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# S,S′-dinitrosobucillamine, a new nitric oxide donor, induces a better vasorelaxation than other S-nitrosothiols

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ABSTRACT

S-nitrosothiols (RSNO) are considered as potential drugs for delivering nitric oxide (•NO) or related species in cardiovascular disorders associated with decrease in •NO bioavailability. We have synthesized a new RSNO, *i.e.* S,S′-dinitrosobucillamine ($BUC(NO)_2$), which combines in its structure two S-mononitrosothiols, S-nitroso-N-acetylpenicillamine (SNAP) and S-nitroso-N-acetylcysteine (NACNO). Synthesized $BUC(NO)_2$ was structurally characterized using high-performance liquid chromatography/mass spectrometry (HPLC/MS), $^1$H nuclear magnetic resonance ($^1$H NMR), infrared (IR) and UV–visible spectroscopies, and thermal analysis; resulting data are consistent with the expected structure. The vasorelaxant effect of $BUC(NO)_2$ was evaluated using isolated rat aortic rings and compared to SNAP, NACNO, and to an equimolar mixture of NACNO plus SNAP in order to mimic the number of •NO contained in a $BUC(NO)_2$ molecule. $BUC(NO)_2$ ($pD_2 = 7.8 \pm 0.1$) was more potent in vasorelaxation than NACNO ($pD_2 = 6.4 \pm 0.2$), SNAP ($pD_2 = 6.7 \pm 0.1$) and the mixture of SNAP plus NACNO ($pD_2 = 6.7 \pm 0.2$). The release of •NO from $BUC(NO)_2$ was 6-fold that of the basal value and significantly higher than the release of •NO from the SNAP plus NACNO mixture (4-fold increase *versus* basal value). Finally, the role of protein disulfide isomerase (PDI) in $BUC(NO)_2$ metabolism was investigated. Vasorelaxant effect ($pD_2 = 6.8 \pm 0.2$) and •NO release decreased in the presence of a PDI inhibitor (both $P < 0.05$ *versus* $BUC(NO)_2$). In conclusion, $BUC(NO)_2$ releases a larger amount of •NO into the aorta, partially through PDI activation, and induces vasorelaxation at lower concentrations than other RSNO previously reported.



## 1. Introduction

Nitric oxide (•NO) plays an important role in many physiological processes, especially in vascular tone homeostasis. In the vessel wall, •NO released from endothelial cells diffuses to the smooth muscle cells layer and activates cytosolic soluble guanylate cyclase (sGC) to produce cyclic guanosine monophosphate (cGMP), which in turn mediates smooth muscle cells relaxation (Arnold et al., 1977; Furchgott and Zawadzki, 1980). Decreased •NO bioavailability has been described under ageing and numerous pathological conditions, particularly in cardiovascular diseases (Förstermann and Münzel, 2006). •NO donors such as organic nitrates and sydnonimines are widely used as therapeutic agents (Thatcher, 1998). However, these treatments are known to provide a fast •NO release concomitant with the induction of oxidative stress and frequently tolerance (Bauer and Fung, 1991; Parker and Gori, 2001). S-nitrosothiols (RSNO) have been proposed as •NO donors because they do not present the drawbacks of organic nitrates. S-nitrosothiols are characterized by an *in vitro* half-life ranging from 10 to 38 h (Mancuso et al., 2003). In vessels, RSNO induce vasorelaxation (Alencar et al., 2003), prevent platelet aggregation (Furlong et al., 1987), and are currently thought to play a major role in the endogenous storage and transport of •NO (Al-Sa'doni and Ferro, 2004).

