-nitroarginine methyl ester; MNO, MahmaNonoate; Mn<sup>III</sup>TSPP, manganese(III) meso-(tetrakis(4-sulfonato-phenyl)) porphyrinate; PE, phenylephrine; NAC, *N*-acetyl-L-cysteine; NO, nitric oxide; NAP, *N*-acetyl-DL-penicillamine; ODQ, 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one; sGC, soluble guanylate cyclase; SNP, sodium nitroprusside; SOD, superoxide dismutase; TP, thiophenol.

## Scheme 1



Development of structurally distinct HNO donors with diverse release rates would complement the majority of studies using AS and form the basis of more efficient formulations.

Previously, we reported acyloxy nitroso compounds as new hydrolytic HNO donors that relax precontracted rat aorta (Scheme 1).<sup>36</sup> Here, we further characterize the kinetics, products, mechanism of HNO release, and the vasoactive properties of three (**1–3**, Scheme 1) acyloxy nitroso compounds. These results provide detailed decomposition rate information and reveal the ability of acyloxy nitroso compounds to directly react with thiolate ions to form oximes and disulfides. The chemical reactivity of this new class of HNO donors provides a basis to explain the observed ability of these compounds to relax precontracted rat aorta.

## Materials and Methods

**Chemistry.** General. Cyclohexanone oxime, 2,2-dimethylpropanoic acid, lead(IV) tetraacetate, [bis(trifluoroacetoxy)iodo]benzene, reduced glutathione (GSH), oxidized glutathione (GSSG), *N*-acetyl-DL-penicillamine (NAP), *N*-acetyl-L-cysteine (NAC), dithiothreitol (DTT), and thiophenol (TP) were purchased from Sigma-Aldrich Chemical Co. and used as received. Analytical thin layer chromatography (TLC) was performed on silica gel plates with C-4 Spectroline 254 indicator. Visualization was accomplished with UV light and 20% phosphomolybdic acid solution in EtOH. Solvents for extraction and purification were technical grade and used as received. <sup>1</sup>H NMR and <sup>13</sup>C NMR spectra were recorded using a Bruker Avance 300 MHz NMR spectrometer. Chemical shifts are given in ppm ( $\delta$ ); multiplicities are indicated by s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), and br (broadened). HT Laboratories, San Diego, CA, performed ESI analyses. Combustion elemental analysis (performed by Atlantic Microlab, Inc., Norcross, GA) provided > 95% purity of any newly described compounds. UV-vis spectrometry was performed on a Cary 100 Bio UV-vis spectrophotometer (Varian, Walnut Creek, CA).

**1-Nitrosocyclohexyl Acetate (NCA, **1**).**<sup>36</sup> Synthesized as previously described to give **1** as a blue oil (4.67 g, 57%).

**1-Nitrosocyclohexyl Pivalate (NCP, **2**).** A solution of cyclohexanone oxime (4.88 g, 43.16 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (50 mL) was added dropwise with stirring to a solution of lead(IV) tetraacetate (19.14 g, 43.16 mmol) and 2,2-dimethylpropanoic acid (44.08 g, 43.16 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (200 mL) at 0 °C. A blue color appeared with the addition of the oxime. The mixture was stirred at 0 °C for 1 h and at room temperature for 3 h. Water (50 mL) was added, the layers separated, and the CH<sub>2</sub>Cl<sub>2</sub> layer was washed with saturated sodium bicarbonate (10 × 50 mL), dried over MgSO<sub>4</sub>, filtered, and concentrated. The crude residue was purified by passing through a short pad of silica gel (20:1 hexanes:EtOAc, *R<sub>f</sub>* = 0.77) to give **2** as a blue oil (4.32 g, 47%). UV/vis (MeOH):  $\lambda_{\max}$  = 665 nm,  $\epsilon$  = 20.7 M<sup>-1</sup> cm<sup>-1</sup>; <sup>1</sup>H NMR (benzene-*d*<sub>6</sub>) 1.2 (s, 9H) 1.2–1.8 (m, 10H). <sup>13</sup>C NMR (benzene-*d*<sub>6</sub>) 21.9, 24.9, 27.2, 29.3, 39.2, 122.8, 175.5. ESI *m/z* 212 [100%, M – 1]. Anal. Calcd. For C<sub>11</sub>H<sub>19</sub>NO<sub>3</sub>: C, 61.95; H, 8.98, N, 6.57. Found: C, 61.71, H, 9.01, N, 6.45.

**1-Nitrosocyclohexyl Trifluoroacetate (NCTFA, **3**).** A solution of cyclohexanone oxime (0.57 g, 5 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (10 mL) was

added dropwise with stirring to a solution of [bis(trifluoroacetoxy)iodo]benzene (2.15 g, 5 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (10 mL) at 0 °C. A blue color appeared with the addition of the oxime. After 1 h at 0 °C, the reaction mixture was allowed to warm to room temperature and stirred for another 1 h. After disappearance of the oxime as judged by TLC, the solvent was evaporated and the residue was purified through a short pad of silica gel (hexanes) to give **3** as blue oil (0.74 g, 65% yield) and characterized as previously described.<sup>36</sup>

**Gas Chromatographic Detection of Nitrous Oxide.**<sup>36</sup> Substrate (0.1 mmol) was placed in a 25 mL round-bottom flask, sealed with rubber septa, and flushed with argon. Solvent (2 mL, either MeOH, 1:1 MeOH:Tris buffer (50 mM, pH = 7.6), 1:1 MeOH:0.1 M NaOH, 1:1 MeOH:0.1 M KOH, or 1:1 MeOH:1 M HCl) was added via syringe. In some samples, GSH or NAP (0.2 mmol) was added to the solvent before injection. After certain times, headspace (250  $\mu$ L) was injected into a 7890A Agilent Technologies gas chromatograph equipped with a thermal conductivity detector and a 6 ft × 1/8 in. Porapack Q column. The oven operated at 40 °C for 5 min and then 150 °C for 4.5 min. The purged packed inlet with a total flow (He carrier gas) at 18 mL/min and a septum purge flow of 3 mL/min was kept constant at 140 °C, and the back detector with a reference flow of 9 mL/min and a makeup flow of 6 mL/min was kept constant at 150 °C. The retention time of N<sub>2</sub>O was 2.5 min, and the yields were calculated from a standard curve of N<sub>2</sub>O purchased from Matheson Tri-Gas. This experiment was repeated measuring headspace injections every 30 min for 9 h to evaluate kinetic measurements.

**UV–Vis Assay for the Decomposition of **1** and **2**.**<sup>36</sup> A solution of Tris buffer (40 mM, pH = 7.0, 1.0 mL) was added to a solution of **1** (70  $\mu$ mol, 12 mg) in MeOH (1 mL) at room temperature in a sealed cuvette. UV–vis measurements were taken every 5 min for 60 min at 665 nm. In other experiments, a solution of NaOH (70 mM, 1 mL) was used and similar measurements made. Similarly, a solution of Tris buffer (40 mM, pH = 7.0, 0.5 mL) was added to a solution of **2** (51  $\mu$ mol, 10.9 mg) in MeOH (1 mL) at room temperature in a sealed cuvette. UV–vis measurements were taken every 30 min for 4 h at 665 nm. In other experiments, a solution of NaOH (0.1, 0.2, 0.5 M, 1 mL) was used and similar measurements made.

