Para-Substituted O-Benzyl Sulfohydroxamic Acid Derivatives as Redox-Triggered Nitroxyl (HNO) Sources

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Abstract: Nitroxyl shows a unique biological profile compared to the gasotransmitters nitric oxide and hydrogen sulfide. Nitroxyl reacts with thiols as an electrophile, and this redox chemistry mediates much of its biological chemistry. This reactivity necessitates the use of donors to study nitroxyl’s chemistry and biology. The preparation and evaluation of a small library of new redox-triggered nitroxyl sources is described. The condensation of sulfonyl chlorides and properly substituted O-benzyl hydroxylamines produced O-benzyl-substituted sulfohydroxamic acid derivatives with a 27–79% yield and with good purity. These compounds were designed to produce nitroxyl through a 1, 6 elimination upon oxidation or reduction via a Piloty’s acid derivative. Gas chromatographic headspace analysis of nitrous oxide, the dimerization and dehydration product of nitroxyl, provides evidence for nitroxyl formation. The reduction of derivatives containing nitro and azide groups generated nitrous oxide with a 25–92% yield, providing evidence of nitroxyl formation. The oxidation of a boronate-containing derivative produced nitrous oxide with a 23% yield. These results support the proposed mechanism of nitroxyl formation upon reduction/oxidation via a 1, 6 elimination and Piloty’s acid. These compounds hold promise as tools for understanding nitroxyl’s role in redox biology.

Keywords: nitroxyl (HNO); Piloty’s acid; redox triggered 1, 6 elimination; gasotransmitters; redox signaling; aromatic nitro/azide reduction; aromatic boronate oxidation

1. Introduction

The one-electron reduction and protonation of nitric oxide (NO), a well-known biological signaling agent characterized as a gasotransmitter, formally produces nitroxyl (HNO) [1]. These structural/electronic differences give HNO a distinct chemistry from NO, as HNO dimerizes to hyponitrous acid (H$_2$N$_2$O$_2$) that dehydrates to nitrous oxide (N$_2$O) [2]. This reactivity necessitates the use of HNO donors and highlights the extreme electrophilic and oxidizing character of HNO [3]. HNO demonstrates different biological properties from NO [4,5], and at least three drugs that chemically release HNO have been used clinically or evaluated in trials for the treatment of cancer (hydroxyurea), alcoholism (cyanamide) and congestive heart failure (Cimlanod), showing the clinical potential of HNO donors [6–8]. Much of our understanding of the pharmacology and therapeutic potential of HNO comes from experiments utilizing HNO donors [3,9]. The most common HNO donors include Angeli’s salt (AS, Na$_2$N$_2$O$_3$) and Piloty’s acid (PA, PhSO$_2$NHOH), which are commercially available solids that rapidly and cleanly release HNO under neutral conditions [3,9].

In addition to dimerization, HNO reacts with thiols, generating a N-hydroxsulfenamide intermediate that can rearrange to a sulfinamide or further react with more thiol to yield a disulfide and hydroxylamine [10]. HNO similarly reacts with hydrogen sulfide (H$_2$S), a second recognized gasotransmitter [1], to give short-chain hydrogen polysulfides (H$_2$S$_n$) or S$_8$ depending on the relative concentrations of HNO and H$_2$S [11]. Based on this chemistry, HNO can influence thiol-mediated biochemistry and potentially H$_2$S-controlled reactions.
For example, HNO inhibits enzymes with active site cysteines, such as aldehyde dehydrogenase (AIDH) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [12,13]. HNO modifies cardiac myoflament proteins affecting myocardial contractility by increasing calcium cycling and sensitizing myocardial tissue responsiveness to calcium [14,15]. HNO directly activates the chemosensory TRPA1 channel [16]. In bacteria, the addition of HNO to Staphylococcus aureus increases the formation of the persulfide (RSSH) of the predominant low-molecular-weight thiol, bacillithiol (BSH) [17]. This oxidized thiol derivative may control transcription factors responsible for directing the overall sulfur metabolism in these bacteria [18]. Under oxidative conditions, the addition of HNO to Bacillus subtilis synergistically enhances hydrogen peroxide (H2O2)-mediated cell killing. Angeli’s salt (AS), which decomposes to HNO and nitrite (NO2−) at neutral pH, was used as the HNO source in these redox investigations in B. subtilis [3,19].

