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Synthesis and Chromatographic Determination of S-Nitrosopantetheine: Exploring Reactivity and Stability in Different Aqueous Solutions

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Abstract: S-nitrosothiols (RSNOs) are a group of sulfur-containing compounds biologically involved in nitric oxide (NO˙) release and signalling pathway. NO˙ plays important physiological and pharmacological activities, particularly in vasodilation and in inducing muscle relaxation. Several RSNO compounds have been detected in biological systems, and many of them have been chemically synthesized in the laboratory. To date, no works describing the synthesis of the S-nitrosopantetheine (SNOPANT) are reported in the literature. Taking into account that pantetheine is a biological thiol with a crucial function in metabolism, its nitrosylation in vivo could play a role in various metabolic signalling pathways. In this paper, the synthesis and the chromatographic determination of SNOPANT is reported for the first time, as well as a brief investigation of its reactivity in aqueous solutions in the presence of factors known to affect its stability.

Keywords: S-nitrosothiols; nitric oxide; pantetheine; synthesis and stability; sulfur-containing compounds; chromatography; S-nitrosopantetheine

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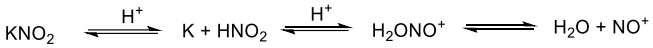
1. Introduction

Nitric oxide (NO˙) is an important gasotransmitter that, among other functions, can elicit smooth muscle relaxation through the activation of the soluble form of the enzyme guanylate cyclase (sGC) [1]. Nitric oxide activates sGC by binding to the ferrous form of a regulatory heme group on the enzyme, leading to a dramatic increase in the rate of cGMP formation [1]. An important component involved in NO˙ signaling is represented by S-nitrosothiols (RSNOs), which can be formed by the addition of a nitroso group to a sulfur atom of cysteine residues or of other biological thiols [2,3]. RSNOs have been shown to have the same physiological role as nitric oxide itself. As with NO˙, RSNOs play a role in host defense mechanisms, affecting both viruses and bacteria [4]. Levels of RSNOs in respiratory airways are affected by diseases such as asthma and pneumonia, and it is believed that RSNOs act as bronchodilators and relax bronchial smooth muscle and thus could potentially have a role in the treatment of these diseases. S-nitroso-glutathione (GSNO) is already being administered at lower concentrations than those required for vasodilation in coronary angioplasty clinical trials, where it can be used to reduce blood clotting. GSNO has also been successful in treating the hemolysis, elevated liver enzymes, and low platelets (HELLP) syndrome in pregnant women [5]. RSNOs, together with NO˙, have also been implicated in the suppression of HIV-1 replication through the S-nitrosylation of the cysteine residues in the HIV-1 protease [6]. All of these and other pharmacological features of RSNOs make

them ideal candidates for the development of novel drugs in different therapeutic fields. This notwithstanding, the most relevant pharmaceutical problem related to RSNOs' use as drugs is their poor stability in aqueous environment, which limits their potential as effective therapeutic agents [7–9].

Most RSNOs display a reddish to pink color due to their UV-visible absorbance at around 550–600 nm. This characteristic absorbance is often used to monitor their formation and decomposition [10]. Chemically, RSNOs can be easily obtained by treating thiols with nitrite in aqueous acidic media [11,12]. In this paper, we used this strategy to synthesize S-nitrosopantetheine (SNOPANT) for the first time, starting from potassium nitrite and pantetheine (Figure 1).



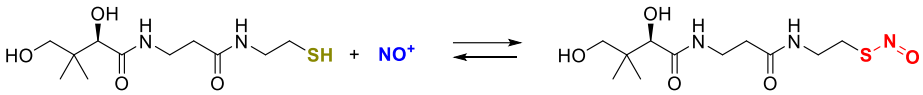


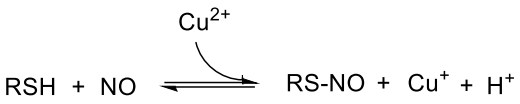
Figure 1. S-nitrosopantetheine formation from pantetheine and nitrite in acidic conditions.

