

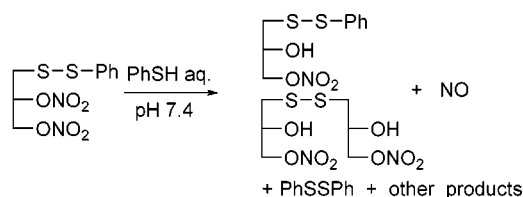
Nitrate Esters as Nitric Oxide Donors:
SS-NitratesSergei I. Zavorin, Jennifer D. Artz, Adina Dumitrascu, Adrian Nicolescu,
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ABSTRACT



The important biological secondary messenger NO can be generated from exogenous nitrovasodilators and NO donors. Nitrate esters are nitrovasodilators and NO mimetics, believed to be biotransformed to NO in vivo. On the basis of a mechanistic hypothesis, nitrates have been synthesized that release NO at significant rates in neutral aqueous solution in the presence only of added thiol. The novel masked β -mercaptonitrates reported (SS-nitrates), provide information on possible sulfhydryl-dependent biotransformation mechanisms for nitrates in clinical use.

Nitric oxide (NO) is a biological messenger molecule produced in response to cell-specific external stimuli, with important biological roles.^{1,2} Physiologically NO is produced by nitric oxide synthase (NOS).³ NO from endothelial NOS mediates effects including vasodilation. Neuronal NOS is involved in neurotransmission in the central and peripheral nervous systems. Inducible NOS produces NO as part of the body's immune response.

In addition to endogenous sources of NO, various exogenous NO donors have been reported, including several classes of nitrovasodilators. Of these, nitroglycerin (GTN) and the organic nitrate vasodilators hold special significance, having been used clinically in treatment of angina for 125 years.^{4,5}

The biological effects of organic nitrates are NO-mimetic, but unambiguous evidence for NO generation from simple chemical reactions of organic nitrates has not been reported.^{5,6} In contrast, other nitrovasodilators, for example, the diazeniumdiolates, DEA/NO, and Sper/NO,⁷ readily release NO in aqueous solution. Nevertheless, it is widely held that nitrates must be biotransformed to give NO and often argued that the clinically significant observation of "nitrate tolerance" is linked to this biotransformation pathway.⁸

Biotransformation pathways proposed for GTN have largely been heme-dependent or sulfhydryl-dependent.^{5,8} The latter include enzymic (e.g., involving glutathione *S*-transferase) or nonenzymic pathways (e.g., involving free glutathione (GSH) or cysteine). In the presence of cysteine, in aqueous phosphate buffer, the rate of NO release from GTN is below the threshold of electrochemical detection, but NO release can be observed by chemiluminescence detection, although reaction is slow and inhibited by metal ion

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(3) Marletta, M. A. *Cell* **1994**, *78*, 927.

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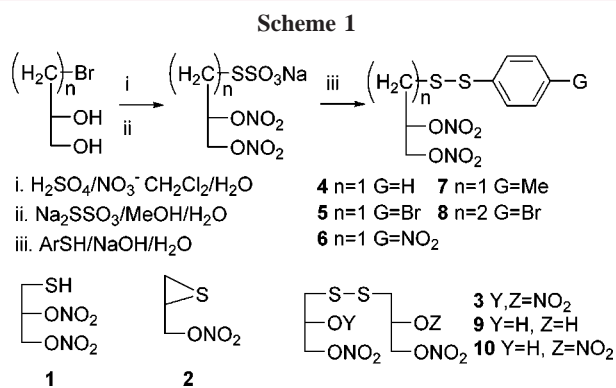
(8) Bennett, B. M.; McDonald, B. J.; Nigam, R.; Simon, W. C. *Trends Pharmacol. Sci.* **1994**, *15*, 245.

chelators.⁹ Of course, binding of GTN in the hydrophobic cleft of a protein proximal to a cysteine moiety may hypothetically, greatly accelerate an otherwise slow nonenzymic reaction, through approximation.

There are few mechanistic studies of organic nitrate reactivity. However, such studies, in particular of the transformation of the nitrate functional group to NO, are crucial for many reasons: first, to gain clues as to which enzymes may be responsible for biotransformation in vivo; second, to understand tolerance; and last, to develop new organic nitrate therapeutic agents.

