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C-Nitroso Donors of Nitric Oxide

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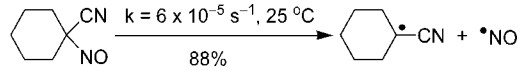
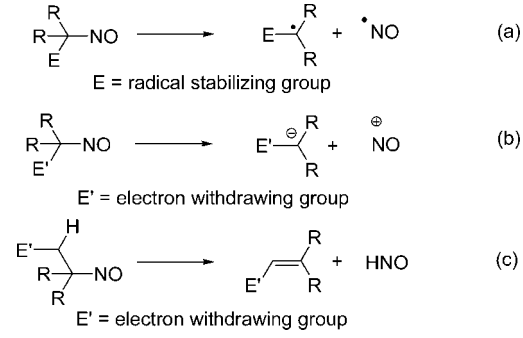


A complete understanding of the biological activity of nitric oxide (NO) is complicated by the different reactivity profiles of its various species and by the often complex decomposition behavior of the NO progenitors in common use. Here, we report that appropriately substituted *C*-nitroso compounds act solely as donors of neutral nitric oxide through a first-order homolytic C−N bond scission to release up to 88% nitric oxide in DMSO at 25 °C. The reaction produces a carbon radical, and the yield of nitric oxide is dependent on the availability of radical traps. *C*-Nitroso compounds are sources of biologically active neutral NO and display potent NO bioactivity in a rabbit aortic ring assay.

Although the involvement of nitric oxide (NO) in biological processes is by now clear, many aspects of its activity remain enigmatic. [1] The varying and often contradictory activities of nitric oxide have in some instances been attributed to differences in the biological activities of its redox-related congeners: neutral NO radical, the +1 cation (nitrosonium), and the one-electron reduced form, HNO (nitroxyl).[2] A clearer and more complete understanding of the physiological activity of NO requires precursors that selectively produce NO at predictable, controlled, and well-defined rates and in a single oxidation state; ideal progenitors for single redox forms of NO are largely lacking.

Several classes of NO/NO$^+$/HNO generators and/or surrogates are in common use, including nitrate esters, inorganic complexes of nitric oxide, *S*-nitrosothiols, various nitrogen heterocycles, and diazenium diolates.[3] In many cases, however, the release of nitric oxide is both mechanistically complex and dependent on the availability of exogenous agents as simple as protons or as complex as enzymes. Decomposition often releases NO in more than one redox state in ratios dependent on environmental conditions. Single-redox sources of NO that release at a predictable rate via well-understood mechanisms are thus vital to a complete understanding of the biological activity of NO.

SCHEME 1. Rational Design of *C*-Nitroso Donors of NO in Various Redox States



C-Nitroso compounds offer the potential for highly selective delivery of a single redox form of NO, facilitating exquisite control of the electronic and steric characteristics of the carbon atom bearing the nitroso group (Scheme 1).[4] Stabilization of an incipient carbon radical should facilitate homolytic scission of the C−N bond and release of neutral nitric oxide (Scheme 1a), while stabilization of the corresponding carbanion would promote the activity of an NO$^+$ equivalent, (Scheme 1b)

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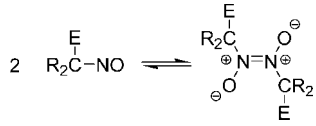
TABLE 1. Calculated Dimerization Energies for *C*-Nitroso Compounds



species	R_2	E	$\Delta E_{dimerization}$ [a]	$\Delta G_{dimerization}$ [b]
1	H	H	−18.9 (**−20.3**)[c]	−8.8 (**−9.9**)[d]
2	CH_3	CH_3	−10.2	+2.1
3	H	CN	−12.7 (**−15.4**)[c]	−1.6 (**−4.0**)[d]
4	CH_3	CN	−9.2	+3.3
5	$-(CH_2)_4-$ (cyclopentyl)	CN	−9.3	+2.8
6	$-(CH_2)_5-$ (cyclohexyl)	CN	−10.0	+1.9
7	nitrosoethylene		−14.2 (**−15.9**)[c]	−3.7 (**−5.1**)[d]
8	nitrosobenzene		−3.9	+7.1

