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Aza-Peptidyl Michael Acceptor and Epoxide Inhibitors—Potent and Selective Inhibitors of *Schistosoma mansoni* and *Ixodes ricinus* Legumains (Asparaginyl Endopeptidases)

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Aza-peptide Michael acceptors and epoxides with the general structure of YCO-Ala-Ala-AAsn-*trans*-CH=CHCOR and YCO-Ala-Ala-AAsn-EP-COR, respectively, are shown to be potent inhibitors of asparaginyl endopeptidases (legumains) from the bloodfluke, *Schistosoma mansoni* (SmAE), and the hard tick, *Ixodes ricinus* (IrAE). Structure−activity relationships (SARs) were determined for a set of 41 aza-peptide Michael acceptors and eight aza-peptide epoxides. Both enzymes prefer disubstituted amides to monosubstituted amides in the P1′ position, and potency increased as we increased the hydrophobicity of the inhibitor in this position. Extending the inhibitor to P5 resulted in increased potency, especially against IrAE, and both enzymes prefer small over large hydrophobic residues at P2. Aza-peptide Michael acceptor inhibitors are more potent than aza-peptide epoxide inhibitors, and for some of these compounds, second-order inhibiton rate constants are the fastest yet discovered. Given the central functions of these enzymes in both parasites, the data presented here may facilitate the eventual design of selective antiparasitic drugs.

Introduction

Asparaginyl endopeptidases (AEs) or legumains (EC. 3.4.22.34) belong to the C13 family of clan CD[a] cysteine proteases, which also include caspases, gingipains, clostripain, and separase.[1] AEs are acidic lysosomal enzymes that cleave substrates specifically after an asparagine residue in the P1 position.[2] Legumains were first discovered in the human bloodfluke, *Schistosoma mansoni*,[3] then in plants,[4] mammals,[5−7] and most recently in the ticks, *Ixodes ricinus*[8] and *Haemaphysalis longicornis*.[9] Plant legumain functions as a processing enzyme of storage proteins during seed germination.[10] Mammalian legumain is involved in the inhibition of osteoclast formation and bone resorption.[11] It has also been shown that human legumain is highly expressed in many tumors, including carcinomas of the breast, colon, and prostate.[12] Recently, it was found that mice lacking legumain develop disorders resembling hemophagocytic syndrome.[13]

The helmintic disease, schistosomiasis, infects over 200 million people in approximately 70 countries, with 779 million at risk, 85% of whom are in Africa alone.[14] Immature and adult bloodflukes live in the cardiovascular system, ingest red blood cells, and utilize hemoglobin as a source of amino acids for growth, development, and reproduction. In the parasite gut, cysteine and aspartic proteases contribute to the proteolytic degradation of hemoglobin.[15] Among these are a legumain (SmAE) that, in vitro, degrades hemoglobin[16] and processes and *trans*-activates the major gut protease cathepsin B.[17] As a central digestive enzyme, therefore, SmAE may represent a useful target for selective inhibitors that impair the parasite's ability to feed.

Hard ticks of the genus *Ixodes* are vectors of Lyme disease caused by the spirochetes *Borrelia burgdorferi sensu lato*. The multiplication of spirochetes in the tick gut and subsequent infection of the host requires unimpaired bloodmeal uptake and digestion.[18] As for SmAE, a gut-associated AE in *Ixodes ricinus* (IrAE) is thought to contribute to hemoglobin degradation either directly or indirectly by the *trans*-activation of other digestive cysteine and aspartic proteases.[8,19] Accordingly, IrAE may represent a drug or vaccine target to prevent or retard transmission of disease.