Redox-triggered HNO donors may possess value in probing biological crosstalk between HNO and other small redox active signaling agents, such as H2S or H2O2. The use of AS as a HNO donor is limited by the co-production of NO2−, a relatively fast and pH-insensitive release rate (t1/2 ~2.8 min, pH 4–8) and limitations in the synthesis of new donors [3]. Piloty’s acid (PA), another common HNO donor, decomposes to HNO and phenyl sulfonic acid (PhSO2H) at a neutral pH [3]. The aryl portion of PA tolerates structural modification while still supporting HNO release, which allows for the installation of designed redox-sensitive elements to initiate a 1, 6-elimination [20]. Scheme 1 shows Piloty’s acid derivatives designed to release HNO via a 1, 6-elimination mechanism upon exposure to either reductants or oxidants. These processes convert the azide, nitro or boronate ester groups into either the aniline or phenol derivative that should decompose to Piloty’s acid with the release of p-quinone methide or its imine (Scheme 1).

![Scheme 1. Designed redox-triggered HNO donors.](image)

Similar constructs have found extensive use for the detection or generation of other signaling agents, such as H2S, but, to the best of our knowledge, have not been applied to HNO release under redox conditions [21]. A hydrogen-peroxide-based prodrug system that releases a structurally similar hydroxamic acid as a histone deacetylase inhibitor has been described [22]. Such compounds permit HNO formation under specific redox conditions and would form the basis of an improved understanding of the role that HNO plays in redox biochemistry. We describe the preparation and characterization of a small library of redox-triggered HNO donors and an evaluation of their ability to produce HNO under specific redox conditions.

2. Results

2.1. Synthesis

Possible redox-triggered HNO donors (1a–d and 2a–c) were prepared with a 27–79% yield by the condensation of the properly substituted O-benzyl hydroxylamine derivative with either p-toluene or methyl sulfonyl chloride under basic conditions (Scheme 2) [23,24]. The variable yields likely arise from mixtures of O and N-mono alkylated products and N, O-dialkylated products as reported [23,24].
This sequence followed by extraction generally gave the desired products with excellent purity, as judged by proton and carbon nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS). Individual compounds could be further purified by recrystallization or silica gel flash chromatography if necessary.

The substituted O-benzyl hydroxylamines required in Scheme 1 were purchased (R = -H or -NO₂) or prepared using reported literature procedures for the azide and the pinacol-derived boronate ester [22,25]. Scheme 3 summarizes the preparation of the azide-containing hydroxylamine (6) from p-toluidine through a four-step sequence that features diazotization/azide substitution, bromination, N-hydroxyphthalimide substitution to install the N-O bond and hydrazine deprotection through intermediates 3–5 (Scheme 3) [25].

The Mitsunobu coupling of N-hydroxyphthalimide with the commercially available pinacol ester of 4-hydroxymethylphenylboronic acid followed by hydrazine deprotection yields the boronate-ester-substituted hydroxylamine (8) via 7 (Scheme 3) [22]. These intermediates were characterized by NMR spectroscopy and MS and purified by normal phase flash chromatography (see Supplementary Materials).

2.2. HNO Donation Ability

Piloty’s acid (PhSO₂NHOH), a common HNO donor, decomposes to HNO and phenyl sulfinic acid (PhSO₂H) at a neutral pH. Compounds 1b–d and 2b,c were designed to decompose upon either reduction or oxidation as depicted in Scheme 1 to yield a reactive intermediate that should release a Piloty’s acid derivative (pCH₂CH₂SO₂NHOH or CH₃SO₂NHOH) that fragments to HNO and the corresponding sulfinic acid. The gas chro-
matographic (GC) headspace measurement of nitrous oxide (N₂O), the dimerization and dehydration product of HNO, provides a rapid and simple measure of HNO formation from these transformations [26]. The decomposition of the PA derivative pCH₃C₄H₆SO₂NHOH, a known HNO donor, in methanol/buffer generates 76% of N₂O by this measure after 24 h (Table 1). The sensitivity of N₂O formation to the addition of glutathione (GSH), which rapidly reacts with HNO blocking N₂O production [10], provides evidence for HNO’s intermediacy (Table 1). Compounds 1a and 2a do not contain reduction or oxidation-sensitive functional groups, preventing the proposed decomposition to a Piloty’s acid derivative, and do not produce N₂O under these conditions (Table 1).

