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Development of *N*-Substituted Hydroxylamines as Efficient Nitroxyl (HNO) Donors

Daryl A. Guthrie, Nam Y. Kim, Maxime A. Siegler, Cathy D. Moore, and John P. Toscano*

Department of Chemistry, 3400 North Charles Street, Johns Hopkins University, Baltimore, Maryland 21218, United States

Supporting Information

ABSTRACT: Due to its inherent reactivity, nitroxyl (HNO), must be generated *in situ* through the use of donor compounds, but very few physiologically useful HNO donors exist. Novel *N*-substituted hydroxylamines with carbon-based leaving groups have been synthesized, and their structures confirmed by X-ray crystallography. These compounds generate HNO under nonenzymatic, physiological conditions, with the rate and amount of HNO released being dependent mainly on the nature of the leaving group. A barbituric acid and a pyrazolone derivative have been developed as efficient HNO donors with half-lives at pH 7.4, 37 °C of 0.7 and 9.5 min, respectively.

Nitroxyl (HNO) has been shown to have biological activity distinct from that of its redox cousin, nitric oxide (NO), and related nitrogen oxides.[1−11] Much of the recent interest in HNO has been catalyzed by research suggesting that it may be a novel therapeutic for the treatment of heart failure.[1−5] At neutral pH in the absence of chemical traps, HNO efficiently dimerizes ($k = 8 \times 10^6$ $M^{-1}s^{-1}$) to hyponitrous acid (HON=NOH), which subsequently dehydrates to nitrous oxide (N_2O).[12] Given this inherent reactivity, HNO cannot be used directly; donor molecules are required for the generation of HNO *in situ*.

The most commonly used HNO donor is Angeli's salt (**AS**) (Figure 1), a compound that has been known since 1896.[13]

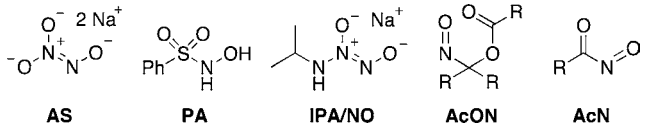
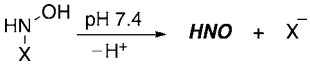


Figure 1. Previously reported classes of HNO donors.

That same year, another HNO donor, Piloty's acid (**PA**), was also reported.[14] In the intervening 115 years, very few other classes of HNO donors suitable for use under physiological conditions have been developed.[15,16] These include primary amine-based diazeniumdiolates such as **IPA/NO**,[17,18] acyloxy nitroso compounds (**AcON**),[19,20] and precursors to the acyl nitroso compounds (**AcN**),[21−23] which themselves are unstable. Indeed, several recent reviews of HNO chemistry and biology have made pleas for new donors.[6−8]

Herein, we are pleased to report a new class of HNO donors based on the general strategy shown in Scheme 1 for *N*-substituted hydroxylamines where X is a good leaving group. Piloty's acid and its derivatives,[24] with sulfinate leaving groups, are classic examples of this strategy. We have employed good carbon-based leaving groups such that HNO is released along with a stable carbanion at neutral pH.

Scheme 1. General Strategy for HNO Release



N-Hydroxycyanamide (HONHCN) is an *N*-substituted hydroxylamine with a carbon-based leaving group (cyanide) and a proposed intermediate in the oxidative bioactivation of cyanamide (H_2NCN), an alcohol deterrent used clinically in Europe, Canada, and Japan.[25−27] Cyanamide has been shown to generate HNO, which is a potent inhibitor of aldehyde dehydrogenase, following metabolic activation. However, *N*-hydroxycyanamide is unstable and has not yet been isolated to confirm its reactivity. In addition, evidence exists that it can be further oxidatively metabolized to a nitrosyl cyanide intermediate (O=NCN) that can also generate HNO following hydrolysis of the nitrile group to form an acyl nitroso compound.[26] An *N*,*O*-dibenzoyl derivative of *N*-hydroxycyanamide has been reported, but this precursor releases HNO not via *N*-hydroxycyanamide, but presumably via an acyl nitroso intermediate, and only following treatment with esterase or base.[28]

We have synthesized and examined alternative *N*-substituted hydroxylamines with carbon-based leaving groups suitable for HNO generation at neutral pH without enzymatic activation. In addition, these derivatives importantly avoid the release of toxic cyanide.

