Pergamon

Tetrahedron 57 (2001) 7173–7180

TETRAHEDRON

Synthesis, electrochemical, and spectroscopic studies of novel *S*-nitrosothiols

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Received 4 April 2001; revised 5 June 2001; accepted 4 July 2001

Abstract—A series of *S*-nitrosothiol compounds, structurally related to the NO-donor *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP) that contain amidin groups were synthesised by *S*-nitrosation of the corresponding thiols and characterised. The kinetics of decomposition were investigated and showed that the two adenine-based thionitrites exhibited an unusual stability in aqueous solution compared to SNAP, suggesting that these compounds may complex the traces of free copper ions present in solution, which is known to catalyze the decomposition of thionitrites. The electrochemical behaviour of these compounds and their nitric oxide-releasing potential were studied by means of cyclic voltammetry techniques on mercury and glassy carbon electrodes.

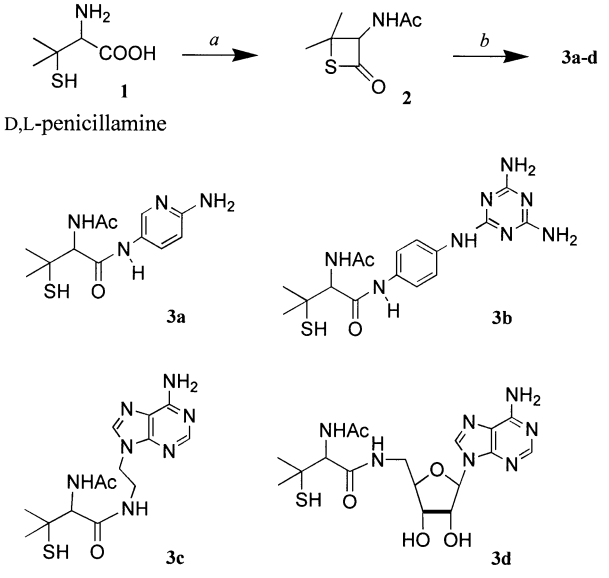
1. Introduction

A large body of evidence suggests that nitric oxide contributes to antiparasitic defence mechanisms during the infection, and the detrimental effect of NO$^{\cdot}$ from NO$^{\cdot}$-donors including *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP) on the growth of several parasites such as *Trypanosoma cruzi* or *Leishmania major* has become established.[1] This effect is mainly attributable to the near diffusion-controlled reaction of nitric oxide and superoxide ($O_2^{\cdot-}$) to form peroxynitrite ($ONOO^-$), a potent oxidant capable of generating highly reactive species.[2] In a preceding study,[3] we detailed the evaluation of a series of nitric oxide-releasing compounds that contained amidin groups for their ability to inhibit the trypanosomal P_2 transporter[4]-mediated uptake of adenosine on *Trypanosoma equiperdum*. These compounds were specifically designed to deliver nitric oxide into the parasite via this purine transporter, so accumulating and displaying its cytotoxic effect. The *S*-nitrosothiol (thionitrite) derivatives that bear a melaminyl, adenine or adenosine moiety were found to have a high affinity for the P_2-transporter suggesting that these recognition structures could be considered as an import signal for such drugs. In what follows, we extend this study to the details of the synthesis, the decomposition in aqueous solution and the electrochemical characterisation of these compounds.

2. Results and discussion

2.1. Synthesis

2.1.1. Synthesis of thiols 3a–d (Scheme 1). Commercially



Scheme 1. (a) Acetic anhydride, pyridine, 25°C, 20 h, 44%. (b) **3a**: 2,5-Diamino-pyridine, $CHCl_3$, 5 h, 25°C, 61%; **3b**: 2-(4-Aminophenylamino)-4,6-diamino-1,3,5-triazine (**5**), DMF, 25°C, 18 h, 30%; **3c**: 9-(2-Aminoethyl)-9*H*-purin-6-ylamine (**6**), $CHCl_3$/1N NaOH, 25°C, 2 h, 61%; **3d**: (i) 5′-amino-2′,3′-(*O*-isopropylidene)-5′-deoxyadenosine, CH_2Cl_2, 25°C, 5 h, 85%; (ii) $HCOOH/H_2O$, 25°C, 48 h, 100%.

Keywords: thionitrites; nitric oxide; purine; electrochemistry.

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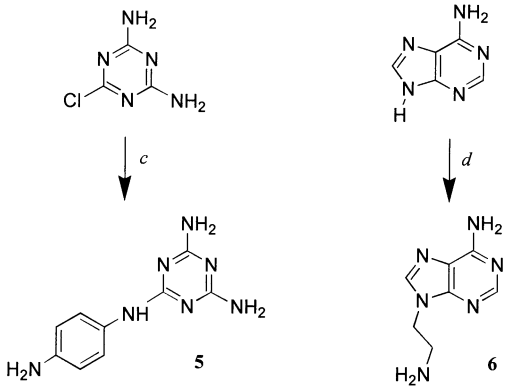
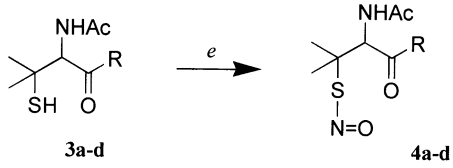
PII: S0040-4020(01)00694-9



Scheme 2. (c) (i) 4-Aminoacetanilide, H_2O, NaOH 1 equiv., 4.5 h, 100°C, 92%; (ii) aq. HCl, 1.5 h, 100°C, 37%. (d) (i) 2-Bromo-1-[(*tert*-butyloxycarbonyl)amino]ethane, K_2CO_3, DMF, 18 h, 25°C (59%); (ii) TFA, CH_2Cl_2, 25°C, 16 h, 100%.

available D,L-penicillamine **1** was converted to 3-acetamido-4,4-dimethylthietan-2-one **2** via cyclisation and concomitant acetylation reaction. This reaction provided a mixture of the desired thietanone **2** (yield: 44%) and of an apolar product corresponding to 4-isopropylidene-2-methyloxazol-5(4*H*)-one which were easily separated. This activation of penicillamine via its thietanone derivative had previously been described by Al-Zaidi et al.[5] and used by others for coupling to a number of nucleophiles.[6,7] Reaction of **2** with 2,5-diamino pyridine in chloroform at room temperature gave thiol **3a**. Thiol **3b** was synthesised from thietanone **2** and melaminyl derivative **5** (Scheme 2), which was obtained from 2-chloro-4,6-diamino-1,3,5-triazine and 4-aminoacetanilide and subsequent deacetylation. Reaction of **2** with adenine derivative **6** (Scheme 2) gave thiol **3c**. Thiol **3d** was obtained from 5′-amino-2′,3′-(*O*-isopropylidene)-5′-deoxyadenosine[8] and subsequent deacetylation.