Table 1. Control N₂O Production at 24 h from 1a−d and 2a−c (CH₃OH/100 mM PBS buffer (pH = 7.4), 1:1, 2 mL).

| Compound          | % N₂O | +GSH (5 equiv.) |
|-------------------|-------|-----------------|
| pCH₃C₄H₆SO₂NHOH  | 76    | 0               |
| 1a                | 1     |                 |
| 1b                | 1     |                 |
| 1c                | 1     |                 |
| 1d                | 1     |                 |
| 2a                | 1     |                 |
| 2b                | 1     |                 |
| 2c                | 1     |                 |

The incubation of 1b-d and 2b-c in a methanol/100 mM PBS buffer showed ~1% N₂O formation, indicating essentially no formation of HNO over 24 h from these compounds, which was as expected, in the absence of any reducing/oxidizing agents. The formation of small amounts of N₂O could arise from the slow hydrolysis/methanolysis of the benzyl group producing pCH₃C₄H₆SO₂NHOH or CH₃SO₂NHOH or from the presence of small amounts of these sulfohydroxamic acids in these samples, which could form from the condensation of residual hydroxylamine potentially present in the commercial or synthetic O-benzylhydroxylamine derivatives.

Table 2 summarizes N₂O formation at 24 h from the treatment of 1a−d and 2a−c with various reducing and oxidizing agents. The addition of sodium borohydride, a reducing agent capable of nitro-to-amine reduction [27], to compounds 1b and 2b generates N₂O with a 25 and 51% yield, respectively, providing evidence for initial HNO formation (Table 2). The lower observed amounts of N₂O from 1b may reflect the poor solubility of 1b, which did not completely dissolve in 1:1 CH₃OH:H₂O (2 mL), possibly due to the presence of two para-substituted aromatic rings, including one with the polar nitro group. Compound 1b dissolved with the addition of another 0.5 mL of CH₃OH (1.5:1 CH₃OH:H₂O, 2.5 mL), and the results reported in Table 2 for 1b were obtained under these conditions. Thin-layer chromatography (TLC) and an MS analysis of the reduction of 1b with sodium borohydride provides evidence for the formation of p-amino benzyl alcohol, the product of water addition to the imine of p-quinone methide. The addition of sodium borohydride/copper (II) sulfate, a mixture known to reduce azides to amines [28], to 1c and 2c produces 89 and 92% N₂O, respectively (Table 2). These results support Scheme 1 and suggest a reduction of the nitro and azide groups to the amine followed by decomposition to the Piloty’s acid derivative. As expected, the treatment of 1a and 2a with sodium borohydride or sodium borohydride/copper (II) sulfate under these conditions did not generate N₂O (Table 2).
Table 2. N$_2$O Production from 1a−d and 2a−c at 24 h (1 h) upon reduction/oxidation.

| Treatment (% N$_2$O) |  |  |  |  |  |  |  |
|----------------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
| **Compound** | NaBH$_4$ | NaBH$_4$/Cu(II)SO$_4$ | GSH | H$_2$S 2.5 equiv. | H$_2$S 5 equiv. | H$_2$O$_2$ |
| 1a | 0 | 1 | - | - | - | 0 |
| 1b | 25(7) | - | - | - | - | - |
| 1c | - | 89(14) | 0 | 6 | 0 | - |
| 1d | - | - | - | - | - | 23(5) |
| 2a | 1 | 1 | - | - | - | 0 |
| 2b | 51(2) | - | - | - | - | - |
| 2c | - | 92 | 0 | 5 | 0 | - |

The incubation of 1c and 2c with GSH (2.5 or 5 equivalents) does not produce N$_2$O, but the treatment of 1c and 2c with H$_2$S (2.5 equivalents) generated small reproducible amounts (6% and 5%, respectively) of N$_2$O. These results suggest H$_2$S-mediated azide reduction to the amine as described, followed by HNO formation [29]. The lower amounts of N$_2$O observed are likely due to the competition between HNO dimerization and H$_2$S addition. Increasing the amounts of H$_2$S to five equivalents abolished N$_2$O formation, supporting this explanation. Table 2 also shows hydrogen peroxide (H$_2$O$_2$)-mediated N$_2$O release from 1d, a boronate-containing compound designed to release HNO via boronate oxidation to the phenol followed by decomposition. The incubation of 1d with H$_2$O$_2$ resulted in the formation of N$_2$O with a 23% yield, presumably indicating the formation of HNO upon oxidation to the phenol. The treatment of 1a and 2a with H$_2$O$_2$ did not produce N$_2$O (Table 2). Table 2 also shows that the amount of N$_2$O produced increases from the 1 h to 24 h measurements, likely reflecting the kinetics of these model oxidations and reductions.

