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Hydrolysis of Acyloxy Nitroso Compounds Yields Nitroxyl (HNO)

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Abstract: Nitroxyl (HNO/NO^-), the reduced form of nitric oxide, has gained attention based on its separate chemistry and biology from nitric oxide. The inherent reactivity of HNO requires new and mechanistically unique donors for the detailed study of HNO chemistry and biology. Oxidation of cyclohexanone oxime with lead tetraacetate yields 1-nitrosocyclohexyl acetate, whereas oxidation of oximes in the presence of excess carboxylic acid gives various acyloxy nitroso compounds. These bright blue compounds exist as monomers as indicated by their infrared, proton, and carbon NMR spectra, and X-ray crystallographic analysis reveals the nitroso groups possess a "nitroxyl-like" bent configuration. Hydrolysis of these compounds produces nitrous oxide, the dimerization and dehydration product of HNO, and provides evidence for the intermediacy of HNO. Both thiols and oxidative metal complexes inhibit nitrous oxide formation. Hydrolysis of these compounds in the presence of ferric heme complexes forms ferrous nitrosyl complexes providing further evidence for the intermediacy of HNO. Kinetic analysis shows that the rate of hydrolysis depends on pH and the structure of the acyl group of the acyloxy nitroso compound. These compounds relax pre-constricted rat aortic rings similar to known HNO donors. Together, these results identify acyloxy nitroso compounds as a new class of HNO donors.

Introduction

Nitroxyl (HNO/NO^-), the reduced form of nitric oxide (NO), has emerged as an important constituent in nitric oxide chemistry and biochemistry in recent years.[1,2] HNO and NO^- form an acid/base pair with NO^- being isoelectronic with dioxygen. HNO possesses a singlet ground state (^{1}HNO) and a triplet excited state (^{3}HNO) with an energy gap of about 18 kcal/mol, whereas NO^- exists in a triplet ground state ($^3NO^-$) and a singlet excited state ($^1NO^-$) with a singlet−triplet energy gap around 17−21 kcal/mol.[3,4] The pK_a value of ^{1}HNO/$^3NO^-$ has recently been corrected to 11.4 making ^{1}HNO the exclusive species under physiological conditions. In addition, the high negative reduction potentials ($NO/^3NO^- = -0.8(\pm0.2)$ V and $NO/^1NO^- = -7(\pm0.2)$ V), make reduction of NO to $^3NO^-$ and $^1NO^-$ extremely unfavorable.[4] HNO and NO^- both react with O_2, NO, thiols, and metal ions with HNO also undergoing rapid dimerization.[3,5−13] The chemistry and biochemistry of HNO/NO^- have recently been extensively reviewed.[14−16]

Recent studies indicate the potential of nitroxyl donors for the treatment of heart failure, a condition characterized by the heart's inability to maintain adequate blood circulation to the peripheral tissues and the lungs.[16,17] Heart failure remains one of the most lethal diseases in the United States with more than 50 000 deaths each year, which exceeds the deaths from all forms of cancer combined.[17] New work shows that nitroxyl donors enhance cardiac inotropy and increase plasma calcitonin gene-related peptide (CGRP) levels but do not affect plasma levels of cyclic guanylate monophosphate (cGMP). These results suggest that nitroxyl may influence cardiovascular function through a different mechanistic pathway than nitric oxide.[18,19]

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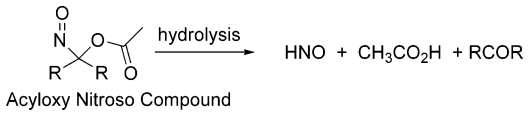
Scheme 1



Also, nitroxyl donors precondition cardiac tissue significantly improving post-ischemic contractility (compared to NO donors) with no adverse effect on systemic blood pressure.[18−20] Nitroxyl donors appear to be unique entities for the protection and stimulation of the cardiovascular system during heart failure.

Due to its inherent reactivity, HNO must be generated in situ from donor compounds with the most common being Angeli's salt ($Na_2N_2O_3$) and Piloty's acid ($PhSO_2NHOH$). However, the structural simplicity of Angeli's salt does not allow extensive variation for the modification of its release properties. Angeli's salt also releases nitrite, which possesses its own biological profile.[21,22] Nitroxyl release from Piloty's acid occurs only under basic conditions and decomposition of Piloty's acid under physiological conditions may release NO.[23] Acyl nitroso compounds react with nucleophiles to yield nitroxyl but these highly reactive intermediates must be generated in situ.[24] Given the renewed interest in HNO chemistry and the discovery of the unique biological properties of HNO, new and mechanistically unique HNO donors are sorely needed.