[a] B3LYP/6-31G* + ZPE (0 K) values. [b] B3LYP/6-31G* values at 298.15 K, 1 M. [c] CBS-QB3 values (0 K) in bold in parentheses. [d] CBS-QB3 values (298.15 K, 1 M) in bold in parentheses. All values in kcal·mol^{-1}. The 1 M standard state values are as defined in ref 10.

effecting nitrosation of suitable nucleophiles. Finally, activation of hydrogen atoms vicinal to the NO function via an electron-withdrawing group (Scheme 1c) promote extrusion of HNO via β-elimination. Here, we provide experimental evidence for the suitability of α-cyano substituted *C*-nitroso compounds as donors of neutral NO. Such compounds decompose by homolytic scission to produce nitric oxide.

We envisioned that selective production of neutral nitric oxide could occur via homolytic scission of a properly activated C−N bond of an alkylnitroso compound which was suitably substituted in order to avoid nitrosonium or HNO formation. Tertiary nitroso species are required, as *C*-nitroso compounds bearing α-hydrogen atoms exist as the thermodynamically favored oxime.[5] The biological activities of a few *C*-nitroso compounds, including species bearing α-cyano substituents, have previously been reported.[6] Although these compounds showed measurable (μM) activity, only small amounts of nitric oxide were released, (<19%) and no kinetic characterization of the release was reported.[6]

C-Nitroso compounds undergo dimerization to the corresponding azodioxy species,[7] and the rate of NO release is dependent not only on the rate of C−N bond homolysis but also on the rate of monomer−dimer interconversion. To probe the effect of cyano substitution on dimerization and homolysis, the thermodynamics of both processes were calculated (Tables 1 and 2, respectively) using density functional theory (B3LYP/6-31G*) and the CBS-QB3 model of Petersson;[8] the latter approach has been shown to reproduce experimental reaction energetics to within ± 2 kcal/mol, including processes involving NO dissociation.[9]

Population of the dimer is significantly disfavored by increasing steric bulk, inductive electron withdrawal, and introduction

TABLE 2. Bond Dissociation Energies for *C*-Nitroso Compounds

species	R	E	C−N BDE (calc.)[a]	C−N BDE[b]
1	H	H	36.0	37−40
2	CH_3	CH_3	40.8	34−40
3	H	CN	26.2	
4	CH_3	CN	26.8	22.7
5	$-(CH_2)_4-$ (cyclopentyl)	CN	26.0	
6	$-(CH_2)_5-$ (cyclohexyl)	CN	28.1	
9	H	CHO	28.0	
10	CH_3	CHO	25.7	
11	CH_3	Br		43.0
12	CH_3	NO_2		29.2
13	CH_3	OAc		36.1

[a] CBS-QB3 values (0 K); this study. [b] Reference 13.

of delocalizing substituents at the nitroso carbon. This observation is in accord with the known resistance of conjugated *C*-nitroso species, such as PhNO (**8**), to undergo dimerization.[11,12] From the CBS-QB3 results on model systems **1**, **3**, and **7** and the known experimental ΔG of dimerization of +0.61 kcal·mol^{-1} for **2** (CCl_4 solution, 26 °C),[11] the predicted (B3LYP/6-31G*) free energy of dimerization appears to be too large (positive) by ca. 1.1 to 2.4 kcal·mol^{-1}. An approximate range of ca. −0.5 to +2.2 kcal·mol^{-1} is therefore predicted for the ΔG of dimerization of α-cyano species **4**−**6** at 1 M and 25 °C, with dimerzation increasingly favored at higher concentrations.

C-Nitroso bond dissociation and activation energies have been reported for a small number of aryl and tertiary *C*-nitroso species.[13] These studies yield C−N homolytic bond dissociation energies near ca. 40 kcal·mol^{-1}, depending on substitution.[4] To supplement these literature values and obtain accurate energetic parameters for homolysis of the species of interest in this study, C−N BDE values were computed using the CBS-QB3 method.