Bacterial nitroreductases using NADH as a co-substrate act as competent reducing agents of aromatic nitro groups, with the subsequent release of desired compounds via a 1, 6-elimination [30,31]. The treatment of 1b or 2b with *Escherichia coli* nitroreductase (Sigma) in the presence of NADH failed to generate N$_2$O as initially expected. A previous report indicates that NADH reduces HNO to hydroxylamine, thus blocking N$_2$O formation and suggesting that the lack of observed N$_2$O in these experiments results from HNO reduction by the NADH co-substrate [32]. Further investigation will be necessary to define the practicality of nitroreductase-triggered HNO donors, but these results suggest that HNO may exert a portion of effects through NADH/NADPH depletion.

3. Materials and Methods

All materials and solvents used for extraction and purification were purchased from commercial vendors and used as received. $^1$H and $^{13}$C NMR spectra were recorded using a Bruker Avance 400 MHz NMR spectrometer. Mass spectra were obtained using a Bruker Amazon SL ion trap. An Agilent Technologies 7890A gas chromatograph equipped with a micro-electron capture detector and a 30 m × 0.32 m (25 µm) HP-MOLSIV capillary column was used for the gas chromatographic analysis of N$_2$O. CAUTION: Any experiments preparing alkyl or aryl azides should be performed in a well-ventilated fume hood and behind a blast shield. Sodium azide should not be mixed with strong acids.

3.1. Synthesis of Piloty’s Acid Derivatives

The Piloty’s acid derivatives were prepared by sulfonyl chloride condensation with an O-benzylhydroxylamine derivative using a modified literature procedure [33].

$N$-(benzyloxy)-4-methylbenzenesulfonylamide (1a). O-benzylhydroxylamine hydrochloride (287 mg, 1.80 mmol) was added to a solution of p-toluenesulfonyl chloride (378 mg, 1.98 mmol) in pyridine (5 mL). The reaction mixture reacted overnight at room temperature and was quenched with 2M HCl (25 mL) and extracted with ethyl acetate (25 mL). The
organic layer was washed with water (10 mL), brine (10 mL), dried over MgSO₄ and concentrated using rotary evaporator to give 396 mg, 79.4% yield of 1a as white solid. ¹H NMR (400 MHz, CDCl₃): δ 7.82 (d, J = 8.0 Hz, 2H), 7.33–7.35 (m, 6H), 6.88 (s, 1H), 4.97 (s, 2H), 2.44 (s, 3H). ¹³C NMR (101 MHz, CDCl₃): δ 148.02, 145.33, 142.47, 133.33, 129.90, 129.59, 128.56, 123.75, 77.79, 21.72. ESI-MS (positive mode) (m/z) calculated mass for C₁₄H₁₄NO₃SNa [M+Na]+ 300.34, found mass 300.13.

4-methyl-N-(4-nitrobenzyl)oxy)benzenesulfonamide (1b). 1-[Aminooxy)methyl]-4-nitrobenzene hydrochloride (368 mg, 1.80 mmol) was added to a solution of p-toluene sulfonyl chloride (378 mg, 1.98 mmol) in pyridine (5 mL). The reaction mixture reacted overnight at room temperature and was quenched with 2M HCl (25 mL) and extracted with ethyl acetate (25 mL). The organic layer was washed with water (10 mL), brine (10 mL), dried over MgSO₄ and concentrated under reduced pressure to give 428 mg, 73.8% yield of 1b as white solid. ¹H NMR (400 MHz, CDCl₃): δ 8.20 (d, J = 8 Hz, 2H), 7.80 (d, J = 8 Hz, 2H), 7.49 (d, J = 8 Hz, 2H), 7.35 (d, J = 8 Hz, 2H), 6.96 (s, 1H), 5.09 (s, 2H), 2.45 (s, 3H). ¹³C NMR (101 MHz, CDCl₃): δ 147.96, 145.32, 142.56, 133.31, 129.89, 129.54, 128.55, 123.72, 79.74, 21.71. ESI-MS (positive mode) (m/z) calculated mass for C₁₄H₁₄NO₃SNa [M+Na]+ 345.06, found mass 345.20.