Acyloxy nitroso compounds (Scheme 1) have been reported by several research groups.[25−34] Although Rehse and Herpel showed these compounds inhibit platelet aggregation and thrombus formation (indicative of NO release), they generate only small amounts (<1%) of NO and HNO (judged by N_2O formation) under neutral conditions.[32] We propose that hydrolysis of the acyl portion of these molecules will generate an unstable intermediate that decomposes to HNO and the corresponding ketone (Scheme 1). Here, we report the synthesis and structural characterization of various acyloxy nitroso compounds that release HNO upon hydrolysis.

Results

Preparation and Evaluation of 1-Nitrosocyclohexyl Acetate as an HNO Donor. Oxidation of cyclohexanone oxime with lead tetraacetate (LTA) gives 1-nitrosocyclohexyl acetate in 52% yield (**1**; Scheme 2).[25,26,31] 1-Nitrosocyclohexyl acetate (**1**) is a bright blue liquid with strong UV−vis absorption at 667 nm and IR absorption for the carbonyl ($\nu_{C=O} = 1750\ cm^{-1}$) and nitroso groups ($\nu_{N=O} = 1561\ cm^{-1}$).

Scheme 2

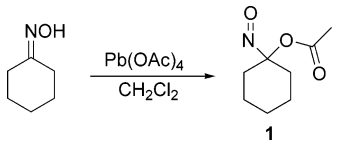


Table 1. Decomposition of 1-Nitrosocyclohexyl Acetate to NO and N_2O under Different Solvent Conditions

solvent conditions[a]	NO formation (% yield)	N_2O formation (% yield)
none	0.5%	0%
phosphate buffer, pH 7.4	0.4%	28%
MeOH + phosphate buffer, pH 7.4	0.4−0.5%	24%
0.1 M NaOH + MeOH	0.4%	58%

[a] Room temperature, 2 h incubation

Table 2. Quenching of N_2O Formation from 1-Nitrosocyclohexyl Acetate by Glutathione and Tetraphenylporphyrin Iron(III) Chloride.

	N_2O formation (% yield)			
conditions	1 h	2 h	3 h	overnight
0.1 M NaOH + MeOH	46%	56%	58%	54%
+ 2 equiv glutathione	0%	0%	0%	0%
+ 2 equiv [Fe^{III}TPP]Cl	14%	13%	8%	11%

Table 1 summarizes chemiluminescence and gas chromatographic assays that respectively reveal the production of NO and HNO from **1** under different solvent conditions. A neat sample of **1** decomposes to a small amount of NO and no N_2O after 2 h. In phosphate buffer, **1** releases a similar amount of NO, but the yield of N_2O, evidence for the intermediacy of HNO, significantly increases. As N_2O arises from the dimerization of 2 HNO molecules, the reported yields are based on the percentage of the theoretical yield of N_2O formation (50% based on **1**). Decomposition of **1** in buffer/methanol solutions (1:1) gives similar results. Under basic conditions, the amount of NO from **1** remains the same but the yield of N_2O further increases suggesting that **1** gives HNO under basic conditions. Chemiluminescence experiments reveal only small amounts (3−4%) of nitrite formation under these conditions. Extraction of the reaction mixture yields cyclohexanone in 60% yield whose structure was confirmed by ^{13}C NMR spectroscopy.

GC competition experiments in the presence of thiols and ferric iron support the existence of nitroxyl under these conditions. The reaction of these species with nitroxyl should compete with dimerization and result in a decrease in the yield of N_2O. The addition of glutathione (2 equiv) to the decomposition of **1** under basic conditions completely suppresses N_2O formation (Table 2).[1,15] Likewise, the addition of tetraphenylporphyrin iron (III) chloride ([Fe^{III}TPP]Cl) to the reaction system significantly decreases the yield of N_2O (Table 2).

Monitoring the disappearance of the strong absorption at 667 nm using UV−vis spectroscopy provides information regarding the kinetics of decomposition of **1**. 1-Nitrosocyclohexyl acetate (**1**) demonstrates relative stability in neutral buffer (Figure 1). Incubation in basic solution results in the apparent exponential decrease of **1** over time with a more rapid decrease in more concentrated base (Figure 1). From these decompositions, half-

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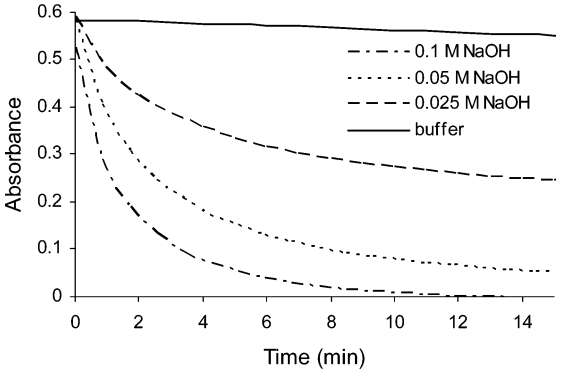


Figure 1. Kinetics of the decomposition of **1** in phosphate buffer (100 mM, pH = 7.4) and basic conditions at room temperature as measured by UV−vis spectroscopy at 667 nm.