A powerful technique in enzyme modeling is to build an intramolecular model, in which reacting groups are positioned in close proximity, such that reaction is greatly accelerated, usually via three-, five-, or six-membered ring transition states.¹⁰ On the basis of a sulfhydryl-dependent mechanism for NO release from GTN, we have synthesized novel nitrates that contain sulfur β to a nitrate group.^{5,11} Some of these nitrates yield NO at rates sufficiently high to be detected electrochemically.⁶ This is the first unambiguous evidence that NO can be a significant product from the reaction of an organic nitrate with thiol in a simple aqueous, chemical system. These important observations support sulfhydryl-dependent organic nitrate biotransformation and allow the proposal of novel mechanisms.

Compound **1** is the archetype β -mercaptonitrate (Scheme 1).¹² When added to a neutral aqueous solution, **1** reacted



rapidly to yield NO, validating its design (Figure 1); however, this thiol is inherently labile.¹² Therefore, the design was modified to synthesize the SS-nitrates (**4–7**) (Scheme 1), a family of masked thiols in which disulfide reduction might lead to unmasking, for example, by addition of free thiol.

Synthesis of the dinitroxypropane derivatives **4–7** rests on the condensation of 1,2-dinitroxypropane-3-thiosulfate (a Bunte salt) with an appropriate thiol under basic conditions (Scheme 1).¹³ The butane derivative **8** can be synthesized in

(9) Diethylenetriaminepentaacetic acid (DTPA) quenches NO release under anaerobic conditions; $d[\text{NO}]/dt = 0.05 \text{ nM/sec}$ for GTN (1mM) + cys (2mM) in aerobic aqueous solution: Artz, J. D.; Toader, V.; Dumitrascu, A.; Zavorin, S.; Bennett, B. M.; Thatcher, G. R. J., submitted for publication.

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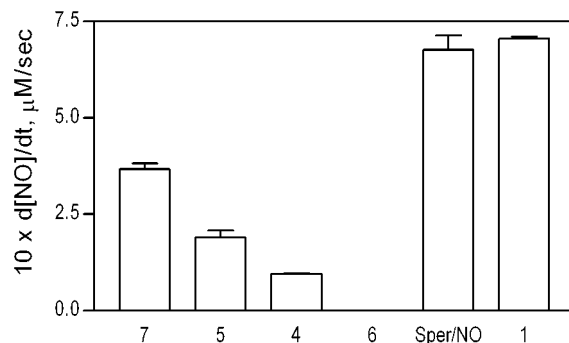


Figure 1. Rates of NO release were measured using a Clark-type, NO-selective electrode (WPI ISO-NO II) at 1 mM nitrate, 2 mM cysteine, 37 °C, in aerobic 40% MeCN/phosphate buffer (100 mM, pH 7.4). Quenching of NO release was not observed under anaerobic conditions nor on addition of DTPA. No cysteine was added for Sper/NO (1 mM) nor for **1** (where DMSO was used in place of MeCN). Measured [NO] increases to a maximum with time and then falls exponentially, as NO is generated and then effuses from the open reaction vessel. The maximal [NO] observed was found to be linearly correlated with $d[\text{NO}]/dt$. Calibration was performed using DEA/NO solutions, under identical reaction conditions ($k_{\text{NO}}(\text{DEA}/\text{NO}) = (148 \pm 1.6) \times 10^{-4} \text{ s}^{-1}$). This method gives apparent initial rates, $d[\text{NO}]/dt$, obtained in quadruplicate [see ref 6 for full details].

a similar manner.¹³ Synthesis of **3** has been described previously.^{11,14}

The reactivity of the SS-nitrates, on addition of various adjuvants, was investigated in aqueous solution (40% acetonitrile/phosphate buffer) at pH 7.4. A Clark-type, NO-