**UV–Vis Assay for the Reaction of **1** and **2** with Thiols.** A solution of GSH (35.1  $\mu$ mol, 10.8 mg) in Tris buffer (40 mM, pH = 7.0, 0.5 mL) was added to a solution of **1** (35.1  $\mu$ mol, 6 mg) in MeOH (0.5 mL) at room temperature in a sealed cuvette. UV–vis measurements were taken every 5 min for 4 h at 665 nm. Similar experiments were performed using varying concentrations of GSH (70.2  $\mu$ mol, 21.6 mg, or 175  $\mu$ mol, 53.7 mg), GSH (175  $\mu$ mol, 53.7 mg) in NaOH (35.1 mM, 0.5 mL), or GSSG (35.1  $\mu$ mol, 21.5 mg). Other experiments were done using NAC (35.1, 70.2, 175  $\mu$ mol). Similarly, a solution of GSH (51  $\mu$ mol, 15.6 mg) in Tris buffer (40 mM, pH = 7.0, 0.5 mL) was added to a solution of **2** (51  $\mu$ mol, 10.9 mg) in MeOH (1 mL) at room temperature in a sealed cuvette. UV–vis measurements were taken every 30 min for 4 h at 665 nm. Similar experiments were performed using varying concentrations of GSH (102  $\mu$ mol, 31.2 mg or 260  $\mu$ mol, 79.8 mg). In other experiments, a solution of NaOH (0.5 M, 1 mL) was used instead of buffer and GSSG (51  $\mu$ mol, 21.5 mg) instead of GSH.

**Product Isolation.** Following UV–vis analysis, the MeOH was removed to leave an aqueous fraction. This fraction was extracted with CH<sub>2</sub>Cl<sub>2</sub> (3 × 10 mL), the organic layers were combined, dried, filtered, and concentrated to give a residue that was analyzed using TLC, and <sup>1</sup>H, and <sup>13</sup>C NMR spectroscopy. In other experiments, DTT (340  $\mu$ mol, 52.4 mg) in MeOH (12 mL) was added and stirred at room temperature for 24 h until the disappearance of the blue color. The MeOH was removed and the residue dissolved in EtOAc and extracted with 1 M NaOH. After the organic layer was dried with MgSO<sub>4</sub>, the products of the reaction were identified by TLC and chemical

shift analysis of the  $^1\text{H}$  and  $^{13}\text{C}$  NMR as compared to the standards: cyclohexanone, cyclohexanone oxime, GSH, GSSG, reduced and oxidized DTT.

**GC-MS Analysis of the Reaction of 1–3 with Thiols.** A solution of acyloxy nitroso compound **1**, **2**, or **3** (0.3 mmol) in solvent (10 mL, MeOH, 1:1 MeOH:Tris buffer (50 mM, pH = 7.6), 1:1 MeOH:1 M NaOH) was stirred overnight. In some experiments GSH, NAC, TP (0.75 mmol), and/or cyclopentanone (1.5, 3, or 6 mmol) was added. The MeOH was evaporated,  $\text{CH}_2\text{Cl}_2$  (2 mL) was added, the organic layer was separated, and an aliquot (1  $\mu\text{L}$ ) was injected onto a 7890A Agilent Technologies gas chromatograph coupled to a 5957C Agilent Technologies inert XL EI/CI MSD with triple axis detector run in electron impact mode, and a 5-MS capillary column (30 m, 0.25  $\mu\text{m}$  film, 0.25 mm OD) were used. The oven operated at 50  $^\circ\text{C}$  for 1 min and ramped to 250  $^\circ\text{C}$  for 24 min for a total run time of 25 min. The purged packed inlet was kept constant at 250  $^\circ\text{C}$  and had a total flow (He carrier gas) of 61 mL/min with a pressure of 7.65 psi and a septum purge flow of 1 mL/min  $^\circ\text{C}$ . Products were identified by retention time analysis compared to standard cyclohexanone, cyclohexanone oxime, and cyclopentanone oxime (0.1 mg/mL) each. The amount of the cyclohexanone and cyclohexanone oxime formed were calculated using area under the peak. For the kinetic analysis of these reactions, MeOH, Tris buffer (50 mM, pH = 7.6), or 1 M NaOH (5 mL) was added to a solution of **1**, **2**, or **3** (0.3 mmol) and cyclopentanone oxime (internal standard, 500  $\mu\text{L}$ , 0.5 mg/mL) in MeOH (4.5 mL). In other experiments, NAC or TP (0.75 mmol) was added. Aliquots (1  $\mu\text{L}$ ) of these solutions were injected into the GC-MS spectrometer using the previous method every 30 min for 24 h. The amount of cyclohexanone, cyclohexanone oxime, and acyloxy nitroso compound was calculated by comparing their area under the peak to the peak of the cyclopentanone oxime internal standard.

**$^{13}\text{C}$  NMR Measurements of the Reaction of 1–3 with Thiols.** A solution of GSH (2.5 mmol, 767.5 mg) in Tris buffer (50 mM, pH = 7.6) or 1 M NaOH (5 mL) and compound (**1–3**, 1 mmol) and cyclopentanone (10 mmol, 884  $\mu\text{L}$ ) in MeOH (5 mL) was stirred overnight at room temperature. The MeOH was evaporated, and the aqueous layer extracted with  $\text{CH}_2\text{Cl}_2$  (2 mL). The organic layer was separated, dried with  $\text{MgSO}_4$ , and evaporated. The products were dissolved in  $\text{CDCl}_3$  for  $^{13}\text{C}$  NMR measurements. The products were identified by chemical shift analysis compared to standards of cyclopentanone, cyclopentanone oxime, cyclohexanone, and cyclohexanone oxime.

**Pharmacology. Materials.** Angeli's salt (AS) and Manganese(III) meso-(tetrakis(4-sulfonato-phenyl))porphyrinate ( $\text{Mn}^{\text{III}}\text{TSP}$ ) were purchased from Cayman Chemicals. Manganese(III) meso-(tetrakis(4-sulfonato-phenyl))porphyrinate ( $\text{Mn}^{\text{III}}\text{TSP}$ ) was purchased from Frontier Scientific. All other reagents were purchased from Sigma Aldrich Chemical Co.. Indomethacin and 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxidecarboxy-PTIO (C-PTIO) were dissolved in 100% EtOH or MeOH, respectively. Acyloxy nitroso compounds (**1** and **3**) were solubilized in 100% dimethyl sulfoxide (DMSO). The concentration of **1** was determined spectrophotometrically ( $\epsilon_{667} = 20.7 \text{ M}^{-1} \text{ cm}^{-1}$ ), while the concentration of **3** was calculated by formula weight.<sup>36</sup> All HNO-donor solutions were diluted in vessel relaxation experiments to a final solvent concentration of 0.1% (v/v). No effects of these solvents were observed on vasoactive responses at these concentrations (not shown). AS and MNO were dissolved in 0.1N NaOH and its concentration determined spectrophotometrically ( $\epsilon_{237} = 6100 \text{ M}^{-1} \text{ cm}^{-1}$  and  $\epsilon_{250} = 8 \text{ mM}^{-1} \text{ cm}^{-1}$  respectively).<sup>37</sup> Krebs–Henseleit buffer (KH) was used for all studies and consisted of the following composition (final concentrations: 118.0 mM NaCl, 27.2 mM  $\text{NaHCO}_3$ , 4.8 mM KCl, 1 mM  $\text{KH}_2\text{PO}_4$ , 1.2 mM  $\text{MgSO}_4$ , 11.1 mM D-glucose, 0.03 mM EDTA) with a pH of 7.4 at 37  $^\circ\text{C}$  and 5%  $\text{CO}_2$  perfusion. Male Sprague–Dawley rats (200–250 g) were purchased from Harlan (Indianapolis, IN, USA). Animals were housed in approved

facilities, exposed to 12 h light/dark cycles and provided food and water ad libitum. All experiments were conducted according to Institutional Animal Care and Use Committee approved protocols.<sup>13</sup>

**Vessel Bioassay Studies.** For all vessel experiments, thoracic aortas from male Sprague–Dawley rats were used as previously described after cardiac puncture.<sup>38</sup> Briefly, aortas were isolated and submerged in KH buffer maintained at room temperature. Vessels were cleansed of fat and connective tissue and segmented into 2–4 mm rings. Vessel rings were placed in vessels bioassay chambers (Radnoti Glass Technologies, Monrovia, CA, USA) containing 15 mL of KH buffer equilibrated at 37  $^\circ\text{C}$  and perfused with a gas mixture comprising of 21%  $\text{O}_2$ /5%  $\text{CO}_2$ /74%  $\text{N}_2$ , pH 7.45. A basal tension of 2 g was applied, and vessel rings were allowed to equilibrate for 1 h. Following equilibration, a hyperpolarizing concentration of KCl (70 mM) was added to the baths to check for viability and assess maximum constriction.