2.1.2. Synthesis and spectral properties of *S*-nitrosothiols 4a–d (Scheme 3). *S*-Nitrosothiols **4a–d** were synthesised in mild conditions by electrophilic nitrosation of their corresponding parent thiols **3a–d**. These nitrosation reactions to obtain the thionitrites required an investigation of various methods. Nitroso groups are commonly introduced using either an organic nitrite or sodium nitrite in acetic conditions. Thionitrite **4a** was obtained by using *tert*-butyl nitrite in chloroform, while **4c** and **4d** were synthesised with the same nitrosating reagent, but in acetone as solvent. We used these two different solvents, which led to precipitation of the desired products. In contrast, attempts to synthesize **4b** by this method were unsuccessful. In that case, the use of sodium nitrite as nitrosating agent in acidic methanolic



3a-d **4a-d**

Scheme 3. (e) **4a**: (i) aq. HCl, ethanol, 2 h, 25°C; (ii) *tert*-butyl nitrite, chloroform, 35 min, 25°C (43%). **4b**: $NaNO_2$, CH_3OH, H_2SO_4, 1 M HCl, 15 min, 25°C (83%). **4c**: *tert*-Butyl nitrite, acetone, 30 min, 25°C (62%). **4d**: *tert*-Butyl nitrite, acetone, 30 min, 25°C (63%).

Table 1. NMR Characterization of *S*-nitrosothiols

	$\Delta\delta$ ($\delta_{4i}-\delta_{3i}$) (ppm)	
i	^{1}H NMR[a]	^{13}C NMR[b]
a	0.52–0.58	12.9
b	0.73–0.74	13.7
c	0.59–0.56	13.5
d[c]	0.60–0.60	13.3
	0.60–0.63	13.2
SNAP	0.67–0.68	12.7

[a] NMR-shifts of the two inequivalent *gem*-methyl groups recorded in D^6-DMSO for all compounds except **4b** in D^5-pyridin.
[b] NMR-shift of $(CH_3)_2$-*C*- recorded in D^6-DMSO for all compounds except **4b** in D^5-pyridin.
[c] Mixture of the two diastereoisomers.

conditions was preferable to the previous method, giving a yield of 83%. All thionitrites were stable at the solid stage and could be stored for long periods. They were characterised by NMR, UV–visible and IR spectroscopies. Compared to the parent thiols, the introduction of the nitroso group caused a high deshielding effect on the *gem*-dimethyl proton resonances, as well as a high downfield shift (about 13 ppm) observed on the carbon bearing the two methyl groups (Table 1).

2.2. Decomposition studies

Decomposition of *S*-nitrosothiol compounds occurs through a homolytic cleavage of the S–N bond as primary process to yield nitric oxide radical and the corresponding thiyl radical, which dimerizes to give the disulfide. The rate of decomposition is strongly dependent on the inherent structure of thionitrites. Most of them are relatively unstable at the solid stage or in solution to allow a full structural characterisation or a therapeutic use, but some of them including SNAP,[9] *S*-nitrosoglutathione (GSNO),[10] certain thiol proteins (such as *S*-nitrosoalbumine) or other structures[11] exhibit significant stability. The decomposition has been shown to be catalyzed by a number of parameters including metal ions, light, pH or temperature; thus as a consequence, stability studies must be investigated under strictly identical conditions so as to be compared. As thionitrites **4a** and **4b** were not water-soluble, we used a mixture of DMSO in a 100 mM phosphate buffer (pH 7.0) to study their decomposition. The kinetics were carried out spectrophotometrically following the disappearance of absorbances at 340 nm for **4b** and at 360 nm for **4a**, which exhibited a shoulder at around 400 nm. In these conditions, without addition of a metal

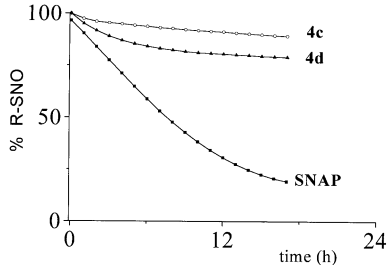


Figure 1. Comparative decomposition plots of SNAP, **4c** and **d** (2 mM) at pH 7.0 (100 mM phosphate buffer) at 25°C in the absence of EDTA.