N-(4-azidobenzyl)oxy)-4-methylbenzenesulfonamide (1c). p-Toluene sulfonyl chloride (340 mg, 1.79 mmol) was added to a solution of O-(4-azido benzyl) hydroxylamine (1.78 g, 10.8 mmol) in pyridine (30 mL). The reaction mixture reacted overnight at room temperature and was quenched with 2M HCl (150 mL) and extracted using ethyl acetate (150 mL). The organic layer was washed with water (100 mL), brine (100 mL), dried over MgSO₄ and concentrated under reduced pressure to give 1.32 g, 38.4% yield of 1c as a light yellow solid. ¹H NMR (400 MHz, CDCl₃): δ 7.73 (d, J = 8 Hz, 2H), 7.26 (d, J = 8 Hz, 4H), 6.93 (d, J = 8 Hz, 2H), 6.85 (s, 1H), 4.87 (s, 2H), 2.37 (s, 3H). ¹³C NMR (101 MHz, CDCl₃): δ 145.00, 140.53, 133.60, 131.96, 131.04, 129.79, 128.55, 119.09, 78.66, 21.69. ESI-MS (positive mode) (m/z) calculated mass for C₁₄H₁₄NO₃SNa [M+Na]+ 341.34, found mass 341.28.

4-methyl-N-(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl)oxy)benzenesulfonamide (1d). p-Toluene sulfonyl chloride (340 mg, 1.79 mmol) was added to a solution of O-(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl) hydroxylamine (410 g, 1.64 mmol) in pyridine (5 mL). The reaction mixture reacted overnight and concentrated under reduced pressure with the addition of excess toluene. The resulting product was acidified using 2M HCl and extracted with chloroform (25 mL). The organic layer was washed with water (10 mL), brine (10 mL), dried over MgSO₄ and concentrated under reduced pressure to give 428 mg, 73.8% yield of 1d as a white solid. ¹H NMR (400 MHz, CDCl₃): δ 7.70–7.75 (m, 4H), 7.24–7.27 (m, 4H), 6.83 (s, 1H), 4.91 (s, 2H), 2.36 (s, 3H), 1.27 (s, 12H). ¹³C NMR (101 MHz, CDCl₃): δ 144.91, 138.23, 134.94, 133.62, 129.76, 128.59, 128.41, 83.92, 79.23, 24.88, 21.68. ESI-MS (positive mode) (m/z) calculated mass for C₂₀H₂₂BNO₃SNa [M+Na]+ 426.15, found mass 426.29.

N-(benzyloxy)methanesulfonamide (2a). O-benzylhydroxylamine hydrochloride (510 mg, 2.50 mmol) was added to a solution of methanesulfon chloride (210 µL, 2.71 mmol) in pyridine (12 mL). The reaction mixture reacted overnight at room temperature and was quenched with 2M HCl (60 mL) and extracted with ethyl acetate (60 mL). The organic layer was washed with water (40 mL), brine (40 mL), dried over MgSO₄ and concentrated using rotary evaporator to give 404 mg, 80.4% yield of 2a as a white solid. ¹H NMR (400 MHz, CDCl₃): δ 7.37–7.41 (m, 5H), 6.86 (s, 1H), 5.00 (s, 2H), 3.04 (s, 3H). ¹³C NMR (101 MHz, CDCl₃): δ 135.02, 129.59, 128.96, 128.88, 79.66, 37.03. ESI-MS (positive mode) (m/z) calculated mass for C₁₉H₁₃NO₃SNa [M+Na]+ 300.34, found mass 299.24.