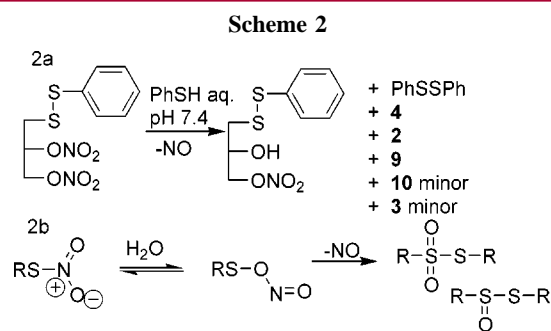
(12) 3-Bromo-1,2-dinitroxypropane (1.92 mmol) was dissolved in acetone (dry, 2 mL) with KSCN (1.2 equiv) and refluxed for 1 h. The residue was concentrated and purified by flash column chromatography on silica gel ($\text{CH}_2\text{Cl}_2/\text{hexane}$ 3:2), to yield 1-thiocyano-2,3-dinitroxypropane in 75% yield. ^{13}C (CDCl_3) 110.99, 76.44, 68.97, 32.03. This thiocyanate (6.73 mmol) was stirred with DTT (10 mmol) in CH_3OH (15 mL) for 30 min at room temperature before concentration and purification by flash column chromatography on silica gel ($\text{CH}_2\text{Cl}_2/\text{hexane}$ 3:2) to yield **2** (40%, ^{13}C (CDCl_3) 76.43, 28.21, 22.55) and **1** as an oil of 95–99% purity (20%, ^{13}C (CDCl_3) 79.4, 69.3, 23.7). The impurities (<5%) identified as **3** and **2** were also formed on standing of **1** over several days (CAUTION: stench).

(13) **General Procedure for 4–8:** Aryl and alkyl mercaptans were obtained commercially or by adaptation of literature procedures. Bunte salts were obtained from the appropriate alkyl bromide by reaction with sodium thiosulfate. Bunte salts (9.67 mmol) were dissolved in distilled water (10 mL). To this solution, a solution of mercaptan (6.46 mmol) in 1 M NaOH (7 mL) was added dropwise. The resulting emulsion was stirred for 1–15 min and then extracted with dichloromethane or ethyl acetate. The combined organic extracts were washed with H_2O , dried over MgSO_4 and concentrated under vacuum. The residue was purified by flash column chromatography on silica gel. ^{13}C NMR (75 MHz, CDCl_3): for **4** (53%) 36.9, 69.8, 77.6, 128.6, 129.5, 129.8, 136.0; for **5** (43%) 36.9, 69.8, 77.4, 122.7, 130.9, 132.9, 135.1; for **6** (9%) 36.9, 69.6, 77.1, 124.5, 126.9, 144.7, 147.0; for **7** (52%) 21.5, 36.8, 69.8, 77.7, 130.5, 130.6, 132.5, 139.2; for **8** (43%) 135.6, 132.2, 129.6, 121.4, 77.0, 70.9, 33.1, 28.0.

(14) Compounds **9** and **10** are prepared in a procedure analogous to that published for **3**, via acid-catalysed oxidation of Bunte salts.⁹ General procedure: The two Bunte salts (each 1.72 mmol) were dissolved in 2 mL of cold H_2O_2 (30%, 0 °C), and then 1 drop of 10% H_2SO_4 was added. The mixture was stirred at 0–5 °C for 20 min. The aqueous layer was discarded, and the remaining oil was dissolved in dichloromethane and washed successively with water, then NaHCO_3 solution, and finally water. The organic solution was dried over MgSO_4 , concentrated, and purified by flash column chromatography (silica gel, $\text{CH}_2\text{Cl}_2/\text{hexanes}$ 70:30). ^{13}C (CDCl_3): for **9** (36%) 76.3, 67.3, 42.7; for **10** (45%) 77.3, 74.2, 69.4, 69.3, 66.9, 66.8, 42.1, 42.0, 36.8, 36.6.

selective electrode was employed to quantify NO release (Figure 1). This method is limited by the detection threshold but has the overriding advantage of selectivity for NO over other possible N,O-containing products.⁶ The electrode was calibrated using diazeniumdiolate salts as previously described.⁶ Products were identified for reaction of the parent SS-nitrate, **4**, with thiophenol, by isolation using reverse-phase HPLC (Beckman ODS2/C18; MeOH/water eluant) and spectrophotometric comparison with independently synthesized compounds.

The SS-nitrates are stable in neutral, aqueous solution but react with added thiols, ionizable at neutral pH, to generate NO.¹⁵ No reaction was seen with nonthiol reducing agents, some of similar electrode potential, including NADH, xanthine, hydroquinone, ascorbic acid, and glucose. The reaction is a thiol–disulfide equilibrium,¹⁶ yielding disulfides, episulfide, and unreacted starting material in the product mixture (Scheme 2a). The observation of the exclusive



requirement for added thiol, over other reducing agents, leans against a radical-initiated or outer sphere electron-transfer process.¹⁷ Nevertheless, 3e⁻ reduction of the nitrate functional group to NO does occur, and therefore a chemically reasonable mechanism must be proposed.