Synthetic AE inhibitors that have been tested against mammalian AEs[20,21] include aza-Asn halomethylketones (Cbz-Ala-Ala-AAsn-CH_2Cl, $k_{obs}/[I]$ = 139 000 M^{-1} s^{-1}), Michael acceptors derived from Asn (Cbz-Ala-Ala-NHCH-(CH_2CONH_2)CH=$CH_2CO_2CH_2CH$=CH_2, $k_{obs}/[I]$ up to 766 M^{-1} s^{-1}), and acyloxymethylketones ($k_{obs}/[I]$ from 769 up to 109 000 M^{-1} s^{-1}). It has also been shown that the

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[a] Abbreviations: AAsn, aza-asparagine; AMC, 7-amino-4-methylcoumarin; Boc, *t*-butoxycarbonyl; Bzl, benzyl, CH_2Ph; C13, a family of enzymes in the CD clan of cysteine proteases; Cbz, benzyloxycarbonyl; CD, a clan of cysteine proteases; $CDCl_3$, deuterated chloroform; DCC, dicyclohexylcarbodiimide; DMF, *N*,*N*-dimethylformamide; DMSO, dimethylsulfoxide; DMSO-d_6, dimethylsulfoxide deuterated; EDC, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride; EP, epoxide (C_2H_2O); EtOAc, ethyl acetate; EtOH, ethanol; HOBt, *N*-hydroxybenzotriazole; *i*BCF, isobutyl chloroformate; IrAE, *Ixodes ricinus* asparaginyl endopeptidase; MeOH, methanol; Mu, morpholinocarbonyl; NMM, *N*-methylmorpholine; OBzl, benzyloxy; Pip, piperadinocarbonyl; Piz, piperazinocarbonyl; SmAE, *Schistosoma mansoni* asparaginyl endopeptidase; TFA, trifluoroacetic acid.

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aspartyl peptidyl fluoromethyl ketones designed for caspase inhibition also moderately inhibit mammalian AE.[22] We recently reported that aza-asparaginyl Michael acceptor inhibitors are potent and selective inhibitors of *S. mansoni*, *I. ricinus*, and *Trichomonas vaginalis* (a parasitic protozoan) AEs.[23] We designed the aza-peptide epoxides and Michael acceptor inhibitors to closely resemble an extended peptide legumain substrate.[24,25] Placement of the carbonyl group of the epoxide moiety or fumarate derivative in the inhibitor places it in a location identical to that of the carbonyl of the scissile peptide bond in a good legumain substrate. This design allows the peptide chain of the inhibitor to exactly match that of a good substrate from the N-terminus across to the scissile peptide carbonyl group. The warhead epoxide or Michael acceptor is then located adjacent to the active site cysteine residue. Aza-peptide epoxides and Michael acceptors have the advantage of being easily extended in the P′ direction, allowing interactions with the S′ subsites of AE.

In our earlier paper, the inhibitor sequence Cbz-Ala-Ala-AAsn was maintained, while substituents on the reactive warhead were changed in order to measure the effects of modifying the P1′ position. SAR studies revealed that the most potent inhibitors have esters or disubstituted amides with aromatic groups in the P1′ position. Work by other investigators with the vinyl sulfone inhibitors of cysteine proteases demonstrated that introduction of the appropriate N-terminal capping groups could increase compound bioavailability.[26] Accordingly, we expand upon our previous design of aza-peptide Michael acceptors and epoxides by introducing groups at the N-terminus in order to both extend the compounds to P4 and P5 and potentially increase bioavailability. In this paper, we report the design and synthesis of forty one aza-peptide Michael acceptors with the general structure Boc-AAsn-*trans*-CH=CHCOR, YCO-Ala-AAsn-*trans*-CH=CHCOR and YCO-Ala-Ala-AAsn-*trans*-CH=CHCOR where YCO- are potentially bioavailable capping groups. In addition, eight epoxide inhibitors with the general structure YCO-Ala-Ala-AAsn-EP-COR, and five aza-peptide substrate analogues, were synthesized and evaluated. Some of the new inhibitors have the fastest inhibition rates thus far measured for these parasitic enzymes.

Chemistry

The syntheses of the aza-peptide fumarate and epoxide analogues are based on the previous syntheses of aza-peptide Michael acceptor[24] and epoxide inhibitors.[27] The inhibitors are obtained by coupling a peptidyl hydrazide to a monoester or amide of fumaric acid or to a monoester or amide of epoxysuccinic acid. Monoethyl fumarate (**2a**) was commercially available, and all of the Michael acceptor warheads were synthesized using this compound as a precursor. The monobenzyl fumarate (**2b**) was formed from monoethyl fumarate and benzyl alcohol using NMM and DCC as the coupling reagent and followed by deprotection of the ethyl ester in ethanol using aqueous NaOH. The fumarate precursors (**2c**−**p**) were prepared from monoethyl fumarate and the corresponding primary or secondary amines by standard mixed anhydride coupling using NMM and iBCF, followed by hydrolysis of the ethyl ester with aqueous NaOH (Scheme 1).