N-(4-nitrobenzyloxy)methanesulfonamide (2b). 1-[Aminooxy)methyl]-4-nitrobenzene hydrochloride (613 mg, 3.0 mmol) was added to a solution of methanesulfon chloride (210 µL, 2.71 mmol) in pyridine (12 mL). The reaction mixture reacted overnight under room temperature and was quenched with 2M HCl (50 mL) and extracted with ethyl acetate (50 mL). The organic layer was washed with water (30 mL), brine (30 mL), dried over MgSO₄ and concentrated using reduced pressure to give 516 mg, 77.5% yield of 2b as
white solid. mp 111–113 °C; IR (film) 3211, 1608, 1517, 1320, 1151 cm⁻¹; ¹H NMR (400 MHz, CDCl₃): δ 8.17 (d, J = 8 Hz, 2H), 7.49 (d, J = 8 Hz, 2H), 7.10 (s, 1H), 5.03 (s, 2H), 3.03 (s, 3H)

¹³C NMR (101 MHz, CDCl₃): δ 148.13, 142.19, 129.74, 123.84, 78.08, 37.09. ESI-MS (positive mode) (m/z) calculated mass for C₁₈H₁₀N₂O₅SNa [M+Na]^⁺ 269.23, found mass 269.06.

N-[(4-azidobenzyl)oxy]methanesulphonamide (2c). O-(4-azidobenzyl) hydroxylamine (1.3g, 7.93 mmol) was added to a solution of methanesulfonyl chloride (675 µL, 8.72 mmol) in pyridine (30 mL). The reaction mixture reacted overnight at room temperature and was quenched with 2M HCl (150 mL) and extracted with ethyl acetate (150 mL). The organic layer was washed with water (100 mL), brine (100 mL), dried over MgSO₄ and concentrated using rotary evaporator to give a crude product that was purified using column chromatography (Pet Ether/Ethyl Acetate, 3:1) to give 530 mg, 27.6% yield of 2c as a white solid. mp 118.85, 20.85.

Sodium azide [25] (6.1g, 92.8 mmol) in 58mL of cold water were added slowly to a solution of 1-azido-4-(bromomethyl)benzene (3.1 g, 14.6 mmol) and 2,2′-Azobis(2-methylpropionitrile) (1 g, 6.1 mmol) were added under N₂ atmosphere to a solution of p-toluidine (10g, 92.8 mmol) in 50mL HCl: H₂O (1:1) at 0 °C. After 1 h of reaction, the mixture was extracted with CHCl₃ (100 mL), the remaining aqueous layer was washed with CHCl₃ (2 × 50 mL) and the combined organic layers were washed with water (100 mL), brine (100 mL), dried over MgSO₄ and concentrated under reduced pressure to give 9.5 g, 79.3% yield of 3 as a brown oil. ¹H NMR (400 MHz, CDCl₃): δ 7.16 (d, J = 8 Hz, 2H), 6.93 (d, J = 8 Hz, 2H), 2.33 (s, 3H). ¹³C NMR (101 MHz, CDCl₃): δ 137.15, 134.63, 130.35, 118.85, 20.85.

1-azido-4-methylbenzene (3). Was prepared following a published procedure [23]. A solution of sodium nitrite (6.3g, 92.8 mmol) was added to 26 mL of cold water and a solution of sodium azide [25] (6.1g, 92.8 mmol) in 58mL of cold water were added slowly to a solution of p-toluidine (10g, 92.8 mmol) in 50mL HCl: H₂O (1:1) at 0 °C. After 1 h of reaction, the mixture was extracted with CHCl₃ (100 mL), the remaining aqueous layer was washed with CHCl₃ (2 × 50 mL) and the combined organic layers were washed with water (100 mL), brine (100 mL), dried over MgSO₄ and concentrated under reduced pressure to give 11.8 g, 84.5% yield of 3 as a white solid. mp 108–110 °C; IR (film) 3207, 3024, 2943, 2111, 1505, 1304, 1157 cm⁻¹; ¹H NMR (400 MHz, CDCl₃): δ 7.89 (d, J = 10 Hz, 2H), 6.98 (s, 1H), 4.96 (s, 2H), 3.05 (s, 3H). ¹³C NMR (101 MHz, CDCl₃): δ 140.78, 131.71, 131.17, 119.19, 78.91, 37.03. ESI-MS (positive mode) (m/z) calculated mass for C₁₈H₁₈NO₂SNa [M+Na]^⁺ 265.04, found mass 265.03.