Several RSNOs have been synthesized by this strategy, such as S-nitrosocysteine, S-nitrosocysteamine and GSNO, which are biologically active S-nitroso compounds [5,9,10,12,13]. To the best of our knowledge, the synthesis of SNOPANT has not been reported to date. Considering that pantetheine is a biological thiol with a crucial role in metabolism, both as the intermediate of the biosynthesis of Coenzyme A and as the most important form of storage of vitamin B5, its nitrosylation in vivo could play an interesting role in various metabolic signaling pathways. Our results are extremely important in view of future in vitro and in vivo studies involving SNOPANT in different biological systems. Furthermore, being a potential natural occurring nitrosothiol, this compound could be considered analogous to GSNO and useful in a clinical setting. However, considering the poor stability of RSNOs in water, our aim was to evaluate the best conditions for SNOPANT formation and stabilization in a simple aqueous system. It has been reported that two of the most important factors influencing RSNOs' formation and degradation are represented by the presence of copper (in different redox states) and by the effect of ascorbic acid/ascorbate [11,12,14,15]. It is well known that, in aqueous solutions, -SH groups are able to reduce Cu^{2+} to Cu^{+} via thiolate anion. Reduced copper is a crucial element that catalyzes NO$^{\bullet}$ release from RSNO (Scheme 1).

$$2Cu^{2+} + 2RS^{-} = 2Cu^{+} + RSSR$$
$$Cu^{+} + RSNO = Cu^{2+} + RS^{-} + NO^{\bullet}$$

Scheme 1. Copper catalyzed nitric oxide release from RSNO.

Conversely, Cu^{2+} catalyzes the scavenge of NO$^{\bullet}$ mediated by thiols, leading to RSNO formation (Scheme 2).



Scheme 2. Cu^{2+} mediated RSNO formation.

It is well known that ascorbic acid is able to reduce RSNOs with a rapid release of NO$^{\bullet}$. However, not all of the RSNOs can be reduced by ascorbate, while, under different conditions, ascorbate can reduce disulfides, maintaining thiol groups in their reduced form,

and thus favoring the reaction with NO• and the subsequent formation of RSNOs. In this paper, we report for the first time the synthesis of SNOPANT and evaluate the effect of ascorbate (as ascorbic acid or ascorbate sodium salt) and copper ions on the formation and stability of SNOPANT in order to pinpoint the most favorable experimental conditions to obtain the highest yield and stability of SNOPANT in aqueous solutions.

2. Results and Discussion

In this work different experimental conditions for SNOPANT formation and decomposition were explored. SNOPANT was synthetized using standard reaction conditions in acidic media and, afterwards, the effect of copper and ascorbate in different aqueous solutions was tested. Figure 2 reports the UV-Vis absorption spectrum of the SNOPANT synthesized with the standard protocol for RSNOs, displaying two λmax, with the first in the near UV at 340 nm and the second between 500 and 600 nm, conferring the typical pink-reddish color characteristic of the visible light absorption (Figure 2 inset).

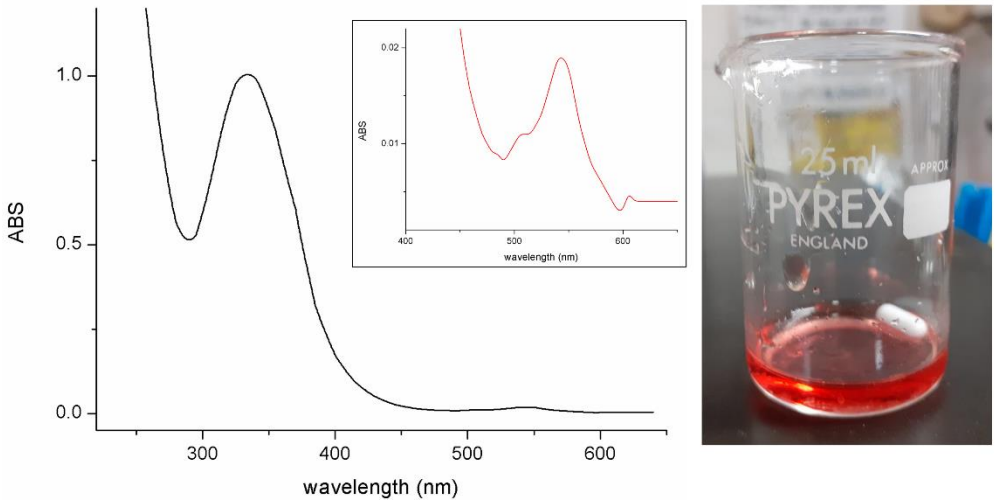


Figure 2. UV-visible absorption spectrum of S-nitrosopantetheine (**left**). Inset shows the peak at 550 nm in the zoomed area. Reaction occurs instantaneously with equimolar amounts of pantetheine and $NaNO_2$ in aqueous HCl at room temperature, giving rise to the typical pink-reddish color of -S=NO chromophore moiety (**right**).

