COMMUNICATION

Synthesis of novel organic nitrate esters: guanylate cyclase activation and tissue relaxation

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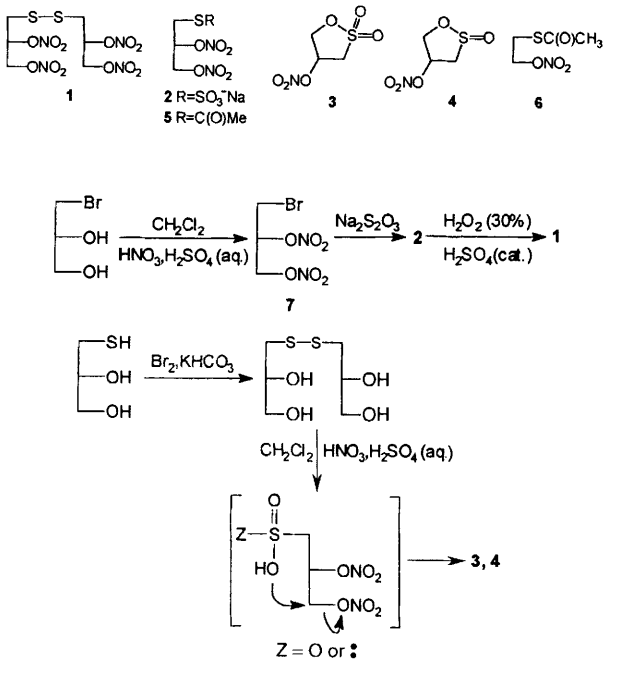
The syntheses of four novel sulfur-containing nitrate esters are reported, together with data for guanylyl cyclase activation and tissue relaxation.

We report the synthesis of four novel nitrate esters bearing a sulfur atom β to a nitrate group (**1**–**4**). These compounds are designed to function as nitrovasodilators and nitric oxide prodrugs based upon the mechanism of action of glyceryl trinitrate (GTN, nitroglycerin). Preliminary data on activation of soluble guanylate cyclase and tissue relaxation suggest that these compounds may represent a novel class of nitrate esters of potential therapeutic significance.

GTN has been in use since 1879 in the treatment of angina pectoris,[1] and is widely believed to exert its therapeutic effect through *in vivo* release of nitric oxide (•NO),[2–4] which itself has been identified as Endothelium Derived Relaxing Factor (EDRF).[5] A small number of simple organic nitrates in addition to GTN (*e.g.* isosorbide dinitrate) are effective and clinically important vasodilators.[6,7] Development of •NO prodrugs is the subject of substantial interest, stimulated by recent evidence suggesting many varied biological roles for NO, extending beyond vasodilation to immune function and neurotransmission.[5,8–11] Design and synthesis of novel nitrate esters may thus lead to new •NO prodrugs and nitrovasodilators that circumvent tolerance.[12]

A substantial body of evidence supports the hypothesis that the vasodilatory activity of organic nitrates is largely the result of activation of guanylate cyclase (GCase) leading to vascular smooth muscle relaxation.[2,7,13,14] GTN must undergo biotransformation *in vivo* and it is proposed that tolerance development may be associated with this need for biotransformation, the exact mechanism of which remains unresolved.[6] Proposed sulfhydryl-dependent pathways include enzymic and non-enzymic biotransformation by a thiol.[15–17] Indeed, the non-enzymic interaction of GTN with a limited range of thiols such as cysteine, *N*-acetylcysteine and thiosalicylic acid leads to activation of GCase with an EC_{50} in the submillimolar range *in vitro*.† Regardless of the exact mechanism of biotransformation of GTN *in vivo*, it may be postulated that if thiol functionalities are incorporated into the structure of nitrate esters, such molecules have the potential to activate GCase and release •NO without reliance on GTN biotransformation pathways. We have chosen to synthesize nitrate esters containing masked thiol groups as progenitors of such mercaptoalkyl nitrates and potential •NO prodrugs.

Three glycerol dinitrate derivatives, **1**, **2** and **5**, were chosen as target molecules. Synthetic difficulties in synthesis of



Scheme 1

polynitrate esters result from: (i) incomplete nitration with all nitration procedures except highly acidic and oxidizing nitrate–sulfuric acid biphasic mixtures,[18] and (ii) the greatly attentuated reactivity towards S_N2 substitution of α-nitro-substituted carbon. For example, the thioester **5** could not be synthesized by reaction of 1-haloglycerol-2,3-dinitrate with thioacetate ion under a variety of conditions and in the presence of crown ethers, although the mononitrate **6** was synthesized in this way, in good yield.

