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# SYNTHESIS OF NITRIC OXIDE RELEASING, VASODILATING AND PLATELET AGGREGATION INHIBITING S-[$^{15}$N]NITROSO COMPOUNDS

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## *Summary*

[$^{15}$N]Nitric oxide ($^{15}$NO) was produced in a "gas-tight" flask from [$^{15}$N]nitrite by reaction with iodide in acetic acid acidified water and purged for 60 min by a continuous nitrogen gas stream applied through an uncoated polytetrafluoroethylene flate membrane into a second flask which contained a methanolic solution of N-acetyl-L-cysteine or N-acetyl-DL-penicillamine. Analysis of these solutions by UV spectroscopy, reversed-phase high-performance liquid chromatography and capillary isotachophoresis showed formation of the corresponding S-nitroso compounds. Gas chromatographic-mass spectrometric analysis for [$^{15}$N]nitrite which was formed by dissolving these compounds in aqueous buffered solutions gave an isotopic purity higher than 95% at $^{15}$N. The S-nitroso compounds were shown to inhibit ADP-induced platelet aggregation.

## *Key words*

Nitric oxide (NO); [$^{15}$N]nitrite; $^{15}$NO; S-[$^{15}$N]nitroso-N-acetyl-L-cysteine; S-[$^{15}$N]nitroso-N-acetyl-DL-penicillamine; platelet aggregation

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## *Introduction*

The endothelium has been recognized for the first time by Furchgott and Zawadski to produce a relaxing factor that has therefore been named endothelium derived relaxing factor (EDRF) [1]. The nature of EDRF is still not clearly identified. NO-containing compounds such as S-nitroso-cysteine more closely resemble the vasorelaxant properties of EDRF [2] than the gaseous molecule nitric oxide (NO) [3]. This is supported by the recent discovery of low and high molecular weight S-nitroso compounds in human plasma which are proposed to be formed by reaction of NO with thiols [4]. Endogenous as well as exogenous S-nitroso compounds such as S-nitroso-L-cysteine and S-nitroso-N-acetyl-DL-penicillamine (SNAP) have been shown in vitro and in vivo to be highly potent vasodilating agents [4-6]. They exert a manifold higher vasodilatatory potency than NO itself and are also essentially longer-living than NO, thus acting possibly as transport carrier for NO [2-6]. Also, S-nitroso-N-acetyl-L-cysteine has been shown to inhibit platelet aggregation in vitro [7]. Despite enormous investigation on EDRF since its identification as NO or S-nitroso compounds the biology of NO-metabolism is still incompletely understood. The main problems in this area are arising from ubiquitously occurring non NO-derived nitrite and nitrate. The use of S-[$^{15}$N]nitroso compounds in combination with mass spectrometry would offer the unique possibility to study EDRF/NO-metabolism in man.

Synthesis of unlabelled S-nitrosothiols by the reaction of thiols with nitrogen dioxide in methanol [2] or with nitrite in aqueous solution [8] has been described. In this paper, we describe a convential procedure to synthesize S-[$^{15}$N]nitroso-N-acetyl-L-cysteine ([$^{15}$N]SNAC) and S-[$^{15}$N]nitroso-N-acetyl-DL-penicillamine ([$^{15}$N]SNAP) chosen as representatives for an endogenous compound and a pharmaceutical, respectively, starting from the corresponding thiols and $^{15}$NO produced from [$^{15}$N]nitrite. UV spectroscopy, reversed-phase high-performance liquid chromatography (RP-HPLC), capillary isotachophoresis (ITP) and gas chromatography-mass spectrometry (GC-MS) were applied to the analysis of the $^{15}$N-labelled S-nitrosothiols.

## *Experimental*

### *Materials*

S-Nitroso-N-acetyl-DL-penicillamine (SNAP; 98%) was obtained from Biomol (Hamburg, Germany). N-Acetyl-DL-penicillamine and pentafluorobenzyl (PFB) bromide were purchased from Aldrich (Steinheim, Germany). N-Acetyl-L-cysteine was from Sigma (Munich, Germany). Sodium nitrite, potassium iodide and acetic acid were obtained from Merck (Darmstadt, Germany). Sodium [$^{15}$N]nitrite (99% at $^{15}$N) was purchased from MSD Isotopes (Montreal, Canada).