To assess the effects of  $\text{O}_2$  on HNO-donor mediated vasodilation, vessels were equilibrated to 21% or 1%  $\text{O}_2$  gas mixtures (containing 5%  $\text{CO}_2$  and balanced with  $\text{N}_2$ ). Gas was precisely delivered by mass flow controllers (Sierra Instruments, California, USA) set to 0.15 L/min as previously described.<sup>38</sup> In all experiments, vessels were pretreated with nitric oxide synthase inhibitor L- $N^G$ -nitroarginine methyl ester (L-NAME, 100  $\mu\text{M}$ ) and indomethacin (5  $\mu\text{M}$ ) to inhibit cyclooxygenase. Vessels were precontracted to approximately 50–75% of maximal KCl constriction with phenylephrine (PE, 100 nM). AS, **1**, or **3** dependent vasodilation was assessed by cumulative administration of increasing concentrations. Experiments were performed in the absence or presence of C-PTIO (200  $\mu\text{M}$ ), superoxide dismutase (SOD, 100 U/mL), GSH (0–1 mM), NAC (1 mM), 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ, 1  $\mu\text{M}$ ), or  $\text{Mn}^{\text{III}}\text{TSP}$  (20  $\mu\text{M}$ ), which were added after PE and 5 min before initiating MNO, AS, **1**, or **3** concentration response. Vasodilatory effects of cumulative MNO, AS, **1**, or **3** additions were determined by measuring the  $\delta$  tension and expressing this as a percent relaxation with respect to the maximal PE constriction. Only one donor was used per aorta preparation with between 1 and 2 concentration response curves evaluated for each vessel segment; no differences in responses observed in consecutive runs (not shown), consistent with recent data showing no tolerance using AS.<sup>17</sup>  $\text{EC}_{50}$ s were obtained by fitting concentration-dependent dilation data nonlinear curve fitting using sigmoidal curve with variable slope algorithm using GraphPad Prism software.

In a subset of experiments, after exposure to different NO/HNO-donors, the effects of sodium nitroprusside (SNP), an endothelium independent vasodilator were evaluated. In all cases SNP was able to fully dilate vessels indicating that different NO or HNO-donors did not compromise the ability of vessels to respond to vasoactive stimuli (not shown).

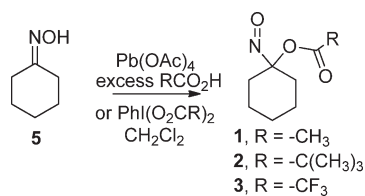
**Statistics.** Concentration-dependent vessel relaxation curves were fitted to sigmoidal dose–response algorithms by nonlinear regression curve fitting and analyzed by 2-way RM-ANOVA with Bonferonni post-test to evaluate significance. When directly comparing measured  $\text{EC}_{50}$ , unpaired *t* test was used. Significance was set at  $P < 0.05$ . All data analysis was performed on GraphPad Prism 5.

## Results

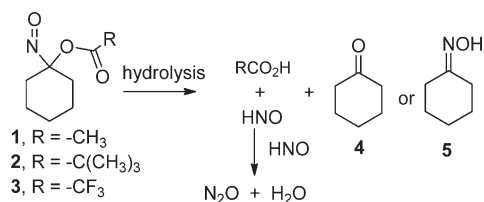
**Synthesis.** Oxidation of cyclohexanone oxime (**5**) with lead tetraacetate generates 1-nitrosocyclohexyl acetate (**1**) and a similar oxidation in the presence of excess 2,2-dimethylpropanoic acid or trifluoroacetic acid (5–10 equiv) yields 1-nitrosocyclohexyl pivalate or trifluoroacetate (**2–3**, Scheme 2). Oxidation of cyclohexanone oxime (**5**) with [bis(trifluoroacetoxy)iodo]benzene provides 1-nitrosocyclohexyl trifluoroacetate (**3**, Scheme 2).<sup>39</sup>  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectroscopy,



## Scheme 2



## Scheme 3



mass spectrometry, and elemental analysis confirm the structure of these acyloxy nitroso compounds, which exist as bright-blue oils.

**Acyloxy Nitroso Compound Decomposition Kinetics and Products.** Previous work shows that 1-nitrosocyclohexyl acetate (**1**) demonstrates reasonable stability at neutral pH.<sup>36</sup> Monitoring the disappearance of the absorption at 667 nm using ultraviolet–visible (UV–vis) spectroscopy or the peak representing the major mass fragment ( $M^+ - \text{NO}$ ) of the acyloxy nitroso compound by gas chromatography–mass spectrometry (GC–MS) provides kinetic information regarding the decomposition of **1**. Incubation of **1** in a 1:1 mixture of MeOH:Tris buffer (50 mM, pH = 7.6) at room temperature results in the exponential decrease of **1** over time with a rate constant of  $k = 8.6 \times 10^{-4} \text{ min}^{-1}$  and a  $t_{1/2} = 800 \text{ min}$  (UV–vis spectroscopy) and  $k = 7.8 \times 10^{-4} \text{ min}^{-1}$  and a  $t_{1/2} = 890 \text{ min}$  (GC–MS). Compound **1** decomposes much slower in MeOH with a rate constant of  $k = 2.1 \times 10^{-4} \text{ min}^{-1}$  and a  $t_{1/2} = 3261 \text{ min}$ . These kinetic results stand in contrast to the previously reported rapid decomposition of **1** in MeOH:0.1 N NaOH ( $t_{1/2} = 0.8 \text{ min}$ ). 1-Nitrosocyclohexyl pivalate (**2**) decomposes very slowly in a 1:1 mixture of MeOH:Tris buffer (50 mM, pH = 7.6) at room temperature as judged by UV–vis or GC–MS experiments with a rate constant  $k = 2.5 \times 10^{-4} \text{ min}^{-1}$  and a  $t_{1/2} = 2268 \text{ min}$ . UV–vis experiments also show that **2** does not appreciably decompose over 4 h in a mixture of MeOH:1 equiv NaOH (Supporting Information). 1-Nitrosocyclohexyl trifluoroacetate (**3**) decomposes immediately upon addition of water or buffer to a MeOH solution of **3** as determined by the visual disappearance of the deep-blue color.

