

N-Nitrosoanilines: A New Class of Caspase-3 Inhibitors

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Abstract—Caspases are a family of cysteine proteases activated during apoptosis. In cultured human endothelial cells, physiological levels of NO prevent apoptosis and interfere with the activation of the caspase cascade. Previous studies have demonstrated that NO inhibits the activity of caspase-3 by *S*-nitrosylation of the enzyme. In this study, the inhibitory effect of a new class of NO donors, *N*-nitrosoaniline derivatives, were examined against caspase-3. Initially eight small molecule inhibitors bearing *N*-nitroso moieties were assayed. It was found that the presence of an electron-donating group on the phenyl ring led to better inhibitory potency, a trend consistent with the results from the previous papain studies. Based on the analysis of the enzyme and substrates' structures, two peptidyl *N*-nitrosoaniline inhibitors [Ac-DVAD-NNO (**1**) and Ac-DV-AMO (**2**)] were designed and synthesized. Both compounds exhibited enhanced inhibitory potency against caspase-3. © 2000 Elsevier Science Ltd. All rights reserved.

Introduction

Nitric oxide (NO), a small potentially toxic diatomic free-radical, has become one of the most studied entities in biological chemistry in the past few years.¹ As a biological messenger, NO was found to be involved in diverse physiological processes such as vasodilatory and antiplatelet effects, macrophage-induced cytotoxicity, and neurotransmission.^{2–9} However, the half-life time of NO in vivo is very short. It easily reacts with molecular oxygen and other reactive oxygen species as well as free thiols, such as glutathione and proteins, to form nitrosothiols. Nitrosothiols functionally mimic NO and are thought to play important roles in vivo.^{10,11} Many studies on cysteine containing enzymes, such as ornithine decarboxylase,¹² vacuolar H⁺-ATPase,¹³ and protein tyrosine phosphatase (PTPase),^{14,15} indicate that thiol residues on these enzymes could be modified by NO donors through an *S*-nitrosylation mechanism and thus influence cellular functions.

Dephostatin, a PTPase inhibitor isolated from the culture broth of *Streptomyces* sp., was recently synthesized in our laboratory, along with its unsubstituted precursor, *N*-methyl-*N*-nitrosoaniline.¹⁶ Besides their competitive inhibition against PTPases,¹⁶ both compounds were also found to be good inhibitors of cysteine protease papain.¹⁷ For a better understanding of this inter-

esting observation, a series of dephostatin analogues, substituted *N*-methyl-*N*-nitrosoanilines, were subsequently synthesized and studied for enzyme inhibition. Compounds substituted at the *para* position by hydroxyl and *N,N*-dimethylamino groups were shown to be more potent than unsubstituted *N*-methyl-*N*-nitrosoaniline in the inactivation. Furthermore, in our recent study on the inactivation of cysteine proteases by peptidyl *N*-nitrosoanilines,¹⁸ we have demonstrated the inhibition was effected through *S*-nitrosylation of the proteins' active site thiol groups by the *N*-nitroso compounds. This kind of direct regulation of protein function by NO, independent of the activation of the soluble guanylyl cyclase and subsequent production of cGMP, has been proposed as an alternative pathway where NO may directly play a critical role in many processes, such as blood pressure regulation, host defense, and neurotransmission.^{19–21}

In connection with our efforts to develop the mechanism based cysteine protease inhibitors mediated by *S*-nitrosylation, caspase-3 was chosen as a target enzyme in the present study. Caspases play a crucial role in the execution of apoptosis. Caspase-3 represents the execution enzyme of the caspase cascade that cleaves the DNase inhibitor ICAD (Inhibitor of Caspase-Activated Deoxyribonuclease) to activate DNA-degrading DNases.²² Within the molecular structure of caspase-3, the catalytic cysteine group that accounts for the proteolytic activity of the enzyme is located at position 163 of the p17 subunit.²³ Recently, the possibility of employing NO to attempt to modulate the activity of the caspase family of cysteine proteases has attracted increasing attention.^{24–28}

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Several studies suggested that NO may inhibit apoptosis via interacting with caspase cysteine protease.^{29–31} As a general feature of its biochemical properties, NO is known to be capable of modifying proteins that contain cysteine residues by *S*-nitrosation of the sulfhydryl group of the respective cysteine.²¹ Indeed, the decrease in caspase-3-like enzyme activity by incubation of the enzyme with NO donors could be specifically assigned to *S*-nitrosylation of the essential cysteine residue at the active site of caspase-3 both in vitro^{26,27} and in vivo.²⁹ This could represent a potential molecular mechanism underlying the functional relationship between NO and inhibition of apoptosis signaling observed in cell culture. The NO donors previously used as the inhibitors of caspase-3 include NONOate, NOBF₄, sodium nitroprusside, and *S*-nitrosoglutathione. However, they are normally unstable, especially in solution, and are photosensitive. This not only makes the delivery and handling of these compounds very inconvenient, but also directly affects the accuracy and reliability of NO research results. In contrast, our peptidyl *N*-nitrosoaniline derivatives are quite stable in physiological systems;¹⁸ therefore, they provide a good platform for designing new agents to specifically deliver NO to the cysteine groups of protein. In this paper, eight small *N*-nitrosoaniline derivatives and two peptidyl *N*-nitrosoanilines were synthesized and their inhibitory abilities against caspase-3 were reported.

Results and Discussion

Bioassay of small molecular *N*-nitrosoaniline derivatives

Eight small molecule *N*-nitroso compounds (Fig. 1) were used in the initial enzymatic assay against caspase-3. Of those eight non-peptide inhibitors, five (3, 4, 7–9) have been previously tested against papain and other plant cysteine proteases.¹⁷ Compounds 5, 6 and 10 were originally synthesized to target protein tyrosine phosphatase, which prefers a substrate with a carboxylate group to facilitate the binding process. They were used in this caspase-3 assay to determine if a carboxylate

group in a small molecule could help enhance inhibitory potency. The protocol used previously in the papain assay was followed to determine the pseudo-first order rate constant.¹⁸ The activity versus time relationship is shown in Figure 2.

