Peptidyl *N*-Nitrosoanilines: A Novel Class of Cysteine Protease Inactivators[†,‡]

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Received December 10, 1997

Abstract: A series of peptidyl *N*-nitrosoanilines were designed, synthesized, and evaluated as inactivators of cysteine protease papain and serine protease chymotrypsin. These new compounds exhibited different inhibitory activities toward the cysteine protease papain in a time- and concentration-dependent manner with second-order rate constants (k_{inact}/K_I) ranging over 2 orders of magnitude from 0.604 M^{-1} s^{-1} (**1**) to 100.36 M^{-1} s^{-1} (**7**). No inactivation was observed for serine protease chymotrypsin. Formation of the *S*-NO bond in papain is supported by several lines of evidences from both spectroscopic studies and chemical analyses. The pH profile study on inactivation of papain by compound **1** was conducted over the pH range 3.2−9.2 to provide more insight into the mechanism of the inactivation process. The *covalent* yet *recoverable* cysteine protease inactivation process offers mechanistic implications and endows this new family of inactivators with special properties that are suitable for the development of stable and potent cysteine protease inhibitors.

Introduction

Cysteine proteases (EC 3.4.22) play an important role in various biological processes.[1] Since many disease states such as muscular dystrophy, inflammation, and rheumatoid arthritis are associated with elevated proteolytic activity of cysteine proteases, much attention has been paid to the rational design and synthesis of selective inhibitors for these types of enzymes.[2] Inhibitors currently in wide use include (1) reversible inhibitors or transition-state analogue inhibitors, exemplified by peptidyl aldehydes,[3] nitriles,[4] and α-ketocarbonyl compounds,[5] and (2) irreversible inhibitors or covalent inhibitors, such as peptidyl halomethyl ketones,[6] diazomethyl ketones,[7] acyloxymethyl ketones,[8] ketomethylsulfonium salts,[9] and various epoxysuccinyl compounds.[10] These inhibitors have been extensively used in the characterization of the binding sites, catalytic functional groups, and transition-state geometries of cysteine proteases.

Dephostatin, a protein tyrosin phosphatase (PTP) (EC 2.7.1.112) inhibitor isolated from the culture broth of *streptomyces* sp.,[11a,b] was recently synthesized in our laboratory, together with its unsubstituted precursor, *N*-methyl-*N*-nitrosoaniline (R = *p*-H) (Figure 1).[11c] Besides their anticipated competitive inhibition against *Yersinia* PTP and mammalian PTP1, both compounds were found to be unexpected inhibitors of cysteine protease papain (EC 3.4.4.10). To further our understanding of this interesting phenomenon, a series of dephostatin analogues, substituted *N*-methyl-*N*-nitrosoanilines, were subsequently synthesized. Compounds substituted at the para position by hydroxyl and *N*,*N*-dimethylamino groups were shown to be more

[†] The preliminary results of this project was presented in the ACS meeting, Orlando, FL, August 1996.

[‡] Abbreviations: amino acids are written in their three-letter codes; EDC, 1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide; HOBt, 1-hydroxybenzotrizole; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

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S0002-7863(97)04187-5 CCC: $15.00
Published on Web 04/02/1998

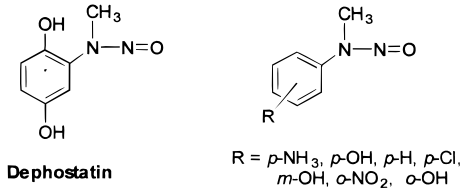


Figure 1. Natural product dephostatin and its analogues.

potent than unsubstituted *N*-methyl-*N*-nitrosoaniline in the inhibition of cysteine proteases papain and bromelain (EC 3.4.22.4).[12] Preliminary studies on enzyme kinetics and the resultant products of the inhibition process enabled us to propose that the inhibition was effected through *S*-nitrosation of the protein active site thiol groups by the *N*-nitrosoanilines.[12] More importantly, this class of compounds provide a novel platform for designing new agents to specifically deliver nitric oxide (NO) to the cysteine groups of proteins.

Being a versatile biological messenger, NO is involved in a vast array of physiological processes such as vasodilatory and antiplatelet effects, macrophage-induced cytotoxicity, and neurotransmission.[13] Over the past few years interest in the *S*-nitroso moiety carried by biological macromolecules has grown due to the existence of overwhelming evidence that NO is stabilized in the form of *S*-nitroso protein in mammalian plasma which preserves the biological activity of NO and prolongs its physiological half-life.[14−17] Biomolecules bearing thiol groups such as serum albumin appear to be likely candidates for this role.[17] Since an *S*-nitrosylated cysteine protease is the sole product of the inactivation process in our study, a myriad of questions are raised as to the possible roles cysteine proteases play in both nitric oxide biological functions and nitrosamine carcinogenic processes. The fact that *S*-nitrosylated enzymes can regain activity upon addition of thiol compounds such as glutathione[12] makes this class of compounds a template suitable for the development of potent *recoverable* and *covalent* inhibitors. Three disparate lines of research in the fields of nitrosamine, cysteine protease, and nitric oxide seamlessly converged in this study.

We report herein the design and synthesis of specific *S*-nitrosation agents, namely peptidyl *N*-nitrosoanilines, their inhibitory potencies against cysteine protease, and detailed mechanistic studies on the inactivation process which revealed a concerted catalytic behavior between active site residues Cys-25 and His-159. Incorporating a functional moiety into the framework of a substrate for the targeted enzyme constitutes a general approach to inhibitor design. Hence, by including into our inhibitor structure a peptide sequence corresponding to the substrate specificity of papain, the inhibitory potency of nitrosoaniline moiety toward thiol protease could be significantly enhanced (Figure 2). Papain, the most thoroughly studied cysteine protease,[18] was again employed to serve as a mechanistic and structural model to evaluate these inhibitors.

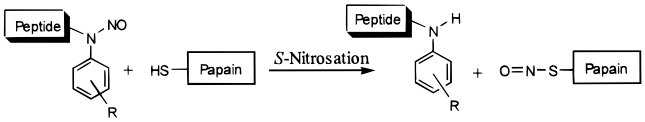


Figure 2. *S*-Nitrosation of papain by peptidyl *N*-nitrosoanilines.

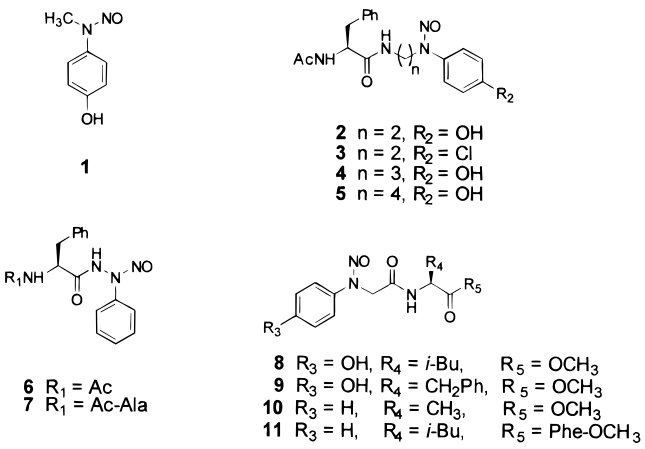
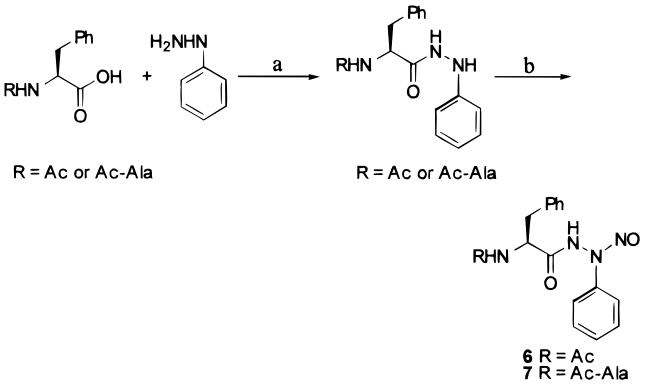


Figure 3. Structures of the peptidyl *N*-nitrosoanilines **2**−**11** designed on the basis of *p*-hydroxyl-*N*-methyl-*N*-nitrosoaniline (**1**).

Scheme 1. Synthetic Approach toward Compounds **6** and **7**[a]



[a] (a) EDC, HOBt, Et_3N, CH_2Cl_2. (b) $NaNO_2$, MeOH/HOAc, 0 °C.