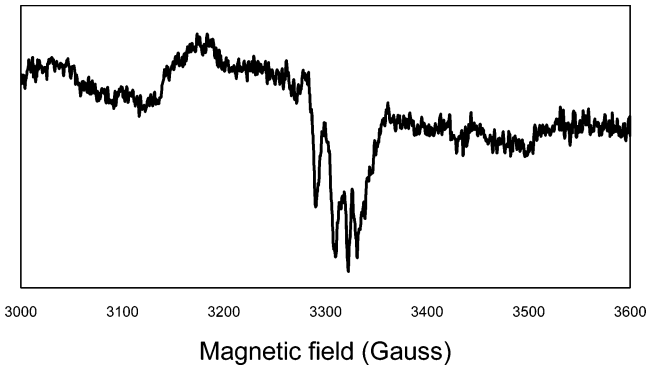
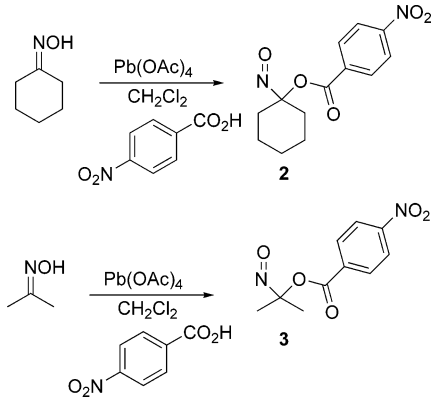


Figure 2. EPR spectrum of a mixture of hemin and **1** in 1:1 MeOH:1 M NaOH at 110 K.

Scheme 3



lives of 0.8, 1.7, and 8 min were estimated for each condition and provide rough estimates for the stability of **1**. A further and more rigorous kinetic examination of this process is underway.

Incubation of **1** with methemoglobin in phosphate buffer (pH 7.4) fails to produce the expected ferrous nitrosyl hemoglobin complex as determined by both UV/vis and EPR spectroscopy.[1,15] Under basic conditions, methemoglobin is not a suitable HNO trap. Incubation of **1** (50 mM) with hemin (50 μM), a nonprotein porphoryrin ferric iron complex soluble in both basic aqueous solutions and methanol, in 1:1 methanol:1 M NaOH yields the ferrous nitrosyl complex as judged by EPR spectroscopy (Figure 2). The conversion of this ferric heme group to a ferrous nitrosyl complex provides further evidence for the release of HNO from the decomposition of **1**.

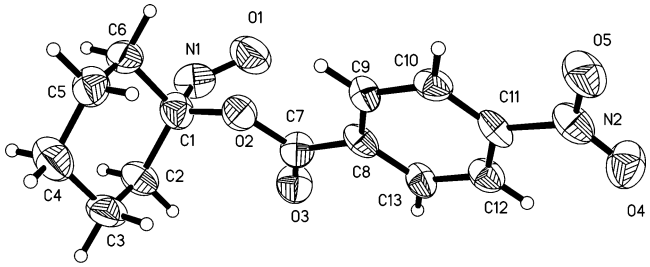


Figure 3. X-ray crystallographic structures of **2**.

Scheme 4

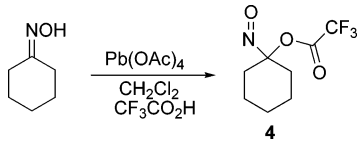


Table 3. Decomposition of 1-Nitrosocyclohexyl Trifluoroacetate to N_2O under Different Solvent Conditions

	N_2O formation			
solvent conditions	1 h	2 h	3 h	overnight
MeOH	0%	0%	0%	0%
phosphate buffer, pH 7.4	21%	34%	50%	56%
MeOH + buffer	52%	72%	74%	ND[a]
+ 2 equiv glutathione	0%	0%	0%	0%
+ 2 equiv $K_2Ni(CN)_4 \cdot H_2O$	3%	18%	24%	8%

[a] ND = no data

Preparation and Evaluation of 1-Nitrosocyclohexyl 4-Nitrobenzoate and 2-Nitrosopropan-2-yl 4-Nitrobenzoate as HNO Donors. Oxidation of cyclohexanone oxime or acetone oxime with LTA in the presence of 4-nitrobenzoic acid (10 equiv) gives the 4-nitrobenzoic acid derived nitroso compounds 1-nitrosocyclohexyl 4-nitrobenzoate (**2**) and 2-nitrosopropan-2-yl 4-nitrobenzoate (**3**) as blue solids in 20−30% yield after recrystallization (Scheme 3).[26,32] Similar to **1**, GC and EPR assays reveal the ability of **2** and **3** to release HNO, especially under basic conditions (Supporting Information). Recrystallization of **2** provides material suitable for X-ray crystallographic analysis. These studies confirm the structure of the reaction product and show that the N=O groups of these compounds exist in a "nitroxyl-like" bent configuration (C−N−O bond angle = 114.2° for **2**) with a N=O bond length of 1.183 Å (Figure 3). In addition, these studies show that these compounds do not predominately exist as nitroso dimers in the solid state.