ion chelator (such as EDTA), reasonable first-order plots were obtained with half-life values of around 3 h for both compounds, i.e. the same order of stability as SNAP in identical experimental conditions. Decomposition studies of thionitrites **4c** and **4d** were followed spectrophotometrically at 25°C under stirring by checking the decreasing absorbance at 340 nm in a 0.1 M phosphate buffer (pH 7.0) in the absence of EDTA. The kinetic experiments showed that these two adenine-related thionitrites were more stable than SNAP (Fig. 1), but unlike SNAP, typical first-order behaviours were not observed for these two compounds. The spontaneous first-order decomposition of SNAP is probably a reaction, which is catalyzed by traces of metal ions present in solution. Indeed, a number of reports demonstrated that the presence of metal ions (such as Cu^{+}, Cu^{++}) significantly accelerates the decomposition of certain thionitrites such as SNAP and its derivatives.[12] Nevertheless, this effect is less marked on *S*-nitrosoglutathione whose decomposition proceeds either by a transnitrosation reaction or by enzymatic cleavage of the peptide bond.[10] The Cu^{+}-catalyzed decomposition of *S*-nitrosocarboxylic acids including SNAP is thought to occur via an intermediate in which copper would be complexed via the carboxylate and the nitrogen of the S–N=O group.[13] In compounds **4c** and **4d**, as well as in other SNAP derivatives such as glyco-*S*-nitrosothiols,[7] the carboxylic group is involved in an amidic bond that should prevent copper coordination, and thus accounting for the higher stability of such derivatives compared to the parent molecule SNAP. On the other hand, based on the observation that copper(II) ions reversibly denature DNA, it has been shown that adenine can form cupric complexes,[14] suggesting that **4c** and **4d** could complex some of the free Cu^{2+} present in solution, thus increasing the apparent stability of these two adenine-based thionitrites. To further support this hypothesis, we used spectrophotometric measurements to evidence the formation of complexes on the parent molecules (compounds **3c** and **3d**) of the two thionitrites **4c** and **4d**. The changes in the absorption spectra were very similar for both compounds: λ_{max} and ϵ_{max} values significantly changed in acetonitrile when the thiols were in presence of 10 equiv. of copper ion (**3c**: 260 nm (11,750 M^{-1} cm^{-1}), **3c**/Cu^{2+}: 265 nm (5280); **4c**: 259 nm (12,300), **4c**/Cu^{2+}: 266 nm (7292)). Such variations were a clear indication of the involvement of the purine group in the coordination of copper, that might account for the higher stability of these two thionitrites.

2.3. Redox behaviour of SNAP and *S*-nitrosothiols 4a–d

The direct measurement of nitric oxide in solution from decomposition of NO-donors is difficult owing to its low concentration due to both a slow decomposition reaction and a high reactivity of the radical $NO^{\cdot}$ with a number of species. As a consequence of its redox properties, the electrochemical-based detection methods are preferred since they are suitable for real-time measurements of $NO^{\cdot}$ with adequate sensitivity.[15] The electrochemical behavior of $NO^{\cdot}$ was studied on glassy carbon and mercury surfaces by cyclic voltammetry. Nitric oxide is oxidised at glassy carbon (GC) or graphite electrodes leading to the formation of the nitrosonium cation (NO^{+}) at around +0.9 to +1.0 V (vs Ag/AgCl) in accordance with the equation:

$$NO^{\cdot} \leftrightarrow NO^{+} + e^{-} \qquad \text{(i)}$$

However, in aqueous solution the following equilibrium exists:

$$NO^{+} + H_2O \leftrightarrow HNO_2 + H^{+} \qquad \text{(ii)}$$

nitrous acid being the prevalent species in acidic diluted solutions.[16]

Hence, the overall electrode reaction leading to the NO_2^{-} formation in the phosphate buffer may be described by the equation:

$$NO^{\cdot} + H_2O \leftrightarrow HNO_2 + H^{+} + e^{-} \qquad \text{(iii)}$$

Nitrite, which is also a degradation product of $NO^{\cdot}$ in solutions containing oxygen, is electroactive at a very similar potential to $NO^{\cdot}$ and is converted in a further step to nitrate. To improve sensitivity and selectivity, modified electrodes with electropolymerised films of metalloporphyrins[17] or phthalocyanine[18] and surfaces covered with Nafion™ and/or cellulose acetate were employed. Nevertheless, the use of Nafion to suppress the oxidation of nitrite could lead to misleading results since, with relatively thick Nafion layers, the transport of $NO^{\cdot}$ to the electrode surface is restricted and lower currents are obtained. On the other hand, a direct reduction of $NO^{\cdot}$ on bare glassy carbon is not observed, except in the presence of electrocatalysts such as iron substituted polyoxotungstates[19] or iron–alizarin complexone.[20] Depending on the experimental conditions, the electroreduction of nitric oxide leads to the formation of ammonia or other intermediate compounds.

So as to check the redox response of thionitrites **4a–d** and obtain information about their $NO^{\cdot}$ generating properties, cyclic voltammograms on a wide potential window (−1500 to 1300 mV) were recorded using glassy carbon electrodes. Firstly, the glassy carbon electrode response was checked on pure $NO^{\cdot}$ solutions obtained by direct bubbling gaseous nitric oxide in phosphate buffer electrolyte (0.1 M, pH 7.0). Only one irreversible oxidation wave was observed at 1000 mV (Fig. 2). A linear relationship of peak current and concentration was obtained. Addition of nitrite ions to the solution resulted in the appearance of a peak at a more cathodic potential (830 mV). In contrast,

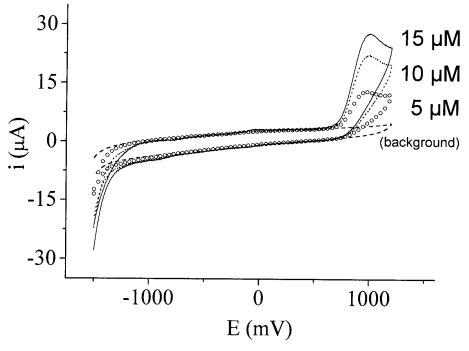


Figure 2. Oxidation of pure nitric oxide solutions at different concentrations on glassy carbon electrode in 0.1 M phosphate buffer pH 7.0.

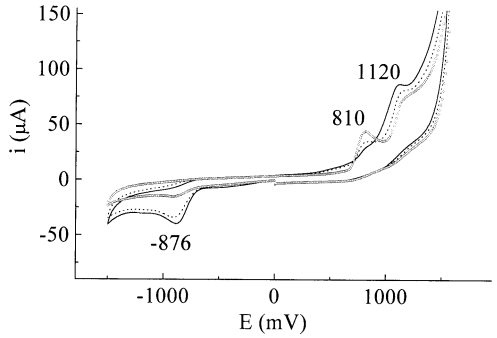


Figure 3. Time course of SNAP (1 mM) in 0.1 M phosphate buffer (pH 7.0), 10% DMSO on glassy carbon electrode at a scan rate of 100 mV s^{-1} (— 5 min; - - - 35 min; ○○○ 90 min).

addition of sodium nitrate, another oxidative product of NO· did not induce any detectable signal.