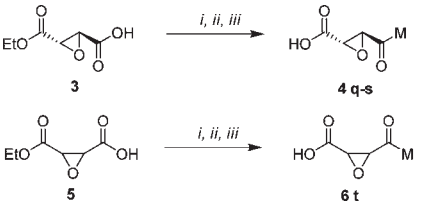
A previously described procedure[27] was used for the synthesis of (2*S*,3*S*)-oxirane-2,3-dicarboxylic acid monoethyl ester (**3**). The epoxide warheads (**4r**, **4s**) were synthesized from

Scheme 1. Synthesis of Fumarate Precursors[a]

a = -OEt
b = -OBzl
c = -NH-cyclopropyl
d = -NHCH$_2$-cyclohexyl
e = -NHCH$_2$-2-furyl
f = -NHBzl
g = -NHCH$_2$CH$_2$Ph
h = -NHCH$_2$CH$_2$CH$_2$Ph
i = -NHCH$_2$Ph-3,4-OCH$_3$
j = -tetrahydroquinoline
k = -tetrahydroisoquinoline
l = -N(Bzl)$_2$
m = -NHCH$_2$-1-naphthyl
n = -N(CH$_3$)CH$_2$-1-naphthyl
o = -N(Bzl)-CH$_2$-1-naphthyl
p = -N(CH$_2$-1-naphthyl)$_2$

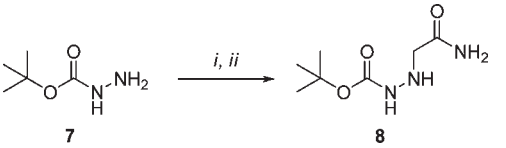
[a] (*i*) NMM, iBCF, CH_2Cl_2, HNR_1R_2; (*ii*) NaOH; (*iii*) HCl, EtOH.

Scheme 2. Synthesis of Epoxysuccinate Derivatives[a]



[a] (*i*) NMM, iBCF, CH_2Cl_2, HNR_1R_2; (*ii*) KOH; (*iii*) HCl, EtOH.

Scheme 3. Synthesis of the Aza-Asparagine Precursor[a]

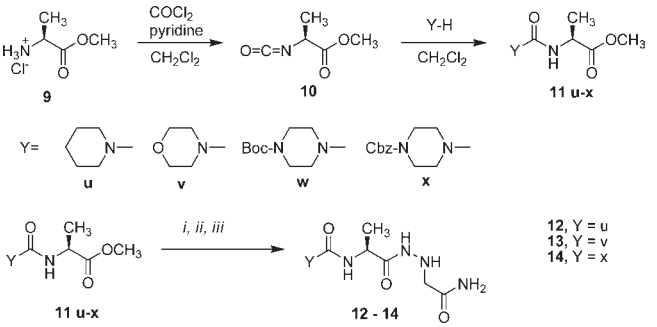


[a] (*i*) $BrCH_2COOEt$, NMM, DMF; (*ii*) NH_3, cat. NaCN, MeOH, DMF.

monoethyl epoxysuccinate and the corresponding primary or secondary amines using the standard mixed anhydride coupling procedure with NMM and iBCF, followed by deprotection of the ethyl ester in ethanol using aqueous KOH (Scheme 2). The monoethyl ester of *cis*-oxirane-2,3-dicarboxylic acid monoethyl ester (**5**) was synthesized following a previously described procedure.[28] Coupling of the dibenzylamine to the monoethyl ester epoxysuccinate to form compound **6t** was performed using the standard mixed anhydride coupling procedure and followed by deprotection as described above. The disubstituted aromatic amines were synthesized by reductive amination starting with an aromatic aldehyde precursor and an aromatic primary amine.