We first considered Meldrum's acid derivative **1a**, which was expected to generate HNO as shown in Scheme 2a. This precursor was easily synthesized without the need for chromatographic purification by formation of the corresponding bromide followed by reaction with *N*,*O*-*bis*(*tert*-butoxycarbonyl) hydroxylamine and subsequent acid deprotection, as shown in Scheme 3. The other precursors reported here were synthesized analogously, and all structures were confirmed by X-ray crystallography. (See Supporting Information (SI) for experimental details concerning the synthesis and characterization of all precursors.)

Compound **1a** was examined for HNO generation by gas chromatographic (GC) headspace analysis to quantify the

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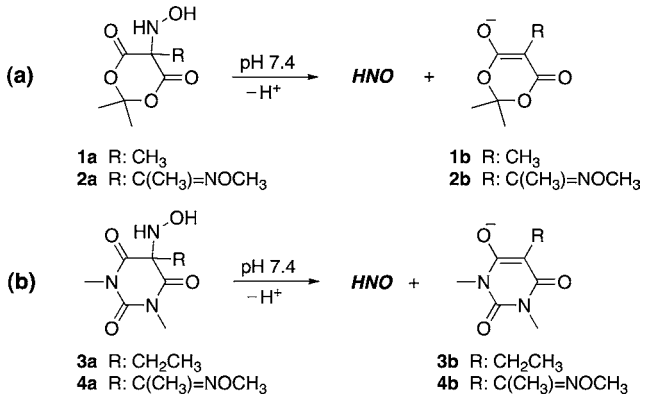
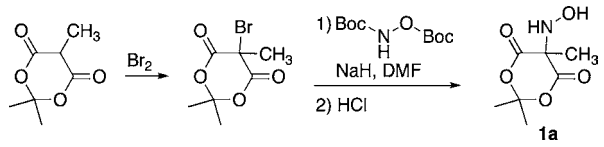
Scheme 2. Potential HNO Release Pathways from (a) Meldrum's Acid Derivatives 1a and 2a and (b) Barbituric Acid Derivatives 3a and 4a



Scheme 3. Synthesis of Meldrum's Acid Derivative 1a



amount of its dimerization product, N_2O, formed following decomposition in pH 7.4 phosphate buffered saline (PBS) at 37 °C. (See SI for experimental details.) Unfortunately, only trace amounts of N_2O are observed (Table 1). Meldrum's acid (pK_a = 4.8)[29] is completely ionized at pH 7.4, and its 5,5-dimethyl derivative has a hydrolysis half-life of about 12 h under physiological conditions.[30] Nonetheless, the major product observed for **1a** by ^{1}H NMR spectroscopy is acetone (SI), indicative of a dominant ring-opening reaction pathway.

Table 1. Decomposition of HNO Donors

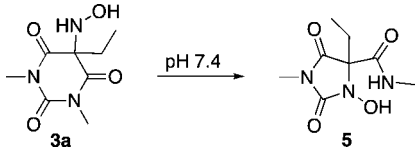
donor	% HNO[a]	% carbanion[b]	$t_{1/2}$ (min)[c]
1a	2	4[d]	*f*
2a	25	34[d]	0.9
3a	2	6[e]	*f*
4a	110	>95	0.7
6a	110	>95	9.5

[a]Donor compounds (0.1 mM) were incubated at 37 °C in PBS, pH 7.4. HNO yields are reported relative to the standard HNO donor, Angeli's salt, as determined by N_2O headspace analysis (SEM ± 5%; $n \geq 3$). N_2O production was completely quenched with added glutathione (0.2 mM). [b]Yields of carbanions **1b**−**4b**, **6b** were determined by ^{1}H NMR spectroscopy. [c]Determined from UV−vis kinetic experiments. [d]Relative to the major byproduct, acetone. [e]Relative to rearrangement byproduct **5**. [f]Not determined.