Gas chromatographic headspace analysis of N<sub>2</sub>O, the dimerization and dehydration product of HNO, provides evidence for HNO intermediacy during the decomposition of **1–3** (Scheme 3, Table 1). The results with **1** and **3** appear similar to those previously reported under neutral and basic conditions (entries 1, 2, and 7–10).<sup>36</sup> Under buffered conditions (entry 1), the rate of N<sub>2</sub>O formation matches the rate of decomposition of **1** for approximately the first 3 h but decreases as decomposition of the acyloxy nitroso compound continues (Supporting Information). Under basic conditions, N<sub>2</sub>O formation occurs within the first hour, consistent with the observed decomposition kinetics (entry 2, Supporting Information). Acid-catalyzed hydrolysis of **1** appears slightly less efficient compared to basic hydrolysis in terms of

Table 1. Product Formation from the Decomposition of **1–3**

entry	compd	conditions	N <sub>2</sub> O (%)		organic 4:5
			2 h	24 h	
1	<b>1</b>	MeOH/Tris (50 mM, pH 7.6)	12	39	84:16
2	<b>1</b>	MeOH/0.1 NaOH	50	36	97:3
3	<b>1</b>	MeOH/HCl	14	29	91:9
4	<b>2</b>	MeOH/Tris (50 mM, pH 7.6)	2	4	86:14
5	<b>2</b>	MeOH/0.1 NaOH	2	10	85:15
6	<b>2</b>	MeOH/HCl	2	8	94:6
7	<b>3</b>	MeOH/Tris (50 mM, pH 7.6)	57	54	97:3
8	<b>3</b>	Tris (50 mM, pH 7.6)	8	17	
9	<b>3</b>	MeOH	1	10	100:0
10	<b>3</b>	MeOH/0.1 NaOH			100:0

HNO formation (entry 1 vs entry 3). Addition of GSH (1 or 2 equiv) to these incubations quenches N<sub>2</sub>O production in each case (<2%), providing further evidence for the intermediacy of HNO in these reactions. 1-Nitrosocyclohexyl pivalate (**2**) does not produce N<sub>2</sub>O (HNO) under neutral buffered, basic, or acidic conditions (entries 4–6) (Supporting Information).

A combination of TLC, NMR spectroscopy, and GC–MS experiments reveals the formation of cyclohexanone (**4**) and cyclohexanone oxime (**5**) from the decomposition of **1–3** over time (much longer for **2**) in MeOH/buffer and MeOH/basic conditions (Scheme 3, Table 1). In general, cyclohexanone (**4**) represents the major product from the decomposition of **1–3** with smaller amounts of cyclohexanone oxime (**5**) being formed (Table 1). No other cyclohexanone-derived products were identified, and starting material was completely consumed during these incubations as judged by both UV–vis spectroscopy and GC–MS.

**Acyloxy Nitroso Compound Decomposition Kinetics and Products in the Presence of Thiols.** Kinetic UV–vis spectroscopic experiments monitoring the disappearance of the absorption at 667 nm show that the addition of GSH to a solution of **1** in MeOH:TRIS buffer (40 mM, pH = 7.0) increases the rate of acyloxy nitroso compound decomposition (Supporting Information). The addition of GSH (5 equiv) completely consumes **2**, which does not appreciably decompose under neutral or basic conditions over 4 h (Supporting Information). Table 2 summarizes more detailed kinetic information regarding the decomposition of **1** in the presence of thiols from GC–MS experiments monitoring the disappearance of the major mass fragment ( $M^+ - \text{NO}$ ) of the acyloxy nitroso compound. Reaction of **1** occurs much more rapidly in a MeOH:buffer mixture than in MeOH alone (Table 2, entries 1 and 5). Addition of NAC approximately doubles the rate of decomposition in MeOH:buffer and has little effect on the rate in MeOH (Table 2, entries 2, 3, 6 and 7). Addition of thiophenol (TP) to the reaction mixtures increases the rate of decomposition nearly 200-fold in MeOH:buffer and 30-fold in MeOH (Table 2, entries 4 and 8). Similar to **1**, treatment of **2** with TP results in its exponential decrease over time with a rate constant of  $k = 1.5 \times 10^{-1} \text{ min}^{-1}$  and a  $t_{1/2} = 4.6 \text{ min}$ .

Addition of thiols to the decomposition of **1–3** under buffered conditions dramatically alters the observed reaction products by (1) abolishing N<sub>2</sub>O formation, (2) generating a disulfide product, and (3) changing the ratio of cyclohexanone (**4**):cyclohexanone oxime (**5**) produced (Scheme 3). Table 3 shows the ratio of **4**:**5** for the decomposition of **1–3** in the presence of different thiols in a mixture of MeOH:Tris buffer

**Table 2.** Kinetics of the Decomposition of **1** in the Presence of Thiols

entry	conditions	thiol (equiv)	$k$ ( $\text{min}^{-1}$ )	$t_{1/2}$ (min)
1	MeOH/Tris (50 mM, pH 7.6)		$7.8 \times 10^{-4}$	890
2	MeOH/Tris (50 mM, pH 7.6)	NAC (2.5)	$1.5 \times 10^{-3}$	453
3	MeOH/Tris (50 mM, pH 7.6)	NAC (5)	$1.6 \times 10^{-3}$	434
4	MeOH/Tris (50 mM, pH 7.6)	TP (2.5)	$1.5 \times 10^{-1}$	4.6
5	MeOH		$2.1 \times 10^{-4}$	3261
6	MeOH	NAC (2.5)	$2.1 \times 10^{-4}$	3261
7	MeOH	NAC (5)	$5.2 \times 10^{-4}$	1324
8	MeOH	TP (2.5)	$6.0 \times 10^{-3}$	113

**Table 3.** Product Formation from the Decomposition of **1–3** in the Presence of Thiols

entry	compd	thiol (2.5 equiv)	4:5	4:5 (with cyclopentanone 10 equiv)
1	<b>1</b>	GSH	26:74	87:13
2	<b>1</b>	TP	7:92	25:75
3	<b>1</b>	NAC	5:95	95:5
4	<b>2</b>	GSH	5:95	88:12
5	<b>2</b>	TP	18:82	33:67
6	<b>2</b>	NAC	4:96	90:10
7	<b>3</b>	GSH	73:28	98:2

(50 mM, pH 7.6). In general and in contrast to reactions in the absence of thiol (Table 1), cyclohexanone oxime (**5**) represents the major product from these decompositions of **1** and **2** with smaller amounts of **4** being formed (Table 3). The decomposition of **3** in the presence of GSH produces cyclohexanone (**4**) as the major product, although the percentage of **5** formed increases compared to the nonthiol reaction (Table 3, entry 7 and Table 1, entry 7). No other cyclohexanone-derived products were identified, and starting material was completely consumed during these incubations as judged by both UV-vis spectroscopy and GC-MS. Kinetic GC-MS experiments reveal the formation of **4** and **5** occurs at the same rate as the disappearance of starting material (Supporting Information).

Addition of cyclopentanone to these reactions further influences the ratio of cyclohexanone (**4**):cyclohexanone oxime (**5**) produced (Table 3). Incubation of **1** or **2** with GSH or NAC under in the presence of cyclopentanone produces **4** as the major product with only small amounts of **5** (Table 3, entries 1, 3, 4, and 6) and increases the amount of **4** formed during the decomposition of **3** in the presence of GSH (Table 3, entry 7). Incubation of **1** or **2** with TP in the presence of cyclopentanone increases the amount of **4** formed, but **5** remains the major component of the reaction mixture (Table 3, entries 2 and 5). GC-MS and  $^{13}\text{C}$  NMR experiments confirm the formation of cyclopentanone oxime in these reactions (Supporting Information).

Similar experiments conducted in a 1:1 mixture of MeOH:1.0 N NaOH reveal further changes in the ratio of cyclohexanone (**4**):cyclohexanone oxime (**5**) produced in the presence and absence of cyclopentanone (Table 4). Generally, incubation of **1** or **2** with GSH, TP, or NAC under basic conditions produces cyclohexanone oxime (**5**) as the major product with only small amounts of cyclohexanone (**4**) being formed (Table 4, entries 1–6). Exposure of **3** to these conditions (with GSH) yields mainly cyclohexanone (**4**) (Table 4, entry 7). Unlike the results from reactions in buffered solution, the addition of cyclopentanone to these reactions did not significantly increase the amount of **4** produced and had little change on the overall ratio of **4**:**5** formed (Table 4).