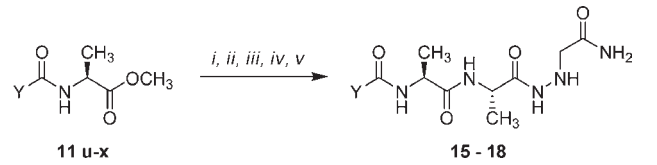
The preparation of the aza-asparagine precursors (Boc-NHNHCH$_2$CONH$_2$, Y-CO-Ala-NHNHCH$_2$CONH$_2$, Y-CO-Ala-Ala-NHNHCH$_2$CONH$_2$, and Cbz-Ala-Val(Ile, Phe)-NHNHCH$_2$CONH$_2$) are shown in Schemes 3, 4, 5, and 6. The hydrazide Boc-NHNHCH$_2$CONH$_2$ was synthesized by the monoalkylation of *tert*-butyl carbazate (**7**) with ethyl bromoacetate, and the conversion of the ethyl ester to the amide by ammonolysis was done with catalytic amounts of NaCN according to the procedure of Hogberg et al. (Scheme 3).[29] For the synthesis of acyl dipeptide aza-asparagine precursors (**12**−**14**), we first prepared alanine methyl ester isocyanate (**10**) by reacting the hydrochloride salt of alanine (**9**) with phosgene according to the procedure by Nowick et al.[30] Alanine methyl ester isocyanate (**10**) was then reacted with piperidine, morpholine, *tert*-butoxycarbonylpiperazine, and benzyloxycarbonylpiperazine to form

Scheme 4. Synthesis of the Acyl Dipeptide Aza-Asparagine Precursors[a]



[a] (*i*) H_2NNH_2, MeOH; (*ii*) $BrCH_2COOEt$, NMM, DMF; (*iii*) NH_3, cat. NaCN, MeOH, DMF.

Scheme 5. Synthesis of the Acyl Tripeptide Aza-Asparagine Precursors[a]



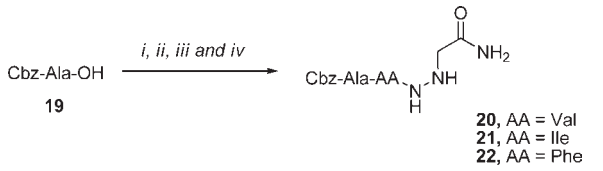
[a] (*i*) NaOH then HCl; (*ii*) HCl·H-Ala-OCH_3, HOBt, DCC, DMF; (*iii*) H_2NNH_2, MeOH; (*iv*) $BrCH_2COOEt$, NMM, DMF; (*v*) NH_3, cat. NaCN, MeOH, DMF.

Y-CO-Ala-OCH_3 (**11u**−**x**), respectively.[31] Piperidine and morpholine were commercially available, however; *tert*-butoxycarbonylpiperazine[32] and benzyloxycarbonylpiperazine[33] were synthesized according to the literature. The ester Y-CO-Ala-OCH_3 (**11u**−**x**) was then reacted with excess hydrazine (NH_2NH_2) in methanol to form Y-CO-Ala-NH-NH_2. To introduce the aza-asparagine side chain, Y-CO-Ala-NH-NH_2 was reacted with ethyl bromoacetate and NMM. The ethyl ester group was then reacted with 7 N NH_3 in methanol in the presence of catalytic NaCN to form the acyl dipeptide aza-asparagine precursors (Y-CO-Ala-NHNHCH$_2$CONH$_2$, **12**−**14**) (Scheme 4). For the synthesis of the acyl tripeptide aza-asparagine precursors (**15**−**18**), we first hydrolyzed the esters **11u**−**x** (Y-CO-Ala-OCH_3) with 1 M NaOH in methanol to form acyl alanine derivatives (Y-CO-Ala-OH). The acyl alanine then was coupled to alanine methyl ester using HOBt and DCC to form the dipeptides Y-CO-Ala-Ala-OCH_3. The dipeptide esters Y-CO-Ala-Ala-OCH_3 were then reacted with excess hydrazine, and the asparagine side chain was introduced as mentioned previously to form the acyl tripeptide aza-asparagine precursors (Y-CO-Ala-Ala-NHNHCH$_2$CONH$_2$, **15**−**18**) (Scheme 5).