Compound 4 possesses the strongest inhibition potency with a pseudo-first order rate constant (k_{obsd}) of 0.195 min⁻¹, followed by 3 (0.129 min⁻¹) and 5 (0.109 min⁻¹). Compounds 6–10 exhibited weak inhibition with k_{obsd} ranging from 0.009 min⁻¹ to 0.076 min⁻¹. The inhibition constants observed in this assay were very similar to those in the papain assay.¹⁷ Although compound 4 showed better inhibition than other carboxylate-containing compounds in this series, its inhibition potency is not comparable to that of peptide based inhibitor for caspase-3.³⁷ Furthermore, it was found that the carboxylate group in the small molecules did not play an important role in the binding process which is considered to be important in the initial long range interaction between the inhibitor and the enzyme.

Design and synthesis of peptidyl *N*-nitrosoaniline inhibitors

Inhibitors of the caspase enzymes are now becoming a popular area of study. In general, good substrates of the caspase enzymes are tetrapeptide molecules (except for caspase-2 inhibitors). The enzyme cleaves its substrate at the C-terminus of the aspartic acid residue. This specificity is what distinguishes caspase from other proteases. The P₄ specificity distinguishes the members of the different caspase groups from each other. An aspartic acid residue is required at the substrate's P₁ position as is abbreviated in the name given to this family of enzymes.^{32,33}

The active site of caspase-3 has a catalytic dyad, consisting of Cys 163 and His 121. This enzyme has a preference for substrates with an aspartic acid residue at the P₄ position.^{32,33} C-terminal aldehydes, nitriles, and ketones have been found to be potent inhibitors.^{33,34} The enzymatic pockets for residues P₂ and P₃ in caspase-3 are relatively open and solvent exposed while the P₄ residue has a defined pocket.³⁵ A crystal structure of the enzyme

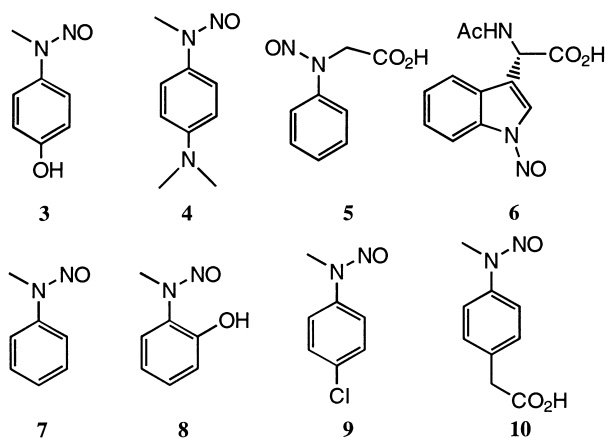


Figure 1. Non-peptide based *N*-nitroso compounds.

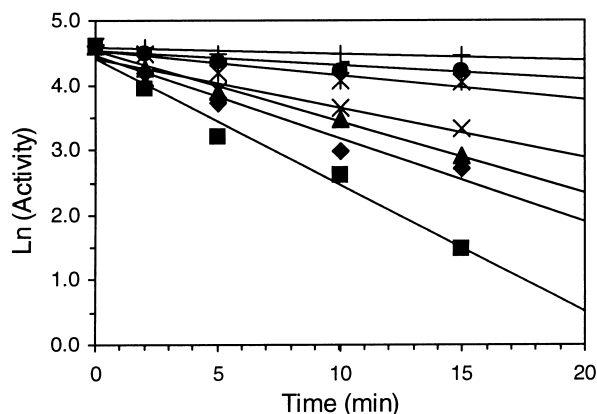


Figure 2. Inactivation of caspase-3 (250 ng/mL, 200 μL) with *N*-nitrosoanilines (5 mM, 200 μL) in 10 mM Tris buffer (pH 7.5, 1 mM EDTA). Pseudo-first order rate constants (min⁻¹): 3 (◆ 0.129); 4 (■ 0.195); 5 (▲ 0.109); 6 (× 0.076); 7 (* 0.038); 8 (● 0.023); 9 (+ 0.009); 10 (– 0.023). Trendline of 10 overlaps with that of 8.

with the substrate in the active site revealed that the P₄ aspartic acid has a polar interaction with an amide nitrogen of the enzyme at the Phe 350 residue and the gamma2 nitrogen of the Asn 208 residue.

Poly-(ADP-ribose) polymerase (PARP), a DNA repair enzyme, has been implicated as a substrate for caspase-3 during apoptosis as it is cleaved into two fragments at the onset of apoptosis.³⁶ Other caspase homologs including caspase-1 cleave PARP at much higher protein

concentration and much more slowly. Tetrapeptide inhibitors designed and synthesized on the basis of sequences at the precursor of Interleukin-1 β and PARP cleavage sites have been used to inhibit caspase-3. Ac-DEVD-CHO, based on PARP cleavage sequence, is very potent against caspase-3 with a K_i of 0.52 nM.³⁷ Synthetic inhibitors of the caspase enzymes map the tetrapeptide sequence of known substrates and replace the C-terminal carboxylic acid, for instance, with an aldehyde. Inhibition operates by the enzyme undergoing

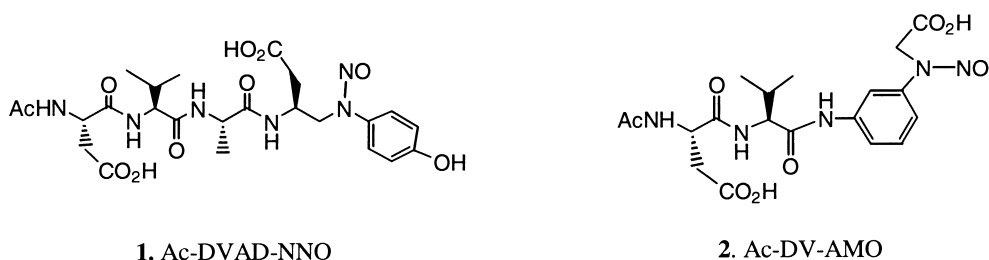


Figure 3. Structures of Ac-DVAD-NNO (1) and Ac-DV-AMO (2).

