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S-NITROSATION OF PROTEINS BY *N*-METHYL-*N*-NITROSOANILINES

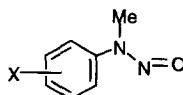
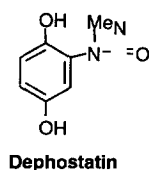
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Abstract: Substituted *N*-methyl-*N*-nitrosoanilines are found to function as stable, potent, nitrosating agents for *S*-nitrosation and inactivation of cysteine proteases such as papain, bromelain and aldehyde dehydrogenase under physiological conditions in a time and concentration dependent manner. Spectroscopic studies on the reaction products have proved that the mechanism of the inactivation is a *S*-nitrosation of the protein.

Nitric oxide (NO) has been implicated in diverse physiological processes, including vasodilatory and antiplatelet effects, macrophage-induced cytotoxicity, and neurotransmission.¹ The nitrosation of protein thiols is believed to be a general way to store, transport, and finally release NO.² *S*-nitroso proteins have been detected in human airway, plasma, and platelets, and have been shown to exhibit endothelium-derived relaxing factor (EDRF)-like effects.³ Currently, some commercially available nitrosating agents for *S*-nitrosation of proteins such as sodium nitroprusside (SNP)⁴ and 3-morpholinosydnonimine (SIN-1)⁵ require preactivation, other NO donors such as *S*-nitrosothiols (e.g., (±)-*S*-nitroso-*N*-acetylpenicillamine, SNAP)⁶ and nucleophile complexes of NO (NONOates)⁷ depend on spontaneous decomposition in aqueous solution. Herein we report our latest finding that substituted *N*-methyl-*N*-nitrosoanilines serve as stable NO⁺ donors for *S*-nitrosation of proteins.

This work was based on our previous syntheses and bioassay of a novel protein tyrosine phosphatase (PTPase, EC 2.7.1.112) inhibitor, dephostatin, a natural product isolated from the culture broth of *streptomyces* sp.⁸ We found that not only did dephostatin inactivate both the recombinant *Yersinia* and mammalian PTPases, but also the compound **3** *N*-methyl-*N*-nitrosoaniline exhibited weak inhibition. A possible inactivation mechanism is a *S*-nitrosation of PTPases by these *N*-nitroso compounds. Subsequently, we synthesized a series of substituted *N*-methyl-*N*-nitrosoanilines and studied their inhibition ability against thiol-dependent enzymes.⁹



#	posi.	X
1	<i>p</i>	NMe ₂
2	<i>p</i>	OH
3	<i>p</i>	H
4	<i>p</i>	Cl
5	<i>m</i>	OH
6	<i>o</i>	OH
7	<i>o</i>	NO ₂

We first chose papain (EC 3.4.22.2) as an example of thiol protease. Papain has only one cysteine residue (Cys-25) in the active site.¹⁰ The results of the enzymatic assay revealed that the substituted *N*-methyl-*N*-nitrosoanilines exhibited different inhibition activities against papain (Figure 1).¹¹

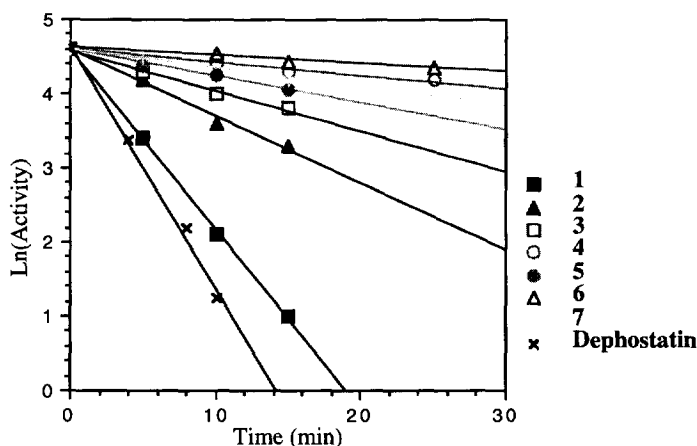


Figure 1. Inactivation of papain (0.25 mg / mL, 500 μ L) with nitrosamines (10 mM, 500 μ L) in 50 mM sodium phosphate buffer (pH 7.0, 10% acetonitrile, 1 mM EDTA). Pseudo-first order rate constants (min^{-1}): dephostatin (0.328); **1** (0.243); **2** (0.090); **3** (0.057); **4** (0.017); **5** (0.036); **6** (0.011); **7** (0.026).

