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

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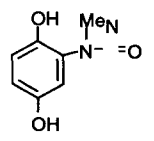
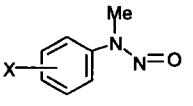
Abstract: Substituted *N*-methyl-*N*-nitrosoanilines are found to function as stable, potent, nitrosating agents for *S*-nitrosation and inactivation of cysteine proteases such as papain, bromelain and aldehyde dehydrogenase under physiological conditions in a time and concentration dependent manner. 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]



Dephostatin



#	posi.	X
1	*p*	NMe_2
2	*p*	OH
3	*p*	H
4	*p*	Cl
5	*m*	OH
6	*o*	OH
7	*o*	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]

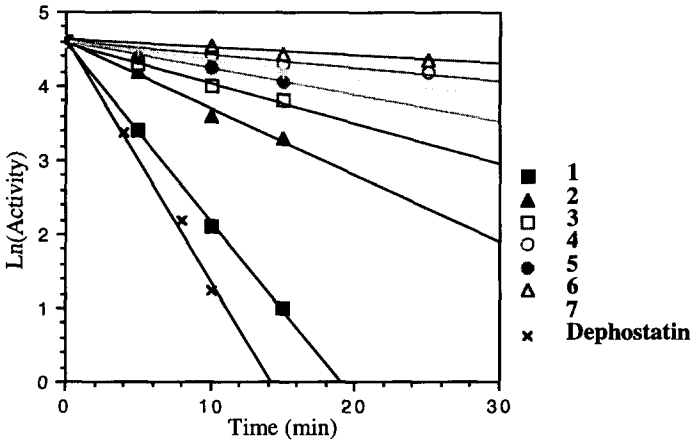


Figure 1. Inactivation of papain (0.25 mg / mL, 500 μL) with nitrosamines (10 mM, 500 μL) in 50 mM sodium phosphate buffer (pH 7.0, 10% acetonitrile, 1 mM EDTA). Pseudo-first order rate constants (min^{-1}): dephostatin (0.328); **1** (0.243); **2** (0.090); **3** (0.057); **4** (0.017); **5** (0.036);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

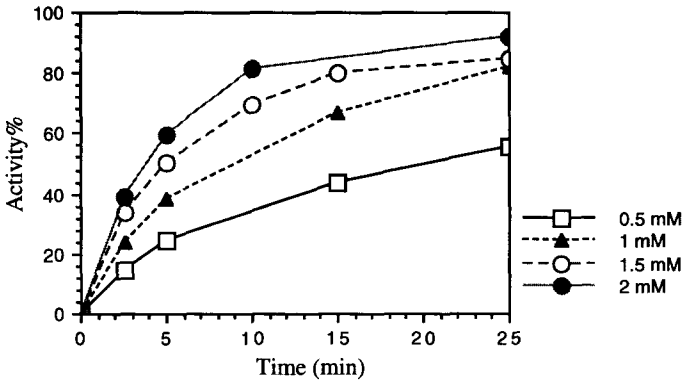


Figure 2. Re-activation of *S*-nitroso papain (1 mg/mL) with glutathione of different concentrations.

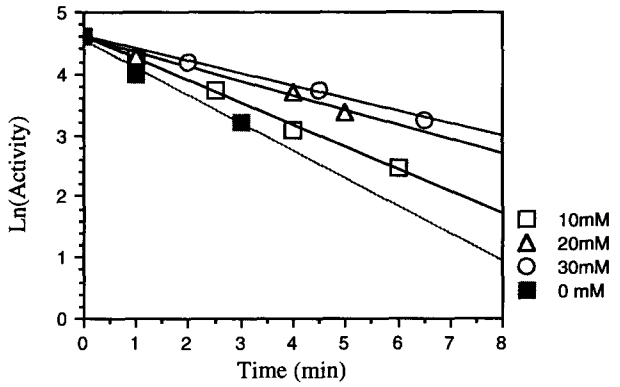
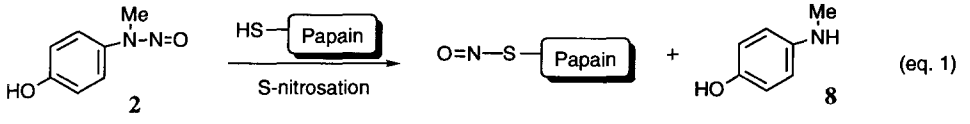


Figure 3. Concentration effect of competitive inhibitor Gly-Gly-Tyr-Arg on the inactivation of papain (250 μg/mL) by compound **2** (3 mM)

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).