The reaction of organic nitrate with thiol to yield NO has been proposed to proceed via nucleophilic attack of thiol at nitrogen to yield a thionitrate ester (RSNO₂).^{5,18,19} We have previously shown that the hydrolytic reaction of a thionitrate ester yields NO plus thiosulfonate and thiosulfinate as products, probably via a caged radical pair rearrangement (Scheme 2b and Scheme 3, route b).¹⁹ Under these conditions, there is no disulfide product, but on addition of thiol,

(15) Rates for NO release (d[NO]/dt) from reaction of thiols (2 mM) with **4** (1 mM), measured under conditions given in Figure 1, relative to reaction with cysteine: cysteine (1.0 ± 0.07); thiophenol (2.2 ± 0.17); aminoethanethiol (0.86 ± 0.09); glutathione (0.67 ± 0.28); cysteine Me-ester (1.3 ± 0.1); penicillamine (0.38 ± 0.03); DTT (0.14 ± 0.01); mercaptosuccinic acid (<0.01).

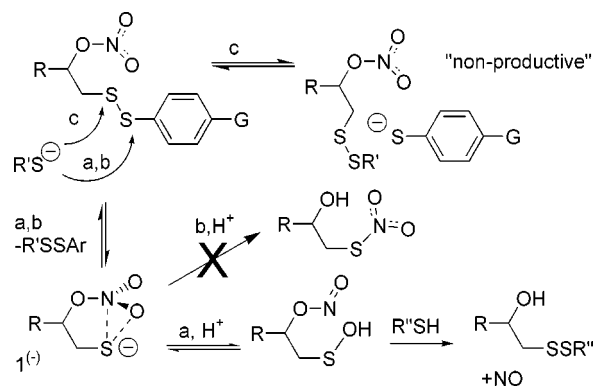
(16) Singh, R.; Whitesides, G. M. In *The Chemistry of Sulfur Containing Functional Groups*; Patai, S., Rappoport, Z., Eds.; Wiley: New York, 1993; p 633.

(17) Although the involvement of specific sulfur radicals generated from added thiol/thiolate is quite possible, for example, disulfide radical anion, thiyl, sulfonyl, sulfanyl and their peroxy radicals.

(18) Yeates, R. A. *Arzneim.-Forsch.* **1992**, *42*, 1314.

(19) Cameron, D. R.; Borrajo, A. M. P.; Bennett, B. M.; Thatcher, G. R. J. *Can. J. Chem.* **1995**, *73*, 1627; Artz, J. D.; Yang, K.; Lock, J.; Sanchez, C.; Bennett, B. M.; Thatcher, G. R. J. *Chem. Commun.* **1996**, 927.

Scheme 3^a



^a a, b and c refer to alternative reaction routes.

rapid nucleophilic attack on the thionitrate sulfur gives NO₂⁻ and disulfide as sole products, with no release of NO. This suggests that the thionitrate mechanism is unlikely, in a reaction that yields significant NO as product, in the presence of high thiol concentrations. An alternative proposal is required.

A mechanism may be written that proceeds, like the thionitrate mechanism, via an initial 2e⁻ reduction and thiolate nucleophilic attack on the nitrate group. Intuitively, the initial encounter of thiolate with nitrate via an nS⁻ → π*(ONO₂) interaction would be expected to involve nucleophilic attack at nitrogen. However, it is apparent from high level MO calculations that the lipophilic nitrate functionality contains little charge separation. If the initial interaction of thiolate HOMO with nitrate LUMO leads to S–O bond formation rather than S–N bond formation, two possible outcomes are (i) N–O bond cleavage to form the sulfenyl nitrite (RSNO) (not shown) or (ii) formation of both a sulfenate and a nitrite ester (Scheme 3, route a).

This mechanism, resulting from reaction of SS-nitrate with added thiolate to generate the conjugate base **1**⁽⁻⁾, is consistent with the observed products (Schemes 2 and 3) since (a) a sulfenate intermediate would be labile toward disulfide formation; and (b) nitrite esters are labile toward NO release in aqueous solution at a kinetically competent rate.⁶ Further, the nitrite ester from GTN (NGDN) has been shown to be especially reactive.²⁰ Alternatively, a nitrosothiol, a well studied NO donor, may be formed from reaction of thiol with the nitrite ester intermediate.^{2,21} The episulfide **2** and NO₃⁻ are expected products from cyclization of **1**⁽⁻⁾; thus this mechanism is compatible with the observed reaction products.