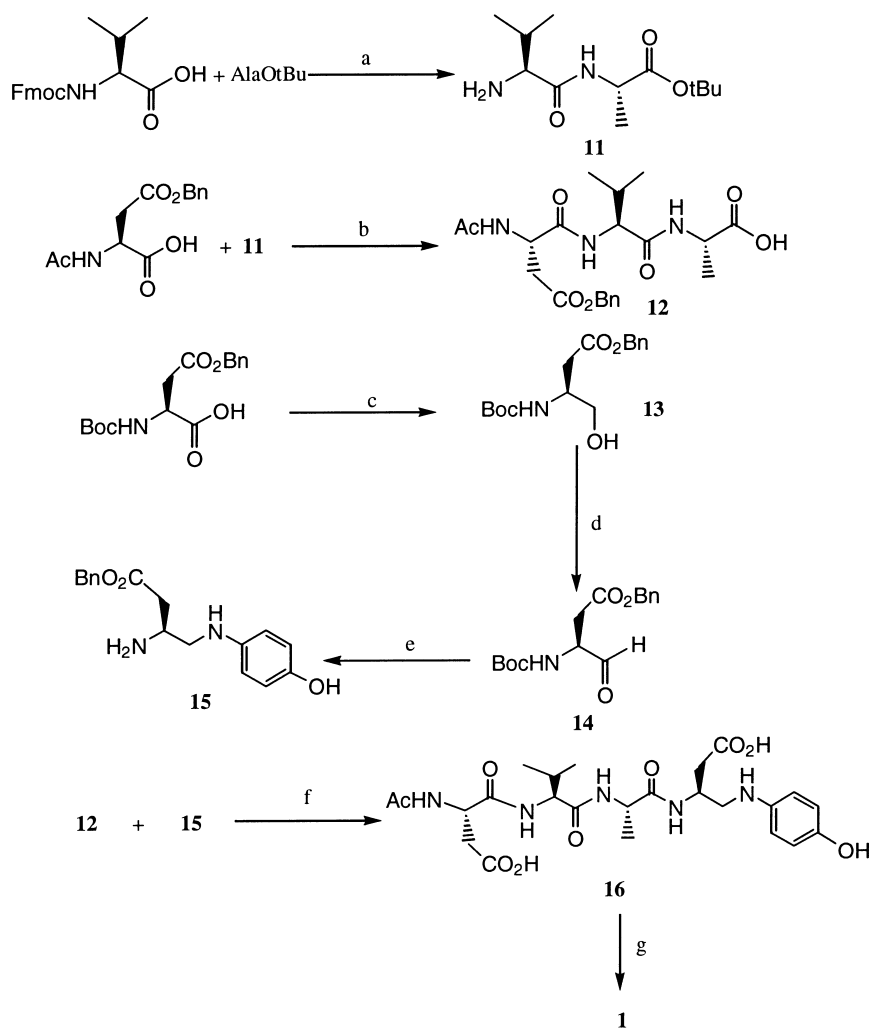


Figure 4. Synthetic approach toward inhibitor 1. (a) (i) EDC, HOBT/CH₂Cl₂; (ii) Et₃NH, DMF; (b) (i) EDC, HOBT/CH₂Cl₂; (ii) TFA, CH₂Cl₂; (c) (i) *t*-BuOCOCl, NMM, TFA; (ii) NaBH₄, MeOH, THF; (d) (COCl)₂, DMSO, Et₃N, CH₂Cl₂; (e) (i) 4-aminophenol, NaCNBH₃, MeOH; (ii) TFA, DCM; (f) (i) EDC, Et₃N, CH₂Cl₂; (ii) H₂/Pd-C; (g) NaNO₂, HCl/H₂O.