Electron-withdrawing substituents and substituents capable of radical stabilization through delocalization (CN, CHO, NO_2) lower C−N homolytic BDEs to 26−29 kcal·mol^{-1}. By comparison, alkyl *S*-nitrosothiols, known to be thermally stable to S−N homolysis at or near room temperature, possess predicted S−N BDEs of 31−32 kcal·mol^{-1} at the same level of theory, with measured activation energies for homolysis ca. 1−2 kcal·mol^{-1} lower than the predicted BDE.[9] Species **4**−**6** are predicted to show significantly more facile NO dissociation (3−5 kcal·mol^{-1}) than *S*-nitrosothiols and might thus afford pure neutral NO at physiologically relevant temperatures.

In principle, electron-withdrawing substituents should also increase the nitrosonium (NO^+) donating potential. However, pK_a's of simple cyanoalkanes are near 35,[14] and CN substitution is not predicted to lower carbon acidity such that nitrosonium donation is competitive. We thus set out to prepare tertiary α-cyano *C*-nitroso compounds and to evaluate their behavior as NO donors.

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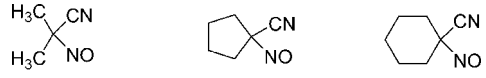
TABLE 3. NO Release from α-Cyano *C*-Nitroso Compounds at 25 °C



4 **5** **6**

compd	initial concn (mM)	conditions	% NO formed
4	10	dI water[a]	<2[b]
4	1.25	DMSO	71
5	1.0	dI water[a]	31
6	1.0	dI water[a]	28
6	1.25	DMSO	88
6	1.0	PBS[c]	23
6	1.0	PBS[c]	35
6	1.0	dI water + O_2[d]	30
6	0.01	dI water + O_2[d]	89

[a] Deionized water. [b] Head space. [c] Phosphate-buffered saline, pH 7.4. [d] Oxygen (1 mL) was injected into the solution at the start of experiment.

TABLE 4. Measured Rate Constants for C−N Homolysis in Various Solvents at 25 °C

compd	solvent	$k \times 10^5$ (s^{-1})	$\Delta G^{\ddagger}$ (kcal·mol^{-1})
4	DMF	6.1 ± 1.9	+23.2 ± 7.2
5	DMF	5.5 ± 0.6	+23.3 ± 2.5
6	DMF	5.5 ± 0.9	+23.3 ± 3.8
6	DMSO	6.0 ± 2.1	+23.2 ± 8.1
6	CH_3CN	8.5 ± 3.5	+23.0 ± 9.4
6	$CHCl_3$	8.0 ± 2.3	+23.0 ± 6.6

Compounds **4**−**6** were prepared according to literature methods and isolated as blue or bluish white solids.[15,16] The solid-state infrared spectrum of **6** indicated the presence of both the *trans* dimer (1292 cm^{-1}) and monomer (1565 cm^{-1}).[6] During storage at −20 °C, the bluish-white solids **4** and **6** become colorless, presumably the result of dimer formation.[6]

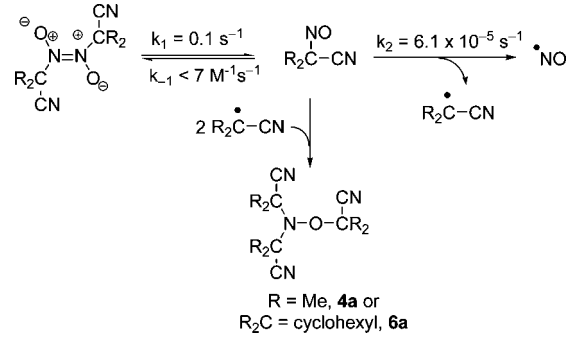
When compound **4** was dissolved in DMSO, it quickly produced a deep blue color ($\lambda_{max} = 650$ nm, $\varepsilon = 2 \times 10^{-2}$ M^{-1} cm^{-1}) characteristic of monomeric *C*-nitroso compounds. The kinetics of dimer dissociation were monitored at 25 °C, yielding a first-order rate constant of 0.1 ± 0.03 s^{-1} and a $\Delta G^{\ddagger}$ of 16.9 kcal·mol^{-1}. The rate of dissociation is solvent independent, and essentially equivalent rates were observed in acetonitrile, methanol, and DMF.