Preparation and Evaluation of 1-Nitrosocyclohexyl Trifluoroacetate as an HNO Donor. Oxidation of cyclohexanone oxime with LTA in the presence of trifluoroacetic acid (10 equiv) in methylene chloride yields 1-nitrosocyclohexyl trifluoroacetate (**4**) as a bright blue liquid (Scheme 4).[33]

Gas chromatographic assays show that **4** is stable in methanol but rapidly decomposes in buffer to yield N_2O (Table 3). Incubation of **4** in buffer/methanol (1:1) produces an even larger amount of N_2O (Table 3). Competition experiments show that the addition of glutathione completely suppresses N_2O formation and the addition of potassium tetracyano-nicklate(II) hydrate ($K_2Ni(CN)_4 \cdot H_2O$) (2 equiv) significantly decreases N_2O formation (Table 3).

Absorption measurements show the characteristic Soret absorbance of methemoglobin (15 μM in phosphate buffer) at 407 nm shifts to 416 nm upon the addition of **4** (45 mM) in

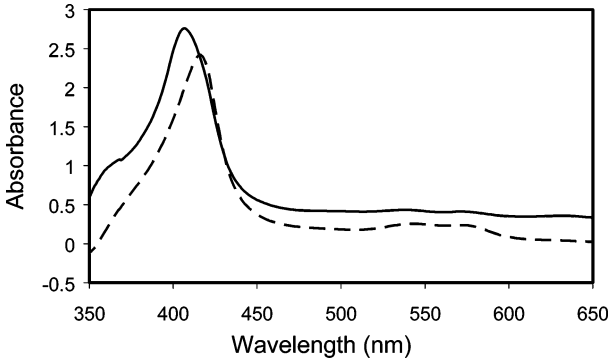


Figure 4. Absorbance spectrum of methemoglobin (solid line), methemoglobin, and **4** (long dashed line).

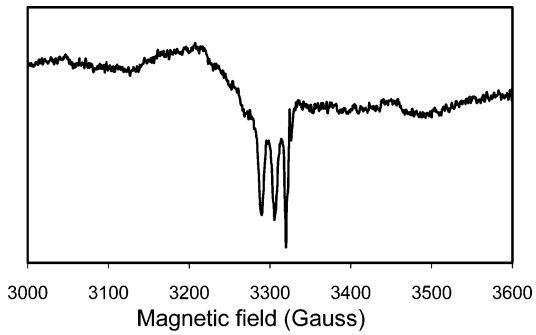


Figure 5. EPR spectrum of a mixture of methemoglobin and **4** at 110 K.

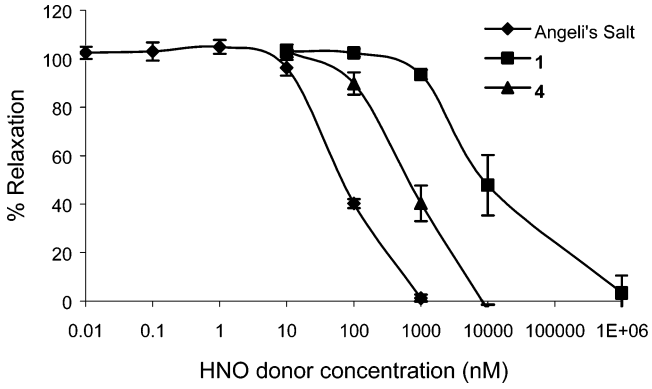


Figure 6. Dose dependent relaxation of pre-constricted rat aortic rings by Angeli's salt, **1** and **4**.

methanol, which indicates the formation of a ferrous nitrosyl complex (Figure 4). Similarly, EPR spectroscopy shows the typical three-line EPR signal of a ferrous nitrosyl complex after the incubation of **4** with methemoglobin (Figure 5). These UV−vis and EPR studies provide strong evidence for the intermediacy of HNO in these reactions.

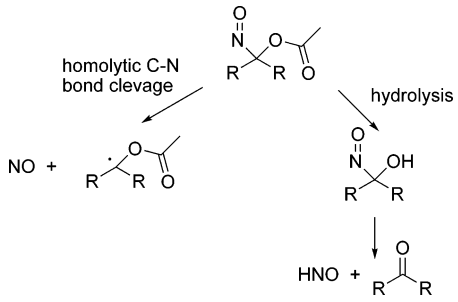
Biological Results

Figure 6 shows the ability of compounds **1** and **4** to vasodilate pre-constricted rat aortic rings in a dose-dependent fashion. Estimated EC_{50}'s (effective concentration to elicit 50% response) were approximately 10 000 nM for **1** and 885 nM for **4**. As a control, the known HNO donor, Angeli's salt, demonstrates a similar profile with an approximate EC_{50} of 82 nM.

