# Reactivity of thionitrate esters: putative intermediates in nitrovasodilator activity

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***tert*-Butyl thionitrate decomposes rapidly in neutral aqueous solution to yield nitric oxide, sulfonyl and sulfinyl species, but does not activate soluble guanylyl cyclase.**

Organic nitrates, including isosorbide dinitrate and glycerol trinitrate (GTN), are known to be effective vasodilators.[1] Although GTN has been used since 1879 in treatment of angina pectoris, its mechanism of action is not fully understood.[2] Nitrate action is mediated by activation of guanylate cyclase (GCase), causing an increase in the intercellular levels of cGMP, leading to vascular smooth muscle relaxation, but the organic nitrate ester requires biotransformation. Evidence has been provided to support the theory that organic nitrates are biotransformed to nitric oxide. Several proposed mechanisms for a sulfhydryl-dependent biotransformation pathway involve thionitrate esters as intermediates, but no thionitrate ester has previously been examined in aqueous solution. Here we report a kinetic and product distribution analysis of the breakdown of *tert*-butyl thionitrate in aqueous solution for comparison with thermochemical calculations and enzyme activation data for soluble GCase.

It is believed that nitric oxide (·NO) is the endogenous endothelium derived relaxing factor (EDRF)[3] and most biotransformation pathways proposed for GTN require conversion to ·NO.[4] Sulfhydryl-dependent biotransformation pathways have been proposed involving either glutathione-*S*-transferase or non-enzymic biotransformation by a free thiol.[5] Certainly, *in vitro*, the interaction of GTN with cysteine leads to activation of GCase with a submillimolar $EC_{50}$ (*vide infra*). An early proposal posited a role for thiol in reaction with $NO_2^-$,[4a] but since conclusive debunking of this theory,[6] pathways have been proposed that almost exclusively require reaction of thiol with GTN to yield a thionitrate ester ($RSNO_2$). Liberation of ·NO from this thionitrate has been proposed to occur *via* conversion to either a nitrosothiol or sulfinyl nitrite.[4c,7] The rearrangement of thionitrate to sulfenyl (RSONO) or sulfinyl [RS(O)NO] nitrite is, indeed, chemically reasonable. However, high level MO calculations contraindicate a concerted rearrangement of thionitrate, although ·NO liberation from a sulfenyl nitrite is calculated to be facile.[8] Oae and co-workers have isolated a limited number of thionitrate esters, which with the exception of *tert*-butyl thionitrate, **1** are highly labile species.[9] We have prepared **1** and the corresponding nitrosothiol **2** (Bu$^t$SNO), in order to examine reaction in aqueous solution at physiological pH.‡

Decomposition of **1** is relatively rapid and pH-independent in the neutral pH region, Table 1. In order to determine the products of reaction, **1** (10 mmol $dm^{-3}$) was stirred for 10 min at room temperature in a 2:3 mixture of acetonitrile–aqueous phosphate buffer (pH 7.4, 50 mmol $dm^{-3}$, 0.1 mol $dm^{-3}$ KCl). After evaporation of the acetonitrile, extraction with diethyl ether, drying and concentration, the sole carbon-containing reaction products were identified as di-*tert*-butyl thiosulfinate **3** and di-*tert*-butyl thiosulfonate **4**, Scheme 1. Characterization of these products rests upon: (1) $^1$H NMR and $^{13}$C NMR shift values compared to literature values;[11]§ (2) GC–MS analysis; and (3) electrospray-MS (ESMS) analysis. GC–MS analysis revealed two parent ions at *m/z* 139 and 146, corresponding to *tert*-butyl sulfonic acid $+H^+$ and di-*tert*-butyl sulfone, respectively. These are reasonable high temperature, vapour phase products from isobutene and $SO_2$ expulsion from **3** and **4**, respectively. Indeed, the ESMS chromatogram revealed both **3** and **4** as the sole primary reaction products, at *m/z* 117 ($M + H^+$), 217 ($M + Na^+$) and 233 ($M + Na^+$), respectively. The intensity of signals in the $^{13}$C NMR product spectrum showed the sulfinate to be the major product by a factor of 4–9 in quadruplicate experiments. Furthermore, in the $^{13}$C NMR spectrum of the crude MeCN–buffer reaction mixture, the only signals present were those of **3** and **4**.