In contrast to the solid-state IR spectra, no IR bands attributable to dimer were observed for dissolved samples. In conjunction with the observation of a single set of ^{13}C resonances, these data suggest that the dimer is populated only in neat samples and at low temperatures.

The blue color of the α-cyano *C*-nitroso solutions gradually disappeared during several hours, and the yield of neutral nitric oxide was determined by chemiluminescence (Table 3). All three compounds produce significant quantities of nitric oxide, although the yields are dependent on both solvent and initial concentration of the *C*-nitroso species.

Having identified neutral nitric oxide as the major inorganic product of decomposition, we fit loss of *C*-nitroso compound to first-order decays, obtaining first-order rate constants for C−N bond homolysis (Table 4). The measured rate constants for decay of compounds **4**−**6** lie in a fairly narrow range of (5.5−8.5) × 10^{-5} s^{-1}, with corresponding $\Delta G^{\ddagger}$ values of ca. 23 kcal·mol^{-1}.[9] The observed invariance in rate as a function of both solvent and structure lends strong support to a homolytic mechanism of NO release.

SCHEME 2. Proposed Mechanism of Nitric Oxide Release from α-Cyano *C*-Nitroso Compounds[15,16]



The yields of nitric oxide reported in Table 3 differ significantly from those reported by Rehse and Herpel, who in all cases observed yields of ≤13% for **6**.[6a] Gasco and co-workers report a 19% yield of NO from **6**,[6b] suggesting that alternative, presumably bimolecular, pathways of *C*-nitroso decomposition might be operative. *C*-Nitroso compounds react rapidly with radicals to form stable adducts. Both Gregor and Gowenlock have reported isolation of the spin-trapped adducts **4a** and **6a** in 20−29% yields during decomposition of **4** and **6**,[15,16] suggesting that spin trapping may be responsible for collateral consumption of α-cyano *C*-nitroso compounds.[17] To test this hypothesis, a 10 μM solution of **6** was prepared in deionized (dI) water pretreated with O_2 (g). The solution decomposed to yield 89% nitric oxide, suggesting that oxygen effectively competes with spin-trapping at low concentrations (Table 3).

A mechanism for nitric oxide release is shown in Scheme 2. Rapid dissociation of the dimer in solution generates 2 equiv of monomer, which undergoes bond homolysis to produce neutral nitric oxide and the corresponding cyanoalkyl radical (R•). Reaction of R• with additional *C*-nitroso compound forms the spin-trapped adduct, limiting the yield of neutral nitric oxide in the absence of efficient radical scavengers.[17]

Our goal at the outset was to prepare useful donors of neutral nitric oxide. To ensure that α-cyano *C*-nitroso compounds do not act as donors of nitrosonium, DMSO and acetonitrile solutions of **6** were treated with excess propane thiol and sodium propanethiolate.[18] In no case was *S*-nitrosothiol, the product of nitrosonium ion transfer, observed. Furthermore, negligible N_2O (<1%) was detected following decomposition of **6**,[6] suggesting that HNO is not a decomposition product of α-cyano *C*-nitroso compounds.

Having prepared compounds that act exclusively as donors of neutral nitric oxide, we examined biological activity of the expressed nitric oxide using the rabbit aortic ring assay. All three compounds relax precontracted aortic rings with nanomolar potencies. The most active of the three, 1-cyano-1-nitrosocyclohexane (**6**), shows an IC_{50} of roughly 20 nM, while the least active of the three, 2-cyano-2-nitrosopropane (**4**), shows an IC_{50} value roughly 10-fold higher than that of **6** (Figure 1). The origin of the difference in activity is unclear, since rates of homolytic

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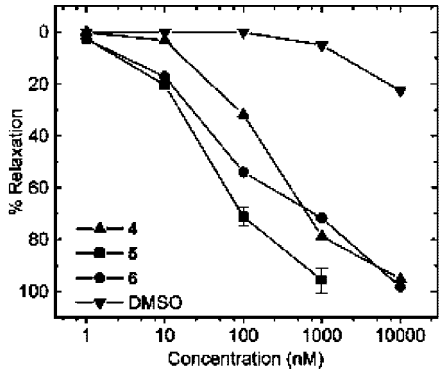

FIGURE 1. Relaxation activity of *C*-nitroso compounds **4**−**6** on precontracted rabbit aortic rings.

scission are predicted to be equivalent in all three compounds. The rate of monomer−dimer interconversion is sufficient such that a Curtin−Hammett condition applies, and population of the dimer cannot account for differences in activity. Differences in solubility or cellular penetration may play a role in the observed differences in biological activity.