To confirm nitrosylation and SNOPANT synthesis, we performed high-resolution (HRESIMS) and tandem mass spectrometry (MS/MS) analyses. The HRESIMS analysis in a positive ionization mode confirmed SNOPANT formation, showing a pseudomolecular ion at 308.1275 $[M + H]^+$ m/z with a corresponding molecular formula of $C_{11}H_{22}O_5N_3S$ in agreement with the theoretical molecular formula for the protonated SNOPANT (calc. for $C_{11}H_{22}O_5N_3S$ $[M + H]^+$, 308.1275 m/z, found 308.1275 m/z). The CID tandem mass spectrometry fragmentation of the precursor ion at m/z 308 $[M + H]^+$ (Figure 3) gives rise to the denitrosylated ion (pantetheine) at 278 m/z $[M + H]^+$ and other daughter ions according to the possible fragmentation of SNOPANT (290 m/z; 177 m/z and 148 m/z ions, Figure 3). Further MS/MS fragmentation of the 278 m/z ion generates a daughter ion at 247 m/z corresponding to the loss of the thiol moiety, and another daughter fragment at 149 m/z resulting from the C–N bond cleavage on the pantothenic moiety.

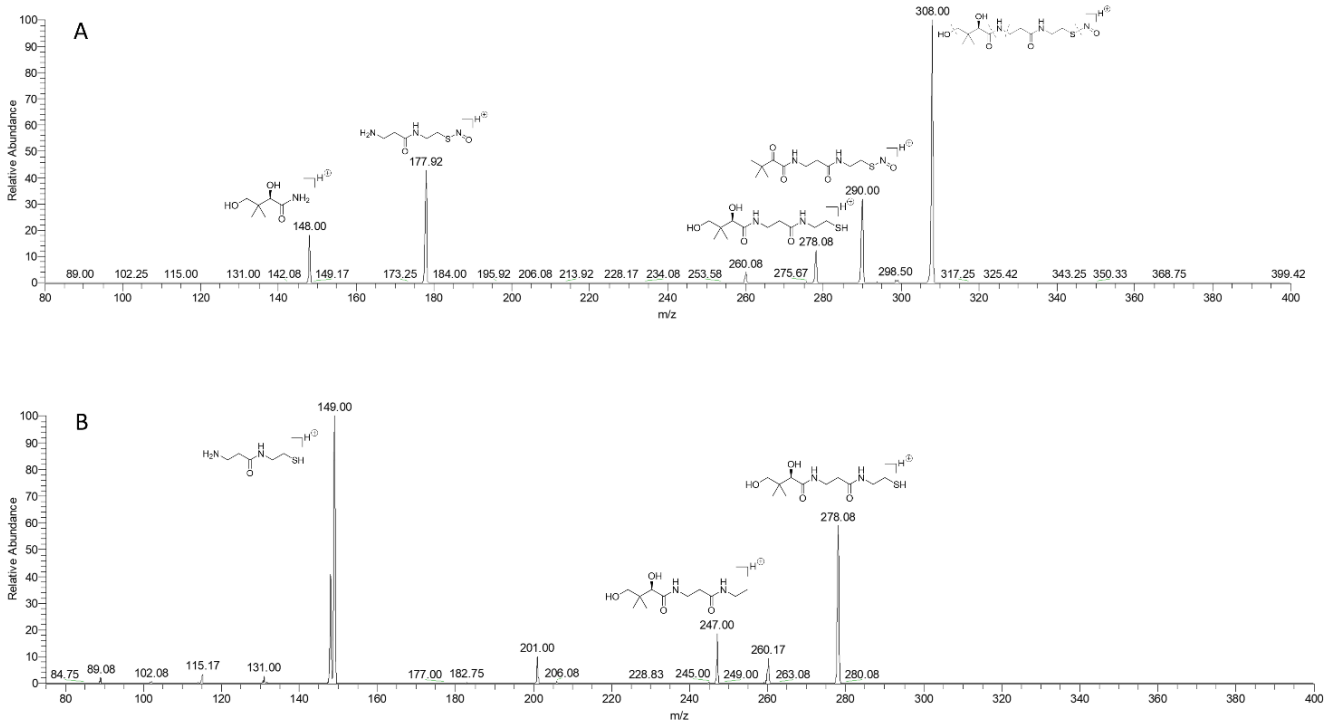


Figure 3. S-nitrosopantetheine MS/MS fragmentation patterns (positive ionization mode). Collision induced dissociation of the pseudomolecular ion of SNOPANT at 308 m/z (**A**) and MS/MS fragmentation [308→278] m/z of the denitrosylated ion at 278 m/z (**B**).