For the synthesis of Cbz-Ala-Val(Ile, Phe)-NHNHCH$_2$-CONH$_2$, we reacted Cbz-Ala-OH with valine methyl ester, isoleucine methyl ester, or phenylalanine methyl ester. The peptidyl methyl ester was then reacted with hydrazine, and finally, the asparagine side chain was attached by alkylation with ethyl bromoacetate followed by ammonolysis (Scheme 6).

The aza-asparagine precursors (**8**, **12**−**14**, **15**−**18**, **20**−**22**) were then coupled to a variety of substituted fumarate analogues (**2a**−**p**) and epoxide moieties (**4q**−**s** and **6t**) using HOBt and EDC to complete the synthesis of the aza-peptidyl inhibitors (Scheme 7).

Scheme 6. Synthesis of Cbz-Ala-AA-NHNHCH$_2$CONH$_2$[a]



[a] (*i*) HCl·H-Val-OCH_3, HCl·H-Ile-OCH_3 or HCl·H-Phe-OCH_3, HOBt, DCC, DMF (*ii*) H_2NNH_2, MeOH, (*iii*) $BrCH_2COOEt$, NMM, DMF; (*iv*) NH_3, cat. NaCN, MeOH, DMF.

Aza-peptide Michael acceptors and epoxides with the Boc-Piz-Ala-Ala-AAsn peptide sequence (**29**, **36a**−**r**) were treated with TFA in methylene chloride to form the simple piperazine derivatives Piz-Ala-Ala-AAsn (**37**, **38a**−**r**).

Results and Discussion

Synthetic Design. Aza-peptide Michael acceptors and epoxide inhibitors are selective and potent inhibitors of SmAE and IrAE.[23,25] Previously synthesized inhibitors have the peptide sequence of Cbz-Ala-Ala-AAsn as a template since Cbz-Ala-Ala-Asn-NHMec (NHMec = 7-(4-methyl)-coumarylamide) is an optimal substrate sequence for legumain.[34] In this study, we used Boc-NHNHCH$_2$CONH$_2$, YCO-Ala-NHNHCH$_2$CONH$_2$, and YCO-Ala-Ala-NHNH-CH$_2$CONH$_2$ peptide sequences where the Y groups are piperidine, morpholine, pipezarine, *tert*-butoxypiperazine, and benzyloxycarbonyl piperazine. These groups were chosen to increase the bioavailability of the inhibitors and to study the interactions of the P4 (YCO-Ala-NHNH-CH$_2$CONH$_2$) and P5 (YCO-Ala-Ala-NHNHCH$_2$CONH$_2$) positions of the inhibitor with SmAE and IrAE. For the inhibitor design, the α carbon of the asparagine (Asn) residue is replaced by nitrogen. This replacement results in an aza-peptide containing the aza-asparagine (AAsn) residue. The presence of an aza-asparagine residue at P1 makes the synthesis of aza-peptide Michael acceptor and epoxide inhibitors easier compared to peptide Michael acceptor and epoxide inhibitors as the synthesis involves simple coupling of an acid and an aza-peptide precursor. Aza-peptides are ideal inhibitors because they are resistant to cleavage by proteases in vivo[35,36] and can incorporate a reactive warhead. We chose the epoxide and Michael acceptor double bonds as these warheads can be easily modified in the P′ position to study interactions with the S′ subsites of the enzymes.

Inhibition of SmAE and IrAE with Aza-Peptidyl Michael Acceptors. The IC_{50} values of 41 aza-peptide Michael acceptor inhibitors are listed in Table 1. We synthesized four (Boc-protected aza-asparagine) inhibitors (**23a**,**d**,**f**,**h**) with different Michael acceptor warheads. Only the monoethyl ester derivative (SmAE IC_{50} > 2000 nM, IrAE IC_{50} = 750 nM) inhibited the enzymes, whereas no inhibition was detected with the other three compounds.