A route to the disulfide **1** was selected *via* the Bunte salt, **2** (Scheme 1). Synthesis of **2** proceeded from 3-bromopropane-1,2-diol (90 mmol) with nitration by dropwise addition into a cold mixture of nitric acid (68–70%, 4.0 equiv.) and sulfuric acid (95%, 4.0 equiv.) in CH_2Cl_2 (50 ml). After further reaction at room temp. for 30 min., the organic layer was separated, washed, dried and concentrated to yield a yellow oil, purified by silica gel flash chromatography to give 3-bromopropane-1,2-diol dinitrate **7** in 45% yield. The Bunte salt **2** was produced in 65% yield from the dinitrate, by reaction with an equimolar portion of $Na_2S_2O_3$ in 3:1 MeOH–H_2O at 50 °C for 10 h and subsequent purification by silica gel flash chromatography. Oxidation of **2** with a small molar excess of H_2O_2 (30%) in EtOH–H_2O (1:1) proceeded for 2 days with a catalytic amount of H_2SO_4. Extraction with CH_2Cl_2 and concentration gave the

† However, •NO release was not detected from GTN with nitrate and thiol concentrations of up to 25 and 100 $mmol^{-1}$, respectively employing a Clark-type •NO-selective electrode. Questions remain as to the function of GTN as an •NO prodrug.[4]

Table 1 Melting point, NMR, IR and mass spectral characteristics of nitrates **1–4**[a]

Compd.	Mp/°C	δ_H(ppm)[b]	δ_C(ppm)[b]	ν_{max}/cm^{-1}[c]	m/z (fragment, %)[d]
1	Liquid	5.43–5.55 (2 H, m) 4.84–4.93 (2 H, m) 4.60–4.69 (2 H, dd, *J* 6, 13) 2.97–3.16 (4 H, m)	77.08/77.00 69.33/69.29 37.05/36.89	1634, 1270, 1042, 995, 855	429 (M + Cl, 100), 393 (M − 1, 10)
2	86 (dec.)	5.75–5.80 (1 H, m) 4.99–5.07 (1 H, dd, *J* 3, 6) 4.77–4.86 (1 H, dd, *J* 6, 13) 3.20–3.23 (2 H, d, *J* 7)	79.02 70.97 32.04	1638, 1449, 1378, 1351, 1290, 1210, 1042, 654	323 (M + Na, 53)
3	65–66	5.80–5.87 (1 H, m) 4.67–4.75 (1 H, dd, *J* 5, 11) 4.50–4.57 (1 H, dd, *J* 2, 11) 3.69–3.80 (1 H, dd, *J* 8, 15) 3.35–3.44 (1 H, dd, *J* 3, 15)	76.95 69.88 48.66	1651, 1344, 1286, 1139, 937	218 (M + Cl, 100)
4	64–65	5.84–5.90 (1 H, m) 4.98–5.06 (1 H, dd, *J* 4, 12) 4.77–4.83 (1 H, d, *J* 12) 3.50–3.58 (1 H, dd, *J* 2, 15) 3.31–3.42 (1 H, dd, *J* 7, 15)	80.49 75.07 64.04	1649, 1339, 1287, 1122, 926	202 (M + Cl, 100)

[a] All compounds were characterized by elemental analysis or high resolution mass spectrometry, HPLC and NMR analysis for homogeneity. [b] $CDCl_3$ as solvent, except for **2** [$(CD_3)_2SO$]. *J* Values in Hz. [c] Run as KBr discs, except **1** which was run as a neat film. [d] All run as chemical ionization (CI, Cl^-) except **2** (ES^+, Na).

Table 2 Summary of guanylyl cyslase activation dose–response curves for nitrates **2–4** in the presence and absence of thiols (2 mmol l^{-1}) relative to GTN + 2 mmol l^{-1} cysteine[a]

	GTN + cys	**2** (no thiol)	**2** + cys	**2** + DTT	**3** + cys	**4** + cys	**4** + DTT
% Maximal response[b]	100 (5)	105 (12)	293 (15)	164 (23)	1087 (200)	98 (16)	174 (30)
Concentration at max. response (mmol l^{-1})[c]	1.0	0.5	5.0	5.0	1.0	5.0	1.5
GTN equivalence concentration (mmol l^{-1})[d]	1.0	0.4	0.7	2.0	0.1	5.0	0.8

[a] Average of 4–6 experiments using 2–3 separate preparations. Various nitrates gave no response above basal concentrations: GTN, GTN + DTT, **1**, **3**, **3** + DTT, **4**, glycerol-1,2-dinitrate + cysteine. Response to **1** (+DTT or cys) was discernible but < 20%. Several responses do not plateau, thus EC_{50} values cannot be quoted. [b] Maximal response to GTN + cys ranged from 360–540 pmol min^{-1} mg^{-1} in separate experiments and was set at 100%. Other responses relative to maximal GTN + cys response are expressed as percentages with standard errors. [c] Nitrate concentration at which maximal response is observed: concentrations did not exceed 5 mmol l^{-1} (1 mmol l^{-1} for **3**). [d] Nitrate concentration at which response reaches maximal response to GTN + cysteine (*i.e.* 100%).

tetranitrate **1** in 47% yield after purification by silica gel flash chromatography. The ^{13}C NMR spectra of **1** and **2** reveal 6 and 3 signals, respectively, as expected from the presence of two chiral centres in **1** and only one in **2** (Table 1). The ^1H NMR spectra of glycerol dinitrate derivatives (**1**, **2** and **7**) are of interest because of the large geminal coupling at the nitrated methylene and small or unobservable geminal coupling at the other methylene position (Table 1).[19]