Dephostatin is the most active inhibitor with a pseudo first order rate constant K_i of 0.328 min^{-1} , which is followed by compounds **1** and **2** with K_i of 0.243 and 0.009 min^{-1} , respectively. For example, when papain (50 $\mu\text{g/mL}$, 200 μL) was incubated with compound **2** (50 μM , 200 μL) in 50 mM sodium phosphate buffer (pH 7.0, 10% acetonitrile) for 20 min, 92% of the enzyme activity vanished. Detailed kinetic measurements showed that K_i and k_i were 2.813 mM and 0.102 min^{-1} , respectively, for the inhibition of papain with nitrosamine **2**.¹² Chlorine, as an electron withdrawing group at the *para* position of aniline, makes compound **4** less active than the unsubstituted compound **3**. Substitution at either the *meta* **5** or the *ortho* position **6** and **7** results in poor inhibitors. Attempts to correlate the inhibiting activities with σ of the Hammett equation or the additivity of multiple substituents failed to produce meaningful results, suggesting that factors like binding of inhibitors with the enzyme may play a role in the process of the inactivation.

The inactivated papain (1 mg/mL, 100 μL) can be re-activated upon the addition of cysteine or glutathione (Figure 2). Furthermore, the inactivation by compound **2** was partially prevented in the presence of Gly-Gly-Tyr-Arg, a competitive inhibitor of papain (Figure 3). These observations strongly suggest that the inactivation with nitrosamines takes place within the active site of the enzyme and the inactivation process is *S*-nitrosation of papain in which the *N*-methyl-*N*-nitrosoanilines function as a NO^+ donor.

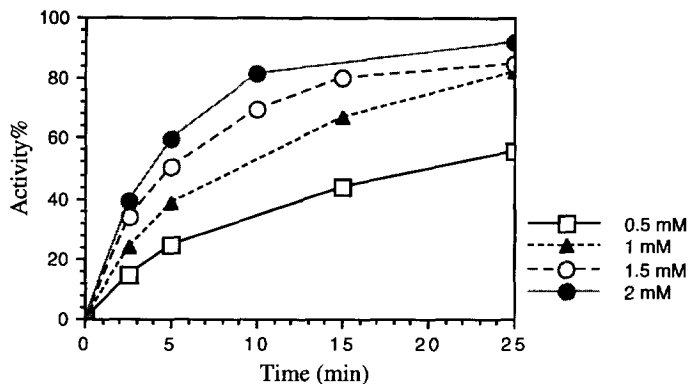


Figure 2. Re-activation of *S*-nitroso papain (1 mg/mL) with glutathione of different concentrations.

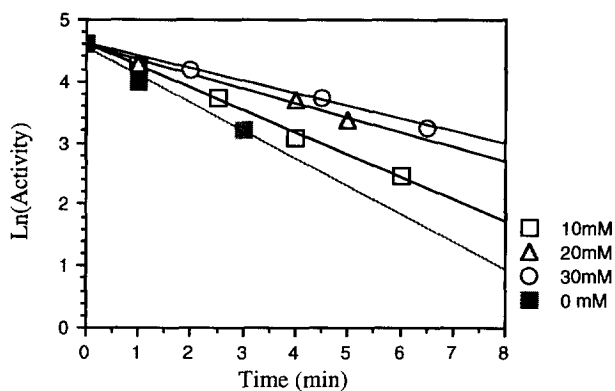
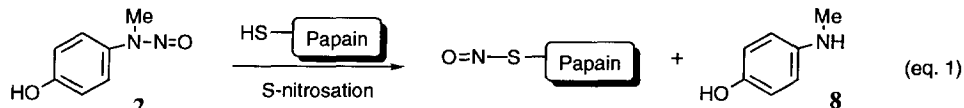