**Reaction of HNO with Acyloxy Nitroso Compounds.** Kinetic UV-vis spectroscopic experiments show the disap-

**Table 4.** Product Formation from the Decomposition of **1–3** in the Presence of Thiols under Basic Conditions

entry	compd	thiol (2.5 equiv)	4:5	4:5 (with cyclopentanone 10 equiv)
1	<b>1</b>	GSH	2:98	12:88
2	<b>1</b>	TP	21:79	21:79
3	<b>1</b>	NAC	10:90	10:90
4	<b>2</b>	GSH	9:91	9:91
5	<b>2</b>	TP	14:86	11:89
6	<b>2</b>	NAC	2:98	6:94
7	<b>3</b>	GSH	97:3	

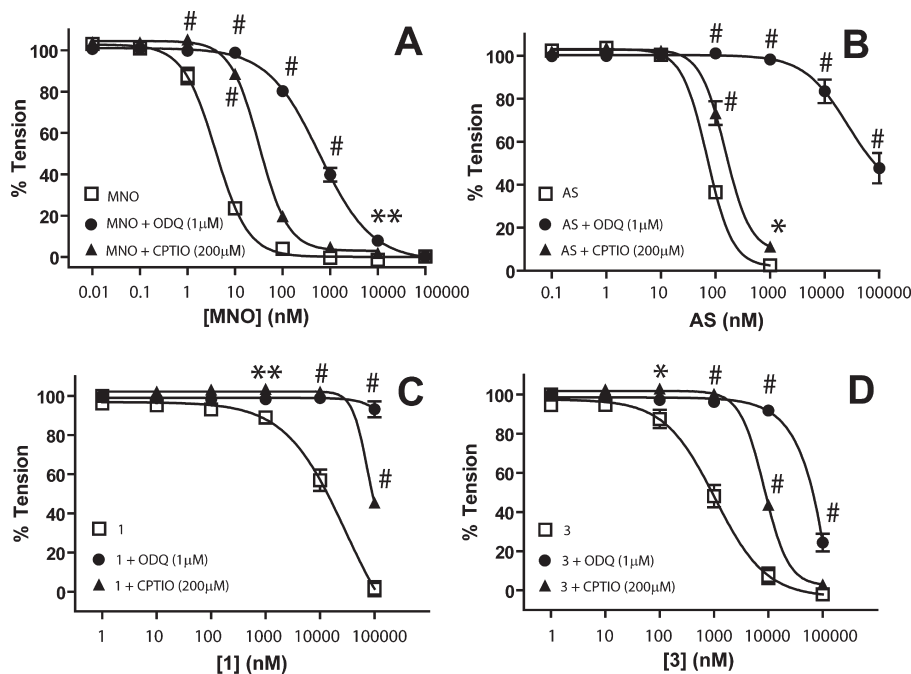
pearance of the absorption at 667 nm of **2** upon the addition of AS within 20 min at room temperature in MeOH:buffer (Supporting Information). Control experiments show that nitrite, another product of AS decomposition, does not affect the UV-vis absorbance of **2**. Addition of copper(II) sulfate to this reaction mixture generates a species with a UV-vis absorbance at 653 nm, similar to the spectrum of Cu(II) sulfate and Cupferron (Supporting Information).

**Vasodilation Assays.** Figure 1 shows that Angeli's salt, **1**, **3**, and MNO stimulated vasodilation in a concentration dependent manner with the potency being MNO > AS > **3** > **1** consistent with our previous studies.<sup>36</sup> Figure 1 shows that in each case, vasodilation occurs by NO-dependent activation of soluble guanylate cyclase (sGC) as indicated by inhibition of relaxation in the presence of either the sGC inhibitor ODQ or the NO-scavenger C-PTIO.

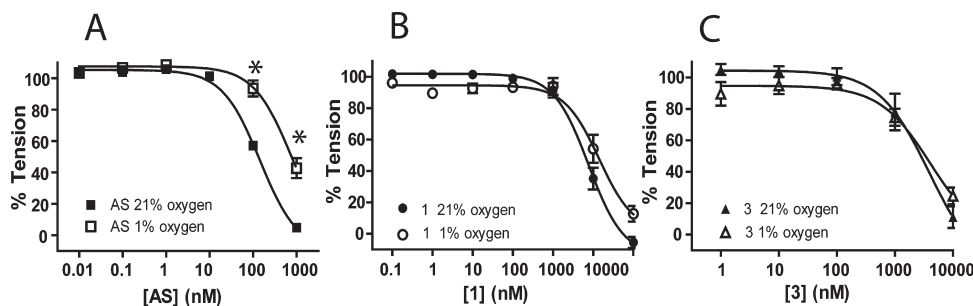
**Effect of Oxygen on HNO-Donor Dependent Dilatation.** Recent studies underscore the importance of varying oxygen tensions and specifically lower oxygen tensions (that are more consistent with in vivo situations) in modulating vasodilatory mechanisms of nitrovasodilators.<sup>38</sup> HNO represents an intriguing nitrovasodilator due to the potential competing reaction between HNO and oxygen, leading to the formation of reactive nitrogen species that could affect HNO signaling.<sup>40,41</sup> Figure 2 shows the HNO-donor dependent dilatation assessed at 21% O<sub>2</sub> and 1% O<sub>2</sub> and reveals that lowering oxygen tensions inhibited AS dependent dilatation but had no effect on **1** or **3** dependent-vasodilation.

**Effects of SOD on HNO-Dependent Relaxation at High and Low Oxygen Tension.** Previous studies suggest that the cupric ion (Cu<sup>2+</sup>) in SOD oxidizes HNO to NO, possibly mediating NO-signaling from HNO.<sup>25</sup> A second distinct mechanism by which SOD may potentiate HNO-dependent vasodilation is superoxide scavenging, thereby preventing rapid NO-scavenging (and peroxynitrite formation) and improving NO-dependent vasodilation.<sup>42</sup> Figure 3 shows the effects of SOD on AS, **1**, and **3** dependent-vasodilation. SOD significantly potentiated vasodilation by AS (indicated by leftward shift in concentration-dependent relaxation) but did not significantly change the vasodilation efficiency of **1** or **3**.

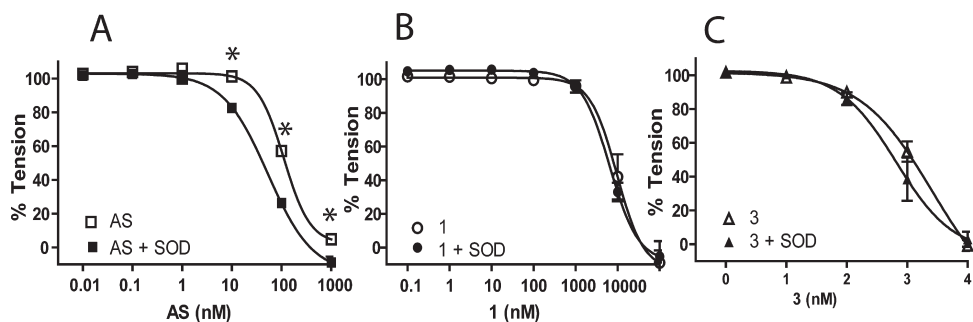
**Selective Effects of Thiols toward HNO-Donor Dependent Vasodilation.** Rapid and selective reactivity with reduced thiols likely underlies many of the biological effects of HNO, and thiols have been used experimentally to inhibit and implicate a role for HNO. Parts A and B of Figure 4 show the effects of NAC on MNO, AS, **1**, and **3** dose-dependent vasodilation. Figure 4C shows the respective EC<sub>50</sub> for vasodilation in the presence and absence of NAC. NAC had no significant effect on MNO nor **1** mediated vasodilation and elicited small but significant right shifts in AS and **3** mediated vasodilation, as indicated by significant increases in EC<sub>50</sub>. To further probe thiol dependence of HNO-donor dependent stimulation of



**Figure 1.** Role of NO in HNO-donor mediated vasodilation. MNO (A), AS (B), **1** (C), or **3** (D) dependent vasodilation was assessed using rat thoracic aorta in the absence (□) or presence of either ODQ (1  $\mu$ M, ●) or CPTIO (200  $\mu$ M, ▲). Data show mean  $\pm$  SEM ( $n = 3-4$ ) by \* $P < 0.05$ , \*\* $P < 0.01$ , # $P < 0.001$  by 2-way RM-ANOVA with Bonferroni post-test for ODQ effect relative to control condition. NAC effect was not significantly different relative to control. In some cases, error bars are not observed and were smaller than symbol size.