Results

Inhibitor Synthesis. Compounds **2**−**11** were synthesized, and their inhibitory potencies against papain were evaluated and compared with that of parental compound **1** (Figure 3). The peptide portions of these peptidyl *N*-nitrosoanilines were synthesized by standard solution phase peptide coupling reactions in which the amide bond was formed through the use of coupling reagent EDC.[19] In compounds **2**−**5**, between the Phe

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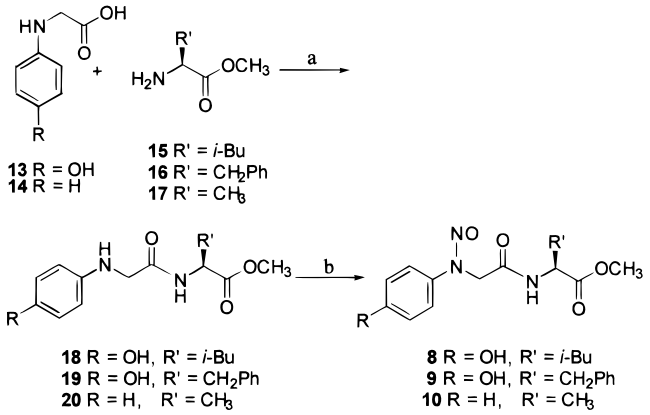
Scheme 2. Synthetic Approach toward Compounds **8**, **9**, and **10**[a]



[a] (a) EDC, HOBt, Et_3N, CH_2Cl_2. (b) $NaNO_2$, MeOH/HOAc, 0 °C. (**14** and Leu-Phe-OCH_3 are used in step a to synthesize **11**.)

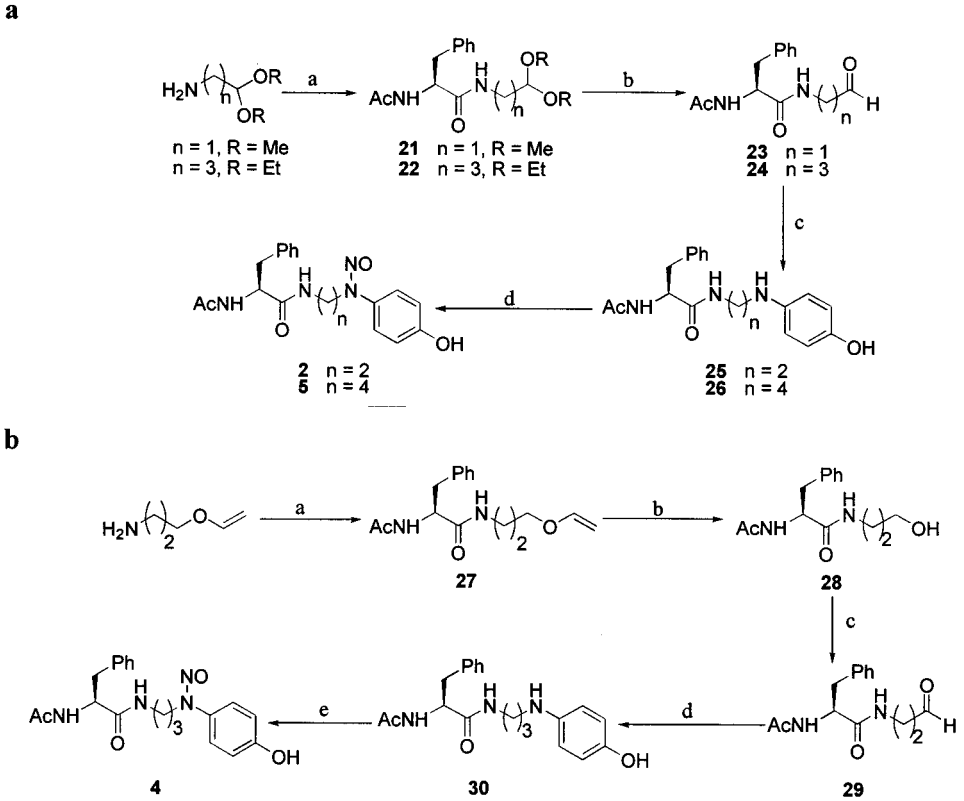
residue and the nitrosoaniline moiety were placed carbon chain spacers varying from two to four carbons. When *n* equals 2 in the carbon chain linker (**2** and **3**), the aminoethyl component is spatially equivalent to a single peptide residue. Peptidyl *N*-nitrosophenylhydrazines (**6** and **7**) were synthesized to further study the effect of carbon linker length on enzyme inhibition (Scheme 1). On the other hand, compounds **8**−**11** were distinct from each other mainly in the size of the side chains R_4 of *C*-terminal residues (Scheme 2). They were expected to interact with the S_1' or S_2' leaving group binding sites. Nitrosation of aniline nitrogens in the last steps yielded the corresponding peptidyl *N*-nitrosoanilines. Unlike the straightforward peptide coupling strategy in the synthesis of compounds **6**−**11** (Schemes 1 and 2), the synthetic procedure for compounds **2**−**5**, as illustrated in Scheme 3, involved the C−N bond formation through reductive amination of the corresponding aldehyde and primary amine. The aldehyde functions are generated either from hydrolysis of acetal **21** and **22** (Scheme 3a) or from Swern oxidation of terminal hydroxyl group **28** (Scheme 3b).

Stability of Peptidyl *N*-Nitrosoanilines in Aqueous Solution. The stability of each peptidyl *N*-nitrosoaniline compound in aqueous solution was examined before being used in the enzymatic assay. No decomposition of peptidyl *N*-nitrosoaniline was detected over a 10-h incubation period in 50 mM acetate buffer over the range pH 3.0−5.6, in 50 mM phosphate buffer from pH 6.0 to 7.8 or in 100 mM borate buffer from pH 8.0 to 10.0 when the UV/vis absorption peak at 281 nm was monitored. All buffers contained 1 mM EDTA and 20% (v/v) acetonitrile. In addition, parallel tests were carried out in the presence of low molecular weight thiols (2 M) such as cysteine and glutathione. Denitrosation was not detectable for either of these thiols.

Inactivation of Enzymes. The serine protease chymotrypsin was preincubated with each peptidyl *N*-nitrosoaniline in 50 mM phosphate buffer (pH 7.0, 1 mM EDTA and 10% (v/v) acetonitrile) at 25 °C. After 1 h of incubation, no loss of enzymatic activity was detected in the presence of inhibitor up to a concentration of 10 mM.

The activated cysteine protease papain was also preincubated with each peptidyl *N*-nitrosoaniline in 50 mM phosphate buffer (pH 7.0, 1 mM EDTA and 10% (v/v) acetonitrile) at 25 °C. The assay revealed that inactivation of papain was time- and concentration-dependent except for compounds **9** and **11**, which showed no inhibition effect. The inactivation process can be analyzed in terms of eq 1, where K_I and k_{inact} represent the

Scheme 3. Synthetic Approaches toward Compounds **2**, **4**, and **5**[a]



[a] Part a (top): (a) Ac-Phe-OH, EDC, HOBt, Et_3N, CH_2Cl_2. (b) TFA, 0 °C. (c) $NaCNBH_3$, MeOH, *p*-$OHC_6H_4NH_2$. (d) $NaNO_2$, MeOH/HOAc, 0 °C. (*p*-$ClC_6H_4NH_2$ is used in step c to synthesize **3**.) Part b (bottom): (a) Ac-Phe-OH, EDC, HOBt, Et_3N, CH_2Cl_2. (b) HOAc, HCl, H_2O. (c) DMSO, $(COCl)_2$, CH_2Cl_2. (d) $NaCNBH_3$, MeOH, *p*-$OHC_6H_4NH_2$. (e) $NaNO_2$, MeOH, HOAc, 0 °C.

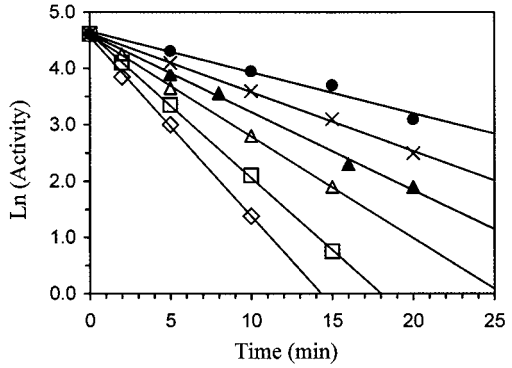


Figure 4. Time course of inactivation of papain by inhibitor **7**, in 50 mM phosphate buffer (pH 7.0, 10% (v/v) acetonitrile and 1 mM EDTA) at 25 °C. Inhibitor concentration: (◇) 400 μM; (□) 200 μM; (△) 100 μM; (▲) 50 μM; (×) 25 μM; (●) 12.5 μM.