The high-performance liquid chromatography (HPLC) chromatographic profile and the respective calibration curves for SNOPANT are shown in Figure 4. The elution was performed isocratically on a C18 reverse phase column with an aqueous/organic mobile phase containing heptane sulfonic acid (see materials and methods section for details). In these conditions, SNOPANT shows a retention time of 6.4 min.

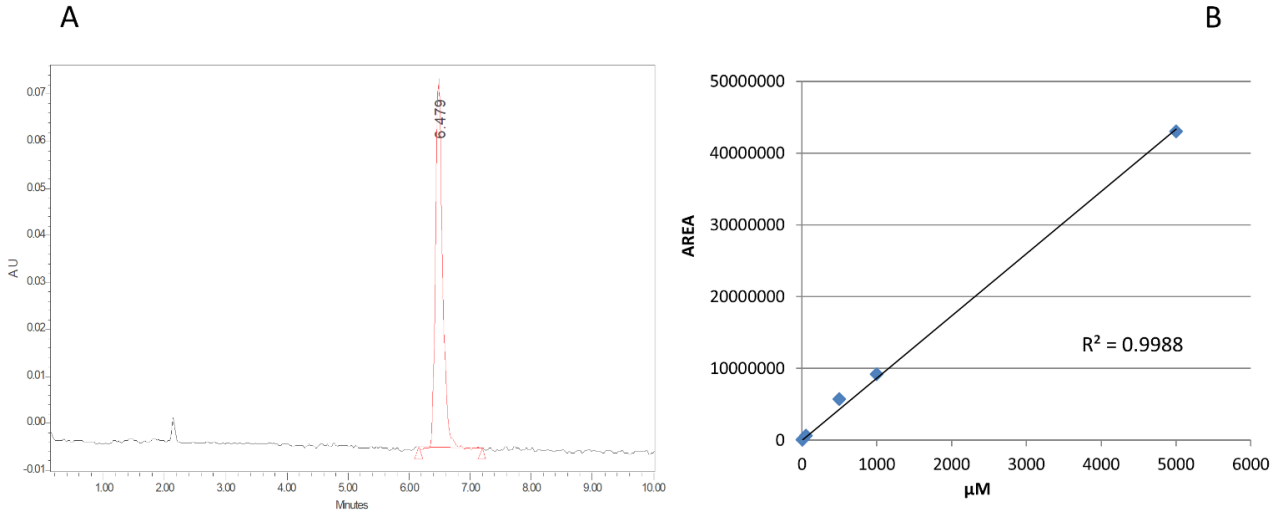


Figure 4. RP-HPLC representative chromatogram of SNOPANT (**A**) and calibration curve (**B**). The elution is carried out isocratically with sodium phosphate containing heptansulfonic acid and acetonitrile as organic solvent (detection wavelength 340 nm).

Given the high instability of nitrosothiols in solution, we evaluated the formation and decomposition of SNOPANT in different aqueous environments. We tested the effect of ascorbic acid (or sodium ascorbate) on the S-nitrosylation rate of pantetheine and its stability

at room temperature. Figure 5 shows the formation of the SNOPANT in two aqueous solutions containing the same amount of ascorbic acid (solution *a*) or sodium ascorbate (solution *b*).