We next examined Meldrum's acid derivative **2a**, equipped with an electron-deficient *O*-methyloxime moiety. Relative to **1a**, an enhanced HNO yield is observed (Table 1), attributed to **2b** being a better leaving group. However, acetone is still the major product, indicating that the non-HNO producing ring-opening reaction pathway remains competitive with the desired pathway. Evidently, a more robust ring system that disfavors alternative decomposition pathways is necessary.

To explore the impact of a different ring system, we evaluated barbituric acid derivatives **3a** and **4a** (Scheme 2b). Barbiturate **3a** unexpectedly produced very little HNO (Table 1). Following a large-scale decomposition, the major organic byproduct was isolated and identified by X-ray crystallography, revealing that barbiturate **3a** primarily undergoes an intramolecular rearrangement to compound **5** in pH 7.4 buffer solutions (Scheme 4).

Scheme 4. Major Reaction Pathway for Barbiturate 3a



Given the positive impact that an electron-deficient *O*-methyloxime group had on HNO production from Meldrum's acid derivatives above, we analyzed an analogous substitution on the barbituric acid ring system. Satisfyingly, exchanging the ethyl group in **3a** with an *O*-methyloxime in barbiturate **4a** strongly favors the generation of HNO, as reflected by the high yield of N_2O observed following decomposition (Table 1). HNO was confirmed as the source of N_2O for this and the other precursors examined by quenching with glutathione, a known efficient trap for HNO.[31,32]

The decomposition of **4a** was monitored by ^{1}H NMR spectroscopy in PBS (pH 7.4, room temperature), and the only detectable organic byproduct was the expected carbanion **4b** (Figure 2a). With an estimated pK_a of ca. 4 (Figure 3a), the byproduct is completely ionized at neutral pH. Influenced by the *O*-methyloxime group, this pK_a is slightly lower than that of *N*,*N*-dimethyl barbituric acid (pK_a = 4.7).[33] Although the

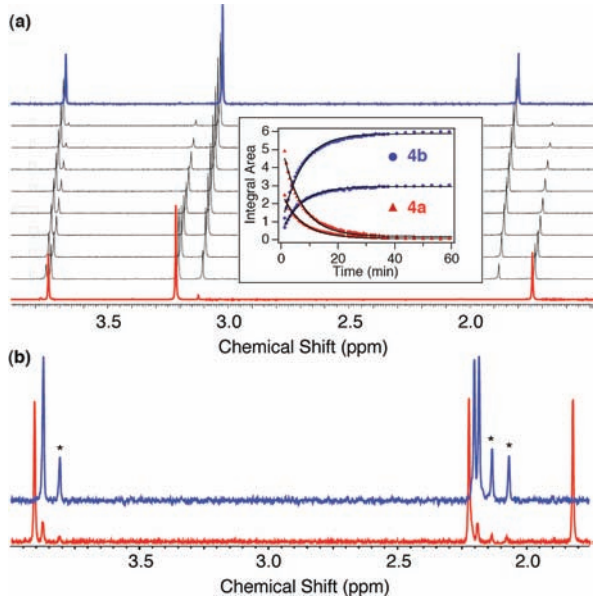


Figure 2. ^{1}H NMR analysis of the decomposition of (a) **4a** to **4b** and (b) **6a** to **6b** in 10% D_2O, PBS, pH 7.4 at room temperature. In each case, the red spectrum was collected at the start of the experiment, and the blue spectrum after complete decomposition. In the insert of (a), the kinetics of decomposition are shown. The red triangles represent the N-CH_3's (6H) and the oxime C-CH_3 (3H) of **4a**, and the blue circles represent the N-CH_3's (6H) and the oxime C-CH_3 (3H) of carbanion **4b**. The solid curves are calculated best fits to a single exponential function ($k = 2.4 \times 10^{-3}$ s^{-1} for each fit). In (b), the asterisks (*) indicate signals due to the minor *anti*-**6b** isomer.