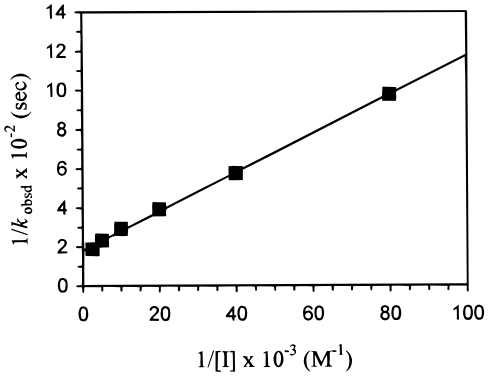


Figure 5. Kitz−Wilson plot for the reaction of papain with inhibitor **7** in 50 mM phosphate buffer (pH 7.0, 1 mM EDTA and 10% (v/v) acetonitrile) at 25 °C.

dissociation constant for the breakdown of enzyme−inactivator complex (E:I) and the inactivation rate constant, respectively.

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

$$v = \frac{d[E']}{dt} = \frac{k_i[I]([E]_0 - [E-I])}{K_I + [I]} \tag{2}$$

$$\frac{1}{k_{obsd}} = \frac{K_I}{k_i[I]} + \frac{1}{k_i} \tag{3}$$

The overall rate expression corresponding to the formation of *S*-nitrosylated protein is given in eq 2, while eq 3 shows the relationship of the observed first-order rate constant (k_{obsd}) to the concentration of inactivator.[20] One typical inactivation process using compound **7** is shown in Figures 4 and 5. Processing of the data according to eq 3 generated the corresponding kinetic parameters which are summarized in Table 1.

Recovery of Enzyme Activity. The papain inactivated by peptidyl *N*-nitrosoanilines could not be recovered after it was subjected to extensive dialysis or a gel permeation column. Nevertheless, almost 90% of the original activity of papain can be regained after treatment of the inactivated enzyme with 2 mM cysteine or glutathione. This was consistent with our previous observations.[12]

Spectroscopic and Chemical Analysis of *S*-Nitroso Papain. The formation of the *S*-nitrosothiol bond in papain has been confirmed by several lines of evidences from spectroscopic and chemical analysis. First, the UV/vis spectra of *S*-nitroso papain demonstrated in Figure 6 exhibited a broad absorption maxima in the range 330−370 nm, which is characteristic of an *S*-N═O functionality.[21] *S*-nitroso papain was purified from the incubation mixture of papain (4 mg/mL) with compound **7** (5 mg/mL) by Sephadex G-25 gel permeation chromatography. Papain derivatized at active site Cys25 with iodoacetamide was prepared to serve as a control sample.

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Table 1. Kinetic Parameters for the Inactivation of Papain by Peptidyl *N*-Nitrosoanilines

inhibitor	k_{inact} (min^{-1})	K_I (mM)	k_{inact}/K_I (M^{-1} s^{-1})
1	0.102	2.818	0.605
2	0.157	0.697	3.754
3	0.121	0.783	2.576
4	0.067	0.617	1.810
5	0.032	0.802	0.665
6	0.339	0.661	8.562
7	0.330	0.055	100.36
8	0.098	1.017	1.606
10	0.087	1.235	1.174

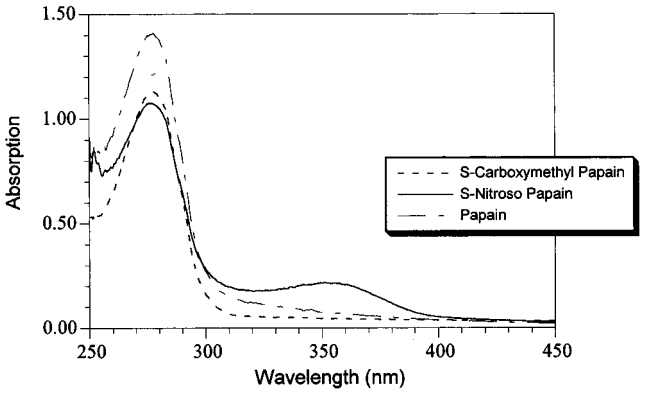

Figure 6. UV/vis spectra of papain, *S*-nitroso papain, and *S*-carboxymethyl papain (50 mM phosphate buffer, pH 7.0, 0.1 mM EDTA).

Second, ^{15}N NMR was used to validate the formation of an *S*-NO bond. ^{15}N-enriched compound **12** was synthesized in the same way as compound **1** with the exception that in this case $Na^{15}NO_2$ was used in the final nitrosation step. The chemical shift of **12** was 492 ppm when referenced to $Na^{15}NO_2$ standard assigned at 570 ppm. The *S*-^{15}NO papain was synthesized by incubating activated papain (80 mg/mL) with compound **12** (60 mg) in 3 mL of D_2O (20% acetonitrile-d_3) at 25 °C. The chemical shift of the observed peak (725 ppm relative to $Na^{15}NO_2$) was consistent with that of an *S*-nitrosothiol bond (Figure 7).[22]

Third, the formation of an *S*-NO bond was verified by FT-IR spectroscopy. In this method, *S*-nitroso papain was desiccated under vacuum and prepared for Fourier transform IR spectroscopy. The spectrum showed a peak in the range 1130−1160 cm^{-1}, which is indicative of the S−N bond formation.[23]

Fourth, *S*-nitrosothiol content in the *S*-NO papain was quantified by the method of Saville.[24] The stoichiometry for

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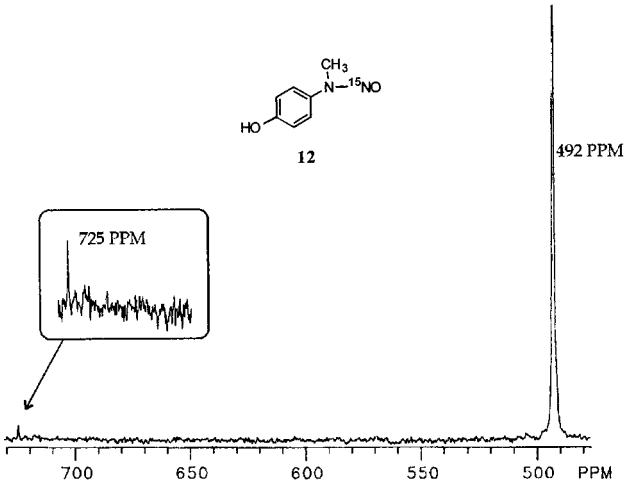
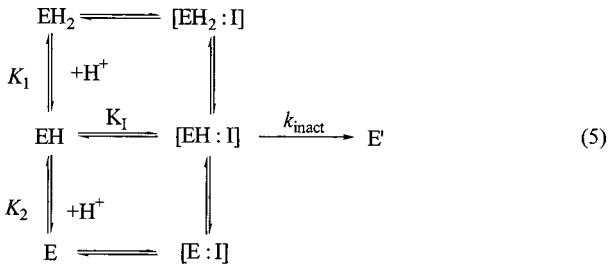


Figure 7. ^{15}N NMR spectrum of *S*-^{15}NO papain.

the *S*-NO papain (mol/mol) was determined to be 0.91 ± 0.06 ($n = 3$). The ratio calculation is based on the concentration of the free thiol group of Cys-25 in the papain active site, as determined by Ellman's reagent (DTNB).[25,26]

The pH Profile of Papain Inactivation. The inactivation of papain was studied in buffers under different pH conditions. The data obtained (Figure 8) can be described by eq 4, which is derived from the model described in eq 5.

$$k_2 = \frac{k_{inact}/K_I}{1 + \frac{[H^+]}{K_1} + \frac{K_2}{[H^+]}} \quad (4)$$



(5)

The scheme above relates the apparent rate constants with the states of dissociation of the sulfhydryl and other groups in papain. Nonlinear regression of the data in Figure 8 to eq 4 yielded the best fit theoretical curve shown in Figure 8 and the calculated values for pK_1, pK_2, K_I, and k_{inact} listed in Table 2. The variation of the second-order rate constant k_2 with pH at 25.0 °C is bell-shaped, with rate constants at a maximum near neutrality, approaching zero at both low and high pH's.

Discussion

Molecular Design. Papain prefers to have a peptide substrate with bulky residue such as Phe at the S_2 subsite and a small hydrophobic residue at the S_1 subsite.[27] The *S*-nitrosation process could therefore be facilitated by directing the nitrosoaniline moiety into the active site through the binding of P_1 and P_2 residues to S_1 and S_2 subsites. On the other hand, utilization of the interaction between the enzyme leaving group

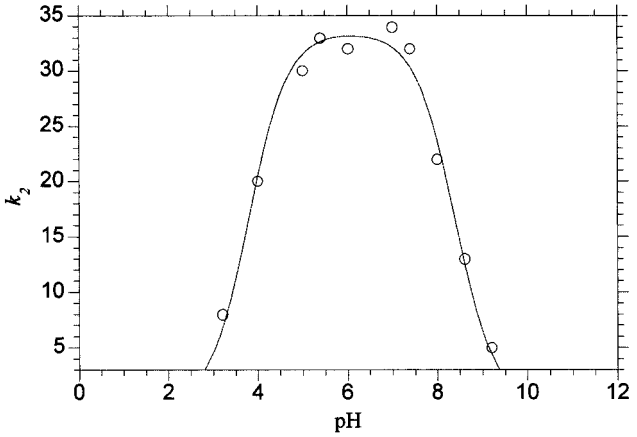


Figure 8. Apparent second-order rate constant k_2 vs pH for the reaction of papain with compound **1** at 25.0 °C. The circles are experimental points, while the solid line is a computer-generated curve fit. Data were gathered in 50 mM acetate buffer over the range pH 3.2−5.8 in 50 mM phosphate buffer from pH 6.0 to 7.8 and in 100 mM borate buffer from pH 8.0 to 9.2. All buffers contained 1 mM EDTA and 10% (v/v) acetonitrile.