To test this mechanism, the reaction of **8** with cysteine was studied. The sulfenate mechanism requires intramolecular reaction of **8** involving attack of S on O via an unfavorable seven-membered ring; in contrast reaction by

(20) Buckell, F.; Hartry, J. D.; Rajalingam, U.; Bennett, B. M.; Whitney, R. A.; Thatcher, G. R. J. *J. Chem. Soc., Perkin Trans. 2* **1994**, 401.

(21) Williams, D. L. H.; Patel, H. M. S. *J. Chem. Soc. Perkin Trans. 2* **1990**, 37.

the thionitrate mechanism allows reaction via a favorable six-membered ring.²² NO is not detected as a product of this reaction by the electrochemical method, supporting the sulfenate mechanism (Scheme 3, route a) over the thio-nitrate mechanism (Scheme 3, route b).

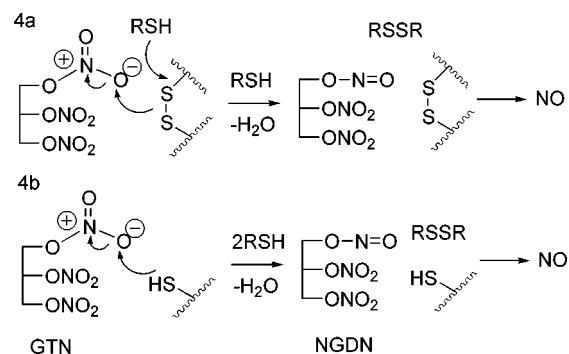
The mechanism proposed in Scheme 3 can also account for the observed rates of NO release (Figure 1). The initial attack of thiolate on the disulfide moiety of the SS-nitrate may be productive (route a) or “nonproductive” (route c). The effects of the ring substituent, G, will be counterpoised; an electron-withdrawing group will favor the nonproductive pathway but will also accelerate the overall rate of reaction with thiolate. Thus, **6** does not yield detectable levels of NO on reaction with cysteine because reaction is channeled along the “nonproductive” pathway, whereas **4**, **5**, and **7** all react to release significant quantities of NO (Figure 1).

Further study is required to define the mechanism of reaction of the SS-nitrates in particular and nitrates in general. However, the SS-nitrates represent the first biomimetic models for sulfhydryl-dependent biotransformation of an organic nitrate to NO. The rapid reaction of SS-nitrates with biologically important thiols generates NO, demonstrating that binding of GTN proximal to a cysteine residue (or bound GSH), at a hydrophobic site in a protein, may lead to biotransformation to NO (Scheme 4b). Binding may or may not be at an enzyme active site, but the presence of a basic residue, to generate the thiolate, will accelerate reaction. Furthermore, binding of GTN proximal to a protein disulfide in the presence of endogenous thiols provides a novel, potential biotransformation mechanism that is supported by this biomimetic model (Scheme 4a).

The design of nitrates that react with thiols in neutral aqueous solution to yield NO, at a significant rate, is an

(22) Organic nitrates have been reported in which cysteine is welded to an alkyl nitrate via an amide linkage: Liu, G. L.; Christopher, T. A.; Lopez, B. L.; Gao, F.; Guo, Y.; Gao, E.; Knuettel, K.; Feelisch, M.; Ma, X. L. *J. Pharmacol. Exp. Ther.* **1998**, *287*, 527. In these, thiol and nitrate groups are too far removed for favorable intramolecular reaction.

Scheme 4. Mechanisms for Sulfhydryl-Dependent Reaction of GTN with (a) Protein–Disulfide or (b) Protein–Thiol Yielding NO via an Organic Nitrite (NGDN)^a



^a Reaction to give 1,2-NGDN is statistically favored (shown), but reaction to give 1,3-NGDN is chemically favored (not shown)

important breakthrough. Nitrates are NO mimetics that may function as NO donors and also, if biotransformation proceeds via an intermediate such as a nitrite ester, as NO⁺ donors. Simple nitrates require biotransformation in order to release NO. The primary therapeutic activity of GTN as a venodilator must exploit, even though fortuitously, a selective biotransformation pathway. Opportunity exists to design nitrates as enzyme- and tissue-targeted NO mimetics and prodrugs, with structures engineered to optimize selectivity. The SS-nitrates represent one approach in this direction, with clear therapeutic potential already apparent.²³

Acknowledgment. NSERC Canada, GoBang Therapeutics Ltd., and Universities Medical Discoveries Inc. are thanked for financial support.

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