Discussion

This work demonstrates the ability of acyloxy nitroso compounds to act as HNO donors. Oxidation of cyclohexanone

Scheme 5



oxime with lead tetraacetate yields 1-nitrosocyclohexyl acetate (**1**).[25,26,31] Lead tetraacetate oxidation of oximes in the presence of excess carboxylic acid gives the acyloxy nitroso compounds (**2**−**4**) similar to previous reports.[32] In these reactions, the excess carboxylic acid presumably exchanges with the acetate groups of lead tetraacetate to form new lead tetraacyl complexes. This protocol should allow for the preparation of numerous acyloxy nitroso compounds based on different oxime and carboxylic acid structures. The bright blue color of these compounds and their infrared, proton and carbon NMR spectra indicate they exist as nitroso monomers and not dimers. The first X-ray crystallographic studies of these compounds confirm their monomeric structures and reveal the nitroso groups possess a "nitroxyl-like" bent configuration with a normal N═O bond length. The bond the C−N−O bond angle and the N═O appear similar to other organic C-nitroso compounds whose structures have been determined crystallographically [35,36]

Previous work shows that 1-nitrosocyclohexyl 4-nitrobenzoate releases only very small amounts (<1%) of NO or HNO under neutral conditions (phosphate buffer, ethanol, or mixtures of both).[32] NO release from these compounds requires homolytic C−N bond cleavage and chemiluminescence analysis reveals little NO (or nitrite) release from **1** under neutral or basic conditions (Table 1) indicating that homolytic cleavage to give NO does not constitute a predominant decomposition pathway for **1** (Table 1 and Scheme 5).

Under conditions that favor ester hydrolysis, **1** acts as a competent HNO donor. Hydrolysis of these compounds forms a nitroso-hydroxy species (the adduct of HNO with a ketone) that decomposes to HNO and the ketone. Kinetic analysis of the hydrolysis of **1** clearly shows the base-dependent decrease in absorbance (attributed to the nitroso group) supporting such a mechanism. Also, examination of the reaction products from the basic decomposition of **1** reveals the formation of cyclohexanone, which provides further support for this pathway. Early work shows the treatment of 2-nitroso-2-benzoyloxypropane with 10 M KOH yields N_2O, evidence for HNO, and acetone and also supports this idea.[32] Based in Scheme 5, modification of the acyl group of the acyloxy nitroso compounds gives **2**−**4**, which should hydrolyze and release HNO faster than **1**. Although **2** and **3** still require basic hydrolysis to release HNO, **4** rapidly hydrolyzes in buffer to yield HNO at room temperature as judged by N_2O formation and the identification of ferrous nitrosyl complexes. These results further strengthen a hydrolytic pathway for the release of HNO from acyloxy nitroso com-

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pounds and also suggest the possibility of biological HNO release through the action of esterases. The ability of **4** to release HNO at neutral pH clearly separates these compounds from Piloty's acid as HNO donors.

Direct HNO detection remains a difficult analytical problem and alternative complimentary detection methods strongly imply the intermediacy of HNO during the hydrolysis of **1**−**4**. Gas chromatographic analysis of the reaction headspace of these reaction mixtures demonstrates the formation of N_2O and provides evidence for the intermediacy of HNO as nitroxyl dimerizes to hyponitrous acid that dehydrates to give nitrous oxide (N_2O).[37,38] Addition of glutathione or oxidative metal complexes to these hydrolyses either abolishes or greatly diminishes N_2O formation. Given that nitroxyl rapidly reacts with thiols to form disulfides and hydroxylamine or sulfinamides and reduces oxidative metal complexes, the addition of these species would be expected to decrease N_2O formation by competing with HNO dimerization.[39,40] The results from these trapping experiments further lend evidence for the intermediacy of HNO in these reactions. Alternative support for HNO formation from **1**−**4** comes from the ability of these compounds to reductively nitrosylate ferric heme groups yielding the relatively stable ferrous nitrosyl complexes as judged by UV/vis and EPR spectroscopy. Obviously, the development of direct and sensitive methods of HNO detection remains an important concern in the field.

Compounds **1** and **4** also relax pre-constricted rat aortic rings demonstrating their ability to elicit biological effects. These compounds behave similarly to the known HNO donor Angeli's salt in this assay reinforcing the ability of these compounds to act as HNO donors.[41] The lower potency of **1** and **4** compared to Angeli's salt may be related to (1) lower solubility and (2) slower HNO release rates (it should be noted that **4** is more potent than **1**). Although relaxation of vascular tissue generally reflects a response elicited by an NO donor, the observed effects remain consistent with Angeli's salt, a known HNO donor.[41] These results may indicate the conversion of donor-derived HNO to NO in this system.