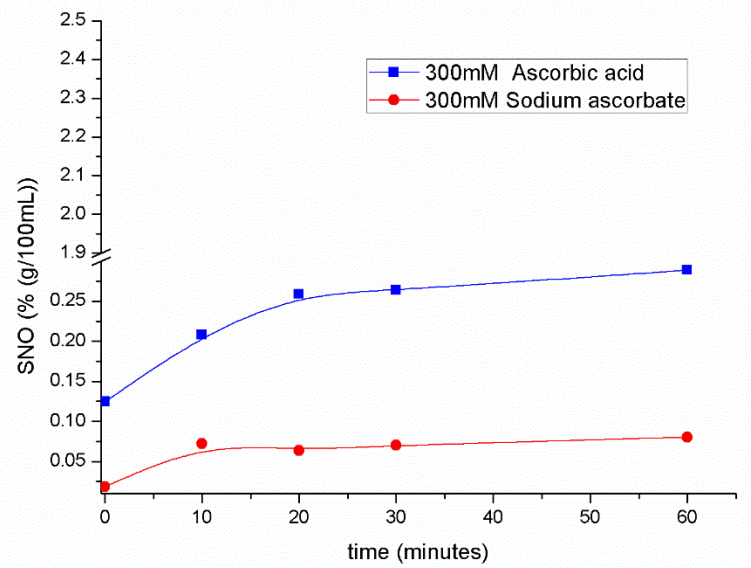


Figure 5. SNOPANT formation during 60 min in the solution (*a*), blue line, and solution (*b*), red line. PANT and $NaNO_2$ were added simultaneously as the last components in equimolar amount with a final theoretical product concentration of 2.5% (w/v).

As shown in Figure 5, the reaction yield is very low for both solution (*a*) with ascorbic acid and (*b*) with sodium ascorbate compared to the expected theoretical value. In the two solutions, the nitrosylation rate is slow and, after 20 min, reaches a plateau with a small increase in production within one hour. The use of the ascorbic acid provides better results than the respective sodium salt in terms of product formation; however, the reaction yield is still less than 10% of the theoretical value within the first 10 min. As for the sodium ascorbate solution, the little amount of SNOPANT produced after 20 min seems to remain stable at the same concentration within 60 min of the reaction. This can be explained by the fact that the use of ascorbic acid instead of the ascorbate can prevent the oxidation of the SH moiety due to a slightly acidic condition that can also favor the S-nitrosylation process. Conversely, it is known that RSNOs can react with ascorbic acid in aqueous solution following two distinct routes. At low concentrations, ascorbic acid can produce nitric oxide and disulfide in a Cu^{2+}-mediated reaction, whereas, at high concentrations, it can act as a nucleophile and undergo electrophilic nitrosation by nitrosothiols, leading to $NO^{\bullet}$ and thiol formation in a reaction that is not affected by the presence of copper.

Catalytic traces of Cu^{2+} are present in ultrapure bidistilled water, and this is one of the reasons for the poor stability of nitrosothiols in nonacidic aqueous solutions. Copper(II) ions are reported to be crucial for RSNOs' formation and degradation. It is reported that Cu^{2+} is reduced to Cu^{+} by -SH groups and that the metal remains in the reduced form during -SH oxidation to S–S. Cu^{+} is the real agent that drives the catalytic $NO^{\bullet}$ release from the RSNO moiety, meaning that, in aqueous non-buffered pH solutions, -SH/S- group can reduce Cu^{2+} ions and generate Cu^{+} ions spontaneously in a catalytic amount sufficient for the rapid $NO^{\bullet}$ release from S-nitrosothiols. While it is known that Cu^{+} ions can catalytical break the S–NO bond, it is also known that RSNO formation is favored by the presence of Cu^{2+} ions. The redox state of copper determines either the $NO^{\bullet}$ release (Cu^{+}) from S-nitrosothiols or the $NO^{\bullet}$ scavenging and incorporation by thiol groups (Cu^{2+}).

Afterwards, we evaluated the formation/degradation of SNOPANT in the same experimental conditions; however, we did this in the presence of Cu^{2+} ions such as copper sulfate to verify the simultaneous effect of ascorbate and copper.

Figure 6 reports the formation/decomposition rate of SNOPANT within 30 min of the reaction in the other two solutions. Solution (*c*) contains 6% ascorbic acid and 0.16% copper sulfate, and solution (*d*) contains 300 mM sodium ascorbate and 10 mM of copper sulfate. Compared to the previous experiments, it is important to notice the kinetics of the reactions since the yields remain almost negligible compared to the complete SNOPANT theoretical production of 2.5% (w/v). In these two conditions, the complete S-nitrosylation of pantheteine is inhibited; however, the reactions seem to follow an opposite trend of formation/decomposition in respect to the solutions (*a*) and (*b*), enhancing the effect of denitrosylation with respect to nitrosylation. These results are in good agreement with the data reported in literature for other RSNOs. The ascorbate can act directly on an RSNO by determining the release of NO, and can simultaneously reduce Cu^{2+} to Cu^{+}, which will amplify the already existing effect of ascorbate on degradation. The use of ascorbic acid should also reduce the S–S groups oxidized by Cu^{2+} favoring a reduced thiol form. We focused on the concentrations of ascorbate and copper, noting that, both in the first two solutions (*a* and *b*) and in the second ones (*c* and *d*), the amount of ascorbate was enormously higher than that of copper, especially considering that fact that catalytic traces of copper are present in ultrapure water. With all these considerations, and taking into account the catalytic nature of these two processes, we decided to lower the concentration of both components and, above all, to invert the molar ratios between ascorbate and copper (1 mM copper sulfate and 0.3 mM ascorbic acid).