### *Apparatus for production of $^{15}$NO and preparation of S-[$^{15}$N]nitroso compounds*

The apparatus used for production of $^{15}$NO and preparation of S-[$^{15}$N]nitroso compounds is shown schematically in Fig. 1. All solutions were freshly prepared and bubbled for 15 min with a stream of nitrogen gas. In flask A (5 ml) equipped with a rubber-stopper a solution of sodium [$^{15}$N]nitrite (200 mM) dissolved in double-distilled water (1 ml) was introduced via syringe A followed by introduction of glacial acetic acid (1 ml) under continous bubbling of nitrogen gas via syringe B. In flask B (3 ml) a methanolic solution of N-acetyl-L-cysteine (100 mM) or N-acetyl-DL-penicillamine (100 mM) were placed. Methanol was dried over mole sieve. Flasks A and B were connected via a Nalgene™ polytetrafluoroethylene (PTFE) flate membrane (0.2 μm pore size) with a polypropylene housing (Nalge Company, NY, USA). Reaction was started by introduction of a saturated solution of potassium iodide in double-distilled water (1 ml) in flask A via syringe A. By this procedure only gaseous materials ($^{15}$NO) reached flask B while contact of the liquids in flasks A and B was completely avoided. After 60 min of reaction the solutions placed in flasks B were transferred into silanized 1.5-ml glas flask and stored at -20°C without any other treatment. Aliquots from these solutions were analyzed as described below.

### *Reversed-phase high-performance liquid chromatography and UV spectroscopy*

RP-HPLC analyses were performed on an LKB solvent delivery system model 2150 coupled with a variable UV-VIS LKB detector model 2151 (Bromma, Sweden), and a Shimadzu integrator model C-R3A (Kyoto, Japan). The stationary phase consisted of a column (250 x 4.6 mm I.D.) packed with ODS Hypersil, 5 μm particles

size (Shandon, GB). The mobile phase consisted of acetonitrile/water, 20/80, v/v, and 10 mM potassium phosphate buffer the pH of which was adjusted to 2.2 by addition of phosphoric acid. The flow rate was 1.0 ml/min and the effluent was monitored at 340 nm. UV spectra were generated on a UVIKON 930 from Kontron Instruments (Zürich, Switzerland). Analytical capillary anionic ITP was carried out on an LKB tachophor, model 2127, fitted with a PTFE capillary (250 x 0.5 mm I.D.), a UV detector (254 nm filter), a conductivity detector and an LKB 2120 line recorder. The electrolyte system and the working conditions used are described elsewhere [9].

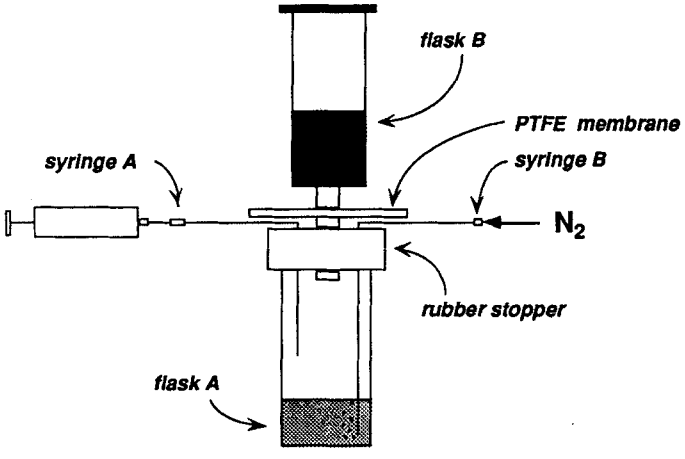


*Figure 1. Exprerimental set up used for the production of $^{15}NO$ and preparation of S-[$^{15}N$]nitroso compounds*

## *Results and discussion*

Original RP-HPLC chromatograms from the direct analysis of aliquots taken from the methanolic solutions of N-acetyl-L-cysteine and N-acetyl-DL-penicillamine are shown in Fig. 2a and Fig. 2b, respectively. Fig. 2c shows a chromatogram from the RP-HPLC analysis of the commercial unlabelled SNAP. In these figures the UV spectra generated from the corresponding RP-HPLC peaks are inserted.