(eq. 1)

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 CaF_2 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 substilisin 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 Zn^{2+} 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 H_2SO_4 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 $NaNO_2$/HOAc. Compound **3** ^1H NMR (400 MHz, $CDCl_3$) δ 3.43 (s, 3H), 7.33-7.52 (m, 5H), MS calcd for $C_7H_8N_2O^+$ (M^+), 136, found 136, IR, ν_{max} (cm^{-1}) 1450, 1480 (N-N=O), elemental analysis calcd for $C_7H_8N_2O$: C, 61.75; H, 5.91, found: C, 61.74; H, 5.91; compound **4** ^1H NMR (400 MHz, $CDCl_3$) δ 3.44 (s, 3H, N-methyl), 7.47 (q, 4H, Ph), MS calcd for $C_7H_7N_2OCl^+$ (M^+), 170, found 170, IR, ν_{max} (cm^{-1}) 1450, 1465, 1505 (N-N=O), elemental analysis calcd for $C_7H_7N_2OCl$: C, 49.28; H, 4.14, found: C, 49.22, H, 4.18; compound **7** ^1H NMR (400 MHz, $CDCl_3$) 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 $C_7H_7N_3O_3^+$ (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 BBr_3 and subsequent *N*-nitrosation. ^1H NMR (400 MHz, $(CD_3)_2CO$) 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 $C_7H_8N_2O_2^+$ (M^+), 152, found 152, IR, ν_{max} (cm^{-1}) 1460, 1475 (N-N=O),elemental analysis calcd for $C_7H_8N_2O_2$: 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 ($CDCl_3$) δ 7.25 (d, 2H, J = 9.2 Hz, Ph), 6.68 (d, 2H, J = 9.2 Hz, Ph), 3.40 (m, 3H, CH_2), 2.91 (s, 6H, $N(CH_3)_2$), MS calcd for $C_9H_{13}N_3O$ (M^+), (m/e) 180 (M^+), found 180, IR, ν_{max} (cm^{-1}) 1465 (N-N=O), elemental analysis calcd for $C_9H_{13}N_3O$: 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 $NaCNBH_3$ prior to *N*-nitrosation. Compound **5** ^1H NMR (400 MHz, $CDCl_3$) 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 $C_7H_8N_2O_2^+$ (M^+), 152, found 152, IR, ν_{max} (cm^{-1}) 1486 (N-N=O),elemental analysis calcd for $C_7H_8N_2O_2$: C, 55.25; H, 5.30, found: C, 55.17; H, 5.31; compound **6** ^1H NMR (400 MHz, $CDCl_3$) δ 3.58 (s, 3H), 7.05 (m, 1H), 7.20 (m, 2H),7.25 (m, 1H), MS calcd for $C_7H_8N_2O_2^+$ (M^+), 152, found 153 (M+1), IR, ν_{max} (cm^{-1}) 1430, 1465 (N-N=O), elemental analysis calcd for $C_7H_8N_2O_2$: C, 55.25; H, 5.30, found: C, 55.31; H, 5.32.

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11 In a typical papain enzymatic assay, papain was first activated by 50 mM sodium phosphate buffer (pH 7.0, 0.3 mM cysteine, 1 mM EDTA) and then was passed through Sephadex G-10 column, eluted with 50 mM sodium phosphate buffer (pH 7.0, 1 mM EDTA). The enzyme obtained in this way excluded any unexpected effect of cysteine which was necessary for initial activation. Nitrosamines were prepared in 50 mM sodium phosphate buffer (pH 7.0, 20% acetonitrile, 1 mM EDTA). Papain activity was measured spectrophotometrically at 410 nm with either UV/Visible spectrophotometer (SHIMADZU) or Spectronic Genesys 2 (Milton Roy) using chromogenic substrate N-Cbz-Glycine *p*-nitrophenyl ester (25 mM, 10 μL), in 1mL of 50 mM sodium phosphate buffer (pH 7.0, 1mM EDTA, 7% (v/v)acetonitrile).

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$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E:I \xrightarrow{k_i} E\text{-}I\,,\ K_I = k_{-1}/k_1\,,\ k_i\text{: } k_{inact}$$

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