Due to this result, we synthesized acyl dipeptide aza-asparagine Michael acceptor inhibitors. Monosubstituted amides (**24k**, **25c**,**e**,**i**,**h**,**k**, **26j**,**k**) were either poor or non-inhibitory. Replacement of the monosubstituted amides with the disubstitued amides (**24k**, **25k**, **26j**−**k**) yielded increased potency. Inhibitors **24k**, **25k**, and **26k** have the same isoquinoline moiety in their P1′ position; however, they have different acyl groups. Both enzymes seem to favor the presence of piperidine residue in the P3 position rather than

Scheme 7. Coupling of Fumarate Precursors and Epoxide Moieties to Aza-Asparagine Precursors

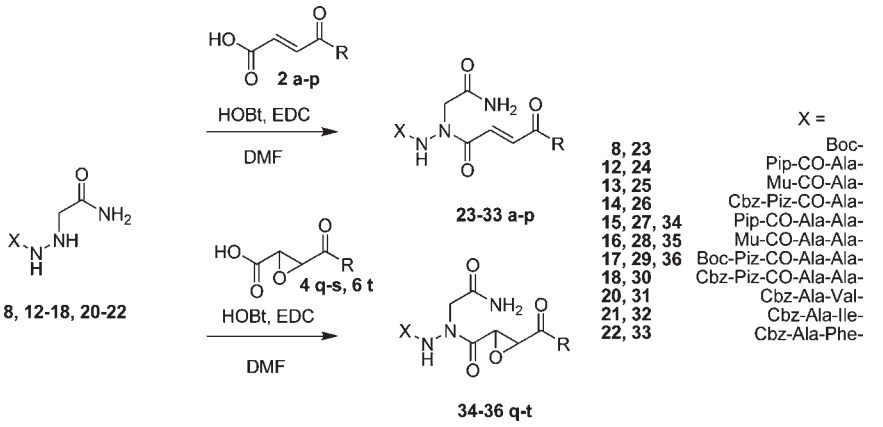


Table 1. Inhibition of SmAE and IrAE by Aza-Peptide Michael Acceptors[a]