Most of RSNO previously synthesized are S-mononitrosothiols based on two different thiol moieties, either penicillamine or cysteine (Megson et al., 1997; Al-Sa'doni and Ferro, 2004). Currently,

*Abbreviations:* AlbSNO, S-nitrosoalbumin; BUC(SH)2, bucillamine; BUC(NO)2, S,S′-dinitrosobucillamine; BUC(NO)1, mononitrosobucillamine; cGMP, cyclic guanosine monophosphate; DAF-2, 4,5-diaminofluorescein; DAF-2 DA, 4,5-diaminofluorescein diacetate; DAN, 2,3-diaminonaphthalene; DSC, differential scanning calorimetry; ESI, electrospray ionization; FT-IR, Fourier transform infrared; GSNO, S-nitrosoglutathione; HPLC, high-performance liquid chromatography; L-AT, L-amino acid transport; MS, mass spectrometry; NACNO, S-nitroso-N-acetylcysteine; NMR, nuclear magnetic resonance; •NO, nitric oxide; ODQ, oxadiazolo [4,3-a] quinoxalin-1-one; PHE, phenylephrine; $pD_2$, negative logarithmic of drug concentration inducing 50% of maximum effect; PDI, protein disulfide isomerase; RSNO, S-nitrosothiol; SNAP, S-nitroso-N-acetylpenicillamine; SNOPC, S-nitrosophytochelatins; S.E.M., standard error of mean; sGC, soluble guanylate cyclase

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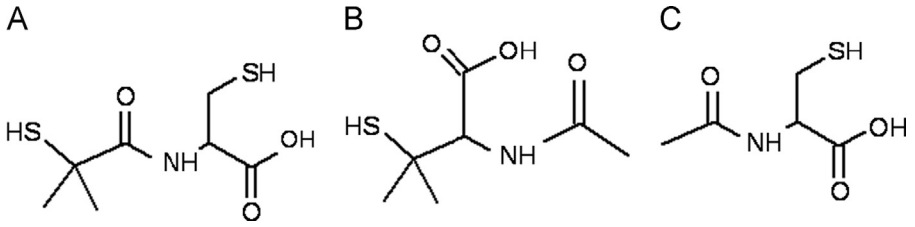


**Fig. 1.** Chemical structure of the different thiols presently studied for nitrosation: bucillamine (A), *N*-acetyl-D,L-penicillamine (B) and *N*-acetylcysteine (NAC) (C).

research focuses on the development of di- or poly-*S*-nitrosothiols in order to increase the payload of compounds releasing •NO, thus limiting the drug concentration.

We have presently selected the dithiol bucillamine ($BUC(SH)_2$) as an interesting •NO carrier because it combines in its structure *N*-acetylcysteine and *N*-acetyl-D,L-penicillamine moieties at the same time (Fig. 1). Moreover, $BUC(SH)_2$ is a commercially available drug used in rheumatoid arthritis; it presents anti-inflammatory and anti-oxidant properties through metallic ion chelation (Mazor et al., 2006), and it has undergone some preliminary investigations in experimental studies of ischemia–reperfusion (Amersi et al., 2002). We have investigated the vasorelaxant properties of $BUC(NO)_2$ using an *ex vivo* aortic ring model and compared them to those of the two constitutive *S*-mononitrosothiols, *S*-nitroso-*N*-acetylpenicillamine (SNAP) and *S*-nitroso-*N*-acetylcysteine (NACNO), and to an equimolar mixture of SNAP plus NACNO (which mimics the number of •NO moles contained in a $BUC(NO)_2$ molecule). We hypothesized that $BUC(NO)_2$ and SNAP plus NACNO would be more pharmacologically potent than the constitutive *S*-mononitrosothiols.

A further objective was to examine the denitrosation pathway of $BUC(NO)_2$. Most of the RSNO are unable to cross the cell membranes and require enzymatic transformations. Protein disulfide isomerase (PDI) is an important enzyme involved in the formation and breakage of disulfide bonds. In the endoplasmic reticulum, PDI is used to regulate protein folding through disulfide formation and correction (Wilkinson and Gilbert, 2004). PDI is also located at the cytoplasmic membrane and has already been pointed out in •NO release from RSNO (Xiao et al., 2005; Heikal et al., 2011). We investigated the contribution of PDI to the $BUC(NO)_2$-induced vasorelaxant effect in isolated rat aorta using bacitracin, *i.e.* a PDI inhibitor.