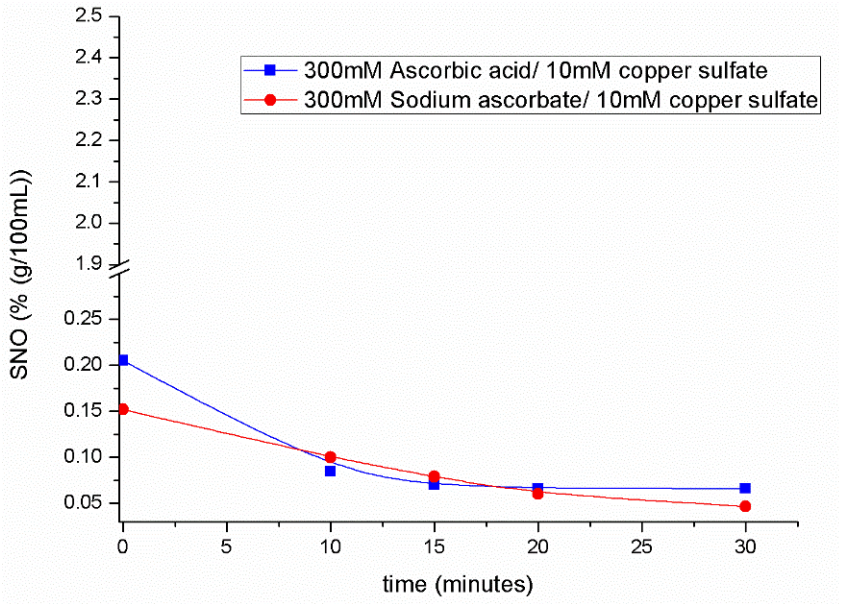


Figure 6. SNOPANT formation and degradation during 30 min in solution (*c*), blue line, and solution (*d*), red line. PANT and $NaNO_2$ were added simultaneously as the last components in equimolar amount with a final theoretical product concentration of 2.5% (w/v).

We then tested the ability of the new formulation (*e*) to promote SNOPANT formation and its stability within one hour. As shown in Figure 7, this formulation gives the best result in terms of yields and stability. We observed very good results in terms of product stability an approximate yield of about 90% with respect to the expected theoretical value. This result was best obtained in our experimental conditions and could be attributed to both effects of ascorbate and copper though in the opposite directions with respect to the previous experiments. It is possible that the low ascorbate concentration can decrease the direct reduction of Cu^{2+} to Cu^{+} and, at the same time, can reduce the S–S moieties maintaining SH groups. To explain our results, it is also possible to assume that the little amount of Cu^{+} formed can reduce the oxidized ascorbate, giving rise to a catalytic cycle in which Cu^{2+} and ascorbic acid are regenerated.