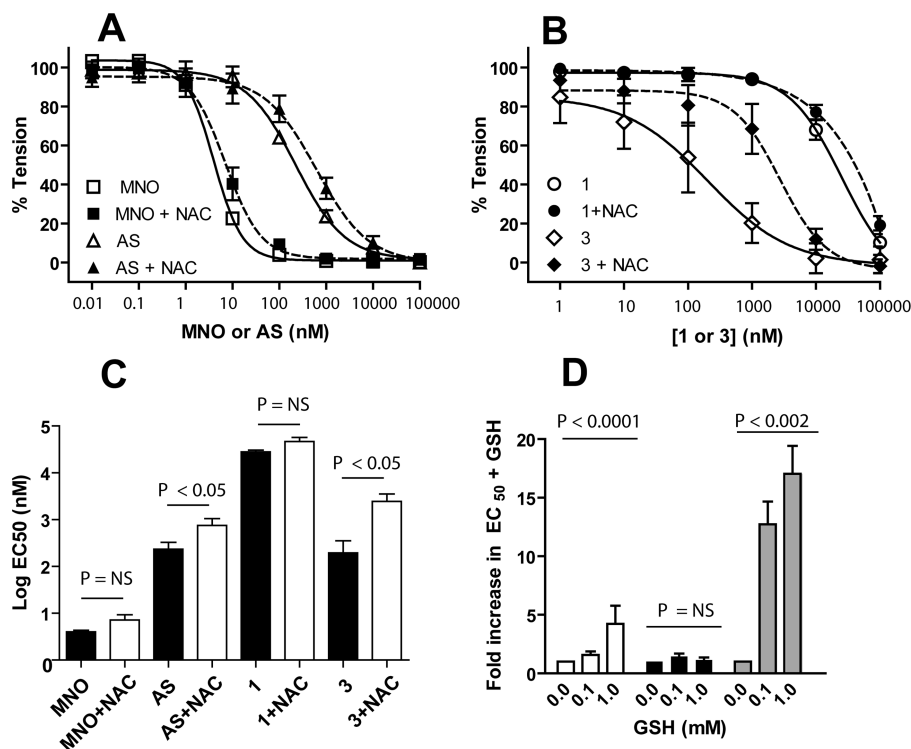
**Figure 2.** Effect of oxygen tension on HNO-donor mediated vasodilation. AS (A), **1** (B), or **3** (C) mediated vasodilation was assessed at either 21% O<sub>2</sub> (filled symbols) or 1% O<sub>2</sub> (open symbols). Data show mean  $\pm$  SEM ( $n = 3$ ). \* $P < 0.001$ , by 2-way RM-ANOVA with Bonferroni post-test relative to 21% O<sub>2</sub> condition.



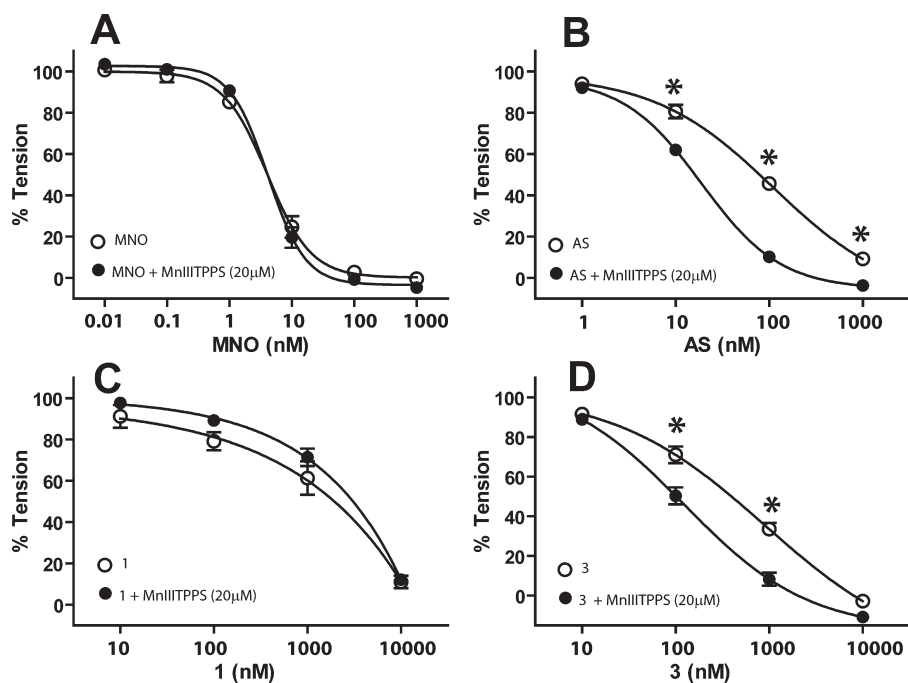
**Figure 3.** Effect of SOD on HNO-donor mediated vasodilation. AS (A), **1** (B), or **3** (C) mediated vasodilation was assessed at 21% O<sub>2</sub> in the presence (filled symbols) or absence of (open symbols) SOD (100U/ml). Data show mean  $\pm$  SEM ( $n = 3-4$ ). \* $P < 0.001$  by 2-way RM-ANOVA with Bonferroni post-test relative to with SOD condition.

NO-signaling, we assessed the effects of differing doses of GSH, which were added to vessel bioassay chambers prior to HNO donors. Figure 4D shows that in a concentration dependent manner, GSH inhibited (indicated by increased EC<sub>50</sub>) vasodilation stimulated by AS and **3** but had no effect on **1** mediated dilation.

**Effects of Mn<sup>III</sup>TPPS on MNO and HNO Donor Mediated Vasodilation.** Figure 5 shows that the proposed HNO scavenger Mn<sup>III</sup>TPPS in fact potentiates vasodilation elicited by AS and **3** but had no effect on **1** nor MNO dependent response, paralleling the distinct vasodilation mechanisms of AS and **3** versus **1** observed in Figure 4.<sup>43</sup>



**Figure 4.** Effects of NAC or GSH on MNO and HNO-donor dependent dilation. Vasodilation induced by the MNO, Angelis salt (AS), **1**, or **3** was determined in the absence or presence of either *N*-acetylcysteine (NAC, 1 mM) (A–C) or varying concentrations of GSH (0–10 mM) (D). (C) EC<sub>50</sub> in the presence and absence of NAC. *P*-values show significance by unpaired *t* test. (D) fold-changes in EC<sub>50</sub> relative to the control (i.e., absence of GSH) with an increase in the fold indicating inhibition of vasodilation. Data show mean ± SEM (*n* = 3–4). *P*-values show significance by 1-way ANOVA. Clear bars = AS, black bars = **1**, gray bars = **3**.



**Figure 5.** Effects of Mn<sup>III</sup>-TPPS on MNO and HNO-donor dependent vasodilation. Vasodilation stimulated by either MNO (A), AS (B), **1** (C), or **3** (D) was assessed in the absence (○) or presence (●) of Mn<sup>III</sup>-TPPS (20 μM). Data show mean ± SEM (*n* = 2–3). \**P* < 0.001 by 2-way RM-ANOVA with Bonferroni post-test relative to corresponding with Mn<sup>III</sup>-TPPS condition.