	inhibitor	SmAE IC_{50} (nM)	IrAE IC_{50} (nM)
23a	Boc-AAsn-CH=CH−COOEt	> 2000	750
23d	Boc-AAsn-CH=CH-$NHCH_2$-cyclohexyl	N.I.	N.I.
23f	Boc-AAsn-CH=CH−CONHBzl	N.I.	N.I.
23h	Boc-AAsn-CH=CH−$CONHCH_2CH_2CH_2Ph$	N.I.	N.I.
24k	Pip-Ala-AAsn-CH=CH−CO-tetrahydroisoquinoline	600	150
25c	Mu-Ala-AAsn-CH=CH−CONH-cyclopropyl	N.I.	N.I.
25e	Mu-Ala-AAsn-CH=CH−$CONHCH_2$-furyl	N.I.	N.I.
25i	Mu-Ala-AAsn-CH=CH−$CONHCH_2$Ph-3,4-OCH_3	> 2000	> 2000
25h	Mu-Ala-AAsn-CH=CH−$CONHCH_2CH_2CH_2Ph$	> 2000	N.I.
25k	Mu-Ala-AAsn-CH=CH−CO-tetrahydroisoquinoline	750	600
26j	Cbz-Piz-Ala-AAsn-CH=CH−CO-tetrahydroquinoline	750	850
26k	Cbz-Piz-Ala-AAsn-CH=CH−CO-tetrahydroisoquinoline	750	800
27a	Pip-Ala-Ala-AAsn-CH=CH−COOEt	80	15
27g	Pip-Ala-Ala-AAsn-CH=CH−$CONHCH_2CH_2Ph$	750	400
27l	Pip-Ala-Ala-AAsn-CH=CH−$CON(Bzl)_2$	80	17
27o	Pip-Ala-Ala-AAsn-CH=CH−CON(Bzl)-CH_2-1-naphthyl	100	2.4
27p	Pip-Ala-Ala-AAsn-CH=CH−$CON(CH_2$-1-naphthyl$)_2$	57	8
28a	Mu-Ala-Ala-AAsn-CH=CH−COOEt	101	0.23
28h	Mu-Ala-Ala-AAsn-CH=CH−$CONHCH_2CH_2CH_2Ph$	1450	> 2000
28k	Mu-Ala-Ala-AAsn-CH=CH−CO-tetrahydroisoquinoline	103	65
28p	Mu-Ala-Ala-AAsn-CH=CH−$CON(CH_2$-1-naphthyl$)_2$	45	3.3
37a	Piz-Ala-Ala-AAsn-CH=CH−COOEt	300	8.5
37b	Piz-Ala-Ala-AAsn-CH=CH−COOBzl	250	13
37l	Piz-Ala-Ala-AAsn-CH=CH−$CON(Bzl)_2$	90	31
37n	Piz-Ala-Ala-AAsn-CH=CH−$CON(CH_3)CH_2$-1-naphthyl	40	3.8
37p	Piz-Ala-Ala-AAsn-CH=CH−$CON(CH_2$-1-naphthyl$)_2$	70	2.6
29a	Boc-Piz-Ala-Ala-AAsn-CH=CH−COOEt	80	8.5
29b	Boc-Piz-Ala-Ala-AAsn-CH=CH−COOBzl	45	7.5
29l	Boc-Piz-Ala-Ala-AAsn-CH=CH−$CON(Bzl)_2$	45	30
29n	Boc-Piz-Ala-Ala-AAsn-CH=CH−$CON(CH_3)CH_2$-1-naphthyl	58	20
29p	Boc-Piz-Ala-Ala-AAsn-CH=CH−$CON(CH_2$-1-naphthyl$)_2$	68	5
30a	Cbz-Piz-Ala-Ala-AAsn-CH=CH−COOEt	70	0.14
30n	Cbz-Piz-Ala-Ala-AAsn-CH=CH−$CON(CH_3)CH_2$-1-naphthyl	70	0.35
30p	Cbz-Piz-Ala-Ala-AAsn-CH=CH−$CON(CH_2$-1-naphthyl$)_2$	50	0.37
31a	Cbz-Ala-Val-AAsn-CH=CH−COOEt	100	65
31m	Cbz-Ala-Val-AAsn-CH=CH−$CONHCH_2$-1-naphthyl	1050	350
31j	Cbz-Ala-Val-AAsn-CH=CH−CO-tetrahydroquinoline	138	13
32a	Cbz-Ala-Ile-AAsn-CH=CH−COOEt	280	0.52
33e	Cbz-Ala-Phe-AAsn-CH=CH−$CONHCH_2$-furyl	> 2000	1800
33j	Cbz-Ala-Phe-AAsn-CH=CH−CO-tetrahydroquinoline	> 2000	500
33k	Cbz-Ala-Phe-AAsn-CH=CH−CO-tetrahydroisoquinoline	> 2000	500

[a] AE zymogens from *Ixodes ricinus* and *Schistosoma mansoni* were expressed in *Pichia pastoris* and activated as described previously.[8,40] Inhibition assays were performed in 0.1 M citrate-phosphate, 4 mM DTT, pH 6.8, < 1% DMSO for SmAE, or 0.1 M citrate-phosphate, 4 mM DTT, 0.1 M NaCl, pH 5.5 for IrAE, < 1% DMSO, with a final concentration of 10 μM Cbz-Ala-Ala-AAsn-AMC (Cbz = $PhCH_2$−OCO−, AMC = 7-amino-4-methyl-coumarin) as substrate.

morpholine or benzyloxypiperazine. Probably, the oxygen of the morpholine is disturbing the H-bond network around the active site of the AEs, whereas the benzyloxypiperazine is not accommodated in the P3 position due to the bulkiness of the

Table 2. Inhibition of SmAE and IrAE by Aza-Peptide Epoxides[a]

	inhibitor	SmAE IC_{50} (nM)	IrAE IC_{50} (nM)
34q	Pip-Ala-Ala-AAsn-EP(*S*,*S*)-COOEt	220	30
34r	Pip-Ala-Ala-AAsn-EP(*S*,*S*)-$CON(Bzl)_2$	72	12
34s	Pip-Ala-Ala-AAsn-EP(*S*,*S*)-$CONHCH_2$-1-naphthyl	160	8.5
35s	Mu-Ala-Ala-AAsn-EP(*S*,*S*)-$CONHCH_2$-1-naphthyl	60	6
35r	Mu-Ala-Ala-AAsn-EP(*S*,*S*)-$CON(Bzl)_2$	90	1.7
35t	Mu-Ala-Ala-AAsn-EP(*cis*)-$CON(Bzl)_2$	N.I.	N.I.
38r	Piz-Ala-Ala-AAsn-EP(*S*,*S*)-$CON(Bzl)_2$	150	11
36r	Boc-Piz-Ala-Ala-AAsn-EP(*S*,*S*)-$CON(Bzl)_2$	80	270