Summary

The recent interest in HNO chemistry, biochemistry, and biology highlights the need for new and mechanistically unique HNO donors. Oxidation of oximes in the presence of carboxylic acids yields acyloxy nitroso compounds that exist as blue monomeric C-nitroso compounds. Hydrolysis of these compounds release HNO as judged by gas chromatographic identification of nitrous oxide and the spectroscopic identification of ferrous nitrosyl complexes. The modular synthetic approach allows for alteration of the structure of the acyl group to control the rate of hydrolysis. Compounds **1** and **4** also relax a pre-constricted rat aortic ring similar to Angeli's salt. These results reveal that acyloxy nitroso compounds represent a new class of HNO donors that may provide an alternative to Angeli's salt and Piloty's acid.

Experimental Section

General. Cyclohexanone oxime, acetone oxime, lead tetraacetate, methemoglobin, hemin, and glutathione were purchased from Sigma Chemical Company, St. Louis, MO.

1-Nitrosocyclohexyl Acetate (1). A solution of cyclohexanone oxime (5.46 g, 48.25 mmol) in methylene chloride (50 mL) was added dropwise with stirring to a solution of LTA (21.39 g, 48.25 mmol) in methylene chloride (100 mL) at 0 °C. A blue color gradually appeared with the addition of the oxime. After 1 h at 0 °C, the reaction mixture was warmed to room temperature. After 2 h at room temperature, water (30 mL) was added, and the organic layer was extracted with water (2 × 30 mL) and saturated sodium bicarbonate solution (2 × 30 mL). The organic layer was dried over Na_2SO_4, the solvent evaporated, and the residue purified by column chromatography to give **1** as a bright blue liquid (52% yield): R_f = 0.68 (pentane/ethyl acetate = 20:1); UV/vis (MeOH): λ_{max} = 667 nm, ϵ = 20.7 M^{-1} cm^{-1}. IR (KBr): υ = 1750 cm^{-1} (C═O), 1561 cm^{-1} (N═O); ^{1}H NMR (300 MHz, benzene-d_6) δ 1.1−1.9 (m, 13H); ^{13}C NMR (300 MHz, benzene-d_6) δ 21.0 (CH_2), 22.0 ($2CH_2$), 25.1 ($2CH_2$), 29.7 (CH_3), 123.8 (O−C−N), 168.5 (C═O); Anal. Calcd. for $C_8H_{13}NO_3$: C, 56.11; H, 7.67; N, 8.18. Found: C, 56.46; H, 7.73; N, 7.82.

1-Nitrosocyclohexyl-4-Nitrobenzoate (2). A solution of cyclohexanone oxime (2.66 g, 23.51 mmol) in methylene chloride (50 mL) was added dropwise with stirring to a solution of LTA (10.42 g, 23.51 mmol) and 4-nitrobenzoic acid (39.29 g, 235.1 mmol) in methylene chloride (300 mL) at 0 °C. A blue color gradually appeared with the addition of the oxime. After 1 h at 0 °C, the reaction mixture was warmed to room temperature. After 3 h at room temperature, water (50 mL) was added, and the organic layer was extracted with water (2 × 50 mL) and 3% sodium bicarbonate solution (2 × 50 mL). The organic layer was dried over Na_2SO_4, the solvent was evaporated, and the residue was recrystalized in diethyl ether/petroleum ether (1:1) in a −5 °C freezer to give **2** as bright blue crystals (20−25% yield): R_f = 0.55 (pentane/ethyl acetate = 20:1); UV−vis (MeOH): λ_{max} = 666 nm; IR (KBr): $\nu_{C=O}$ = 1732 cm^{-1}, $\nu_{N=O}$ = 1562 cm^{-1}, ν_{NO2} = 1530 cm^{-1}; ^{1}H NMR (300 MHz, Benzene-d_6) δ 1.17−2.04 (m, 10H), 7.38−7.98 (d, 4H); ^{13}C NMR (300 MHz, Benzene-d_6) δ 22.1 ($2CH_2$), 25.0 (CH_2), 29.7 ($2CH_2$), 123.8 (2Ph-CH), 125.3 (O−C−N), 131.1 (2Ph-CH), 135.3 (Ph-C), 151.1 (Ph-C), 162.6 (C═O).