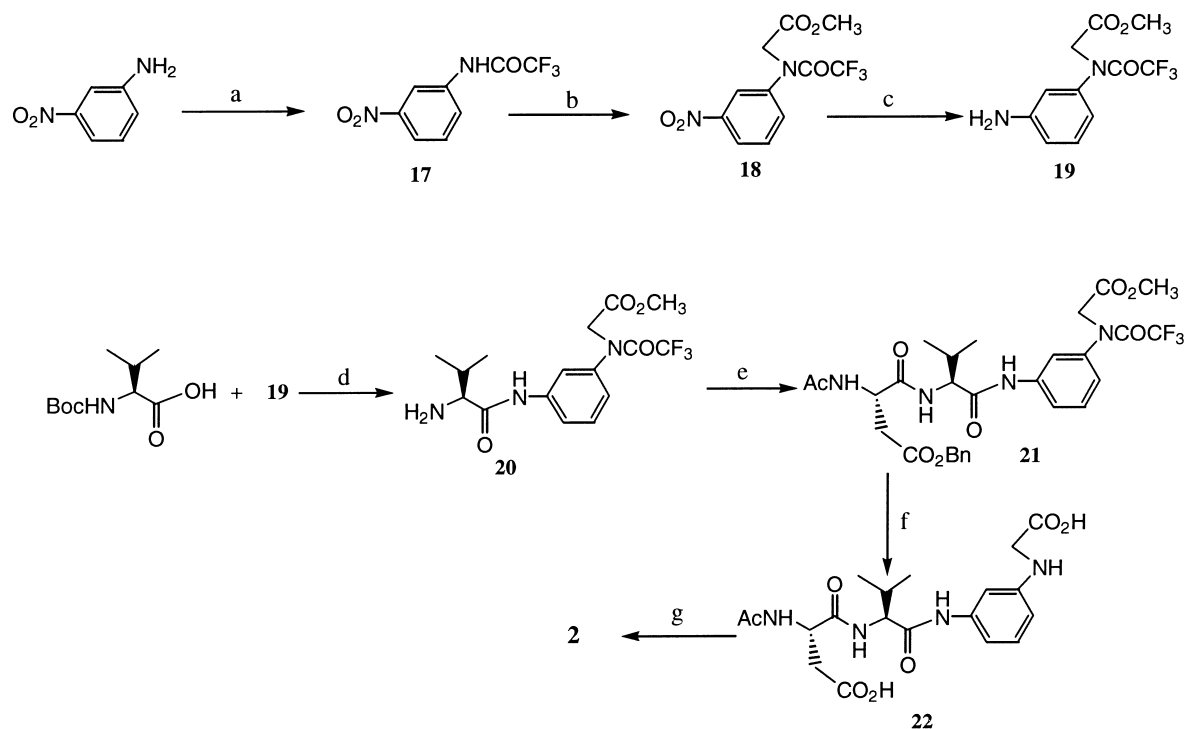


Figure 5. Synthetic approach toward inhibitor **2**. (a) 1.1 equiv $(\text{COCF}_3)_2\text{O}$; (b) $\text{BrCH}_2\text{COOCH}_3$, NaH, DMF; (c) RaNi , 2-propanol; (d) (i) EDC, CH_2Cl_2 ; (ii) DMAP, DMF; (iii) 3 M HCl in EtOAc; (e) Ac-Asp- β -(OBn), EDC, Pyr; (f) (i) K_2CO_3 , MeOH/ H_2O ; (ii) KOH; (g) NaNO_2 , HCl/ H_2O .

a nucleophilic attack at a carboxylic position or at a position alpha to the carboxylic group when a leaving group can be released from the site.³⁸

Based on the preceding analysis, two peptide based caspase-3 inhibitors were designed and their structures are shown in Figure 3. Both compounds were designed to incorporate our *N*-nitrosoaniline moiety into a peptidomimetic structure. Since the PARP-fragment sequence is known to be an effective inhibitor, our compounds were designed to keep a similar spatial arrangement. Compound **1**, Ac-DVAD-NNO, uses a tetrapeptide template to which an *N*-nitrosoaniline fragment is attached on the C-terminus by a C–N bond. The P_4 Asp residue is required for molecular recognition and binding of the inhibitor or substrate. The P_3 position, which is believed to be open to solvent in the caspase-3 active site, was designated as a valine residue since only valine or glutamic acid were experimentally shown to be favored at that site for the caspase-3 enzyme.³⁵ As discussed previously, an aspartic acid residue is required at the P_1 position for this class of enzymes. Since an electron donating *para*-substituted molecule makes NO transfer easier, a hydroxyl group was introduced into the benzene ring. For compound **2**, Ac-DV-AMO, an *N*-nitrosoaniline structure was configured with a similar spatial arrangement to replace the P_2 and P_1 amino acids. It was designed to retain two residues, Asp and Val, from the N-terminus of the tetrapeptide, which is coupled via an amide bond to the aromatic ring of *N*-nitroso-*N*-phenylglycine. In the design of compound **2**, the β -acid of aspartic acid was preserved through an *N*-acetic acid moiety. The phenyl ring in structure **1** is necessary for the susceptibility of the N=N=O motif, but is too bulky for

Table 1. Kinetic parameters for the inactivation of caspase-3

Inhibitor	k_{inact} (min^{-1})	K_{I}	$k_{\text{inact}}/K_{\text{I}}$ ($\text{M}^{-1} \text{s}^{-1}$)
1	0.039	0.002 mM	322
2	0.101	0.022 mM	78
Ac-DEVE-CHO ³⁷	—	0.52 nM	—

the S' subsites. This same group in **2** replaced the Ala residue in the tetrapeptide, resulting in a reduction in the number of rotatable bonds. As in all of the previous inhibitors, the NO is attached to the amino acid residue. If the molecule described here is a good mimetic, cleavage should take place at the nitric oxide position.

Synthetic approach to inhibitor **1** is illustrated in Figure 4. It was synthesized in a convergent manner from both C- and N-terminals with a final coupling reaction between a tripeptide fragment **12** and aniline **15**. Synthesis of the tripeptide portion began with a coupling between an Fmoc-protected Val and an alanine *t*-butyl ester by using standard solution phase peptide coupling method. The free amino group of the dipeptide **11** thus obtained was subsequently coupled with β -benzyl protected aspartic acid to yield a tripeptide **12**. The synthesis of the aniline fragment **15** started with an in situ activation of the commercially available compound *N*-Boc-L-aspartic acid β -benzyl ester with isobutyl chloroformate at -20°C to give a mixed anhydride intermediate which was immediately treated with a suspension of sodium borohydride in THF/methanol at -78°C . The reduction product **13** was then subjected to a Swern oxidation to give an aldehyde **14**, which underwent a reductive

amination with a primary amine 4-aminophenol, then deprotection of the Boc group yielded a secondary amine product **15**. Fragments **12** and **15** were then coupled to give a tetrapeptide **16**. *N*-Nitrosation of **16** in acidic conditions gave the final inhibitor **1**.

The designed inhibitor **2** Ac-DV-AMO was synthesized according to Figure 5, in which *m*-amino-*N*-trifluoroacetyl-*N*-phenylglycine methyl ester **19** was used as a precursor to *N*-nitroso substructure. Starting with 3-nitroaniline, the amino group was first protected using trifluoroacetic anhydride. The resulting *N*-protected nitroaniline **17** was then treated with methyl bromoacetate and sodium hydride to form the *N*-methyl acetate moiety **18**. This compound was subsequently reduced in a solution of Raney nickel in 2-propanol, yielding **19**. Aniline derivative **19** was coupled to the *N*-Boc protected valine through an in situ generation of *N*-Boc-L-valine anhydride, synthesized from the free acid *N*-Boc-L-valine. After deprotection using 3 M HCl in ethyl acetate, the resulting compound **20** was then coupled to *N*-Ac-L-aspartic acid β -benzyl ester to form **21**. Compound **21** was then stepwise deprotected followed by nitrosation using sodium nitrite in HCl to afford inhibitor **2**.