For comparison purposes, we used SNAP as a model compound. On a GC electrode, SNAP exhibited only a single reduction peak at −876 mV (vs Ag/AgCl, 3 M KCl) in phosphate buffer at a scan rate of 100 mV s^{-1} (Fig. 3). This peak was broad and has an irreversible nature, and the potential was found to be independent of solution pH. When the scan rate increased, the reduction potential shifted to more negative potentials indicating a slow heterogeneous electron transfer rate. To further elucidate the electron transfer mechanism of thionitrite decomposition, Wang et al.[21] carried out controlled potential electrolysis experiments and concluded that NO· was formed during the bulk electrolysis, and the amount of NO· generated correlated linearly with the applied potential between −800 and −1150 mV. They also determined that one electron *per* equivalent was transferred during the bulk electrolysis. Thus, on the basis of these previous studies, it can be concluded that this wave at −876 mV is due to the electrochemical cleavage of the S–NO bond. As time progressed, the wave diminished, revealing the spontaneous decomposition of SNAP (Fig. 3). This decrease correlated with the spectrophotometrically spectral change at 340 nm, associated with the first order decomposition reaction. On the oxidation side, two waves were observed which were not affected by pH changes: the wave at E_p=810 mV which increased with time corresponded to nitrite, and the second at E_p=1120 mV was due to NO· oxidation (Fig. 3).

On a mercury electrode, in addition to the cleavage of the S–NO bond (at E_p=−1140 mV), a reversible wave was observed at $E_{1/2}$=−336 mV at pH 7.0, which can be attributed to the formation of a mercury mercaptide (not shown). This was confirmed with *N*-acetyl-penicillamine which have a half wave potential of −338 mV in the same experimental conditions. This reversible wave shifted to negative potentials with increasing pH with a slope of −56 mV/pH, suggesting that one proton is involved in the reaction mechanism. The presence of mercury mercaptide accounts for a chemical rupture of the S–NO bond. Based on our experiments and literature results,[21] the following reaction mechanism is suggested:

$$\text{R-SNO} \rightarrow \text{R-S}^{\cdot} + \text{NO}^{\cdot} \quad (1)$$

$$2\text{R-S}^{\cdot} \rightarrow \text{R-SS–R} \quad (2)$$

$$\text{R-SNO} + e^- \rightarrow \text{R-S}^- + \text{NO}^{\cdot} \quad (3)$$

$$\text{R-S}^- + \text{H}^+ \leftrightarrow \text{R-SH} \quad (4)$$

$$\text{R-SH} + \text{Hg} \leftrightarrow (\text{HgSR})_{ads} + \text{H}^+ + e^- \quad (5)$$

Reaction (Eq. (1)) corresponds to the spontaneous homolytic cleavage of the S–N bond, which is in fact catalyzed by a number of parameters such as metal ions (Cu^+, Cu^{2+}), light, pH or temperature. The thiyl radical then dimerizes to give the corresponding dissulfide (Eq. (2)). The electrochemical reaction (Eq. (3)) results in the formation of nitric oxide and R-S^-, which protonates to form a free thiol (Eq. (4)). In presence of Hg, the thiol reacts to form the mercury mercaptide, which is reversibly oxidised or reduced electrochemically (Eq. (5)).

On these grounds, we then examined the electrochemical properties of thionitrites **4a**–**4d** by recording a series of cyclic voltammograms. As expected, subsequent scans of potential revealed that **4c** and **4d** exhibit a slower chemical decomposition process compared to SNAP, in accordance with the spectrophotochemical studies. Surprisingly, in these electrochemical experimental conditions (i.e. 10% DMSO in 0.1 M phosphate buffer, pH 7.0), **4a** and **4b** also exhibited a greater stability than SNAP, suggesting that their stability is highly affected by the solvent. Indeed, these two compounds were found to be much more stable in organic solvents such as methanol or DMSO. On the oxidation, a single process was observed for the four compounds corresponding to NO_2^- oxidation, in relation with the release of nitric oxide, and its subsequent conversion to NO_2^-. The NO· signal itself could not be observed, probably due to the small concentration reached. On the other hand, on the reduction side, small waves were observed on glassy carbon corresponding to the S–NO cleavage. This electrochemical reaction occurred at more negative potentials than SNAP in the same experimental conditions (around −900 mV, Table 2 and Fig. 4), and smaller current intensity were observed. These data are consistent with a greater stability of these compounds in the experimental conditions compared to SNAP. In addition, a wave at more positive potentials was observed for each compound **4a**-**4d** which might be attributed to their inherent structure or to oxygen traces present in solution (E_p=−610 mV).

Table 2. Electrochemical potentials of *S*-nitrosothiols, nitric oxide and nitrile ion

	Oxidation E_p (mV)	Reduction E_p (mV)
SNAP	810/1120	−876
4a	840	−644/−900
4b	696	−638
4c	897	−698/−962
4d	865	−596
NO·	950	–
NO_2^-	835	–

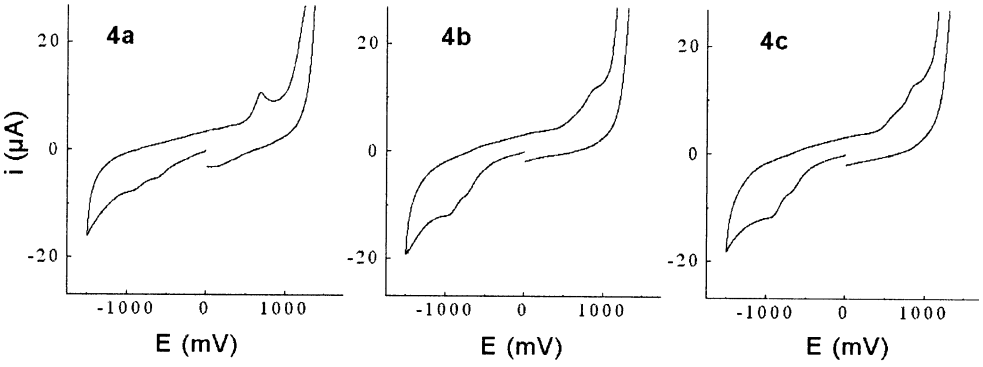


Figure 4. Cyclic voltammograms of compounds **4b–d** on glassy carbon electrode in 0.1 M phosphate buffer (pH 7.0) and 10% DMSO.