Table 2. Kinetic Parameters for the Inactivation of Papain by Compound **1**

pK_1	pK_2	K_I (mM)	k_{inact} (min^{-1})
3.76	8.29	2.90	0.098

binding sites S_1' or S_2' and peptide components has also been demonstrated in the design of several other classes of inhibitors such as E−64 analogues[28] and azapeptides.[29] Inhibitors designed in this manner are equally effective in delivering the functional moiety to the enzyme active site. These inhibitors prefer to bind to the cysteine proteases with moderate size residues such as Leu and Val occupying either S_1' or S_2' binding sites.[2a]

Selective Inhibition of Serine and Cysteine Proteases. Based on the substrate specificity of chymotrypsin, a representative member of serine proteases, the peptidyl *N*-nitrosoanilines synthesized in this work should form the noncovalent enzyme−inactivator complexes with chymotrypsin, placing the *N*-nitroso moiety near active site serine. No irreversible inactivation was observed, however, with these inactivators. This observation was in contrast to the formation of covalent adducts between serine proteases and a variety of peptide-based reversible and irreversible inhibitors such as *N*-nitrosoaminde,[30] peptidyl aldehydes, peptidyl trifluoromethanes, and peptidyl halomethanes.

The peptidyl *N*-nitrosoanilines exhibited time- and concentration-dependent inhibition toward cysteine protease papain in good agreement with covalent inactivation. The kinetic results presented in Table 1 show that the peptidyl *N*-nitrosoanilines are moderate inactivators of papain. The second-order rate constants for inhibition of cysteine proteases by peptidyl *N*-nitrosoanilines were substantially lower than those exhibited by corresponding peptidyl diazomethanes. This interesting observation was partly attributable to higher K_I values of the former inactivators, while in the latter case, the actual dissociation constant for the Michaelis-type complex was significantly lower due to the fast reversible chemical step preceding the alkylation step. The intrinsic reactivity of the nitrosoaniline moiety toward

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the papain thiol group differed in 10 compounds, of which the most potent was inhibitor **7**, followed by **6** and **2**. By comparing the kinetic parameters of **6** and **7**, it appeared that the 12-fold increase in k_{inact}/K_I originated from the decrease in dissociation constants K_I from 0.661 mM (**6**) to 0.055 mM (**7**). This result suggested that the Ala residue in **7** significantly contributed to the stabilization of the inactivator−enzyme complex. Besides the parent *N*-nitrosoaniline compound **1,** compound **5** exhibited the weakest activity. Inhibitory potency decreased as the length of the carbon linkers in compounds **2**, **4**, and **5** increased from two to four carbons. This result strongly suggested that the inactivation happened at the active site of papain and shorter carbon chain brought closer the thiolate of Cys-25 and nitrosoaniline moiety. Variation of the para substitution from hydroxy in **2** to chloro in **3** led to weakened inhibition partly due to electron withdrawing effects, which was consistent with our previous results when dephostatin analogues were used as inactivators.[12] Among those designed to bind the *S′* sites, compounds **9** and **11** had no detectable activities as irreversible inhibitors of papain, while **8** and **10** possessed weak inhibitory potency. When compound **11** was elongated from **8** with a Phe residue at the *C*-terminal, its inhibition against papain disappeared most likely due to the frame shift recognition of the Phe residue by the S_2 site of papain. The same reasoning can also be applied to compound **9**, where the *C*-terminal residue is Phe.

Studies on the pH−Rate Profile. As discussed above, the pH-dependent reaction of papain with compound **1** can be analyzed in terms of the model shown in eq 5. The bell-shaped curve obtained from the k_2−pH profile is remarkably similar to the k_{cat}/K_m−pH profile for the papain-catalyzed hydrolysis of specific substrates.[31] The acid limb of the curve appears to be a dissociation curve from which the pK_1 was calculated to be 3.76. This pK_1 value represents the dissociation of the thiol group of Cys-25. The basic limb of the bell-shaped curve in Figure 8 can be described by pK_2, which has the value of 8.29 at 25.0 °C. This pK_2 can be ascribed to His-159 imidazolium group. This result, instead of the sigmoidal pH dependence, indicated that the enzyme activity toward the *N*-nitroso moiety was dependent upon the states of ionization of both SH and Im-H$^+$ residues.

Mechanistic Implications. In consideration of the selectivity between serine and cysteine proteases, the mechanism for the inactivation of papain by peptidyl *N*-nitrosoanilines is of particular interest. Serine and cysteine proteases are similar in many aspects of their catalytic mechanisms. For example, they both utilize a covalent nucleophilic catalysis and involve formation of an acyl enzyme and two tetrahedral intermediates along the catalytic pathway. Although in protic solutions thiolates have higher nucleophilicity than alkoxides, under nonsolvating conditions, like enzyme−inactivator complexes, alkoxides in serine proteases become better nucleophiles than thiolates in cysteine proteases.[32] Nevertheless, in serine proteases, the nucleophilic attack of the active site serine on the substrate amide bond precedes protonation. In cysteine proteases, on the other hand, protonation of the scissile amide bond occurs prior to or concomitant with the nucleophilic attack.[33a,b]

Scheme 4. Mechanism for the Denitrosation of Nitrosamines in Acidic Conditions

It has been proposed that, at physiological pH, 90% of the papain exists in a form wherein the active site consists of kinetically effective imidazolium thiolate zwitterionic pair.[33c,d] Such initial protonation, a crucial step in the denitrosation of *N*-nitrosoaniline by a nucleophilic group (Scheme 4),[34] is only possible in cysteine proteases due to the existence of a thiolate−imidazolium ion pair in the Michaelis complex and in the free enzyme. On the other hand, despite the higher nucleophilicity of alkoxides than thiolates, initial protonation of *N*-nitrosoaniline is not feasible in serine proteases since the serine and histidine residues are neutral at the corresponding stages. This could account for our observation that peptidyl *N*-nitrosoanilines have no inhibitory potency against serine protease.

Previous studies indicated that strongly acidic conditions are a prerequisite for the denitrosation of *N*-nitrosoaniline-type compounds.[34] However, we observed, for the first time, that the denitrosation process occurred between the papain active site thiol group and *N*-methyl-*N*-nitrosoanilines *under physiological conditions*, which finally led to the formation of *S*-nitroso protein. In analogy with the established mechanism for the denitrosation of nitrosamines under acidic conditions (Scheme 4), a mechanism is suggested for *S*-nitrosation of cysteine protease by peptidyl *N*-nitrosoanilines in which protonation of the *N*-nitroso nitrogen by general acid His-159 is prior to or concomitant with the nucleophilic attack by thiolate group of Cys-25, displacing the aniline moiety with thiolate through a tetrahedral intermediate (Figure 9). The protonation step could be a rate-limiting step and should be aided by electron-donating substitution of *N*-nitrosoanilines. As illustrated in the pH profile (Figure 8), at certain pH values, the Im-H$^+$ group is probably able to protonate the aniline nitrogen and the nucleophilicity of thiolate is strong enough to attack the nitroso nitrogen. This reaction mechanism parallels the nonenzymatic reaction and, at the same time, reflects the cooperative property of the enzyme active site.

Physiological Significance. Existing evidence supports the view that in vivo biological activity of short-lived nitric oxide is stabilized and prolonged by thiol-containing biomolecules[15] in the form of *S*-nitrosothiols,[35] among which *S*-nitrosoalbumin is the predominant form in mammalian plasma.[16,17,36] Although the molecular mechanism of this cGMP-independent pathway is not well understood, alteration and regulation of protein functions through thiol nitrosation has been demonstrated in a tissue-type plasminogen activator[16] which possesses a free thiol group (Cys83) and several thiol-dependent enyzmes such as cathepsin B,[15] glyceraldehyde-3-phosphate dehydrogenase,[37] and *N*-methyl-D-aspartate neuronal receptor.[38] Several commercially available nitrosating agents for *S*-nitrosation purposes are sodium

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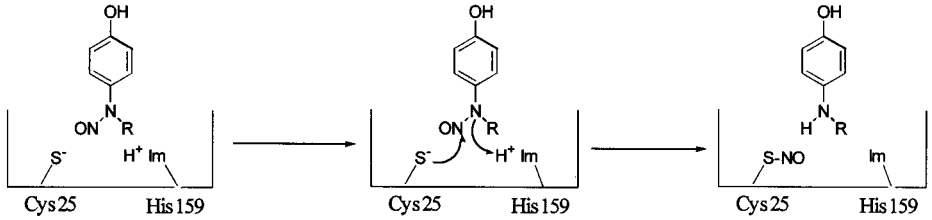

Figure 9. Proposed mechanism for the inactivation of papain by peptidyl *N*-nitrosoanilines.