Caspase-3 assay of inhibitors **1** and **2**

Following the enzymatic assay for eight small molecules, two peptide based *N*-nitroso compounds designed and synthesized in this study were assayed against caspase-3. The stability of each peptidyl *N*-nitrosoaniline compound in aqueous solution was examined before being used in the enzymatic assay. No decomposition of compounds **1** and **2** was detected over a 10-h incubation period in the assay buffer (data not shown; the method was the same as described in our previous report¹⁸). Incubation of caspase-3 with each inhibitor resulted in a time- and concentration-dependent loss of enzymatic activity. The apparent first order inactivation constant (k_{app}) can be calculated by plotting the residual activity versus time and fitting a linear equation.¹⁸ The replots of k_{app} as a function of inhibitor concentration gave a Kitz–Wilson plot.^{18,39} The calculated kinetic parameters are shown in Table 1.

Assay results showed that the designed compounds are effective against caspase-3 with second order rate constants of 322 M⁻¹ s⁻¹ for **1** and 78 M⁻¹ s⁻¹ for **2**. The K_i of **1** is lower than that of **2**, which suggests that **1** has a tighter binding than **2**, whereas the second step of inactivation by **1**, as characterized by k_{inact} , is almost three-fold slower than that of **2**.

Conclusions

N-Nitroso compounds have been extensively studied in the past few years due to their carcinogenic and mutagenic properties,⁴⁰ while substituted *N*-methyl-*N*-nitrosoanilines normally exhibit weak carcinogenicity and almost no mutagenicity to several different animal species.⁴¹ We showed in this study that *N*-nitrosoaniline

derivatives are potent inhibitors of caspase-3. Taken together with our previous studies on the inhibition of papain and protein tyrosine phosphatase by *N*-nitrosoaniline derivatives,^{16–18} it has been shown that this class of compounds seems to be general inhibitors of cysteine dependent enzymes. These NO donating compounds provide a novel platform for the design of more potent inhibitors to specifically deliver NO to the enzyme.

Experimental

Amino acids, amino acid derivatives and other chemical reagents were purchased from Sigma or Aldrich, and used without further purification unless otherwise noted. ¹H and ¹³C NMR spectra were recorded on a Mercury 400 NMR spectrometer. Mass spectra were obtained from a Kratos MS 80 spectrometer using electron impact mode or a Kratos MS 50 spectrometer using fast atomic bombardment. Silica gel plates (Merck F254) and silica gel 60 (Merck; 200–240 mesh) were used in analytical thin-layer chromatography (TLC) and column chromatography, respectively. Small molecule *N*-nitroso compounds (**3**–**10**) were synthesized as in the previous report.^{16–18}

L-Valinyl-L-alanine *t*-butyl ester (11). The title compound was obtained by coupling an Fmoc-Val and an alanine *t*-butyl ester using standard solution phase peptide coupling method (87% yield). ¹H NMR (CD₃OD) δ 0.922 (d, 3H, CH(CH₃)CH₃, J =6.4 Hz), 0.992 (d, 3H, CH(CH₃)CH₃, J =6.4 Hz), 1.44 (d, 3H, CH(CH₃), J =7.2 Hz), 1.47 (s, 9H, (CH₃)₃CO), 4.34 (m, 2H, C α H); EIMS calcd for C₁₂H₂₄N₂O₃⁺ (M⁺) 244, found 245 (M + H).

***N*-Acetyl-L-aspartyl-L-valinyl-L-alanine β -benzyl ester (12).** This compound was obtained by coupling **11** and *N*-acetyl-L-aspartic acid β -benzyl ester using standard solution phase peptide coupling method (75% yield). ¹H NMR (CD₃OD) δ 0.922 (d, 3H, CH(CH₃)CH₃, J =6.4 Hz), 0.992 (d, 3H, CH(CH₃)CH₃, J =6.4 Hz), 1.44 (d, 3H, CH(CH₃), J =7.2 Hz), 1.98 (s, 3H, CH₃CO), 2.64 (AB, 1H, CH₂CO, J_{AB} =13.6 Hz, $J_{H\alpha-B}$ =9.6 Hz), 2.80 (AB, 1H, CH₂CO, J_{AB} =13.6 Hz, $J_{H\alpha-A}$ =6 Hz), 4.27 (d, 1H, CHCH(CH₃)₂, J =6.2 Hz); 4.45 (d, 1H, CHCH₂, J =7.2 Hz), 5.19 (s, 2H, CH₂Ph), 7.39 (m, 5H, CH₂Ph); FABMS calcd for C₂₁H₂₉N₃O₇⁺ (M⁺) 435, found 435.

***N*-Boc-L-aspartanol β -benzyl ester (13).** To a solution of *N*-Boc aspartic acid β -benzyl ester (1 g, 3.10 mmol) in 50 mL of THF at –20 °C was added *N*-methyl morpholine (1.36 mL, 12.3 mmol) followed by isobutyl chloroformate (460 μ L, 3.30 mmol). After 10 min, the mixture was added to a suspension of sodium borohydride (200 mg, 5.29 mmol) in 20 mL of THF and 5 mL of methanol at –78 °C. After being kept at –78 °C for 2 h, the mixture was quenched with hydrochloric acid, diluted with ethyl acetate, and washed with sodium bicarbonate. The organic layers were pooled and dried over anhydrous sodium sulfate, filtered, and concentrated. The residue was chromatographed (EtOAc:hexane, 1:1) to give **13** as a colorless oil (58% yield): ¹H NMR (CD₃OD)