Identical retention times on RP-HPLC, UV spectra with the characteristic maximum wavelength ($\lambda_{max}$) at 340 nm (Fig. 2), and identical reciprocal reference unit (RRU) values (mean ± SD, n=4) calculated relative to the terminating ion from the relative step-heights of the conductivity signals [10] of SNAP (RRU= 3.00 ± 0.01) and

the reaction product obtained from N-acetyl-DL-penicillamine and $^{15}$NO (RRU= 3.02 ± 0.02) strongly suggest the formation of S-[$^{15}$N]nitroso-N-acetyl-DL-penicillamine. The characteristic absorbance in the UV range with $\lambda_{max}$ of 340 nm (Fig. 2a) and the similar RRU value of 3.91 ± 0.04 (mean ± SD, n=4) to the compound formed by reaction of N-acetyl-L-cysteine and nitroprusside sodium (RRU= 3.95 ± 0.06; [9]) strongly suggest the formation of S-[$^{15}$N]nitroso-N-acetyl-L-cysteine.

GC-MS analysis of authentic S-nitroso compounds was not possible because of the thermal lability of these compounds. In order to determine the isotopic purity of the $^{15}$N-labelled materials their solutions in aqueous phosphate buffer were prepared and allowed to stand for 24 h at room temperature. Aliquots of these solutions were treated with PFB bromide as described previously in order to obtain the α-[$^{15}$N]nitrotoluene derivates [11]. In the negative-ion chemical ionization (NICI) GC-MS mass spectrum which was obtained from the PFB alkylation of [$^{15}$N]nitrite formed in the aqueous solution of S-[$^{15}$N]nitroso-N-acetyl-DL-penicillamine, the most intensive mass fragment was observed at m/z 47 ([$^{15}$N]nitrite) which is increased by 1 Da with

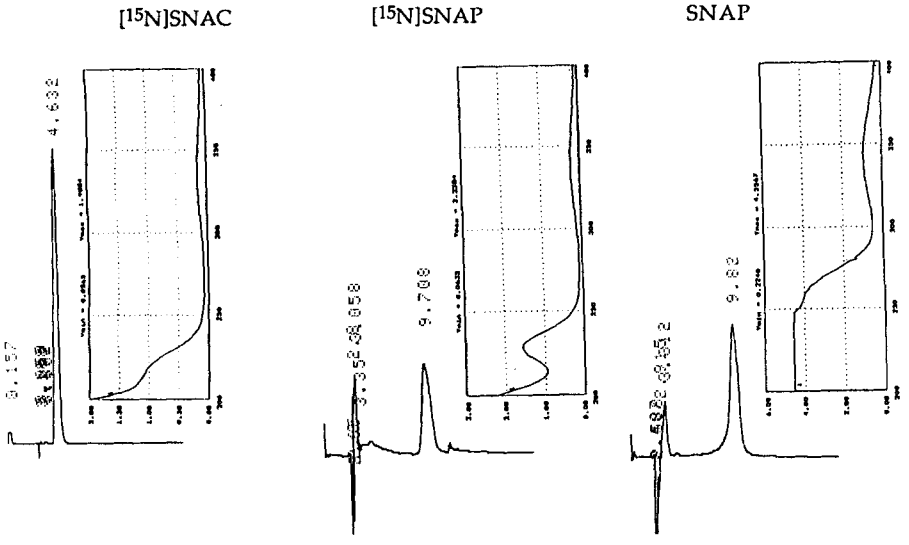


*Figure 2. RP-HPLC chromatograms obtained from separate analysis of the reaction mixtures of (a) N-acetyl-L-cysteine, (b) N-acetyl-DL-penicillamine and of (c) authentic commercial SNAP, and UV spectra from the collected RP-HPLC peaks. [$^{15}$N]SNAP, S-[$^{15}$N]nitroso-N-acetyl-DL-penicillamine; [$^{15}$N]SNAP, S-[$^{15}$N]nitroso-N-acetyl-cysteine*

respect to α-nitro-toluene derived from unlabelled nitrite. Less intensive signals were observed at m/z 227 ($[M-H]^-$), m/z 181 ($[PFB]^-$) and m/z 46 due to unlabelled nitrite present in the chemicals and solvents used. A similar mass spectrum was also obtained from the PFB alkylation of an aqueous solution of S-[$^{15}$N]nitroso-N-acetyl-L-cysteine. Selected ion monitoring at m/z 46 and m/z 47 in the NICI mode gave an isotopic purity of more than 95% at $^{15}$N for the two preparations. Based on the ITP analysis the yield of S-[$^{15}$N]nitroso-N-acetyl-DL-penicillamine was calculated to be 25%. Provided equal coefficients of absorptivity at 340 nm for all S-[$^{15}$N]nitroso-compounds prepared the yield of S-[$^{15}$N]nitroso-N-acetyl-L-cysteine was calculated to be 18%.