2-Nitrosopropan-2-yl 4-Nitrobenzoate (3). A solution of acetone oxime (3.11 g, 42.58 mmol) in methylene chloride (50 mL) was added dropwise with stirring to a solution of LTA (18.88 g, 42.58 mmol) and 4-nitrobenzoic acid (71.16 g, 425.8 mmol) in methylene chloride (300 mL) at 0 °C. A blue color gradually appeared with the addition of the oxime. After 1 h at 0 °C, the reaction mixture was warmed to room temperature. After 3 h at room temperature, water (50 mL) was added, and the organic layer was extracted with water (2 × 50 mL) and 3% sodium bicarbonate solution (2 × 50 mL). The organic layer was dried over Na_2SO_4, the solvent was evaporated, and the residue was recrystalized in diethyl ether/petroleum ether (1:1) in a −5 °C freezer to give **3** as bright blue crystals (25−30% yield): R_f = 0.5 (pentane/ethyl acetate = 20:1); UV−vis (MeOH): λ_{max} = 659 nm; IR (KBr): $\nu_{C=O}$ = 1738 cm^{-1}, $\nu_{N=O}$ = 1563 cm^{-1}, ν_{NO2} = 1529 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.69 (s, 6H), 8.31−8.48 (d, 4H); ^{13}C NMR (300 MHz, $CDCl_3$) δ 21.1 ($2CH_3$), 122.6 (O−C−N), 124.0 (2Ph-CH), 131.4 (2Ph-CH), 135.6 (Ph-C), 151.2 (Ph-C), 162.9 (C═O).

1-Nitrosocyclohexyl Trifluoroacetate (4). A solution of cyclohexanone oxime (4.88 g, 43.16 mmol) in methylene chloride (50 mL) was added dropwise with stirring to a solution of LTA (19.14 g, 43.16 mmol) and trifluoroacetic acid (49.22 g, 431.6 mmol) in methylene chloride (200 mL) at 0 °C. A blue color gradually appeared with the addition of the oxime. After 1 h at 0 °C, the reaction mixture was warmed to room temperature. After 3 h at room temperature, water (50 mL) was

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added, and the organic layer was extracted with water (2 × 50 mL). The organic layer was dried over Na_2SO_4, the solvent was evaporated, and the residue was purified by column chromatography to give **4** as a bright blue liquid (30% yield): R_f = 0.57 (pentane); UV−vis (MeOH): λ_{max} = 648 nm; IR (KBr): $\nu_{C=O}$ = 1792 cm^{-1}, $\nu_{N=O}$ = 1570 cm^{-1}; ^{1}H NMR (300 MHz, Benzene-d_6) δ 1.1−1.9 (m, 10H); ^{13}C NMR (300 MHz, benzene-d_6) δ 19.9 ($2CH_2$), 22.9 (CH_2), 27.4 ($2CH_2$), 113.8 (q, J = 286.2 Hz), 127.2 (O−C−N), 153.7 (q, J = 42.5 Hz); ^{19}F NMR (300 MHz, benzene-d_6) δ −75.7 (CF_3).

Chemiluminescence NO Assay. 1-Nitrosocyclohexyl acetate (**1**) (0.1 mmol) or a solution of **1** (0.1 mmol) in solvent (2 mL) was placed in a 25 mL flask sealed with a rubber septa. Sample aliquots (10 μL) of the reaction headspace were injected over time into the reaction vessel of a Sievers 280 nitric oxide analyzer chemiluminescence detector. This apparatus directly detects NO and the concentrations of NO were determined based on a standard curves.

Gas Chromatographic Detection of Nitrous Oxide. A solution of substrate (0.1 mmol) in solvent (2 mL) was placed in a 25 mL flask sealed with a rubber septa under an argon atomosphere. In some cases, glutathione or Fe(III) were added to the solution. Aliquots of the reaction headspace (250 μL) were injected onto a 6890 Hewlett-Packard gas chromatograph equipped with a thermal conductivity detector, a 6 ft. × 1/8 in. Porapak Q column at an operating oven temperature of 50 °C (injector and detector 150 °C) with a flow rate of 16.67 mL/min (He, carrier gas). The retention time of nitrous oxide was 2.9 min and identical to known samples of N_2O (Aldrich). The yields were calculated based upon a standard curve prepared by injecting known amounts of these gases.

UV−vis Assay for the Decomposition of 1. A solution of sodium hydroxide (0.05 M, 1 mL) was added to a solution of **1** (0.1 mmol, 0.05 M) in methanol (1 mL) at room temperature in a cuvette. UV−vis measurements were taken every 0.74 min at a wavelength of 667 nm on a Cary 100 Bio UV−visible spectrophotometer (Varian, Walnut Creek, CA). Similar experiments were performed using 0.1 and 0.2 M sodium hydroxide with UV−vis measurements being taken every 0.84 min (0.1 M NaOH) and every 1 min for 0.2 M NaOH.

EPR Assay of Ferrous Nitrosyl Complex Formation from the Reactions of 1 with Hemin. A solution of **1** (50 mM) in methanol was added to a solution of hemin (50 μM) in 1 M NaOH at room temperature in a 25 mL flask. Samples (2 mL) for EPR studies were withdrawn every 2 min for 10 min and then at 15 and 20 min and transferred to an EPR tube and frozen in liquid nitrogen (77 K). EPR spectra were taken on a Bruker ER200D spectrometer at 110 K using 10 mW microwave power, 5.0 G modulation amplitude, and 9.3 GHz microwave frequency.