δ 1.47 (s, 9H, $(\text{CH}_3)_3\text{CO}$), 2.50 (AB, 1H, CH_2CO , $J_{\text{AB}}=13.6$ Hz, $J_{\text{H}\alpha\text{-B}}=9.6$ Hz), 2.65 (AB, 1H, CH_2CO , $J_{\text{AB}}=13.6$ Hz, $J_{\text{H}\alpha\text{-A}}=6$ Hz), 3.42 (AB, 1H, CH_2OH , $J_{\text{AB}}=12.6$ Hz, $J_{\text{H}\alpha\text{-B}}=9.6$ Hz), 3.55 (AB, 1H, CH_2OH , $J_{\text{AB}}=12.6$ Hz, $J_{\text{H}\alpha\text{-A}}=6$ Hz), 3.98 (m, 1H, C_αH), 5.16 (s, 1H, CH_2Ph), 7.37 (m, 5H, CH_2Ph). EIMS calcd for $\text{C}_{16}\text{H}_{23}\text{NO}_5^+$ (M^+) 309, found 310 ($\text{M}+\text{H}$).

N-Boc-L-aspartic aldehyde β -benzyl ester (14). At -78°C , to a stirred solution of oxalyl chloride (1.96 mL, 3.91 mmol) in CH_2Cl_2 was added DMSO (0.56 mL, 7.82 mmol), followed by the addition of **13** in CH_2Cl_2 (500 mg, 1.6 mmol). Stirring was continued at -78°C for 10 min followed by addition of the alcohol dissolved in CH_2Cl_2 . The reaction mixture was stirred for 15 min, and Et_3N (2 mL, 14.3 mmol) was added with stirring at -78°C . After 30 min, water was added at room temperature and stirring was continued for 10 min. The organic layer was separated, and the aqueous phase was re-extracted with CH_2Cl_2 . After drying over sodium sulfate, the solution was filtered and concentrated. The residue was chromatographed (EtOAc :hexane, 1:3) to afford **14** as a colorless oil (82% yield): ^1H NMR (CD_3OD) δ 1.47 (s, 9H, $(\text{CH}_3)_3\text{CO}$), 2.50 (AB, 1H, CH_2CO , $J_{\text{AB}}=13.6$ Hz, $J_{\text{H}\alpha\text{-B}}=9.6$ Hz), 2.65 (AB, 1H, CH_2CO , $J_{\text{AB}}=13.6$ Hz, $J_{\text{H}\alpha\text{-A}}=6$ Hz), 4.46 (m, 1H, C_αH), 5.16 (s, 1H, CH_2Ph), 7.37 (m, 5H, CH_2Ph). EIMS calcd for $\text{C}_{16}\text{H}_{21}\text{NO}_5^+$ (M^+) 307, found 308 ($\text{M}+\text{H}$).

4-N-(p-Hydroxyphenyl)-3-(S)-amino-butyric acid benzyl ester (15). To a stirred solution of **14** (246 mg, 0.8 mmol) in anhydrous MeOH (20 mL) was added 4-aminophenol (262 mg, 2.4 mmol) and NaCNBH_3 (51 mg, 0.8 mmol). After the mixture being stirred overnight at room temperature, the solvent was removed in vacuo and the residue was chromatographed with 0–5% MeOH/ EtOAc to afford product **15** (50%): ^1H NMR (CD_3OD) δ 2.50 (AB, 1H, CH_2CO , $J_{\text{AB}}=13.6$ Hz, $J_{\text{H}\alpha\text{-B}}=9.6$ Hz), 2.65 (AB, 1H, CH_2CO , $J_{\text{AB}}=13.6$ Hz, $J_{\text{H}\alpha\text{-A}}=6$ Hz), 3.04 (AB, 1H, CH_2NH , $J_{\text{AB}}=13.6$ Hz, $J_{\text{H}\alpha\text{-B}}=9.4$ Hz), 3.19 (AB, 1H, CH_2NH , $J_{\text{AB}}=13.6$ Hz, $J_{\text{H}\alpha\text{-A}}=9.4$ Hz), 4.46 (m, 1H, C_αH), 5.16 (s, 2H, CH_2Ph), 6.45 (d, 2H, $J=9.0$ Hz, Ph), 6.63 (d, 2H, $J=9.0$ Hz, Ph), 7.37 (m, 5H, CH_2Ph). EIMS calcd for $\text{C}_{17}\text{H}_{20}\text{N}_2\text{O}_3^+$ (M^+) 300, found 301 ($\text{M}+\text{H}$).

3-(S)-N-(N'-Acetyl-L-aspartyl-L-valinyl-L-alanyl)-4-N''-(p-hydroxyphenyl)-butyric acid (16). To a stirring solution of **12** (436 mg, 1 mmol) and **15** (300 mg, 1 mmol) in CH_2Cl_2 (25 mL) were added EDC (201 mg, 1.05 mmol) and Et_3N (0.15 mL, 1.05 mmol), with stirring being continued at room temperature for 24 h. The reaction mixture was worked up to give **16** (65%). ^1H NMR (CD_3OD) δ 0.922 (d, 3H, $\text{CH}(\text{CH}_3)\text{CH}_3$, $J=6.4$ Hz), 0.992 (d, 3H, $\text{CH}(\text{CH}_3)\text{CH}_3$, $J=6.4$ Hz), 1.44 (d, 3H, $\text{CH}(\text{CH}_3)$, $J=7.2$ Hz), 1.98 (s, 3H, CH_3CO), 2.00 (s, 3H, CH_3CO), 2.59 (AB, 1H, CH_2CO , $J_{\text{AB}}=12.6$ Hz, $J_{\text{H}\alpha\text{-B}}=6.6$ Hz), 2.64 (AB, 1H, CH_2CO , $J_{\text{AB}}=12.6$ Hz, $J_{\text{H}\alpha\text{-A}}=6.6$ Hz), 3.04 (AB, 1H, CH_2NH , $J_{\text{AB}}=13.6$ Hz, $J_{\text{H}\alpha\text{-B}}=9.4$ Hz), 3.19 (AB, 1H, CH_2NH , $J_{\text{AB}}=13.6$ Hz, $J_{\text{H}\alpha\text{-A}}=9.4$ Hz), 4.32 (m, 3H, C_αH), 5.20 (s, 2H, CH_2Ph), 6.45 (d, 2H, $J=9.0$ Hz, Ph), 6.63 (d, 2H, $J=9.0$ Hz, Ph); FABMS calcd for $\text{C}_{24}\text{H}_{35}\text{N}_5\text{O}_9^+$ (M^+) 537, found 537.