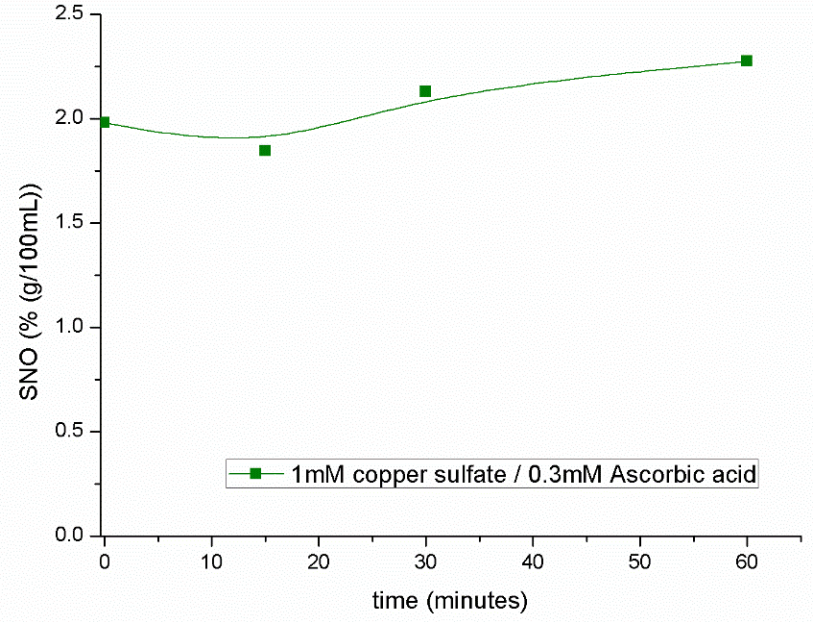


Figure 7. Kinetics of SNOPANT formation/decomposition during 60 min in the solution (*e*). PANT and $NaNO_2$ were added simultaneously as the last components in equimolar amount with a final theoretical product concentration of 2.5% (w/v).

3. Methods and Materials

3.1. Chemicals and Reagents

High-performance liquid chromatography (HPLC) grade methanol, acetonitrile, and ultrapure water were purchased from Carlo Erba (Milan, Italy). *N*-acetylcysteine, pantetheine, ascorbic acid, sodium ascorbate, sodium nitrite, copper sulfate, and analytical-grade solvents were purchased from Sigma–Aldrich (Milan, Italy).

3.2. Synthesis of S-Nitrosopantetheine

S-nitrosothiols were prepared by reacting equimolar amounts of sodium nitrite and thiol in acidic conditions, as reported in literature. To verify pantetheine S-nitrosilation, the described synthesis of S-Nitroso-N-acetylcysteine (SNONAC) was performed in parallel, and both yields were checked. SNONAC was synthesized using sodium nitrite (20 mM $NaNO_2$ in H_2O), *N*-acetylcysteine (10 mM NAC in H_2O), and hydrochloric acid (4M HCl) as reagents. The reaction was carried out at room temperature by mixing 150 µL of bidistilled water (deoxygenated with N_2 stream), 500 µL of NAC (5 mM final concentration), 250 µL of $NaNO_2$ (5 mM final concentration), and 100 µL HCl (0.4 M final concentration). The synthesis of SNOPANT was carried out in a similar way to that of the SNONAC. Sodium nitrite (20 mM $NaNO_2$ in H_2O), pantetheine (10 mM PANT in H_2O), and hydrochloric acid (4M HCl) were used as reagents. The reaction was carried out at room temperature by mixing 150 µL of bidistilled water (deoxygenated with N_2), 500 µL of PANT (5 mM final concentration), 250 µL of $NaNO_2$ (5 mM final concentration), and 100 µL of HCl (0.4 M final concentration). The reaction took place immediately, and it was possible to appreciate it by the rapid color change of the colorless solution to an intense red typical of the nitrosothiols' chromophore. Reaction yield was checked for both products spectrophotometrically using the S-nitroso molar extinction coefficient (ε330 nm = 727 M^{-1} cm^{-1}) and was evaluated to be quantitively 100% after calculating 5 mM of final product. Identity of the product was confirmed by high-resolution and tandem mass spectrometry analysis (HRESIMS and MS/MS). Purity was also checked by HPLC, and both products were stable up to one week at +4 °C in the reaction conditions.

3.3. High Resolution ESI-MS and ESI-MS/MS

High-resolution (HR) mass spectra for exact mass determination were obtained using an Orbitrap mass spectrometer (Q Exactive, Thermo Scientific, Bremen, Germany) equipped with an electrospray ESI ionization source (positive mode), capillary temperature 275 °C, spray voltage 3.5 kV, sheath gas (nitrogen) 10 arbitrary units, capillary voltage 65 V, and tube lens 125 V. Data were collected on Xcalibur (Version 2.2, Thermo Scientific, Bremen, Germany). The tandem mass spectrometry experiments (CID MS/MS) were performed on a linear ion trap mass spectrometer (LTQ mass spectrometer, Thermo Scientific) equipped with an ESI source (positive mode) and a syringe pump. Operating conditions of the ESI source were as follows: ion spray voltage = 4.0 kV; sheath gas = 5 (arbitrary scale); sweep gas = 5 (arbitrary scale); capillary temperature = 275 °C; capillary voltage 10 V, and tube lens voltages 55 V. Samples were diluted in MeOH and were infused via a syringe pump into the ESI source at a flow rate of 5 μL/min. The collision energy employed for collision induced dissociation (CID) spanned between 5% and 20% (arbitrary units of helium flow).