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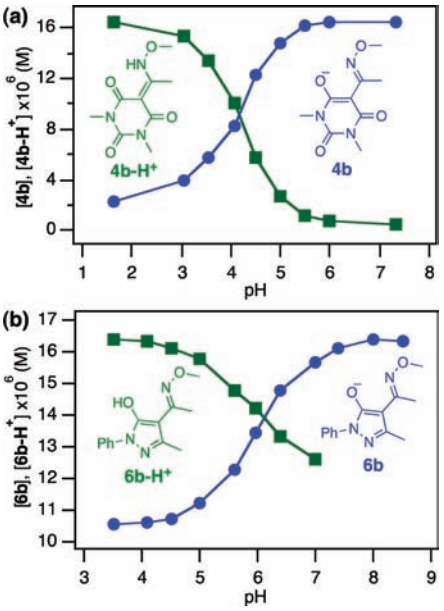


Figure 3. Plot of the concentration of (a) **4b**, **4b**-H^+, and (b) **6b**, **6b**-H^+ as a function of pH. In (a), the green squares represent **4b**-H^+ (λ_{max} = 298 nm), and blue circles represent carbanion **4b** (λ_{max} = 261 nm). In (b), the green squares represent **6b**-H^+ (λ_{max} = 270 nm), where the last three data points were omitted due to spectral overlap, and the blue circles represent carbanion **6b** (λ_{max} = 253 nm). See SI for representative UV–vis spectra of **4b** and **6b** at high and low pH.

pharmacology of **4b** has not yet been evaluated, barbituric acids and their C5-substituted derivatives in general have an expansive history in medicinal chemistry including hypnotic, sedative, antiepileptic, and immunomodulation applications.[34]

The kinetics of decomposition from **4a** to **4b** was easily monitored by UV–vis spectroscopy given the distinctive absorbance of anion **4b** (λ_{max} = 261 nm) (Figure 4a). Analysis of the decomposition rate as a function of pH reveals a sharp increase near pH 8 (Figure 4b). Barbiturate **4a** has a half-life of ca. 1 min at pH 7.4 and 37 °C (Figure 4a) but is relatively stable at pH 4.0 and room temperature, with a half-life of approximately 12 h under these conditions (SI).

To demonstrate the generality of this approach for HNO generation, we have also examined another *N*-substituted hydroxylamine with a suitable carbon-based leaving group. Like barbiturates **3a** and **4a**, pyrazolone **6a** (synthesized analogously to compounds **1a**–**4a**) also takes advantage of the formation of an aromatic byproduct (**6b**) (Scheme 5) and efficiently produces HNO with a half-life of ca. 10 min at pH 7.4, 37 °C (Table 1). Another potentially practical benefit enjoyed by precursor **6a** is that byproduct **6b**, formed along with HNO, is a 4-substituted derivative of edaravone (Scheme 5), a potent antioxidant already in clinical use for the treatment of stroke and cardiovascular disease.[35,36] Moreover, a variety of 4-substituted edaravone analogues have also shown strong antioxidant activity.[37]

The decomposition of **6a** was analyzed similarly to that of **4a** by ^{1}H NMR spectroscopy in PBS (pH 7.4, room temperature) (Figure 2b). The only organic byproducts observed by this analysis are the *syn* (major) and *anti* (minor) isomers of **6b** (Scheme 5), and the relative abundance of these isomers is unchanged at high and low pH. Donors **6a**, **2a**, and **4a** are observed to be all *syn* by NMR analysis and X-ray crystallography.