3-(S)-N-(N'-Acetyl-L-aspartyl-L-valinyl-L-alanyl)-4-N''-(p-hydroxyphenyl)-4-N''-nitroso-butyric acid (1). To a suspension of **16** (500 mg, 0.93 mmol) in 5 mL of H_2O was added 1 N HCl gradually until pH 3. The reaction mixture became homogeneous. At 0°C , NaNO_2 (150 mg, 2.2 mmol) was added to the solution and the product (320 mg) precipitated from the solution in 1 h. ^1H NMR (CD_3OD) δ 0.922 (d, 3H, $\text{CH}(\text{CH}_3)\text{CH}_3$, $J=6.4$ Hz), 0.992 (d, 3H, $\text{CH}(\text{CH}_3)\text{CH}_3$, $J=6.4$ Hz), 1.44 (d, 3H, $\text{CH}(\text{CH}_3)$, $J=7.2$ Hz), 1.98 (s, 3H, CH_3CO), 2.00 (s, 3H, CH_3CO), 2.59 (AB, 1H, CH_2CO , $J_{\text{AB}}=12.6$ Hz, $J_{\text{H}\alpha\text{-B}}=6.6$ Hz), 2.64 (AB, 1H, CH_2CO , $J_{\text{AB}}=12.6$ Hz, $J_{\text{H}\alpha\text{-A}}=6.6$ Hz), 3.04 (AB, 1H, CH_2NH , $J_{\text{AB}}=13.6$ Hz, $J_{\text{H}\alpha\text{-B}}=9.4$ Hz), 3.19 (AB, 1H, CH_2NH , $J_{\text{AB}}=13.6$ Hz, $J_{\text{H}\alpha\text{-A}}=9.4$ Hz), 4.32 (m, 3H, C_αH), 5.20 (s, 2H, CH_2Ph), 7.20 (d, 2H, CH_2Ph , $J=9.0$ Hz), 7.39 (d, 2H, CH_2Ph , $J=9.0$ Hz); ^{13}C NMR (CD_3OD) 173.9, 173.0, 172.2, 171.0, 165.7, 164.4, 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. FABMS calcd for $\text{C}_{24}\text{H}_{34}\text{N}_6\text{O}_{10}^+$ (M^+) 566, found 536 ($\text{M}-\text{NO}$).

N-Trifluoroacetic-3-nitroaniline (17). To a solution of 3-nitroaniline (10 g, 72 mmol) in CH_2Cl_2 :pyridine (1:3, v/v, 80 mL), was added dropwise trifluoroacetic anhydride (11 mL, 78 mmol). When the reaction was complete, the solution was washed with HCl (1 N), then brine and dried (Na_2SO_4). The solvent was removed under reduced pressure affording quantitative yield of a yellowish-orange, needle-like solid. ^1H NMR ($d\text{-CHCl}_3$) δ 8.52 (t, 1H, ar), 8.14 (dd, 1H, ar), 8.06 (dd, 1H, ar), 7.64 (t, 1H, ar). ^{13}C NMR δ 156.51, 149.71, 146.36, 137.47, 131.53, 127.33, 122.08, 116.68. MS calcd for $\text{C}_8\text{H}_5\text{F}_3\text{N}_2\text{O}_3^+$ (M^+) 234, found 234.

m-Nitro-N-trifluoroacetyl-N-phenylglycine methyl ester (18). To a solution of NaH (1.27 g, 53 mmol) in DMF (25 mL) at -78°C was slowly added **17** (4.9 g, 21 mmol) dissolved in DMF (25 mL). After addition of the aniline, the temperature was brought to 0°C . After approximately 1 h, the temperature was reduced to -78°C for the addition of the methyl bromoacetate (6.4 g, 42 mmol). The temperature was then brought to 25°C . It was worked up by pouring the solution into cold HCl (1 N). It was then extracted with EtOAc , washed with brine then dried (Na_2SO_4). The solvent was removed under reduced pressure. Purification by column chromatography with hexane and hexane: EtOAc (20:1, 15:1, 10:1 then 5:1, v/v) afforded a bright yellow crystalline solid (42%). ^1H NMR ($d\text{-CHCl}_3$) δ 8.31 (d, 2H, ar), 7.83 (d, 1H, ar), 7.67 (d, 1H, ar), 4.46 (s, 2H), 3.81 (s, 3H); ^{13}C NMR δ 168.19, 149.40, 141.14, 135.46, 131.30, 127.19, 125.26, 124.43, 116.49, 61.34, 53.43. MS calcd for $\text{C}_{11}\text{H}_9\text{F}_3\text{N}_2\text{O}_5^+$ (M^+) 306, found 306.

m-Amino-N-trifluoroacetyl-N-phenylglycine methyl ester (19). To a slurry of Raney nickel (50% in water, 20 g) in 2-propanol (45 mL) was added compound **18** (1.99 g, 6.5 mmol). After 5 h the solution was carefully filtered to avoid ignition of the metal. The solvent of the filtrate was removed under reduced pressure. Purification by column chromatography with hexane and hexane: EtOAc (20:1, 10:1 then 5:1, v/v) afforded a white, semi-crystalline

solid (53%). The first product to elute from the column was the desired product. ^1H NMR ($d\text{-CHCl}_3$) δ 7.20 (t, 1H, ar), 6.74 (d, 2H, ar), 6.74 (s, 1H, ar), 4.41 (s, 2H), 3.80 (s, 3H), 1.27 (s, 2H, aniline); ^{13}C NMR δ 169.10, 148.61, 141.71, 131.36, 118.63, 116.92, 115.19, 54.16, 53.67, 30.83. MS calcd for $\text{C}_{11}\text{H}_{11}\text{F}_3\text{N}_2\text{O}_3^+$ (M^+) 276, found 276.