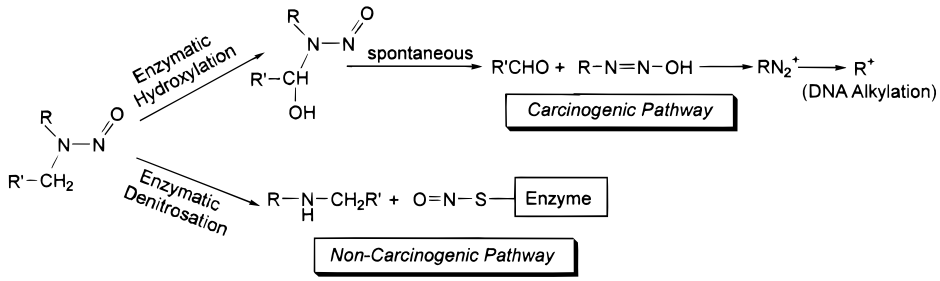

Figure 10. Two distinct pathways for the decomposition of *N*-nitroso compounds.

nitroprusside (SNP),[39] 3-morpholinosydnonimine (SIN-1),[40] (±)-*S*-nitroso-*N*-acetylpenicillamine (SNAP),[41] and nucleophile complexes of NO (NONOates).[42] One salient property shared by these nitric oxide donors is that NO is readily released in vivo and oxidized into nitrosating species N_2O_3. Since NO does not react directly with thiols in the absence of oxygen and transition metals,[43] it is N_2O_3 which actually nitrosates protein thiols to form RS−NO through its nitrosonium-bearing moiety ($ON^{\delta+}-{}^{\delta-}NOO$).[44] In our study, however, the peptidyl *N*-nitrosoanilines were very stable in aqueous solution and no intermediate forms were involved in the *S*-nitrosation process. Unlike low molecular weight *S*-nitrosothiols, under neutral conditions, peptidyl *N*-nitrosoanilines do not react with glutathione which exists in vivo at the 2−10 mM scale. Therefore, this unique type of peptidyl *N*-nitrosoanilines can be labeled as stable, covalent yet recoverable cysteine protease inactivators.

The carcinogenic properties of nitrosamines are not caused by their nitrosating function.[45] In common with many other chemical carcinogens, it is now known that nitrosamines bring about alkylation of specific sites within the cellular DNA. On the basis of our understanding of the properties of *N*-nitrosoanilines, the fate of this class of compounds in vivo is illustrated in Figure 10, which consists of two major pathways, carcinogenic and noncarcinogenic pathways. In the first route, nitrosamines first undergo enzymatic α-hydroxylation and the α-hydroxy nitrosamine breaks down initially to an aldehyde and an unstable primary nitrosamine, from which the alkyl diazonium ion and the alkyl carbocation could react with nucleophilic sites in DNA (and also RNA and proteins). It is also noteworthy that a greater exposure to nitrosamines in man arises from endogenous formation rather than preformed nitrosamines because secondary amines are widespread in foods. In the second route of nitrosamine metabolism, the nitrosamine can react with the cysteine residue of proteases and produce a secondary amine and *S*-nitroso proteins. Although the noncarcinogenic pathway suggested a new route to the detoxification of the nitrosamine compounds by cysteine proteases, its biological relevance awaits further in vivo or ex vivo study.

Conclusions

In the present study, peptidyl *N*-nitrosoanilines were shown as novel selective cysteine protease inhibitors. Selectivity was manifested in the inhibition process between serine and cysteine proteases. Unlike previously reported cysteine protease inactivators, the *S*-nitrosylated proteases can be recovered by the addition of cysteine or glutathione. Mechanistic studies revealed that the inactivation process is through the nucleophilic attack of thiolate ion on the nitroso nitrogen. Besides possible use of these cysteine protease inactivators in the identification of the spatial arrangement of the enzyme active site residues, these peptidyl *N*-nitrosoanilines can also function as stable nitrosonium donors for biomedical and pharmaceutical applications.

Experimental Section

General Methods. Enzymes, amino acids, amino acid derivatives, and other chemical reagents were purchased from commercial suppliers and used without further purification unless otherwise noted. ^1H and ^{13}C NMR spectra were recorded on a Varian VRX 400S NMR or Gemini 300 NMR spectrometer. ^{15}N NMR experiments were performed on a GN 300 NMR spectrometer. High-resolution mass spectra (HRMS) were obtained from mass spectrometer facilities at the University of California, Riverside. The FTIR spectra were recorded on a Perkin-Elmer 2000 with microscope Perkin-Elmer I series. Silica gel plates (Merck F254) and silica gel 60 (Merck; 70−230 mesh) were used in analytical thin-layer chromatography (TLC) and column chromatography, respectively.

Synthesis of Compound 1. 31: To a solution of *N*-methyl-*p*-anisidine (0.209 g, 98%, 1.5 mmol) in anhydrous CH_2Cl_2 (10 mL) at −78 °C was added BBr_3 (3 mL, 1 M in CH_2Cl_2, 3 mmoL). The solution was allowed to warm to room temperature and was stirred for 12 h. Sodium hydroxide solution (1 M) was added to quench the reaction

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until pH 8∼9. The solution was extracted three times with CH_2Cl_2. The combined organic phase was washed with water and brine sequentially and dried over anhydrous $MgSO_4$. The solvent was evaporated and the residue recrystallized in EtOAc/hexane to yield *p*-hydroxyl-*N*-methylaniline (**31**) (0.145 g, 78.6%) as a dark brown solid: ^{1}H NMR (acetone-d_6) **δ** 2.68 (s, 3H), 4.38 (s, 1H), 6.45 (d, 2H, J = 9.0 Hz, Ph), 6.63 (d, 2H, J = 9.0 Hz, Ph), 7.38 (s, 1H); MS calcd for $C_7H_9NO^+$ (M^+) 123, found 123.

1: General Method for Nitrosation Procedure. *p*-Hydroxyl-*N*-methylaniline (**31**) (0.139 g, 1.13 mmol) was dissolved in 1 mL of glacial acetic acid in an ice−water bath. Then 5 mL of water and $NaNO_2$ (0.179 g, 1.14 mmol) were added. The reaction mixture was stirred for 1 h and quenched by saturated $NaHCO_3$. The resulting solution was extracted by EtOAc (3 × 20 mL). The combined organic phase was washed with brine and dried over anhydrous $MgSO_4$. The solvent was removed to afford **1** (0.130 g, 76%) as a dark brown solid: ^{1}H NMR (CD_3OD) δ 3.22 (s, 3H, CH_3), 6.81 (d, 2H, Ph, J = 8.8 Hz), 7.29 (d, 2H, Ph, J = 8.8 Hz); ^{13}C NMR acetone-d_6) 157.73, 122.48, 122.47, 116.74, 32.30; HRMS (CI) calcd for $C_7H_8N_2O_2$ (M^+) 152.0586, found 152.0643

Synthesis of Compound 2. 21: To a stirred solution of *N*-acetyl-L-phenylalanine (1 g, 4.83 mmol) and aminoacetaldehyde dimethyl acetal (0.53 mL, 4.83 mmol) in CH_2Cl_2 (25 mL) was added EDC (0.926 g, 4.83 mmol) and HOBt (0.653 g, 4.83 mmol), with stirring being continued at 4 °C for 24 h. The reaction mixture was worked up and dried over anhydrous $MgSO_4$. The solvent was evaporated to give white solid product **21** (1.067 g, 75%): ^{1}H NMR ($CDCl_3$) δ 2.00 (s, 3H, Ac), 3.02 (AB, 1H, C*H*$_2$Ph, J_{AB} = 13.6 Hz, $J_{H\alpha-B}$ = 9.6 Hz), 3.06 (AB, 1H, C*H*$_2$Ph, J_{AB} = 13.6 Hz, $J_{H\alpha-A}$ = 6 Hz), 3.30 (m, 2H, N*H*CH_2), 3.31 (s, 6H, CH(O*Me*)$_2$), 4.21 (t, 1H, C*H*(OMe)$_2$), 4.68 (q, 1H, C_αH), 6.13 (d, 1H, J = 7.2 Hz, N*H*CH_2), 6.51 (d, 1H, J = 8.0 Hz, AcN*H*), 7.27 (m, 5H, Ph); FABMS calcd for $C_{15}H_{22}N_2O_4$ (M^+) 294, found 295 ($M + H^+$).