## Discussion

A more comprehensive array of HNO donor compounds is needed to provide better experimental tools to study HNO biology and aid in the development of HNO therapeutics. We and others have recently synthesized and characterized novel

HNO donor compounds that are structurally distinct from Angeli's salt and release HNO at different rates upon hydrolysis at neutral pH.<sup>36</sup> Here, we detail the chemical decomposition of acyloxy nitroso compounds to HNO, describe their reactions with thiols and thiolate ions, and compare the



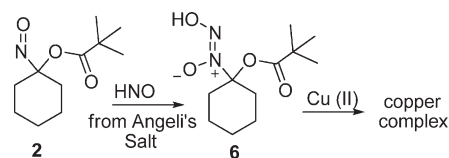
reactivity of **1** and **3** with AS and an NO-donor (MNO) using vasodilation as a biological end point.<sup>17–19,21,22,24</sup>

**Decomposition and HNO Release from Acyloxy Nitroso Compounds.** Acyloxy nitroso compounds (**1–3**) hydrolyze to yield an unstable  $\alpha$ -hydroxy nitroso intermediate that collapses to cyclohexanone and HNO (Scheme 1, Scheme 5, path 1). This mechanism predicts that the rate and amount of HNO formation should directly relate to the ease of ester hydrolysis for **1–3** (with the prediction of **3** > **1** > **2**). Kinetic decomposition studies show the water-sensitive trifluoroacetate group facilitates hydrolysis and **3** rapidly decomposes upon mixing with any aqueous solvent.<sup>44</sup> The *t*-butyl ester resists hydrolysis, and **2** decomposes very slowly in either neutral ( $t_{1/2} = 2268$  min) or basic conditions. 1-Nitrosocyclohexyl acetate (**1**) rapidly hydrolyzes under basic aqueous conditions favoring hydrolysis ( $t_{1/2} = 0.8$  min) but predictably hydrolyzes slowly in neutral buffered conditions ( $t_{1/2} = 800–890$  min).<sup>36</sup> Regardless of the rate, cyclohexanone represents the major organic product for each of these reactions, indicating a hydrolytic pathway (Table 1). These results generally segregate **1–3** into three groups regarding kinetic decomposition (and presumably HNO release) at neutral pH, with **1** being a slow HNO donor, **2** not being an HNO donor (or extremely slow HNO donor), and **3** being a rapid HNO donor similar to Angeli's salt ( $t_{1/2} = 2.3$  min).<sup>45</sup>

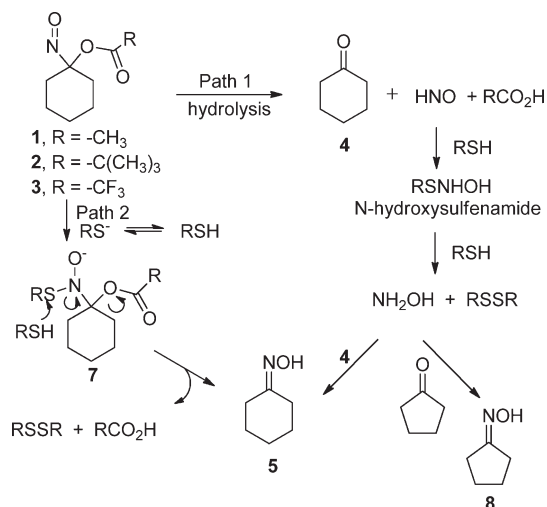
Gas chromatographic headspace identification of N<sub>2</sub>O from the aqueous-based decomposition of **1–3** parallels the kinetic studies. 1-Nitrosocyclohexyl trifluoroacetate (**3**), which decomposes fastest, yields the largest amount of N<sub>2</sub>O (57%) within 2 h in a mixture of MeOH:buffer compared to **1** (12%) and **2** (2%, Table 1). Incubation of **1** under basic conditions facilitates hydrolysis of the acetate group and subsequent N<sub>2</sub>O formation but has little effect on N<sub>2</sub>O release from **2** that contains the hydrolysis resistant *t*-butyl ester (Table 1). Identification of N<sub>2</sub>O, the dimerization/dehydration product of HNO,<sup>6</sup> provides evidence for HNO intermediacy, and N<sub>2</sub>O quenching by glutathione, which rapidly reacts with HNO,<sup>46</sup> supports this assertion. Earlier work also shows potassium ferricyanide reduces N<sub>2</sub>O formation from the decomposition of **1** and **3**, presumably through the iron(III)-mediated oxidation of HNO to NO.<sup>36</sup> The decomposition of **3** in the presence of methemoglobin yields iron(II) nitrosyl hemoglobin, a characteristic reaction of HNO, which supports HNO release from **3**.<sup>36</sup>

While these gas chromatographic results strongly imply HNO release from **1** and **3**, the less than expected quantitative amounts of N<sub>2</sub>O clearly reveals limitations to this indirect method of HNO detection. This assay relies on HNO dimerization, making the rate and amount of N<sub>2</sub>O formation dependent on the square of HNO concentration. As the amount of donor and HNO decrease with time, the rate of dimerization decreases, which may explain the decrease in rate of N<sub>2</sub>O formation compared to rate of donor decomposition observed with **1** (Supporting Information). Also, a very slow HNO donor, such as **2**, would only produce a low steady-state HNO concentration (and low N<sub>2</sub>O formation). The reaction of HNO with other species, including residual oxygen, solvent components, or other organic byproduct, would also reduce N<sub>2</sub>O formation. Indeed, Angeli's salt-generated HNO directly reacts with the nitroso group of the acyloxy nitroso compound (**2**), similar to *C*-nitroso compounds, to give a proposed "cupferron-like" derivative (**6**, Scheme 4).<sup>47</sup> Addition of copper(II) to this complex generates

#### Scheme 4



#### Scheme 5



a species with absorption properties similar to a Cupferron–copper complex. The observation of small amounts of cyclohexanone oxime (**5**) in the neutral decomposition of **1–3** clearly indicates the occurrence of non-HNO producing reactions (Table 1). Such reactions may contribute to decreased N<sub>2</sub>O yields and clearly show the ability of HNO to react with other organic molecules including the parent donor. The gas chromatographic identification of N<sub>2</sub>O provides a reasonable marker for HNO formation, but further evidence should be gathered for new HNO donors and improvements in HNO detection remains a priority for advancing HNO chemistry and biology.

**Reactions of Acyloxy Nitroso Compounds with Thiols.** The addition of GSH to the decomposition of **1–3** under neutral or basic conditions quenches N<sub>2</sub>O formation, presumably through thiol trapping of the nascent HNO. While the decomposition of **1–3** in the absence of thiol gives cyclohexanone (Table 1), the decomposition of **1** and **2** under neutral and basic conditions in the presence of GSH, NAC, or TP forms disulfide and cyclohexanone oxime (**5**) as the major organic products (Tables 3 and 4). Decomposition of **3** in the presence of GSH predominately gives cyclohexanone under both neutral and basic conditions (Table 3, entry 7 and Table 4, entry 7). Scheme 5 (path 1) shows a reasonable pathway for the formation of these products via HNO. Hydrolysis of the acyloxy nitroso compound produces HNO, the corresponding carboxylic acid, and cyclohexanone (**4**). In the presence of GSH or NAC, the thiol reacts with HNO to generate the proposed *N*-hydroxysulfenamide intermediate and further thiol reduction gives disulfide and hydroxylamine (Scheme 5). Hydroxylamine condenses with cyclohexanone to form cyclohexanone oxime (Scheme 5, path 1).