3.4. HPLC Chromatographic Determination of S-Nitrosothiols

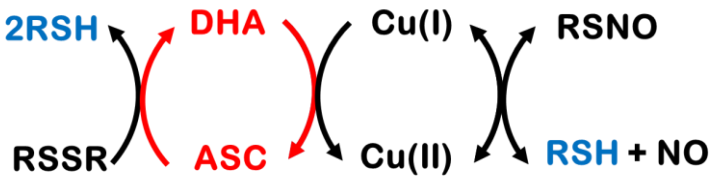
The chromatographic analyses were carried out on a Waters 600 HPLC apparatus consisting of a quaternary gradient pump equipped with a UV-visible photodiode array spectrophotometric detector (PDA mod. 2996). The system is managed by the Millennium[32] software (Version 4.0, Waters, Milford, MA, USA) dedicated to the automatic integration of chromatographic peaks. The elution was carried out isocratically with a flow rate of 1 mL/min on a Waters Symmetry column (C18 reverse phase, 4.6 × 250 mm, 5 μm particle size, 100 Å) coupled with a pre-column (4.6 × 10 mm) of the same material. The mobile phase consisted of 50 mM monobasic sodium phosphate containing 10 mM heptansulfonic adjusted at pH 4.4 with phosphoric acid (A) and acetonitrile (B). The composition of the final mobile phase consisted of 85% of solvent A and 15% of solvent B. Under this chromatographic conditions, SNONAC has a retention time of 2.26 min and SNOPANT 6.4 min. Calibration curves used for quantification showed a good linearity in the range 5 μM to 5 mM (R^2 = 0.9976 for SNONAC and R^2 = 0.9984 for SNOPANT).

3.5. Samples Preparation

All the tested samples were prepared in bidistilled and degassed water. In all the samples, PANT and $NaNO_2$ were added simultaneously as the last components, respectively, at the final concentrations of 2.5 and 1.38% (90 mM) in a final volume of 5 mL. Solution (*a*) contains 300 mM ascorbic acid and solution (*b*) contains 300 mM NaAscorbate. Solution (*c*) contains 300 mM ascorbate and 10 mM copper sulfate; (*d*) contains 300 mM NaAscorbate and 10 mM copper sulfate. Solution (*e*) contains ascorbic acid and copper sulfate in lower concentrations and different molar ratios (1 mM $CuSO_4$ and 0.3 mM ascorbic acid). After preparation, each sample was diluted in the HPLC mobile phase and 50 μL was injected into the column.

4. Conclusions

In this paper, the synthesis and the chromatographic determination of SNOPANT is reported for the first time, as well as a brief investigation of its reactivity in aqueous solutions in the presence of factors known to affect its stability. SNOPANT has been successfully prepared by reacting pantetheine and nitrite under acid conditions. The effect of copper and ascorbic acid/ascorbate has been explored to assess the stability of this novel nitrosothiol. Interestingly, SNOPANT can be obtained in high yields and in a stable form by the mutual and opposite effect of the copper ion and ascorbic acid, avoiding the use of strongly acidic conditions. Catalytic amounts of ascorbic acid protect SNOPANT by decomposition, which acts on the redox cycle of copper. Under our experimental conditions, Cu^{2+} can be continuously regenerated by the ascorbic/dehydroascorbic acid (ASC/DHA) pair, preventing Cu^{+} from mediating SH group oxidation and $NO^{\cdot}$ release from nitrosothiol, according to the Scheme 3.



Scheme 3. Catalytic cycle of copper and ascorbate mediated RSNO formation and degradation.

Our data confirm the importance of ascorbic acid and copper ions in RSNOs' formation and degradation and highlight how pantetheine and other biologically active thiols can be obtained in aqueous solutions in their nitrosylated form through the fine regulation of these two factors.

Author Contributions: Conceptualization, L.M. and A.F.; methodology, D.D.R., R.M., M.T., M.F. and A.F.; resources, L.M. and M.F.; writing—original draft preparation, A.F.; Investigation A.F. and G.M.; Formal analysis A.F. and S.M.; writing—review and editing, L.M., M.F. and A.F.; funding acquisition, A.F. and L.M. All authors have read and agreed to the published version of the manuscript.

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