3. Conclusions

S-Nitrosothiols display a number of pharmacological effects, and their biological activity is mainly attributed to their capacity to release nitric oxide.[22] Moreover, there is an interest in their reactivity and they have been used as reagents in some chemical reactions such as disulfide formation[23] or decarboxylative nitrosation reactions.[24] Consequently, there is an increasing interest in the development of new nitric oxide-releasing compounds capable of generating $NO^{\cdot}$ quantitatively at a predictable rate. The present work deals with the preparation and the decomposition and electrochemical studies of new *S*-nitrosothiols which contain amidin groups that were designed for better targetting. All these compounds were prepared by electrophilic nitrosation of their corresponding parent thiols using either *tert*-butyl nitrite or sodium nitrite in acidic conditions. Two of these compounds (**4c** and **4d**), bearing purine moieties, exhibited a remarkable stability in aqueous solution compared to SNAP, probably due to their capacity to retain trace quantities of Cu^{2+} ions present in aqueous solution, as shown by spectroscopic studies. The electrochemical properties of the different compounds confirm this difference in stability of **4c** and **4d** compared to SNAP; results were similar on mercury and glassy carbon electrodes, demonstrating irreversible processes attributed to the electrochemical oxidation reaction of NO_2^- and to the electrochemical cleavage of S–NO bond. Beyond the specific interest of the physical properties of present nitrogen oxide derivatives, these results will help to define the best compromise between the relative stability of these NO donors in solution, and their ability to deliver this species within the cell in the presence of metal catalysts and/or under the action of enzymes. This aspect is at present being investigated.

4. Experimental

4.1. General

^{1}H NMR and ^{13}C NMR spectra were recorded on Brucker AC200, AC250 or AC300 spectrometers. Ultraviolet spectra were recorded on a HP8453 spectrophotometer (Hewlett Packard), and IR spectra on a Perkin–Elmer 1600 FTIR spectrophotometer. Silica gel 60 (70–230 mesh, Merck) was used for column chromatography and silica plates (60F254, Merck) were used for thin layer chromatography. All chemicals were obtained from Aldrich, and used without further purification. 2-Bromo-1-[(*tert*-butyloxycarbonyl)-amino]ethane was synthesised from 2-bromoethylamine hydrobromide and di-BOC dicarbonate. Elemental analyses were performed by the *Ecole Nationale Supérieure de Chimie de Toulouse, France*.

Electrochemical measurements were carried out on a BAS CV-50 Electrochemical Analyzer (Bioanalytical Systems Inc.) and a PAR model 273 Potentiostat/Galvanostat (EG&G Princeton Applied Research). A conventional three electrode-cell with a glassy carbon electrode (BAS, 2.5 mm ϕ) as working electrode, Ag/AgCl reference and platinum counter electrode was used. A phosphate buffer (0.1 M, pH 7.0) was used as supporting electrolyte. Compounds were dissolved in DMSO (0.5 mL) and this solution was added to 5 mL of supporting electrolyte. Solutions were deoxygenated by argon bubbling.

4.1.1. 3-Acetamido-4,4-dimethylthietan-2-one (2). To an ice-cooled suspension of (+/−) penicillamine (6.5 g, 43.5 mmol) in dry pyridine (25 mL), acetic anhydride (12.52 g, 123 mmol) was added for 30 min. The reaction mixture was stirred for 20 h at room temperature. The solution was dissolved in chloroforme (250 mL), washed with 0.1 M HCl, water and dried over magnesium sulphate. The solvent was evaporated to dryness and the residue was triturated with light petroleum ether and filtered to yield **2** as a pale yellow crystalline solid (3.31 g, 19.1 mmol, 44%). IR (KBr) 3053, 1758, 1658 cm^{-1}. ^{1}H NMR ($CDCl_3$, 250 MHz): δ 1.59 and 1.82 (s, 6H), 2.02 (s, 3H), 5.66 (d, J=8 Hz, 1H), 6.98 (Br, 1H). ^{13}C NMR ($CDCl_3$, 60 MHz): δ 22.4, 26.1, 30.2, 51.2, 76.4, 169.9, 193.3.

4.1.2. N^1-(6-Amino-3-pyridyl)-2-(acetylamino)-3-methyl-3-sulfanylbutanamide (3a). A solution of 2,5-diaminopyridine (0.155 g, 1.42 mmol) and 3-acetamido-4,4-dimethylthietan-2-one (0.295 g, 1.70 mmol) in chloroform (17 mL) was stirred for 5 h at room temperature. The solvent was evaporated to dryness and the residue was treated with a mixture of ethyl acetate and methanol (85:15). The solution was filtered and the filtrate was evaporated to dryness. The residue was purified by chromatography (ethyl acetate/methanol/32% aq. ammonium hydroxide, 85:15:0.25) to yield **3a** as a pink powder (0.245 g, 61%). ^{1}H NMR (D^6-DMSO, 250 MHz): δ 1.37

(s, 3H), 1.40 (s, 3H), 1.94 (s, 3H), 2.75 (s, 1H), 4.65 (d, *J*=9 Hz, 1H), 5.75 (s, 2H), 6.41 (d, *J*=9 Hz, 1H), 7.55 (dd, *J*=9, 1 Hz, 1H), 8.08–8.12 (m, 2H), 9.91 (s, 1H). ^{13}C NMR (D^6-DMSO, 50 MHz): 22.4, 28.7, 30.0, 46.2, 60.9, 107.4, 124.8, 130.4, 139.7, 156.5, 167.6, 169.4. Mass spectrometry FAB (glycerol/thioglycerol) *m/z* 283 $[M+H]^+$. Anal. Calcd for $C_{12}H_{18}N_4O_2S$: C, 51.04; H, 6.43; N, 19.84. Found C, 50.99; H, 6.41; N 19.81.