23: Compound **21** (0.5 g, 1.21 mmol) in trifluoroacetic acid (10 mL) was stirred at 0 °C for 10 min. The reaction was immediately quenched by dilution with 50 mL of 30% CH_3CN in H_2O. After evaporation of the solvent in vacuo, the residual oil was redissolved into EtOAc (60 mL), washed with cold saturated $NaHCO_3$ (2 × 20 mL), and dried over anhydrous $MgSO_4$. The solvent was removed in vacuo to give yellow oil **23** (0.38 g, 80%): ^{1}H NMR (CD_3OD) δ 1.81 (s, 3H, Ac), 2.76 (AB, 1H, C*H*$_2$Ph, J_{AB} = 10.6 Hz, $J_{H\alpha-B}$ = 9.6 Hz), 2.80 (AB, 1H, C*H*$_2$Ph, J_{AB} = 13.6 Hz, $J_{H\alpha-A}$ = 6 Hz), 3.00 (m, 2H, N*H*CH_2), 4.50 (q, 1H, C_αH), 7.16 (m, 5H, Ph); FABMS calcd for $C_{13}H_{16}N_2O_3$ (M^+) 248, found 248.

25: General Method for Reductive Amination. To a stirred solution of **23** (0.2 g, 0.8 mmol) in anhydrous MeOH (20 mL) was added 4-aminophenol (0.263 g, 2.4 mmol) and $NaCNBH_3$ (0.051 g, 0.8 mmol). After the mixture was stirred at room temperature overnight, the solvent was removed in vacuo and the residue was chromatographed with 0−5% MeOH/EtOAc to afford brown solid product **25** (0.125 g, 50%): ^{1}H NMR (CD_3OD) δ 1.83 (s, 3H, Ac), 2.81 (AB, 1H, C*H*$_2$Ph, J_{AB} = 13.2 Hz, $J_{H\alpha-A}$ = 8 Hz), 2.95 (m, 3H), 3.21 (m, 2H, NCH_2), 4.43 (t, 1H, C_αH, J = 8.4 Hz), 6.46 (d, 2H, J = 8.8, NPh), 6.55 (d, 2H, J = 9.2 Hz, NPh), 7.16 (m, 5H, CH_2*Ph*); CIMS calcd for $C_{19}H_{23}N_3O_3$ (M^+) 341, found 341.

2: ^{1}H NMR (CD_3OD) δ 1.80 (s, 3H, Ac), 2.65 (AB, 1H, C*H*$_2$Ph, J_{AB} = 13.6 Hz, $J_{H\alpha-B}$ = 9.6 Hz), 2.84 (AB, 1H, C*H*$_2$Ph, J_{AB} = 13.6 Hz, $J_{H\alpha-A}$ = 6 Hz), 3.20 (m, 2H, NCH_2), 3.98 (m, 2H, CH_2NNO), 4.35 (t, 1H, C_αH, J = 7.6 Hz), 6.80 (d, 2H, NPh, J = 9.2 Hz), 7.15 (m, 5H, CH_2*Ph*), 7.29 (d, 2H, NPh, J = 9.2 Hz); ^{13}C NMR (CD_3OD) δ 173.9, 173.0, 158.7, 138.5, 135.2, 130.2, 129.4, 127.7, 123.7, 117.0, 56.1, 45.3, 38.8, 37.2, 22.4; HRFABMS calcd for $C_{19}H_{22}N_4O_4$ (M^+) 370.1641, found 371.1714 (M + H).

Synthesis of Compound 3. 3: ^{1}H NMR (CD_3OD) δ 1.85 (s, 3H, Ac), 2.79 (AB, 1H, C*H*$_2$Ph, J_{AB} = 13.6 Hz, $J_{H\alpha-B}$ = 9.6 Hz), 2.88 (AB, 1H, C*H*$_2$Ph, J_{AB} = 13.6 Hz, $J_{H\alpha-A}$ = 6 Hz), 3.18 (m, 2H, NCH_2), 3.95 (m, 2H, CH_2NNO), 4.38 (t, 1H, C_αH, J = 7.6 Hz), 7.17 (m, 5H, CH_2*Ph*), 7.38 (m, 2H, NPh), 7.15 (m, 5H, CH_2*Ph*), 7.48 (m, 2H, NPh); ^{13}C NMR (20% $CDCl_3$/CD_3OD) δ 172.0, 171.0, 139.7, 136.2, 132.9, 129.4, 128.7, 128.3, 126.5, 120.3, 54.2, 42.7, 37.6, 35.5, 22.1; HRFABMS calcd for $C_{19}H_{21}ClN_4O_3$ (M^+) 388.1302, found 389.1377 (M + H).

Synthesis of Compound 4. 27: To a stirred solution of *N*-acetyl-L-phenylalanine (1 g, 4.83 mmol) and 3-amino-1-propanol vinyl ether (0.42 mL, 4.83 mmol) in CH_2Cl_2 (20 mL) was added EDC (0.926 g, 4.83 mmol) and HOBt (0.653 g, 4.83 mmol), with stirring being continued at 4 °C for 24 h. The reaction mixture was worked up and dried over anhydrous $MgSO_4$. The solvent was rotary evaporated to give white solid product **27** (1.1 g, 80%): ^{1}H NMR ($CDCl_3$) δ 1.68 (m, 2H, $NHCH_2$C*H*$_2$), 1.97 (s, 3H, Ac), 2.95 (AB, 1H, C*H*$_2$Ph, J_{AB} = 13.2 Hz, $J_{H\alpha-A}$ = 8 Hz), 3.05 (AB, 1H, C*H*$_2$Ph, J_{AB} = 12.2 Hz, $J_{H\alpha-B}$ = 6 Hz), 3.26 (m, 2H, $NHCH_2$), 3.51 (m, 2H, C*H*$_2$O), 3.97 (d, 1H, J = 8.8 Hz, OCHC*H*$_2$), 4.10 (d, 1H, J = 8.8 Hz, OCHC*H*$_2$), 4.55 (d, 1H, J = 9.2 Hz, H_α), 5.87 (s, 1H, N*H*CH_2), 6.22 (d, 1H, J = 8.0 Hz, AcN*H*), 6.35 (q, 1H, J = 9.2 Hz, OC*H*CH_2), 7.20 (m, 5H); FABMS calcd for $C_{16}H_{22}N_2O_3$ (M^+) 290, found 291 ($M + H^+$).

28: To a glacial acetic acid solution (10 mL) of **27** (500 mg, 1.02 mmol) was added 1 N HCl (15 mL) at 0 °C. After being stirred for 10 min, the reaction mixture was immediately quenched by dilution with 60 mL of 30% acetonitrile in H_2O. After evaporation of the solvent in vacuo, the residual oil was redissolved into EtOAc (60 mL), washed with cold saturated $NaHCO_3$ (2 × 20 mL), and dried over anhydrous $MgSO_4$. The solvent was removed in vacuo to give white solid product **28** (350 mg, 75%): ^{1}H NMR ($CDCl_3$) δ 1.55 (m, 2H, $NHCH_2$C*H*$_2$), 1.97 (s, 3H, Ac), 3.01 (AB, 1H, J = 12.2 Hz, C*H*$_2$Ph), 3.05 (AB, 1H, J = 13.0 Hz, C*H*$_2$Ph), 3.29 (m, 2H, NHC*H*$_2$), 3.47 (t, 2H, J = 6 Hz, C*H*$_2$OH), 4.62 (q, 1H, J = 14.8 Hz, J = 8.4 Hz, C_αH), 6.55 (d, 1H, J = 8 Hz, AcN*H*), 7.24 (m, 5H, Ph); MS calcd for $C_{14}H_{20}N_2O_3$ (M^+) 264, found 265 ($M + H^+$).

30: To a solution of $(COCl)_2$ (0.174 mL, 2 mmoL) in CH_2Cl_2 (20 mL) was added a mixture of alcohol **28** (500 mg, 1.89 mmol) and DMSO (0.355 mL, 5 mmol) diluted with CH_2Cl_2 at −78 °C. The mixture was added within 5 min, and the reaction solution was stirred for additional 15 min. The reaction was quenched with 30 mL of water and extracted with CH_2Cl_2 (3 × 20 mL). The organic layer was washed sequentially with 1 N HCl (2 × 10 mL), 1 N $NaHCO_3$ (2 × 10 mL), and saturated NaCl (2 × 10 mL). The solution was dried over anhydrous $MgSO_4$. Removal of the solvent in vacuo gave the yellow syrup which was directly used for reductive amination (340 mg, 72%): ^{1}H NMR (CD_3OD) δ 1.57 (m, 2H, $NHCH_2$C*H*$_2$), 1.83 (s, 3H, Ac), 2.80 (AB, 1H, C*H*$_2$Ph, J_{AB} = 13.2 Hz, $J_{H\alpha-A}$ = 8 Hz), 2.97 (AB, 1H, C*H*$_2$Ph, J_{AB} = 13.2 Hz, $J_{H\alpha-A}$ = 8 Hz), 3.12 (m, 2H, NCH_2), 4.42 (t, 1H, C_αH, J = 8.4 Hz), 6.51 (d, 2H, J = 6.0 Hz, NPh), 6.55 (d, 2H, J = 6.0 Hz, NPh), 7.14 (m, 5H, CH_2*Ph*); FABMS calcd for $C_{20}H_{25}N_3O_3$+ (M^+) 355, found 356 ($M + H^+$).