UV−vis and EPR Assay of Ferrous Nitrosyl Complex from the Reaction of 4 with Methemoglobin. A solution of **4** (45 mM) in methanol was added to a solution of methemoglobin (15 μM) in phosphate buffer (100 mM, pH 7.4) at room temperature in a cuvette. UV−vis measurements were taken every 10 min for 4 h on a Cary 100 Bio UV−visible spectrophotometer (Varian, Walnut Creek, CA). Similarly, a solution of **4** (45 mM) in methanol was added to a solution of methemoglobin (15 μM) in phosphate buffer (100 mM, pH 7.4) at room temperature in a 25 mL flask. Samples (2 mL) for EPR studies were withdrawn every 2 min for 10 min and then at 15 and 20 min and transferred to an EPR tube and frozen in liquid nitrogen (77 K). EPR spectra were taken on a Bruker ER200D spectrometer at 110 K using 10 mW microwave power, 5.0 G modulation amplitude, and 9.3 GHz microwave frequency.

X-ray Crystallograpy of 2. A blue single crystal of $C_{13}H_{14}N_2O_5$ (dimensions 0.25 × 0.16 × 0.13 mm^3) was, at 193(2) K, monoclinic, space group $P2(1)/c$-C_{2h}^5 (No. 14) with a = 6.210(3) Å, b = 11.183(5) Å, c = 19.130(8) Å, β = 90.723(7)°, V = 1328.5(10) Å^3, and Z = 4 {FW = 278.26, d_{calcd} = 1.391 g cm^{-3}; μ_a(Mo K$\bar{\alpha}$) = 0.108 mm^{-1}}. A total of 11378 reflections having 2Θ(Mo K$\bar{\alpha}$) < 54.98° were collected on a Bruker SMART APEX CCD area detector system using graphite-monochromated Mo K$\bar{\alpha}$ radiation (λ = 0.71073 Å). Of these 11378 reflections, 3032 were unique (R_{int} = 0.089). The structure was solved using "Direct Methods" techniques with the Bruker AXS SHELXTL-PC software package. The final anisotropic full-matrix least-squares refinement on F^2 with 181 variables converged at R1 = 6.31%, for the 2306 observed data and wR2 = 17.7% for all data. The goodness-of-fit was 1.042. Hydrogen atoms were included in the structure factor calculations as idealized isotropic atoms "riding" on their respective carbon atoms. Crystallographic software references include: SMART V5.628 "Program for Data Collection on Area Detectors" BRUKER AXS Inc., 5465 East Cheryl Parkway, Madison, WI 53711-5373; SAINT V6.36 "Program for Reduction of Area Detector Data" BRUKER AXS Inc., 5465 East Cheryl Parkway, Madison, WI 53711-5373; and SHELXTL-PC V6.12, BRUKER AXS Inc., 5465 East Cheryl Parkway, Madison, WI 53711-5373.

Vasorelaxation of Rat Aortic Rings. Isometric tension in isolated rat thoracic aortic ring segments were measured as described previously.[42] Upon sacrifice, the aorta was excised and cleansed of fat and adhering tissue. Vessels were then cut into individual ring segments (2−3 mm in width) and suspended from a force-displacement transducer in a tissue bath (Radnoti). Ring segments were bathed at 37 °C in a bicarbonate-buffered, Krebs-Henseleit (K−H) solution of the following composition (mM): NaCl 118; KCl 4.6; $NaHCO_3$ 27.2; KH_2PO_4 1.2; $MgSO_4$ 1.2; $CaCl_2$ 1.75; Na_2EDTA 0.03; and glucose 11.1 and perfused continuously with 21% O_2/5% CO_2/74% N_2. A passive load of 2 g was applied to all ring segments and maintained at this level throughout the experiments. At the beginning of each experiment, indomethacin-treated ring segments were depolarized with KCl (70 mM) to determine the maximal contractile capacity of the vessel. Rings were then washed extensively and allowed to equilibrate. For subsequent experiments, vessels were submaximally contracted (50% of KCl response) with phenylephrine (PE, 3 × 10^{-8} − 10^{-7} M), and L-NMMA, 0.1 mM, was also added to inhibit eNOS and endogenous NO production. After tension development reached a plateau, HNO donors were added cumulatively to the vessel bath and effects on tension monitored. Real time data were collected, downloaded to an IBM PC, and analyzed using commercially available software. Stock solutions of HNO donors were prepared immediately (<15 min) before addition to vessel bioassay chambers and stored in the dark at 4 °C.

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Supporting Information Available: Experimental details and results for the gas chromatographic and electron paramagnetic resonance analysis for the hydrolytic decomposition of **2** and **3**. Copies of the ^{1}H and ^{13}C NMR spectra of **1**−**4** are also included. Crystallographic Information Files (CIF) for **2** are included. This material is available free of charge via the Internet at http://pubs.acs.org.

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