However, the addition of NAC or GSH (thiol pK<sub>a</sub>s = 9.5 and 8.7, respectively)<sup>48</sup> under buffered conditions to **1** or **2** increases the rate of acyloxy nitroso compound decomposition



as judged by UV–vis spectroscopy (Table 2 and Supporting Information). Addition of thiophenol ( $pK_a = 6.6$ )<sup>49</sup> to **1** or **2** under buffered conditions further increases their rate of decomposition (Table 2). The rapid reaction of **2** under these conditions, which does not produce  $N_2O$  over the same time period in the absence of thiol, suggests a nonhydrolytic (and non-HNO) mediated decomposition pathway. On the basis of the  $pK_a$ s of the thiols examined and the pH of the incubation buffer, these results strongly suggest the direct reaction of thiolates with the acyloxy nitroso compounds (Scheme 5, path 2). Thiolate addition to the nitroso group would disrupt the  $n$  to  $\pi^*$  transition, resulting in decreased absorbance and generate an organic *N*-hydroxysulfenamide intermediate (**7**, Scheme 5, path 2). Attack of a second thiol (or thiolate) on **7** would generate disulfide and cyclohexanone oxime (**5**) without the intermediacy of HNO (Scheme 5, path 2). The increased rate of reaction with TP compared to GSH or NAC provides evidence for thiolate ion involvement. Overall, these results show that in the presence of thiols, acyloxy nitroso compounds can decompose through two different paths, only one of which involves HNO, to form identical products (disulfides and oximes).

Experiments using cyclopentanone as a trap for hydroxylamine provide a method for separating the two decomposition paths of acyloxy nitroso compounds in the presence of thiols. Only path 1 (Scheme 5) includes HNO and hydroxylamine, and the addition of a second ketone (cyclopentanone) could trap any hydroxylamine to generate cyclopentanone oxime (**8**) and preserve any originally generated cyclohexanone (**4**, Scheme 5). The addition of excess cyclopentanone to the decomposition of **1** under neutral buffered conditions in the presence of GSH reverses the ratio of **4**:**5** from 26:74 to 87:13, a similar ratio to decomposition in the absence of thiol (Tables 1 and 3). Similar experiments using NAC also reverse the ratio of **4**:**5** formed from **1** and the addition of cyclopentanone to the decomposition of **2** in the presence of these thiols yields a similar reversal in the ratio of **4**:**5** (Table 3). GC-MS experiments show the formation of **8** during these reactions and provide strong evidence that **1** and **2** primarily decompose through a path that involves free hydroxylamine (Scheme 5, path 1). However, the decomposition of **1** and **2** in the presence of excess cyclopentanone and TP predominantly forms cyclohexanone oxime (**5**, Table 3), which suggests these reactions do not generate hydroxylamine and proceed through the thiolate of thiophenol via path 2. Decomposition of **1** or **2** in the presence of GSH, NAC, or TP under basic conditions (that ensure thiolate formation) yields cyclohexanone oxime (**5**) as the major product regardless of the presence of cyclopentanone, providing strong evidence that acyloxy nitroso compounds directly react with thiolate ions through path 2 (Table 4). Regardless of conditions (pH, presence/absence of thiol, or cyclopentanone), **3** primarily forms cyclohexanone (**4**), suggesting a primarily hydrolytic decomposition (path 1, Scheme 5).

On the basis of these results, the reactivity and HNO-donating ability appears to depend on the structure of the acyloxy nitroso compound, the presence of thiols, the  $pK_a$  of these thiols, and the pH of the reaction medium. Compound **3**, which contains the hydrolytically sensitive trifluoroacetate group, rapidly hydrolyzes (generating HNO) regardless of the reaction conditions or presence of thiols (path 1, Scheme 5). Compounds **1** and **2** hydrolyze (at different rates) to HNO in the absence of thiols or under conditions that favor the thiol (rather than thiolate) ionization state (path 1, Scheme 5).

However, these compounds appear to directly react through thiolate addition to the nitroso group (path 2, Scheme 5) under conditions that favor the thiolate ionization state. These findings indicate acyloxy nitroso compounds may demonstrate different reactivity (including their ability to release HNO) depending on their structure and the presence of thiols/thiolates. This differing reactivity with thiols compared to thiolates could have important implications in targeting specific highly reactive low  $pK_a$  thiol residues in various proteins.

**Vasorelaxation Properties and Structure–Activity Relationships.** Angeli's salt (AS) elicits vasodilation of rabbit thoracic aorta and bovine intrapulmonary arteries through soluble guanylate cyclase (sGC) activation.<sup>50</sup> The sGC inhibitor 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ) and NO-scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide (C-PTIO) block this relaxation, providing evidence for NO-mediated sGC activation, suggesting some oxidative metabolism of HNO to NO.<sup>18,51</sup> While early work indicates that NO is the only nitrogen monoxide capable of sGC activation,<sup>52–54</sup> recent reports provide evidence for HNO-mediated sGC activation,<sup>55</sup> a finding that has also been challenged.<sup>56</sup> Our previous studies show that acyloxy nitroso compounds **1** and **3** relax precontracted rat aorta similar to other HNO donors including AS.<sup>36</sup> Compared to AS ( $EC_{50} = 0.082 \mu M$ ), **1** is much less potent ( $EC_{50} = 10 \mu M$ ) and **3** slightly ( $\sim 10$ -fold) less potent ( $EC_{50} = 0.88 \mu M$ ), indicating some differences in the biological actions of these HNO donors.<sup>50</sup>

The current vasorelaxation studies show differences between the acyloxy nitroso compounds **1** and **3** compared to AS and each other. The very poor water solubility and extremely slow decomposition rate of **2** prevented the reliable assay of its activity. Both ODQ and C-PTIO inhibit vasodilation induced by **1** and **3**, revealing that this activity likely occurs through NO-mediated activation of sGC, similar to AS (Figure 1). Experiments with bovine lung sGC indicate that **3** acts as a competent sGC activator, presumably through HNO formation,<sup>55</sup> but the C-PTIO inhibition observed in these studies suggests NO-mediated activity. Overall, the acyloxy nitroso compounds **1** and **3** appear to elicit vasodilation through NO mediated activation of sGC in this system.

The most surprising and interesting results were the similarity in vasodilatory mechanisms between AS and **3** compared to **1** (Figures 4 and 5). Reduced thiols (NAC and GSH (Figure 4)) and the putative HNO trapping porphyrin ( $Mn^{III}TPPS$ ) differentially altered the observed biological response of AS and **3** compared to **1** (Figures 4 and 5). Multiple studies show that HNO rapidly reacts with thiols with a measured rate constant for reaction with GSH of  $\sim 10^6 M^{-1} s^{-1}$ .<sup>8</sup> Addition of thiols also experimentally discerns HNO and NO-dependent pathways, as thiols selectively scavenge HNO with little effect on NO (confirmed by the lack of any effect of NAC on MNO-dependent vasodilation). Consistent with this concept and supporting previous work,<sup>17,24</sup> NAC and GSH inhibited AS-mediated vasodilation (Figure 4). Surprisingly, these thiols failed to modulate dilation by **1**, suggesting a distinct vasodilatory mechanism for this HNO donor compared to AS. Moreover, GSH did inhibit dilation mediated by **3**, a compound structurally similar to **1** but which releases HNO at a faster rate. Further evidence illustrating a distinct mechanism of action of **1** was provided by the lack of effect of the addition of  $Mn^{III}TPPS$ . This water-soluble porphyrin reacts with HNO via a reductive nitrosylation



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**Supporting Information Available:**  $^1\text{H}$  and  $^{13}\text{C}$  NMR data for compound **2**, along with UV-vis and GC-MS kinetic decomposition data for compounds **1–3**. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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