4: H NMR (300 MHz, acetone-d_6) δ 1.58 (m, 2H, $NHCH_2$C*H*$_2$), 1.84 (s, 3H, Ac), 2.83 (AB, 1H, C*H*$_2$Ph, J_{AB} = 11.1 Hz, $J_{H\alpha-A}$ = 6 Hz), 3.09 (m, 3H), 3.97 (t, 2H, NCH_2), 4.54 (m, 1H, C_αH), 6.93 (d, 2H, N*Ph*), 7.16 (m, 5H, CH_2*Ph*), 7.36 (d, 2H, NPh), 7.72 (s, 1H, N*H*CH_2), 7.79 (d, 1H, AcN*H*), 9.60 (s, 1H, PhO*H*); ^{13}C NMR (CD_3-OD) 172.00, 169.95, 158.40, 138.98, 130.03, 128.83, 126.99, 123.25, 116.78, 55.40, 42.73, 38.77, 37.27, 27.21, 22.82; HRFABMS calcd for $C_{20}H_{24}N_4O_4$ (M^+) 384.1798, found 384.1811.

Synthesis of Compound 5. 22: To a stirred solution of *N*-acetyl-L-phenylalanine (1 g, 4.83 mmol) and 4-aminobutyraldehyde diethyl acetal (0.78 mL, 4.83 mmol) in CH_2Cl_2 (25 mL) was added EDC (0.926 g, 4.83 mmol) and HOBt (0.653 g, 4.83 mmol), with stirring continued at 4 °C for 24 h. The reaction mixture was worked up and dried over anhydrous $MgSO_4$. The solvent was evaporated to give a white solid product **22** (1.09 g, 70%): ^{1}H NMR ($CDCl_3$) δ 1.19 (t, 2H, $NHCH_2$C*H*$_2$), 1.40 (m, 2H, NH$(CH_2)_2$C*H*$_2$), 1.97 (s, 3H, Ac), 2.96 (AB, 1H, C*H*$_2$Ph, J_{AB} = 13.6 Hz, $J_{H\alpha-B}$ = 9.6 Hz), 3.06 (AB, 1H, C*H*$_2$Ph, J_{AB} = 13.6 Hz, $J_{H\alpha-A}$ = 6 Hz), 3.16 (m, 2H, NHC*H*$_2$), 3.46 (m, 6H, CH*(*OCH$_2$C*H*$_3$*)*$_2$), 3.60 (m, 4H, CH*(*OC*H*$_2$CH$_3$*)*$_2$), 4.40 (t, 1H, C*H*(O-Et)$_2$), 4.58 (q, 1H, C_αH), 5.59 (d, 1H, J = 7.2 Hz, N*H*CH_2), 6.41 (d, 1H, J = 8.0 Hz, AcN*H*), 7.26 (m, 5H, Ph); FABMS calcd for $C_{19}H_{30}N_2O_4^+$ (M^+) 350, found 351 ($M + H^+$).

26: H NMR ($CDCl_3$) δ 1.19 (t, 2H, $NHCH_2$C*H*$_2$), 1.40 (m, 2H, NH$(CH_2)_2$C*H*$_2$), 1.97 (s, 3H, Ac), 2.84 (AB, 1H, C*H*$_2$Ph, J_{AB} = 13.2 Hz, $J_{H\alpha-A}$ = 8 Hz), 3.02 (AB, 1H, C*H*$_2$Ph, J_{AB} = 13.2 Hz, $J_{H\alpha-A}$ = 8

Hz), 3.22 (m, 2H, NCH_2), 4.48 (t, 1H, $C_\alpha H$, $J = 8.4$ Hz), 6.56 (d, 2H, $J = 6.0$ Hz, NPh), 6.71 (d, 2H, $J = 6.0$ Hz, NPh), 7.16 (m, 5H,CH_2Ph); FABMS calcd for $C_{21}H_{27}N_3O_3^+$ (M^+) 369, found 370 ($M + H^+$).

5: ^{1}H NMR (CD_3OD) δ 1.19 (m, 2H, $NHCH_2CH_2$), 1.38 (m, 2H, CH_2CH_2N), 1.83 (s, 3H, Ac), 3.02 (AB, 1H, CH_2Ph, $J_{AB} = 11.2$ Hz, $J_{H\alpha-A} = 6$ Hz), 3.25 (AB, 1H, CH_2Ph, $J_{AB} = 11.2$ Hz, $J_{H\alpha-A} = 6$ Hz), 3.48 (m, 2H, CH_2N), 4.06 (m, 2H, $NHCH_2$), 4.91 (t, 1H, $C_\alpha H$, $J = 8.2$ Hz), 6.86 (d, 2H, $J = 6.0$ Hz, NPh), 7.17 (m, 5H, Ph (Phe)), 7.37 (d, 2H, $J = 6.0$ Hz, NPh), 7.40 (d, 1H, Ac*NH*), 9.58 (s, 1H, Ph*OH*); ^{13}C NMR (acetone-d_6) 172.50, 170.10, 158.23, 139.12, 134.32, 130.02, 128.80, 127.99, 123.14, 117.00, 57.10, 44.10, 38.50, 37.60, 27.00, 25.10, 22.92; HRFABMS calcd for $C_{21}H_{26}N_4O_4$ (M^+) 398.1954, found 399.2018 ($M + H^+$).

Synthesis of Compound 6. 6: ^{1}H NMR (300 MHz, CD_3OD) δ 1.94 (s, 3H, Ac), 2.99 (AB, 1H, CH_2Ph, $J_{AB} = 13.6$ Hz, $J_{H\alpha-B} = 9.6$ Hz), 3.26 (AB, 1H, CH_2Ph, $J_{AB} = 13.6$ Hz, $J_{H\alpha-A} = 6$ Hz), 4.83 (t, 1H, $C_\alpha H$), 6.13 (d, 1H, $J = 7.2$ Hz, $NHCH_2$), 7.30 (m, 5H, CH_2Ph); 7.45 (m, 5H, N*Ph*); ^{13}C NMR (75 MHz) (CD_3OD) 171.85, 170.32, 140.84, 136.54, 129.02, 128.84, 128.11, 126.61, 126.46, 116.56, 53.16, 37.28, 20.81; HRFABMS calcd for $C_{17}H_{18}N_4O_3^+$ (M^+) 326.1379, found 327.1468 ($M + H^+$).

Synthesis of Compound 7. 7: ^{1}H NMR (300 MHz, 20% acetone-d_6/CD_3OD) δ 1.28 (d, 3H, CH_3(Ala)), 1.93 (s, 3H, Ac), 3.08 (AB, 1H, CH_2Ph, $J_{AB} = 13.6$ Hz, $J_{H\alpha-B} = 9.6$ Hz), 3.28 (AB, 1H, CH_2Ph, $J_{AB} = 13.6$ Hz, $J_{H\alpha-A} = 6$ Hz), 4.32 (q, 1H, $C_\alpha H$ (Ala)), 4.82 (q, 1H, $C_\alpha H$ (Phe)), 7.32 (m, 5H, Ph (Phe)), 7.47 (m, 5H, NPh); ^{13}C NMR (CD_3OD) 171.57, 169.82, 165.82, 142.45, 138.01, 128.88, 128.55, 127.50, 116.01, 52.31, 36.45, 21.50, 16.31; HRFABMS calcd for $C_{20}H_{22}N_5O_4$+ (M^+) 369.1672, found 370.1721 ($M + H^+$).

Synthesis of Compound 8. 8: ^{1}H NMR (CD_3OD) δ 0.84 (d, 3H, $CHCH_3$, $J = 6.4$ Hz), 0.89 (d, 3H, $CHCH_3$, $J = 6.4$ Hz), 1.55 (m, 2H, $CHCH_2CH$), 1.62 (m, 1H, $CHCH_2CH$), 3.24 (s, 3H, OCH_3), 3.37 (q, 1H, $C_\alpha H$,), 4.53 (d, 1H, NCH_aH_bCO, $J = 16$ Hz), 4.66 (d, 1H, NCH_aH_b-CO, $J = 16$ Hz), 6.82 (d, 2H, Ph, $J = 5.3$ Hz), 7.32 (d, 2H, Ph, $J = 5.3$ Hz); ^{13}C NMR (acetone-d_6) δ 173.39, 165.89, 157.92, 135.59, 128.48, 123.49, 116.56, 52.26, 51.59, 49.13, 41.53, 25.33, 23.16, 21.81; HRFABMS calcd for $C_{15}H_{21}N_3O_5^+$ (M^+) 323.1481, found 323.1567.