1-azido-4-(bromomethyl)benzene (4). Was prepared following a published procedure [25] N-Bromosuccinimide (10.51 g, 59 mmol) and 2,2′-Azobis(2-methylpropionitrile) (1 g, 6.1 mmol) were added under N₂ atmosphere to a solution of 1-azido-4-methylbenzene (8 g, 60.1 mmol) in benzene (200 mL). The reaction mixture was heated under reflux for 3 days and concentrated under reduced pressure. The resulting product was partitioned between CH₂Cl₂ (100 mL) and washed with water (100 mL), brine (2 × 50 mL), dried over MgSO₄ and concentrated under reduced pressure to give 4.2g, 84.5% yield of 4 as a yellow-orange oil. ¹H NMR (400 MHz, CDCl₃): δ 7.40 (d, J = 12 Hz, 2H), 7.02 (d, J = 12 Hz, 2H), 4.50 (s, 2H).

2-(4-azidobenzyl)oxy)-isoindoline-1,3-dione (5). Was prepared following a published procedure [25] K₂CO₃ (2.34 g, 16.9 mmol) was added slowly to a solution of 1-azido-4-(bromomethyl)benzene (3.1 g, 14.6 mmol) and N-Hydroxyphthalimide (2.76 g, 16.9 mmol) in 45 mL DMF. The reaction mixture reacted overnight at room temperature and partitioned between CH₂Cl₂ (150 mL) and H₂O (100 mL). The organic layer was washed with brine (100 mL), dried over MgSO₄ and concentrated under reduced pressure to give 4.2g, 84.5% yield of 5 as a white solid. ¹H NMR (400 MHz, CDCl₃): δ 7.75–7.76 (m, 4H), 7.45 (d, J = 8 Hz, 2H), 6.96 (d, J = 8 Hz, 2H), 5.11 (s, 2H). ¹³C NMR (101 MHz, CDCl₃): δ 163.51, 141.17, 134.51, 131.57, 130.41, 128.83, 123.57, 119.14 79.11.

O-(4-azidobenzyl) hydroxylamine (6). Was prepared following a modified procedure [25] Hydrazine monohydrate (1.04 mL, 32.4 mmol) was added slowly to a solution of 2-(4-azidobenzyl)oxy)-isoindoline-1,3-dione (4.2 g, 14.3 mmol) in CH₂Cl₂ (45 mL) and the reaction mixture reacted overnight at room temperature. The resulting mixture was quenched with 1M NaOH (50 mL) and the separated organic layer was washed with water (30 mL), brine (30 mL), dried over MgSO₄ and concentrated under reduced pressure to give 1.78g, 76.4% yield of 6 as a yellow oil. ¹H NMR (400 MHz, CDCl₃): δ 7.27 (d, J = 8 Hz, 2H), 6.94 (d, J = 8 Hz, 2H), 5.35 (broad s, 2H), 4.56 (s, 2H). ¹³C NMR (101 MHz, CDCl₃): δ 139.75, 134.29, 130.01, 119.06, 77.24.
2-(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenoxy)isoindoline-1,3-dione (7). Was prepared following a published procedure [22]. Diethyl azodicarboxylate (1 mL, 5.2 mmol) was added slowly at 0 °C to a solution of triphenylphosphine (1.23 g, 4.7 mmol), \(N\)-hydroxyphthalimide (0.77 g, 4.7 mmol), and 4-hydroxymethylphenylboronic acid pinacol ester (1 g, 4.3 mmol) in \(\text{CH}_2\text{Cl}_2\) (10 mL). After 1 h of reaction, the reaction mixture was concentrated under reduced pressure and purified by column chromatography (Pet Ether/Ethyl Acetate, 10:1) to give 900 mg, 50.5% yield of 7 as a white solid.

\[\delta_{\text{H NMR}} (400 \text{ MHz, CDCl}_3): \delta 7.75–7.71 (m, 4H), 7.66–7.62 (m, 2H), 7.45 (d, \text{ } J = 8 \text{ Hz, 2H}), 5.15 \text{ (s, 2H)}, 1.26 \text{ (s, 12H)}.\]

O-(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl)hydroxylamine (8). Was prepared following a modified procedure [22]. Hydrazine hydrate (120 \(\mu\)L, 3.75 mmol) was added slowly to a solution of 2-(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenoxy)isoindoline-1,3-dione (900 mg, 3.61 mmol) in ethanol (20 mL). The reaction mixture reacted overnight and the solid was filtered and rinsed with cold ethanol. The filtrate was concentrated under reduced pressure to give 410 mg, 45.6% yield of 8 as a yellow oil.