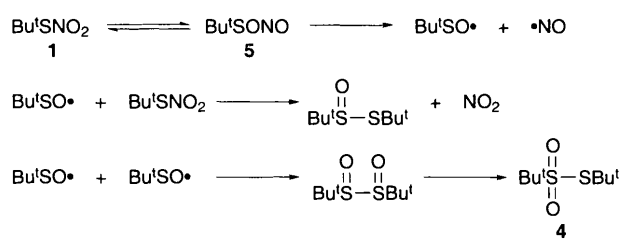
A Clarke-type ·NO-selective electrode was employed to detect ·NO. Substantial concentrations of ·NO were generated from **1** at pH 7.6 in 50 mmol $dm^{-3}$ phosphate buffer, Fig. 1. The maximal concentration of ·NO generated was observed to increase with increasing concentration of **1** and to decrease on addition of increasing concentrations of cysteine.

The spectral characteristics of **1** reported by Oae could conceivably be compatible with the sulfenyl nitrite isomer **5**, which would account for the rapid release of ·NO, since spontaneous release of ·NO from **5** would not be unexpected. The experimental IR spectrum was compared with IR frequencies and intensities for **1** and **5** obtained from normal mode vibrational analysis based upon MO calculations at the HF/6–31G* level. The reasonable correlation obtained is compatible with Oae's structural assignment and not with structure **5**.¶ Further MO calculations at the MP2/6–31G*//HF/6–31G* level were carried out to obtain energy, enthalpy and free energy values for homolysis of **1** and of **5**.[12] These MO calculations

**Table 1** Pseudo first order rate constants ($\times 10^4$ $s^{-1}$) for breakdown of **1**

| pH | 50 mmol $dm^{-3}$ buffer | 20 mmol $dm^{-3}$ buffer | 5 mmol $dm^{-3}$ buffer |
|---|---|---|---|
| 6.4 | 13 ± 1 | 9 ± 1 | 7 ± 1 |
| 7.0 | 16 ± 2 | 10 ± 2 | 7 ± 1 |
| 7.7 | 19 ± 2 | 10 ± 1 | 7 ± 1 |

Triplicate reactions monitored at 277 nm at 21 °C in phosphate buffer, EDTA (10 μmol $dm^{-3}$), KCl (0.1 mol $dm^{-3}$), **1** (50 μmol $dm^{-3}$).




**Scheme 1**

indicate a S–N bond dissociation free energy of 24 kcal $mol^{-1}$ for **1**, which is not incompatible with the observed stability on storage at low temperature (1 cal = 4.184 J). These calculations also predict the facile homolysis of **5** to give ·NO and sulfinyl radical (RSO·) ($\triangle G^{\ddagger}$ = 5 kcal $mol^{-1}$), which is favoured over homolysis to yield $NO_2$ ($\triangle G^{\ddagger}$ = 20 kcal $mol^{-1}$). Sulfinyl radical recombination to yield a thiosulfonate is well-documented.[11] Thus decomposition of **1** *via* rearrangement to **5** and homolysis would account for the observed products: **3**, **4** and ·NO, Scheme 1. Furthermore, when decomposition of **1** was allowed to proceed under identical conditions to those above, but in the presence of 50% labelled $H_2{}^{18}O$, no incorporation of $^{18}O$ was observed in either **3** or **4** by ESMS. This observation is compatible with unimolecular rearrangement of **1** and radical recombination, Scheme 1, but not with dissociation of **1** *via* bimolecular reaction with water and generation of $NO_2^-$ and RSOH. Both (i) the alternative heterolytic decomposition pathway *via* formation of the highly reactive nitronium ion and thiol and (ii) homolysis of **5** or **1** to yield freely dissociated thiyl radical and $NO_2$ are unlikely because of the absence of $Bu^tSH$ and $Bu^tSSBu^t$ as reaction products. The observed fall in ·NO release on addition of cysteine is compatible with the non-productive, competitive reaction with free thiol leading to disulfide formation [eqn. (1)].

$$RSH + Bu^tSNO_2 \rightarrow Bu^tSSR + NO_2^- + H^+ \quad (1)$$

Since thionitrate esters, in particular cysteinyl thionitrate, are proposed as organic nitrate biotransformation intermediates, GCase activation was examined in the presence of **1** by the radioimmunoassay method.[13] No activation of soluble GCase by **1** was detected, in the presence or absence of added thiols. In contrast, under these conditions, the $EC_{50}$ for nitrosothiol **2** was 20 μmol $dm^{-3}$ compared to 100 μmol $dm^{-3}$ for GTN + 2 mmol $dm^{-3}$ cysteine.‖ Furthermore, the maximal response to the nitrosothiol was considerably greater (×3.5) than that measured for GTN in the presence of cysteine. We suggest that the β-carboxylate group of cysteinyl thionitrate is essential for GCase activation by such thionitrate esters. However, the detection of substantial ·NO release, but the absence of GCase activation requires, further study. Preliminary experiments using the GCase assay have not provided evidence for enzyme inhibition by **1**.