It was anticipated that simple nitration of bis(2,3-dihydroxypropyl) disulfide **8** would result in sulfur oxidation and poor yields of **1**, owing to the highly oxidizing conditions of the medium. However, this alternative route was explored, using similar oxidation conditions to those employed above. Only small quantities of two organic nitrate-containing products were isolated from the organic layer of the biphasic nitrate medium. On chromatographic purification of **3** and **4** on silica gel, in yields of 5 and 10%, respectively, it was revealed that neither was the tetranitrate **1**. NMR spectra obtained for these products are highly solvent-dependent and are similar to those of the glycerol dinitrates, but with the significant difference that the large geminal coupling is associated with the upfield rather than the downfield methylene protons (Table 1).[19] Definitive structure identification rested upon mass spectral data: soft chemical ionization with Cl^- ion capture determined **3** and **4** to be the sultone and sultine, respectively. A simple cyclization in the nitration medium is likely (Scheme 1).‡ Durst and co-workers have previously isolated both diastereoisomers of a 4-phenyl-substituted sultine, whilst only one isomer of **4** was isolated.[20]

Activation of soluble GCase by nitrates **1–6** was assayed employing the radioimmunoassay method.§ Dose–response curves were obtained for GCase activation by nitrates **1–4** and GTN in the presence and absence of cysteine and dithiothreitol (DTT; both 2 mmol l^{-1}). The data from these curves are summarized in Table 2, which gives: (i) concentrations of nitrates required to give a response equivalent to the maximal response seen for GTN + cysteine, and (ii) the maximal response measured for each nitrate. The GCase assay data shows that dinitrate **2** activates GCase, with a submillimolar EC_{50}, in the absence of any added thiol, in contrast to GTN which requires added cysteine. Compounds **2** and **4** also activate GCase in the presence of DTT in contrast to GTN. Furthermore, nitrates **2** and **3** are seen to produce substantially elevated responses relative to GTN. The activity of the tetranitrate **1** is very low and entirely equivalent to glycerol-1,2-dinitrate in this assay. No activation of GCase by glycerol mononitrates (at ≤5 mmol l^{-1}) is observed in this assay.

‡ Several alternative cyclizations may be drawn including, most reasonably, intramolecular attack of the C-1 primary alcohol on sulfonyl or sulfinyl S with thiol displacement leading to **3** and **4** respectively.

§ Partially purified enzyme was freshly prepared from rat aorta homogenates. See ref. 21 for full experimental details.

In order to extend the GCase data, the relaxing effects of nitrates **2**, **3** and **4** on rat aortic tissue were examined.¶ Compared to the control experiments, in this intact tissue assay, all three nitrates were observed to cause significant tissue relaxation. The EC_{50} values for **2**, **3** and **4** were 3.94, 3.37 and 9.06 μmol l^{-1} respectively. In similar rat aorta relaxation assays, a nitrosothiol (Bu'SNO) and GTN itself were seen to give EC_{50} values of 5 μmol l^{-1} and 8.3 nmol l^{-1}, respectively.[22]

Three of the four sulfur-containing nitrates reported herein, two mononitrates (**3**, **4**) and one dinitrate (**2**), are shown to activate GCase at a higher maximal level than GTN. In addition, compound **2** does not require added thiol for activation. The significant relaxing effects of **2**, **3** and **4** on rat aortic tissue are compatible with the GCase activation data. The positioning of the sulfur atom β to the nitrate group allows intramolecular reaction of S with the nitrate N *via* a five-membered ring.[23] This design is founded upon a biotransformation theory for GTN in which the intermolecular reaction with cysteine results in formation of a cysteinyl thionitrate which may release •NO or activate GCase directly.[3,13,25] Mechanistic studies are required to determine if the pathways for GCase activation by compounds **1**–**4** involve such intramolecular activation. However, the potential for development of novel nitrate esters, including mono- and di-nitrates, with activity significantly different from glyceryl trinitrate itself is clearly demonstrated.‖

Acknowledgements

The financial support of the Heart & Stroke Foundation of Ontario, Grant #A2259 and the initial involvement of Dr Ralph Whitney are gratefully acknowledged.

¶ Thoracic aortic strips were prepared from male Sprague-Dawley rats (Charles-River, Canada) as described in ref. 22. Tissues were contracted submaximally with phenylephrine (0.1 μmol l^{-1}) and exposed to various concentrations of nitrovasodilator to obtain concentration-response curves.

‖ Two mononitrates which incorporate cysteinyl residues have been reported, however, (i) the S–N spacing does not allow rapid intramolecular reaction *via* a small ring ($\leqslant$6 atoms), and (ii) control compounds without S performed in a comparable fashion to the cysteinyl derivatives.[24]

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Paper 6/02080A
Received 25*th March* 1996
Accepted 2*nd April* 1996