4.1.3. N^1-4-[(4,6-Diamino-1,3,5-triazin-2-yl)amino]phenyl-2-(acetylamino)-3-methyl-3-sulfanylbutanamide (3b). A solution of **5** (0.3 g, 1.38 mmol) and 3-acetamido-4,4-dimethylthietan-2-one (0.285 g, 1.65 mmol) in dimethylformamide (10 mL) was stirred overnight. The solvent was evaporated to dryness. The residue was triturated with hot ethyl acetate. The solution was filtered and the filtrate evaporated to dryness. The residue was purified by chromatography (dichloromethane/methanol/aq. 32% ammonium hydroxide, 90:10:0.25) to yield **3b** as a white powder (0.16 g, 30%). ^{1}H NMR (D^6-DMSO, 200 MHz): δ 1.39 (s, 3H), 1.43 (s, 3H), 1.96 (s, 3H), 2.73 (s, 1H), 4.70 (d, *J*=9 Hz, 1H), 6.19 (s, 4H), 7.45 (d, *J*=8.5 Hz, 2H), 7.67 (d, *J*=9 Hz, 2H), 8.03 (d, *J*=9 Hz, 2H), 8.7 (s, 1H), 10.01 (s, 1H). ^{13}C NMR (D^6-DMSO, 50 MHz): δ 22.4, 28.6, 30.1, 46.3, 61.1, 119.5, 119.7, 132.2, 136.5, 164.6, 167.0, 167.6, 169.4. Mass spectrometry (FAB, glycerol/thioglycerol) *m/z* 391 $[M+H]^+$. Anal. Calcd for $C_{16}H_{22}N_8O_2S$, 1.5 H_2CO_3: C, 43.47; H, 5.21; N, 23.18. Found C, 42.71; H, 5.67; N 22.98.

4.1.4. N^1-[2-(6-Amino-9*H*-9-purinyl)ethyl]-2-(acetylamino)-3-methyl-3-sulfanylbutanamide (3c). A solution of 3-acetamido-4,4-dimethylthietan-2-one (0.273 g, 1.58 mmol) in chloroform (1.8 mL) and a solution of **6** (1.26 mmol) in aq. NaOH 1N (2.7 mL) were stirred for 2 h. The aqueous layer was separated and evaporated to dryness. The residue was treated with a mixture of ethyl acetate/methanol/32% aq. ammonium hydroxide, 75:25:0.25. The solution was filtered, and the filtrate evaporated to dryness. The residue was purified by chromatography (ethyl acetate/methanol/32% aq. ammonium hydroxide, 75:25:0.25) to yield **3c** as a white powder (0.27 g, 61%). IR (KBr) 3293, 3210, 1654, 1602, 648 cm^{-1}. ^{1}H NMR (D^6-DMSO, 200 MHz): δ 1.23 (s, 3H), 1.26 (s, 3H), 1.91 (s, 3H), 2.69 (s, 1H), 3.51–3.53 (m, 2H), 4.21 (t, *J*=6 Hz, 2H), 4.38 (d, *J*=9 Hz, 1H), 7.21 (s, 2H), 7.95 (d, *J*=9 Hz, 1H), 8.02 (s, 1H), 8.13 (s, 1H), 8.31–8.34 (m, 1H). ^{13}C NMR (D^6-DMSO, 50 MHz): δ 22.4, 28.9, 29.7, 38.1, 42.4, 45.6, 60.8, 118.7, 140.9, 149.5, 152.2, 155.8, 169.3, 169.5. Mass spectrometry (FAB, glycerol/thioglycerol) *m/z* 352 $[M+H]^+$, 374 $[M+Na]^+$. Anal. Calcd for $C_{14}H_{21}N_7O_2S$, 1.5 H_2CO_3: C, 41.89; H, 5.44; N, 22.06. Found C, 41.01; H, 5.40; N, 22.06.

4.1.5. N^1-[(3*S*,4*R*)-5-(6-Amino-9*H*-9-purinyl)-3,4-dihydroxytetrahydro-2-furanyl]methyl-2-(acetylamino)-3-methyl-3-sulfanylbutanamide (3d). (i) To a solution of 5′-amino-2′,3′-(*O*-isopropylidene)-5′-deoxyadenosine (0.305 g, 1 mmol) in CH_2Cl_2 (12 mL) was added 3-acetamido-4,4-dimethylthietan-2-one (0.208 g, 1.2 mmol). After 5 h at room temperature, the solvent was evaporated to dryness. The residue was purified by chromatography (ethyl acetate/methanol, 95:5) to yield the 2′,3′-(*O*-isopropylidene) derivative of **3d** as a white powder (0.41 g, 85 %). IR (KBr) 1651, 649 cm^{-1}. ^{1}H NMR ($CDCl_3$, 250 MHz): δ 1.27, 1.34 (s, 3H), 1.30 (s, 3H), 1.42, 1.47 (s, 3H), 1.56 (s, 3H), 2.00 (s, 3H), 2.50, 2.58 (s, 1H), 3.32–3.48, 3.95–4.06 (m, 2H), 4.41–4.46 (m, 1H), 4.58, 4.68 (d, *J*=9 and 9.5 Hz, 1H), 4.78–4.84 (m, 1H), 5.26–5.34, 5.40–5.44 (m, 1H), 5.82, 5.88 (d, *J*=4 Hz, 1H), 6.79, 6.86 (s, 2H), 6.89–6.93 (m, 1H), 7.91, 7.88 (s, 1H), 8.38, 8.65 (s, 1H), 8.76, 8.90–8.93 (m, 1H). ^{13}C NMR ($CDCl_3$, 50 MHz): δ 23.2, 23.3, 25.1, 25.2, 27.4, 28.1, 29.0, 31.3, 31.6, 41.0, 41.5, 45.7, 46.0, 60.8, 81.6, 82.1, 82.5, 82.9, 83.1, 83.8, 92.1, 92.5, 114.6, 114.7, 120.7, 120.8, 140.3, 148.8, 153.1, 153.6, 156.0, 156.1, 170.1, 170.3, 170.7, 170.8. Anal. Calcd for $C_{20}H_{29}N_7O_5S$, 1.5 H_2CO_3: C, 46.57; H, 5.77; N, 18.10. Found C, 46.06; H, 5.84; N, 18.08.