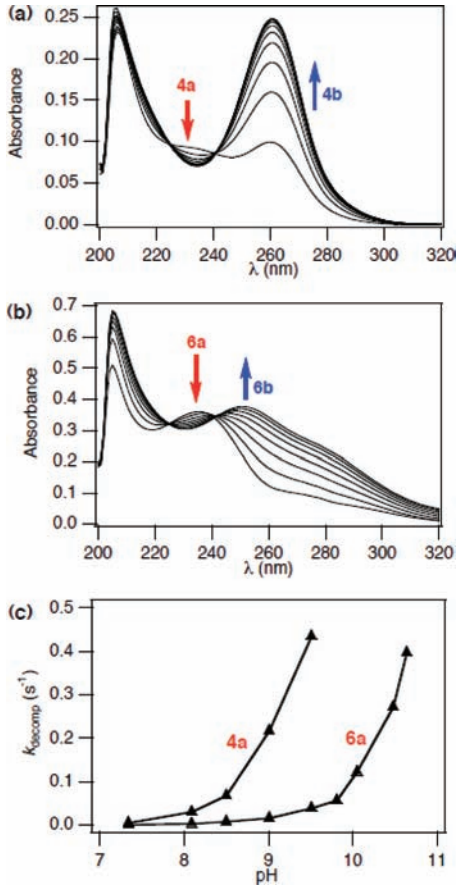
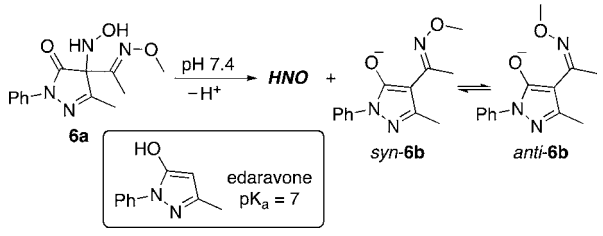


Figure 4. (a) UV–vis analysis of the decomposition of (a) **4a** (time between traces = 30 s) and (b) **6a** (time between traces = 240 s) at 37 °C in PBS, pH 7.4. (c) Plot of UV–vis determined decomposition rate as a function of pH at 25 °C for **4a** (monitored at 261 nm) and **6a** (monitored at 253 nm).

Scheme 5. HNO Release from Pyrazolone Derivative 6a



The pK_a of **6b**/**6b**-H^+ is estimated to be ca. 6 (Figure 3b), shifted below that of edaravone (pK_a = 7) by *O*-methyloxime substitution, indicating that nearly all of this byproduct is ionized at pH 7.4. As was the case for precursor **4a**, the decomposition rate of pyrazolone **6a** is highly pH dependent, here with a sharp increase near pH 10 (Figure 4c); **6a** is much more stable at pH 4.0, with a half-life of about 25 h at room temperature (SI).

The data presented on compounds **1a**–**4a** and **6a** suggest that the ability of these *N*-substituted hydroxylamines to generate HNO is based mainly on the nature of the leaving group. To produce HNO efficiently, the driving force for the formation of a stabilized carbanion must overcome other non-HNO producing reaction pathways. Barbiturate **4a** and pyrazolone **6a**, easily synthesized without the need for chromatographic purification, have been developed as effective

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HNO donors with half-lives at pH 7.4, 37 °C of 0.7 and 9.5 min, respectively. These compounds represent new examples of the general strategy for HNO production from *N*-substituted hydroxylamines with good leaving groups. Future work will involve the further development of this strategy with other suitable carbon-based leaving groups.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures; compound characterization data, including X-ray crystallographic data, details concerning N_2O, NMR, and UV−vis analysis; and complete refs 4 and 17. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

jtoscano@jhu.edu

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