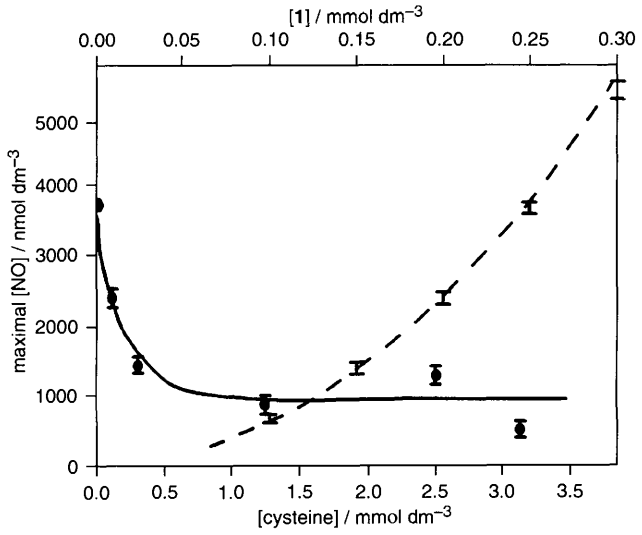



**Fig. 1** Maximal [·NO] generated: (*a*) from **1** (dashed line, upper *x*-scale); and (*b*) from **1** (0.25 mmol $dm^{-3}$) with added cysteine (solid line, lower *x*-scale). Thionitrate in MeCN (to < 5% of total vol.) was injected into pH 7.6 phosphate buffer (50 mmol $dm^{-3}$), EDTA (10 μmol $dm^{-3}$), KCl (0.1 mol $dm^{-3}$), 21 °C, under argon, but not in a pressurized or sealed vessel. Data are average of 2–4 runs. The rate of ·NO release was estimated from the maximal response, calibrated using *in situ* chemical ·NO generation from standard $KNO_2$–KI–$H_2SO_4$–$K_2SO_4$ solutions, according to World Precision Instruments Inc., Sarasota, Florida, USA. GTN + cysteine at concentrations up to 25 mmol $dm^{-3}$ gave no response above the detection limit of 5–10 nmol $dm^{-3}$ ·NO.

In summary, the thionitrate ester **1**, a simple example of a class of compounds of some biological significance, is observed to dissociate rapidly, in neutral aqueous solution, in a pH-independent manner, to yield ·NO and sulfinyl radical. Experimental observations and MO calculations are compatible with a mechanism *via* initial rearrangement to the sulfenyl nitrite **5** and subsequent rapid homolysis. Perplexingly, the addition of the thionitrate to GCase in aqueous solution does not lead to enzyme activation, whereas the corresponding nitrosothiol **2** is a potent activator. Given the proposed importance of thionitrates in biotransformation of nitrovasodilators, elaboration of this preliminary study is justified and in progress.

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## Footnotes

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‡ Compound **1** was synthesized according to the procedure of Oae *et al.*, using $NO_2$ as nitrating agent.[9] Compound **2** was synthesized according to the procedure of Field *et al.*, using acidified $NO_2^-$ as nitrosating agent.[10] Spectral characteristics for both compounds correspond to literature values.[9] By $^1H$ NMR **1** was homogeneous and **2** contained < 8% $Bu^tSH$ and $Bu^tSSBu^t$ by $^1H$ NMR.

§ $^1H$ NMR ($CDCl_3$) For **1** δ = 1.55; for **3** δ = 1.35, 1.53; for **4** δ = 1.44, 1.60. $^{13}C$ NMR ($CDCl_3$). For **3** δ = 24.2, 32.2, 48.7, 59.4; for **4** δ = 23.7, 31.5, 56.3, 68.0.

¶ Observed IR For **1** (neat)/$cm^{-1}$, 829(s), 1254(s), 1301(s) and 1525(vs); calculated IR for **1**/$cm^{-1}$, 803 (m, $NO_2$, b), 1324(s, $NO_2$, ss) 1335(s, $NO_2$, ss), 1527(vs, $NO_2$, sa); calculated IR for **5**/$cm^{-1}$, 795(vs, N–O, s), 895(s, $NO_2$, b), 1707(vs, N=O, s). Calculated frequencies were scaled by 0.84.

‖ Partially purified enzyme was freshly prepared from rat aorta homogenates and assay carried out according to ref. 13. Data presented are from dose–response curves obtained by duplicate analysis in at least two separate assays.

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