\[\delta_{\text{H NMR}} (400 \text{ MHz, CDCl}_3): \delta 7.64 \text{ (d, } J = 8 \text{ Hz, 2H}), 7.45 \text{ (d, } J = 8 \text{ Hz, 2H}), 5.15 \text{ (s, 2H)}, 1.26 \text{ (s, 12H)}.\]

3.2. Gas Chromatographic \(\text{N}_2\text{O}\) Analysis

For headspace analysis, substrate (1a–d, 2a–c, 0.04 mmol) was placed in a 10 mL round-bottom flask with a stir bar and sealed with a rubber septum. Solvent (methanol:water or methanol:PBS (100 mM), pH = 7.4; 1:1, 2 mL) was added using a syringe, and headspace aliquots (0.1 mL) from each experiment were injected at 1 and 24 h onto a 7890A Agilent Technologies gas chromatograph equipped with a micro-electron capture detector and a 30 m × 0.32 m (25 \(\mu\)m) HP-MOLSIV capillary column. The oven was operated at 200 °C for the duration of the run (4.5 min). The inlet was held at 250 °C and run in split mode (split ratio 1:1) with a total flow (\(\text{N}_2\) as carrier gas) of 4 mL/min and a pressure of 37.9 psi. The \(\mu\)ECD was held at 325 °C with a makeup flow (\(\text{N}_2\)) of 5 mL/min. The retention time of nitrous oxide was 3.4 min, and yields were calculated based on a standard curve for nitrous oxide gas (Gasco Precision Calibration Mixtures). To some samples, sodium borohydride (1.1 equivalents), sodium borohydride (1.1 equivalents) and copper (II) sulfate (0.1 equivalents), GSH (5 equivalents) or sodium sulfide (2–5 equivalents) were added. For oxidation of 1d, hydrogen peroxide (5 equivalents) was added to a solution (2 mL) of the substrate in a mixture of 3:2:0.5 acetonitrile, water, PBS (100 mM, pH = 7.4).

4. Conclusions

Nitroxy (HNO) demonstrates a unique biological profile compared to NO that deserves more detailed studies that have been afforded to other nitrogen oxides and gasotransmitters. The high chemical reactivity of HNO requires the use of donors and relatively complex detection methods, which complicate such studies and the confirmation of endogenous HNO production. HNO exhibits rich redox reactivity with thiols, hydrogen sulfide and heme proteins, suggesting a potential role in various redox-mediated processes. We report a small library of derivatives (1a–d and 2a–c) of the HNO donor, Piloty’s acid, that liberate HNO upon oxidation or reduction through a 1, 6 elimination mechanism. These compounds were quickly constructed by the condensation of a sulfonyl chloride and an appropriately substituted hydroxylamine derivative. The nitro and azide-containing molecules (1b, c and 2b, c) demonstrated HNO release, as measured by headspace GC for \(\text{N}_2\text{O}\), upon chemical treatment with reducing agents. Similarly, a boronate ester derivative (1d) generates HNO upon hydrogen peroxide oxidation. Control experiments with 1a and 2a show that only compounds with redox active groups generate HNO under these conditions, supporting the mechanism. Overall, these results show the ability of these redox-sensitive HNO donors to release HNO upon oxidation/reduction, and could find use in further defining the role of HNO in redox-based biological processes.
Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/molecules27165305/s1, Materials; Figure S1. 1H NMR and 13C NMR of 1a; Figure S2. 1H NMR and 13C NMR of 1b; Figure S3. 1H NMR and 13C NMR of 2b; Figure S4. IR of 2b; Figure S5. 1H NMR and 13C NMR of 3; Figure S6. 1H NMR of 4; Figure S7. 1H NMR of 5; Figure S8. 1H NMR and 13C NMR of 6; Figure S9. 1H NMR and 13C NMR of 1c; Figure S10. 1H NMR of 7; Figure S11. 1H NMR of 8; Figure S12. 1H NMR and 13C NMR of 1d; Figure S13. 1H NMR and 13C NMR of 2a; Figure S14. 1H NMR and 13C NMR of 2c; Figure S15. IR of 2c; Figure S16. MS of 1a; Figure S17. MS of 1b; Figure S18. MS of 2b; Figure S19. MS of 1c; Figure S20. MS of 1d; Figure S21. MS of 2a; Figure S22. MS of 2c.

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