The methanolic solutions of S-[$^{15}$N]nitroso-N-acetyl-L-cysteine (rose-collored) and S-[$^{15}$N]nitroso-N-acetyl-DL-penicillamine (green-colored) were found to contain besides the oxidized thiols an approximate three-fold excess of unreacted thiols with respect to S-[$^{15}$N]nitroso compounds as measured by ITP analysis (not shown). S-[$^{15}$N]nitroso compounds were stable for at least two months when their methanolic solutions were stored at -20°C. The half-lives of both S-[$^{15}$N]nitroso-N-acetyl-L-cysteine and S-[$^{15}$N]nitroso-N-acetyl-DL-penicillamine in aqueous buffered solutions of neutral pH at room temperature were of the order of about 5 h as found by RP-HPLC.

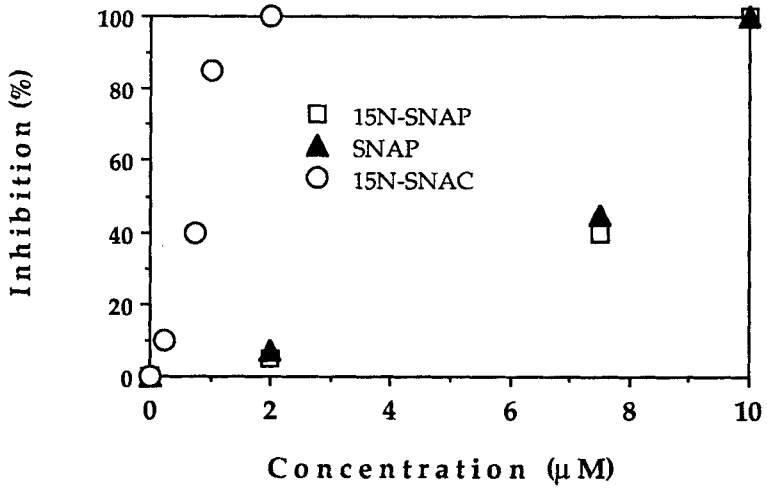


*Figure 3. Inhibitory potency of S-[$^{15}$N]nitroso-N-acetyl-DL-penicillamine ([$^{15}$N]-SNAP), SNAP and S-[$^{15}$N]nitroso-N-acetyl-L-cysteine ([$^{15}$N]-SNAC) on platelet aggregation. Aggregation was induced by ADP (10 μM) and measured according to a previously described method [12]*

The $^{15}$N-labelled compounds prepared here and the commercially available SNAP have been found to exert EDRF-like activity with respect to vasodilatation of bovine aorta and inhibition of platelet aggregation (Fig. 3). The higher inhibitory potency of S-[$^{15}$N]nitroso-N-acetyl-L-cysteine compared to S-[$^{15}$N]nitroso-N-acetyl-DL-penicillamine corresponds to the relative relaxation potency of these compounds on bovine coronary artery [8]. It is interesting that S-[$^{15}$N]nitroso-N-acetyl-L-cysteine, which is the N-acetylated analog of the endogenously produced S-nitroso-L-cysteine, exerts an inhibitory potency on platelet aggregation in vitro at concentrations that have been found in human plasma [4].

## *Conclusions*

We described in this paper a conventional method to prepare biologically active low molecular weight S-[$^{15}$N]nitroso compounds with high isotopic purity starting from commercially available materials. Both S-[$^{15}$N]nitroso-N-acetyl-L-cysteine and S-[$^{15}$N]nitroso-N-acetyl-DL-penicillamine release nitric oxide, relax bovine aorta and inhibit platelet aggregation. The $^{15}$N-labelled analogs of the endogenous S-nitroso-N-acetyl-L-cysteine and of the pharmaceutical agent SNAP should be useful in studying the metabolism as well as the pharmacological action of EDRF/NO and in quantifying the corresponding unlabelled compounds by mass spectrometric techniques.

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