(ii) A solution of the 2′,3′-*O*-isopropylidene derivative of **3d** (0.36 g, 0.75 mmol) in water (1 mL) and formic acid (1.5 mL) was stirred at room temperature for 48 h. The solvent was evaporated to dryness. Several co-evaporatings of the residue with ethanol afforded **3d** as a white powder (yield: 100%). ^{1}H NMR (D^6-DMSO, 250 MHz): δ 1.24, 1.30 (s, 3H), 1.32, 1.33 (s, 3H), 1.87, 1.92 (s, 3H), 2.68, 2.72 (s, 1H), 3.37–3.65 (m, 2H), 3.94–4.07 (m, 2H), 4.51–4.59 (m, 1H), 4.66–4.70 (m, 1H), 5.24 (s, 1H), 5.44 (s, 1H), 5.82, 5.86 (m, 1H), 7.31 (s, 2H), 7.95–8.07 (m, 1H), 8.24, 8.29–8.36 (m, 2H), 8.35–8.46, 8.54–8.65 (m, 1H). ^{13}C NMR (CD_3OD, 50 MHz): δ 22.6, 29.2, 29.7, 30.7, 31.1, 42.2, 42.6, 46.3, 46.4, 63.3, 63.5, 72.9, 73.1, 74.5, 74.6, 85.3, 85.4, 90.2, 90.9, 121.2, 142.4, 150.4, 153.8, 154.2, 157.3, 172.1, 173.2, 173.3. Mass spectrometry (FAB, glycerol/thioglycerol) *m/z* 430 $[M+H]^+$, 462 $[M+Na]^+$.

4.1.6. *S*-Nitrosothiol 4a. To a solution of **3a** (0.15 g, 0.53 mmol) in ethanol (3 mL) was added 12 M hydrochloric acid (0.044 mL). After 2 h at room temperature, the solution was filtered to yield the hydrochloride salt of **3a** as a white powder (0.1 g, 0.32 mmol). To a solution of **3a** hydrochloride in chloroform (4.5 mL) was added *tert*-butyl nitrite (2.08 g, 20.18 mmol). After 35 min at room temperature, the solution was filtered to yield **4a** (0.08 g, 43%). IR (KBr) 1657, 1531, 667 cm^{-1}. UV (50 mM phosphate buffer pH 7.4) λ nm (ϵ, M^{-1} cm^{-1}): 206 (7500), 234 (6930), 400 (sh). ^{1}H NMR (D^6-DMSO, 250 MHz): δ 1.89 (s, 3H), 1.98 (s, 3H), 2.03 (s, 3H), 5.40 (d, *J*=9.5 Hz, 1H), 7.06 (d, *J*=9.5 Hz, 1H), 7.96 (d, *J*=9 Hz, 1H), 8.41 (s, 1H), 8.69 (d, *J*=9 Hz, 1H), 10.94 (s, 1H). ^{13}C NMR (D^6-DMSO, 60 MHz): 22.2, 24.7, 26.4, 59.1, 59.5, 114.1, 124.9, 125.0, 137.6, 151.3, 167.6, 169.5.

4.1.7. *S*-Nitrosothiol 4b. To a solution of **3b** (0.1 g, 0.26 mmol) in methanol (1 mL), concentrated sulfuric acid (1 mL) and 1 M HCl (1 mL), was slowly added a 1 M aqueous solution of sodium nitrite (1 mL). After 15 min under vigorous stirring, the precipitate was filtered and washed with water to yield **4b** (0.090 g, 83%). IR (KBr) 1654, 1510, 668 cm^{-1}. UV (50 mM phosphate buffer pH 7.4/5% methanol) λ nm (ϵ, M^{-1} cm^{-1}): 212 (4380), 276 (3200), 340 (320). ^{1}H NMR (C_5D_5N, 300 MHz): δ 2.12 (s, 3H), 2.17 (s, 3H), 2.24 (s, 3H), 6.16 (d, *J*=9.5 Hz, 1H), 6.57 (Br, 6H), 7.89 (d, *J*=9 Hz, 2H), 7.97 (d, *J*=9 Hz, 2H), 9.53 (d, *J*=9.5 Hz, 1H), 10.74 (s, 1H); 11.73 (s, 1H). ^{13}C NMR (C_5D_5N, 75 MHz): δ 22.9, 25.6, 27.0, 60.0, 61.1, 121.2, 122.2, 134.6, 136.7, 164.4, 168.5, 170.4.

4.1.8. *S*-Nitrosothiol 4c. To a solution of **3c** (0.27 g, 0.77 mmol) in acetone (11 mL) was slowly added *tert*-butyl nitrite (4.91 g, 48 mmol). After 35 min at room temperature, the solution was filtered to yield **4c** (0.18 g, 62%). IR (KBr) 1700, 1655, 1512, 668 cm^{-1}. UV (50 mM phosphate buffer pH 7.4) λ nm (ϵ, $M^{-1}\,cm^{-1}$): 212 (26,460), 260 (18,920), 340 (480). ^1H NMR (D^6-DMSO, 200 MHz): δ 1.82 (s, 6H), 1.85 (s, 3H), 3.59–3.62 (m, 2H), 4.33 (m, 2H), 5.05 (d, J=9.5 Hz, 1H), 8.35 (d, J=9.5 Hz, 1H), 8.43 (s, 1H), 8.45 (s, 1H), 8.59 (m, 1H), 8.77 (Br, 1H), 9.55 (Br, 1H). ^{13}C NMR (D^6-DMSO, 50 MHz): δ 22.2, 24.6, 26.7, 38.2, 43.3, 58.8, 59.1, 117.9, 144.1, 144.6, 148.7, 148.8, 168.6, 169.2.