Figure 3. Concentration effect of competitive inhibitor Gly-Gly-Tyr-Arg on the inactivation of papain (250 µg/mL) by compound **2** (3 mM)

To support the *S*-nitrosation mechanism, papain (10 mg) and compound **2** (10 mg) were incubated in 2 mL of 50 mM sodium phosphate buffer (pH 7.0, 10% acetonitrile, 1 mM EDTA) at room temperature until papain was completely inactivated. From the concentrated reaction mixture, *p*-hydroxy-*N*-methylaniline **8**, a byproduct of the papain *S*-nitrosation with the same R_f as that of authentic *p*-hydroxy-*N*-methylaniline sample (eq.1), was isolated by preparative thin layer chromatography (hexane : ethyl acetate = 2:1) and further proved by mass spectroscopy (calcd for $C_7H_9NO^+$, (M^+), m/e 123, found 123).



In a separate run, the UV-Vis spectrum of the inactivated papain which was separated from reaction mixture by Sephadex G-10 showed absorption maxima in the 330-370 nm wavelength range which are characteristic for *S*-nitrosothiols, whereas nitrosamines have UV absorptions varying from 280 nm to 310 nm depending on specific substituent and papain has absorption at 280 nm.¹³ In this case, the absorption peak for *p*-hydroxy-*N*-methyl-*N*-nitrosoaniline **2** was at 284 nm. Finally, the formation of the S-NO bond was further verified by FTIR spectroscopy. Running on cast films obtained by evaporation of the inactivated papain solution on a CaF₂ crystal, the spectra clearly showed the S-NO bond absorption bands at 1166 cm⁻¹ and 1153 cm⁻¹.¹⁴

We also tested another thiol protease, bromelain (EC 3.4.22.4). It was found that nitrosamines (**1** and **2**) exhibited strong inhibition activities as in the case of papain. In contrast, no inhibition was detected when serine proteases such as α -chymotrypsin (EC 3.4.21.1) and subtilisin BPN' (EC 3.4.21.14) solutions were incubated with these nitrosamines. Besides thiol proteases, other sulfhydryl-dependent enzymes were also inactivated by substituted *N*-methyl-*N*-nitrosoanilines. For example, aldehyde dehydrogenase (EC 1.2.1.5) has a critical cysteine at its active site and can be inactivated by nitrosamine **2**. In contrast, alcohol dehydrogenase (EC 1.1.1.1), in which the Cys-46 and Cys-174 serve as ligands to the Zn²⁺ at the active site, was not inactivated by these nitrosamines.

N-nitroso compounds have been extensively studied in the past thirty years due to their carcinogenic and mutagenic properties.¹⁵ Based on the published biological testing results, substituted *N*-methyl-*N*-nitrosoanilines normally exhibit weak carcinogenicity and almost no mutagenicity to several different animal species.¹⁶ Previous physical organic studies indicated that the transnitrosation reaction between nitrosamines and nucleophiles (including thiols) occurred at strong acidic conditions.¹⁷ For example, Williams and coworkers reported that nitrosamine **3** underwent *S*-nitrosation with cysteine in 0.48 M H₂SO₄ solution.¹⁸ The rate-limiting step in this case was the initial protonation of the nitroso compound. In this work, we observed that properly substituted *N*-alkyl-*N*-nitrosoanilines underwent *S*-transnitrosation with sulfhydryl-dependent enzymes *under physiological conditions*. An electron-donating substitution at the *para*-position of the phenyl ring apparently assists the formation of the protonated reaction intermediate. The higher nucleophilicity of the critical cysteine of the enzyme than that of simple thiols makes the transnitrosation possible.¹⁹ Our finding points out that *N*-alkyl-*N*-nitrosoaniline structure can function as a stable, functional moiety in the design of nitrosating agents for biomedical and pharmaceutical applications.