***m*-(Val-NH)-*N*-trifluoroacetyl-*N*-phenylglycine methyl ester (20).** Boc-Val (3.5 g, 16.1 mmol) and EDC (2.3 g, 12 mmol) was stirred in CH_2Cl_2 for 30 min at 0°C . This solution was then pipetted into a solution of **19** (442 mg, 1.6 mmol) with Et_3N (2 mL). DMAP (3 mmol) was then added and the reaction mixture was stirred for 5 h. Then, the mixture was worked up. The crude product was deprotected by HCl (3 M). After the reaction was complete, the solvent was removed by rotary evaporation. Water was added to the residue and it was washed three times with ethyl ether. The aqueous solution was then neutralized with sodium bicarbonate, then extracted with ethyl acetate. The organic solution was then dried (Na_2SO_4). The solvent was removed under reduced pressure affording a pale-yellow solid (59%). ^1H NMR (CD_3OD) δ 7.82 (s, 1H, ar), 7.65 (d, 1H, ar), 7.40 (t, 1H, ar), 7.17 (d, 1H, ar), 4.46 (s, 2H), 3.76 (s, 3H), 3.23 (d, 1H), 2.03 (m, 1H), 0.99 (dd, 6H); ^{13}C NMR δ 174.39, 168.29, 157.10, 156.74, 140.19, 139.40, 129.53, 123.27, 102.49, 119.38, 117.71, 114.86, 60.92, 52.78, 51.64, 32.38, 18.49, 16.42. MS calcd for $\text{C}_{16}\text{H}_{20}\text{F}_3\text{N}_3\text{O}_4^+$ (M^+) 375, found 375.

***m*-(Ac-L-Asp-L-Val-NH)-*N*-phenylglycine (22).** Compound **20** (188 mg, 0.5 mmol) was dissolved in CH_2Cl_2 (10 mL) and pyridine (2.5 mmol). Ac-Asp- β -(OBn) (186 mg, 0.7 mmol), then EDC (134 mg, 0.7 mmol) were added. When the reaction was complete, the mixture was worked up. The crude product was dissolved in $\text{MeOH}:\text{H}_2\text{O}$ (1:1). K_2CO_3 (2 mmol) was added and, once the trifluoroacetate and benzyl groups were removed, KOH (9 mmol) was added. After the reaction was complete, it was acidified by adding Dowex 50 WX2-100 ion exchange resin (acidic). The solution was then filtered and the resin rinsed with water. The solvent was then removed under reduced pressure. A pale-yellow solid was afforded (43%). ^1H NMR (D_2O) δ 0.912 (d, 3H, $\text{CH}(\text{CH}_3)\text{CH}_3$, $J=6.2$ Hz), 0.982 (d, 3H, $\text{CH}(\text{CH}_3)\text{CH}_3$, $J=6.2$ Hz), 2.01 (s, 3H, CH_3CO), 2.09 (m, 1H, $\text{CH}(\text{CH}_3)_2$), 2.65 (d, 2H, CH_2COOH $J=7.0$ Hz), 2.88 (m, 1H, CHCH_2), 2.92 (m, 2H, CH_2NH), 4.20 (m, 1H, CHCH), 7.66 (t, 1H, Ph), 7.92 (d, 2H, $J=9.2$ Hz, Ph), 8.10 (s, 1H, Ph). MS calcd for $\text{C}_{19}\text{H}_{26}\text{N}_4\text{O}_7^+$ (M^+) 422, found 422.

***m*-(Ac-L-Asp-L-Val-NH)-*N*-nitroso-*N*-phenylglycine (2).** Compound **22** (25 mg, 0.06 mmol) was dissolved in 0.1 N HCl. An aqueous solution of NaNO_2 (5 mg, 0.07 mmol) was added at 0°C . One hour later, the solvent was removed under reduced pressure. The crude mixture was chromatographed on silica gel and eluted with dichloromethane to afford a red solid (64%). ^1H NMR (D_2O) δ 0.910 (d, 3H, $\text{CH}(\text{CH}_3)\text{CH}_3$, $J=6.2$ Hz), 0.985 (d, 3H, $\text{CH}(\text{CH}_3)\text{CH}_3$, $J=6.2$ Hz), 2.04 (s, 3H, CH_3CO), 2.14 (m, 1H, $\text{CH}(\text{CH}_3)_2$), 2.76 (d, 2H, CH_2COOH $J=7.2$

Hz), 2.91 (m, 1H, CHCH_2), 3.30 (m, 2H, CH_2NNO), 4.29 (m, 1H, CHCH), 7.20 (t, 1H, Ph), 7.52 (d, 2H, Ph, $J=9.2$ Hz), 7.96 (s, 1H, Ph). FABMS calcd for $\text{C}_{19}\text{H}_{25}\text{N}_5\text{O}_8^+$ (M^+) 451, found 421 ($\text{M}-\text{NO}$).

Caspase-3 assays

Recombinant human caspase-3 was purchased from PharMingen. Assays were conducted on a 96-well microtiter plate and contained 65 μL of assay buffer (10 mM Tris, 1 mM EDTA, pH 7.5), 10 μL of incubation solution, 5 μL of DMSO containing the inhibitor and 20 μL of substrate Ac-DEVD-pNA. Production of *p*-nitroaniline (pNA) from reaction mixtures was measured by following the absorbance at 405 nm. Second order rate constants for irreversible inhibitors were determined from assays where the reaction buffer containing inhibitor and caspase-3 was incubated at 37°C , and aliquots were used for enzyme activity determination. Kitz–Wilson plots of the kinetic data were prepared to derive the second order rate constants.

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