4.1.9. *S*-Nitrosothiol 4d. To a solution of **3d** (0.27 g, 0.61 mmol) in acetone (8.7 mL) was slowly added *tert*-butyl nitrite (3.93 g, 38 mmol). After 35 min at room temperature, the solution was filtered to give 0.18 g of **4d** (yield: 63%). IR (KBr) 1686, 1654, 1510, 669 cm^{-1}. UV (50 mM phosphate buffer pH 7.4) λ nm (ϵ, $M^{-1}\,cm^{-1}$): 210 (61,400), 258 (23,600), 338 (430). ^1H NMR (D^6-DMSO, 250 MHz): δ 1.84, 1.90 (s, 3H), 1.92, 1.96 (s, 3H), 1.95 (s, 3H), 3.37–3.50 (m, 2H), 3.99–4.10 (m, 2H), 4.60–4.65 (m, 1H), 5.22, 5.24 (d, J=9.5 Hz, 1H), 5.92–5.94 (m, 1H), 8.40–8.53 (m,2H), 8.67–8.73 (m, 2H). ^{13}C NMR (D^6-DMSO, 60 MHz): δ 22.4, 24.5, 24.7, 26.6, 40.0, 40.4, 58.8, 58.9, 59.6, 71.2, 73.2, 83.2, 83.5, 87.5, 87.6, 118.8, 119.0, 142.3, 146.6, 148.4, 148.5,; 151.0, 151.2, 168.5, 169.3.

4.1.10. 2-(4-Aminophenylamino)-4,6-diamino-1,3,5-triazine (5). (i) A solution of 4-aminoacetanilide (10.3 g, 68.7 mmol) and 2-chloro-4,6-diamino-1,3,5-triazine (10 g, 68.7 mmol) in water (250 mL) was heated to reflux. To this mixture, 2×17.5 mL of 2 M NaOH were added successively after 1 and 2 h, and heating was maintained for a total of 4.5 h. After cooling, the precipitate was filtered and washed with water to give 16.41 g of 2-(4-acetylaminophenyl)-4,6-diamino-1,3,5-triazine (92%). ^1H NMR (D^6-DMSO, 250 MHz): δ 2.00 (s, 3H), 6.28 (s, 4H), 7.40 (d, J=7 Hz, 2H), 7.65 (d, J=7 Hz, 2H), 8.75 (s, 1H), 9.77 (s, 1H). ^{13}C NMR (D^6-DMSO, 50 MHz): δ 23.8, 119.2, 119.8, 133.0, 136.0, 164.6, 167.0, 167.7.

(ii) A solution of 2-(4-acetylaminophenyl)-4,6-diamino-1,3,5-triazine (16 g, 62 mmol) in 1.2 M HCl (500 mL) was refluxed for 1.5 h. After cooling, 250 mL 2.5 M NaOH were added, and the precipitate was filtered and washed with hot methanol (150 mL). The filtrate was then evaporated to dryness under reduced pressure to yield 5 g of **6** (37%). ^1H NMR (D^6-DMSO, 250 MHz): δ 4.67 (s, 2H), 6.13 (s, 4H), 6.47 (d, J=7 Hz, 2H), 7.29 (d, J=8.5 Hz, 2H); 8.3 (s, 1H). ^{13}C NMR (D^6-DMSO, 50 MHz): δ 113.7, 122.0, 129.6, 143.5, 164.8, 167.0.

4.1.11. 9-(2-Aminoethyl)-9*H*-purin-6-ylamine (6). (i) A solution of adenine (2 g, 14.8 mmol), 2-bromo-1-[(*tert*-butyloxycarbonyl)amino]ethane (3.32 g, 14.8 mmol) and potassium carbonate (4.09 g, 29.6 mmol) in dry *N*,*N*′-dimethylformamide (60 mL) was stirred at room temperature for 18 h. After filtering, the solvent was evaporated to dryness under reduced pressure. The residue was triturated with water and filtered to give 2.4 g of 9-[2-(*tert*-butoxycarbonylamino)ethyl]-adenine as a white powder (59%). IR (KBr) 3364, 3148, 1686, 1598 cm^{-1}. ^1H NMR (D^6-DMSO, 250 MHz): δ 1.31 (s, 9H), 3.30–3.37 (m, 2H), 4.17 (t, J=6 Hz, 2H), 6.97 (t, J=6 Hz, 1H), 7.17 (s, 2H), 8.00 (s, 1H), 8.13 (s, 1H). ^{13}C NMR (D^6-DMSO, 50 MHz): δ 28.0, 38.1, 42.7, 77.7, 118.6, 140.9, 149.6, 151.9, 155.5, 155.6. Mass spectrometry (DCI/NH_3) *m*/*z* 279 $[M+H]^+$. (ii) A solution of 9-[2-(*tert*-butoxycarbonylamino)ethyl]-adenine (0.35 g, 1.2 mmol) in dichloromethane (4 mL) and trifluoroacetic acid (4 mL) was stirred at room temperature for 16 h. The solvent was removed by evaporation in vacuo and the remaining residue produced, after several co-evaporations with ethanol, 9-(2-aminoethyl)-9*H*-purin-6-ylamine **5** as a white powder (100%). ^1H NMR (D^6-DMSO+D_2O, 200 MHz): δ 3.38 (t, J=6 Hz, 2H), 4.50 (t, J=6 Hz, 2H), 8.34(s, 1H), 8.43 (s, 1H).

Acknowledgements

This work was supported by a CNRS-CONICYT international scientific co-operation program (8726). The authors would like to thank Dr Fethi Bedioui for fruitful discussions.

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