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- 9 Compounds **3**, **4** and **7** were synthesized by *N*-nitrosation of substituted *N*-alkylanilines with NaNO₂/HOAc. Compound **3** ¹H NMR (400 MHz, CDCl₃) δ 3.43 (s, 3H), 7.33-7.52 (m, 5H), MS calcd for C₇H₈N₂O⁺ (M⁺), 136, found 136, IR, ν_{max} (cm⁻¹) 1450, 1480 (N-N=O), elemental analysis calcd for C₇H₈N₂O: C, 61.75; H, 5.91, found: C, 61.74; H, 5.91; compound **4** ¹H NMR (400 MHz, CDCl₃) δ 3.44 (s, 3H, N-methyl), 7.47 (q, 4H, Ph), MS calcd for C₇H₇N₂OCl⁺ (M⁺), 170, found 170, IR, ν_{max} (cm⁻¹) 1450, 1465, 1505 (N-N=O), elemental analysis calcd for C₇H₇N₂OCl: C, 49.28; H, 4.14, found: C, 49.22, H, 4.18; compound **7** ¹H NMR (400 MHz, CDCl₃) δ 3.42 (s, 3H, NMe), 7.55 (m, 1H), 7.62 (m, 1H), 7.80 (m, 1H), 8.08 (m, 1H), MS calcd for C₇H₇N₃O₃⁺ (M⁺), 181, found 181, IR, ν_{max} (cm⁻¹) 1460, 1470 (N-N=O). Compound **2** was synthesized by de-*O*-methylation of *p*-hydroxy-*N*-methylaniline with BBr₃ and subsequent *N*-nitrosation. ¹H NMR (400 MHz, (CD₃)₂CO) δ 3.4 (s, 3H), 6.98 (d, 2H, *J* = 9.0 Hz), 7.42 (d, 2H, *J* = 9.0 Hz), 8.66 (s, 1H), MS calcd for C₇H₈N₂O₂⁺ (M⁺), 152, found 152, IR, ν_{max} (cm⁻¹) 1460, 1475 (N-N=O), elemental analysis calcd for C₇H₈N₂O₂: C, 55.25; H, 5.30, found: C, 55.28; H, 5.32. Compound **1** was synthesized from *N*-nitrosation of *p*-*N*,*N'*-dimethylamino-aniline. ¹H NMR (CDCl₃) δ 7.25 (d, 2H, *J* = 9.2 Hz, Ph), 6.68 (d, 2H, *J* = 9.2 Hz, Ph), 3.40 (m, 3H, CH₂), 2.91 (s, 6H, N(CH₃)₂), MS calcd for C₉H₁₃N₃O (M⁺), (m/e) 180 (M⁺), found 180, IR, ν_{max} (cm⁻¹) 1465 (N-N=O), elemental analysis calcd for C₉H₁₃N₃O: C, 60.31; H, 7.31, found: C, 60.06; H, 7.22. Compounds **5** and **6** were synthesized from *N*-methylation of substituted anilines with formaldehyde and NaCNBH₃ prior to *N*-nitrosation. Compound **5** ¹H NMR (400 MHz, CDCl₃) δ 3.44 (s, 3H, N-methyl), 6.84 (d, 1H, Ph), 7.05 (d, 1H, Ph), 7.28 (s, 1H, Ph), 7.34 (t, 1H, Ph), MS calcd for C₇H₈N₂O₂⁺ (M⁺), 152, found 152, IR, ν_{max} (cm⁻¹) 1486 (N-N=O), elemental analysis calcd for C₇H₈N₂O₂: C, 55.25; H, 5.30, found: C, 55.17; H, 5.31; compound **6** ¹H NMR (400 MHz, CDCl₃) δ 3.58 (s, 3H), 7.05 (m, 1H), 7.20 (m, 2H), 7.25 (m, 1H), MS calcd for C₇H₈N₂O₂⁺ (M⁺), 152, found 153 (M+1), IR, ν_{max} (cm⁻¹) 1430, 1465 (N-N=O), elemental analysis calcd for C₇H₈N₂O₂: C, 55.25; H, 5.30, found: C, 55.31; H, 5.32.

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$$E + I \xrightleftharpoons[k_{-1}]{k_1} E : I \xrightarrow{k_i} E-I, K_I = k_{-1} / k_1, k_i: k_{\text{inact}}$$
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