## 2. Materials and methods

### 2.1. Chemicals and standards

All reagents were of analytical grade and used without further purification. 4,5-Diaminofluorescein (DAF-2), 4,5-diaminofluorescein diacetate (DAF-2 DA) were obtained from Interchim (Montluçon, France). *N*-(2-mercapto-2-methylpropanoyl)-L-cysteine (bucillamine, CAS no: 65002-17-7) was purchased from Discovery Fine Chemicals (Wimborne Dorset, United Kingdom). SNAP, oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), bacitracin, carbachol, protein disulfide isomerase (PDI) from bovine liver, 2,3-diaminonaphthalene (DAN) and all other reagents were from Sigma–Aldrich (Saint Quentin Fallavier, France). Ultrapure deionized water ($>18.2\ M\Omega\ cm$) was used to prepare all solutions.

### 2.2. Synthesis and physico-chemical characterization of S-nitrosothiols

Nitrosation of $BUC(SH)_2$ was performed in acidic medium with a 2-fold molar excess of sodium nitrite using two different protocols (see online Supplementary material). Mononitrosobucillamine ($BUC(NO)_1$) and NACNO were obtained using similar conditions but with a $BUC(SH)_2$ and *N*-acetylcysteine to sodium nitrite ratio of 1:1. Due to the light-sensitivity of RSNO, all obtained solutions of RSNO were protected by aluminum foil.

Physicochemical characterization of $BUC(NO)_2$ was performed either on the oily product or on the unisolated form in solution obtained using the two synthesis protocols. Structure of $BUC(NO)_2$ was studied using high-performance liquid chromatography (HPLC) coupled with mass spectrometry (MS), $^1H$ nuclear magnetic resonance ($^1H$ NMR), Fourier transform infrared (FT-IR) and UV–visible spectroscopies. Thermal analysis was performed by differential scanning colorimetry (DSC) (see online Supplementary material).

The reaction yield of $BUC(NO)_2$ synthesis and the residual nitrite ion ($NO_2^-$) content in the reaction medium were measured using Saville-Griess and Griess methods, respectively (Bryan and Grisham, 2007). Calibration curves were performed in the range $10^{-6}$–$10^{-5}$ M, using SNAP and sodium nitrite as standards for Saville-Griess and Griess assays, respectively. Additional impurities ($BUC(NO)_1$, $BUC(SH)_2$ and $NO_2^-$) in $BUC(NO)_2$ solution which could result from the synthesis process were quantified by HPLC coupled with UV detection at 220 nm, using a calibration curve ($10^{-6}$–$10^{-5}$ M). The $BUC(NO)_2$ solution was injected at a concentration of $10^{-3}$ M (see online Supplementary material).

### 2.3. In vitro stability studies of S,S′-dinitrosobucillamine

The stability of a $10^{-2}$ M $BUC(NO)_2$ solution was monitored as a function of time in three different conditions of temperature and pH: (i) 4 °C, pH 7.4; (ii) 4 °C, pH < 0.5; and (iii) 37 °C, pH 7.4. Data were expressed as percentage of remaining $BUC(NO)_2$ concentration measured by monitoring the absorbance at 334 nm.

### 2.4. Biochemical and vascular studies of S-nitrosothiols

#### 2.4.1. Interaction of S-nitrosothiols with purified protein disulfide isomerase

Interaction between RSNO and PDI was first evaluated *in vitro* by measuring the kinetics of the reaction between $10^{-6}$ M PDI and $5\times10^{-6}$ M of each RSNO in Tris buffer ($10^{-2}$ M; pH 7.4) at room temperature ($22\pm2$ °C). The decrease of fluorescence intensity measured at an excitation wavelength of 280 nm and an emission wavelength of 340 nm was monitored for 30 min. The release of NO-derived species ($NO_X$: sum of $NO_2^- + {}^{\bullet}NO$ + other nitrogen oxides) occurring during this reaction time was determined by 2,3-diaminonaphthalene (DAN) assay, as previously described (Misko et al., 1993). The concentration of $NO_X$ was calculated using a calibration curve ($5\times10^{-8}$–$5\times10^{-7}$ M, fluorescence intensity measured at $\lambda_{exc}=375$ nm and $\lambda_{em}=415$ nm) built with sodium nitrite ($NaNO_2$) as the standard (see online Supplementary material). The experiments were repeated in the presence of $10^{-4}$ M bacitracin as PDI inhibitor.