Synthesis of Compound 9. 9: ^{1}H NMR (CD_3OD) δ 2.92 (AB, 1H, CH_2Ph, $J_{AB} = 16$ Hz, $J_{H\alpha-B} = 8.4$ Hz), 3.09 (AB, 1H, CH_2Ph, $J_{AB} = 16$ Hz, $J_{H\alpha-B} = 5.2$ Hz), 3.24 (s, 3H, OCH_3), 4.48 (d, 1H, NCH_aH_b-CO, $J = 15.6$ Hz), 4.59 (d, 1H, NCH_aH_bCO, $J = 15.6$ Hz), 4.60 (q, 1H, $NHCHCO$), 6.79 (d, 2H, NPh, $J = 5.3$ Hz), 7.18 (m, 5H, CH_2Ph); 7.20 (d, 2H, NPh, $J = 5.3$ Hz); ^{13}C NMR (acetone-d_6) δ 172.18, 165.68, 157.91, 137.64, 135.56, 130.18, 129.19, 128.47, 127.55, 123.35, 116.60, 54.61, 52.33,48.99, 38.26; HRFABMS calcd for $C_{18}H_{19}N_3O_5^+$ (M^+) 357.1325, found 358.1421 ($M + H^+$).

Synthesis of Compound 10. 10: ^{1}H NMR (CD_3OD) δ 1.40 (d, 3H, $CHCH_3$, $J = 7.2$ Hz), 3.30 (s, 3H, OCH_3), 4.39 (q, 1H, $NHCHCO$,), 4.66 (d, 1H, NCH_aH_bCO, $J = 16$ Hz), 4.81 (d, 1H, NCH_aH_bCO, $J = 16$ Hz), 7.39 (m, 1H, NPh H_γ), 7.50 (m, 2H, NPh H_β); 7.58 (m, 2H, NPh H_α); ^{13}C NMR (acetone-d_6) δ 173.41, 165.45, 143.20, 130.14, 128.09, 120.89, 52.35, 49.01, 48.06, 17.78; HRFABMS calcd for $C_{12}H_{15}N_3O_4$ (M^+) 265.1063, found 266.1211 ($M + H^+$).

Synthesis of Compound 11. 11: ^{1}H NMR (CD_3OD) δ 0.82 (d, 3H, CHCH3, $J = 6.8$ Hz), 0.88 (d, 3H, CHCH3, $J = 6.8$ Hz), 1.42 (t, 3H, $CHCH_2CH$), 1.58 (m, 1H, $CH(CH_3)_2$), 3.20 (m, 2H, NCH_2), 3.98 (m, 2H, CH_2NNO), 3.30 (s, 3H, OCH_3), 4.39 (q, 1H, $NHCHCO$,), 4.66 (d, 1H, NCH_aH_bCO, $J = 16$ Hz), 4.81 (d, 1H, NCH_aH_bCO, $J = 16$ Hz), 7.39 (m, 1H, NPh H_γ), 7.50 (m, 2H, NPh H_β); 7.58 (m, 2H, NPh H_α); ^{13}C NMR (acetone-d_6) δ 172.37, 165.58, 143.24, 137.83, 130.16, 130.10, 129.16, 128.13, 127.49, 121.01, 54.53, 54.46, 52.45, 52.41, 52.36, 52.23, 48.45, 42.01, 41.96, 38.18, 38.15, 25.25, 23.37; HRFABMS calcd for $C_{24}H_{30}N_4O_4$ 438.2267, found 439.2311 ($M + H^+$).

Enzymatic Assay. In a typical papain enzymatic assay, papain was first activated by either 2 mM cysteine or 10 mM sodium cyanoborohydride in 50 mM sodium phosphate buffer (pH 7.0, 1 mM EDTA, 100 mM NaCl) and then was passed through a 2.3 cm × 26 cm Sephadex G-25 column (Amicon, Danvers, MA) equipped with Retriever II fraction collector and ISCO UA-5 absorbance/fluorescence detector (ISCO, Inc., Lincoln, NE) at 4−6 °C. The column was equilibrated prior to purification and eluted with 50 mM sodium phosphate buffer (pH 7.0, 1 mM EDTA, 100 mM NaCl). Fractions containing the protein were pooled and lyophilized when necessary. The enzyme obtained in this way excluded any unexpected effects of cysteine and $NaCNBH_3$. Papain activity was measured spectrophotometrically at 410 nm with either UV/vis spectrophotometer (SHIMADZU) or Spectronic Genesys 2 (Milton Roy) using chromogenic substrate *N*-Cbz-Gly *p*-nitrophenyl ester (25 mM, 10 μL) in 1 mL of 50 mM phosphate buffer (pH 7.0, 1 mM EDTA, 7% (v/v) acetonitrile).

A 5 mM peptidyl *N*-nitrosoaniline solution was prepared in 50 mM phosphate buffer (pH 7.0, 20% (v/v) acetonitrile, 1 mM EDTA). To initiate incubation, each peptidyl *N*-nitrosoaniline solution obtained after serial dilution was mixed with activated papain in 50 mM sodium phosphate buffer of equal volume (pH 7.0, 1 mM EDTA, 100 mM NaCl) at room temperature. An aliquot was periodically removed from the incubation mixture and diluted into the enzyme assay solution containing the substrate. The residual enzymatic activity was measured. A control preincubation solution, containing all of the ingredients except for the inhibitor itself, was run and assayed in parallel.

Enzymatic assay protocol for serine protease is similar to that of cysteine protease except that the substrate is *N*-succinyl-Ala-Ala-Pro-Phe *p*-nitroaniline.

Protrein Content. Protrein content of papain preparations was determined according to the method of Lowry.[46]

Protein Thiol Derivatization. Carboxyamidation of protein thiols was carried out with 10-fold molar excess of iodoacetamide in sodium phosphate buffer of neutral pH for 1 h in the dark at 25 °C. Proteins were then dialyzed extensively to remove excess iodoacetamide.

Thiol Content. Thiol content was measured according to a modified[28] Ellman assay.[29] Protein was diluted in 50 mM sodium phosphate buffer containing 6 M guanidine hydrochloride and 1 mM EDTA at pH 7.0. Then, 5,5′-dithiobis(2-nitrobenzoic acid) was added to a final concentration of 0.15 mM. The increase of absorbance at 412 nm was followed until maximal absorption was achieved. The concentration of free thiols was calculated from the molar extinction coefficient of the nitrothiobenzoate in 6 M guanidine ($\epsilon_{412} = 13\,700$ M^{-1} cm^{-1}).

***S*-Nitrosothiol Content.** The molar nitrosothiol content of papain was determined by the method of Saville.[24] A sample containing *S*-nitroso derivatives was first mixed with 0.1% ammonium sulfamate in 0.4 N HCl (total volume = 0.5 mL) for 1 min to remove NO_2^- or HNO_2 from the sample. A solution (0.5 mL) containing 3% sulfanilamide and 0.25% $HgCl_2$ in 0.4 N HCl was then added, followed by 0.5 mL of 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride in 0.4 N HCl. The mixture was incubated at room temperature for 10 min, and the absorbance was read at 540 nm. The nitroso content was calculated according to a calibration curve with 2.5−20 μM $NaNO_2$.

^{15}N NMR Measurements. ^{15}N NMR experiments were done according to the method of Bonnett et al.,[25b] and the spectra were recorded on a GN 300-MHz spectrometer (General Electric). The spectra were referenced to a ^{15}N spectrum of $Na^{15}NO_2$ at 570 ppm. Spectra were recorded at 30.40 MHz, and 20 000 transients were collected with a 30° pulse width and a 8-s relaxation delay. The data obtained were multiplied by a 2-Hz line broadening factor before transformation.

Acknowledgment. This work was generously supported by the research grants from the NIH (GM54074), NSF (BES-9728366), ACS-PRF (30616-G1), American Cancer Society, Florida Division (F95UM-2), American Heart Association, Florida Affiliate (9701760), Mizutani Foundation (138A), Herman Frasch Foundation (449-HF97), and Hercules Incorporation. J.R. thanks the National Cancer Institute for a Predoctoral Fellowship (1F31CA70109) and J.L. thanks the Maytag Fellowship from the University of Miami. We thank Dr. M. Ksebati for his help in performing ^{15}N NMR experiments.

Supporting Information Available: ^{15}N, ^{1}H, and ^{13}C NMR spectra (**1**−**11**) and kinetic data of the enzymatic assay (33 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

JA974187E

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