In summary, we have demonstrated the utility of α-cyano *C*-nitroso compounds as donors of neutral nitric oxide. Although the compounds decompose via a unimolecular process, they exist as stable dimers in pure form and can be stored for significant, perhaps indefinite, periods of time as pure solids. The flexibility afforded by the available range of carbon substitution may be useful in controlling the rate of NO release. Finally, the exclusive production of neutral nitric oxide should permit a better understanding of the biological roles of the various redox states of nitric oxide. We continue our studies on the effect of carbon substitution of the chemical and biological properties of *C*-nitroso compounds and will report our results in due course.

Experimental Section

General Methods. NMR spectra were recorded on a spectrometer operating at 300 MHz for ^{1}H and 75 MHz for ^{13}C. Organic solvents were freshly distilled and degassed before use or used directly as received. Deionized water from the MilliQ filtration apparatus was distilled and purged with nitrogen before use. pH 7.4 phosphate-buffered saline (GIBCO) and DMSO (Hybri-Max, Sigma) in sealed ampules were used as received. All kinetic plots were prepared using *Origin*, and its program for curve fitting was used for rate analysis. Nitric oxide was determined using a chemiluminescence-based detector (Sievers 280 Nitric Oxide Analyzer, NOA). Compounds **4**−**6** were prepared by literature methods.[6,15,16]

Synthesis of C-Nitroso Compounds 4−6. Compounds **4**−**6** were prepared according to literature protocols and showed physical and spectroscopic properties identical to those of the previously reported species.

Kinetics of Dissociation of Dimeric 4. Compound **4** (dimer, 2 mg, 10 mmol) was dissolved in 3 mL of DMSO, and the absorbance at 650 nm was measured. A first-order rate constant of 0.1 s^{-1} was extracted by curve fitting. This procedure was used in the measurement of half-lives in acetonitrile (12 s), acetone (11 s), and methanol (5 s).

Nitric Oxide Analysis. Nitric oxide was quantified by preparing DMSO stock solutions of **4**−**6**. This stock solution was then diluted with DMSO, dI water, or PBS to the desired final concentration. Calibration of the instrument was carried out using 1% w/v potassium iodide in glacial acetic acid into which known concentrations of nitrite was injected, and a calibration curve was generated before each experiment. Injections of suitable volumes of samples into the NOA chamber were carried out to estimate levels of nitric oxide in headspace or solution. The value of % NO formation was calculated assuming 2 mol of NO formed per mole of dimer or 1 mol of NO formed from the monomer, as applicable.

Computational Methodology. All calculations were performed with the *Gaussian 98* program system.[19] Zero-point energies are uncorrected. Where relevant, the conformation of a given compound with the lowest B3LYP/6-31G* energy (UB3LYP for open-shell species) was used in the CBS-QB3 calculations.

Bioassay.[20] The thoracic aorta of freshly sacrificed (CO_2) New Zealand White rabbits (2.5−3 kg) were removed, cleaned of fat and connecting tissue, and cut into 3-mm rings. The rings were mounted under 2 g of resting tension in tissue baths (25 mL) filled with Krebs solution (37 °C) containing 118 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, and 11 mM glucose, pH 7.4. The solution was bubbled with 20% O_2, 5% CO_2, and balance N_2. Changes in isometric tension were recorded with Statham (Hato Rey, PR) transducers and a Grass Instruments (Quincy, MA) polygraph, and contractions were initiated with phenylephrine. The adducts **4**−**6** were first dissolved in DMSO (∼10 mM) and then diluted in PBS (pH 7.4 to give final concentration of approximately 100 μM). From these stock solutions of **4**−**6** and subsequent serial dilutions were generated the concentration−relaxation curves.

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Supporting Information Available: Cartesian coordinates of all species and intermediates described in the manuscript and their absolute energies. This information is available free of charge via the Internet at http://pubs.acs.org.

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