[a] AE zymogens from *Ixodes ricinus* and *Schistosoma mansoni* were expressed in *Pichia pastoris* and activated as described previously.[8,40] Inhibition assays were performed in 0.1 M citrate-phosphate, 4 mM DTT, pH 6.8, < 1% DMSO for SmAE, or 0.1 M citrate-phosphate, 4 mM DTT, 0.1 M NaCl, pH 5.5 for IrAE, < 1% DMSO, with a final concentration of 10 μM Cbz-Ala-Ala-AAsn-AMC (Cbz = $PhCH_2$−OCO−, AMC = 7-amino-4-methyl-coumarin) as substrate.

group. Although the disubstituted amides were better inhibitors than the monosubstituted ones, they were still weak.

In view of these results, acyl tripeptide aza-asparagine Michael acceptors were synthesized. Among the inhibitors with Pip-Ala-Ala-AAsn sequence, the ethyl ester derivative (**27a**) was as effective (SmAE IC_{50} = 80 nM, IrAE IC_{50} = 15 nM) as the dibenzyl amide analogue (**27l**) (SmAE IC_{50} = 80 nM, IrAE IC_{50} = 17 nM). Again, the disubstituted amides (**27l**,**o**,**p**) were more potent than the monosubstituted amide analogue (**27g**). The dinaphthyl derivative (**27p**) was the most potent of the acyl tripeptide inhibitors against SmAE (IC_{50} = 57 nM), whereas the *N*-benzyl-*N*-naphthyl derivative was the best against IrAE (IC_{50} = 2.4 nM).

Among the inhibitors with the Mu-Ala-Ala-AAsn peptide sequence, the ethyl ester analogue (**28a**) was as effective (IC_{50} = 101 nM) as the disubstituted amide analogue (**28k**) (IC_{50} = 103 nM) against SmAE. However, in the case of IrAE, replacement of the ethyl ester (IC_{50} = 0.23 nM) with the disubstituted amide analogue, tetrahydroisoquinoline (IC_{50} = 65 nM), results in 300-fold decrease in potency. This is one of very few examples where the parasite AEs diverged markedly in their SAR. We conclude that, even though IrAE does prefer disubstituted amides, for example, the dinaphthyl analogue (**28p**) (IC_{50} = 3.3 nM), it does not favor constrained amides in the P1′ position. The monoamide analogue (**28h**) (SmAE IC_{50} = 1450 nM; IrAE IC_{50} > 2000 nM) was a poor inhibitor of both enzymes. There can be two reasons for this; either the alkyl spacer puts the phenyl group in an unfavorable position or the presence of a hydrogen bond donor group is not favored in the S1′ position.

Next, we synthesized five inhibitors with the Piz-Ala-Ala-AAsn peptide sequence. The two ester derivatives (ethyl ester, **37a**, and benzyl ester, **37b**) were moderate inhibitors of both SmAE (IC_{50} = 300 and 250 nM, respectively) and IrAE (IC_{50} = 8.5 and 13 nM, respectively). However, the disubstituted amide derivatives (**37l**,**n**,**p**) were better against SmAE (IC_{50} = 90,40, and 70 nM). Because these compounds contain aromatic groups in their warheads, we conclude that the presence of such groups at P1′ improves binding of the inhibitors due to π-stacking in either S1′ or S2′ of SmAE. Piz-Ala-Ala-AAsn-CH=CH−$CON(CH_3)CH_2$-1-naphthyl (**37n**) was the most potent inhibitor against SmAE (IC_{50} = 40 nM) among all the compounds synthesized. Probably, the methyl group interacts with a small hydrophobic pocket in the S1′ subsite, whereas the naphthyl group reaches to a larger hydrophobic pocket in the S2′ subsite.

Next, we synthesized eight compounds with protecting groups on the piperazine; five of these (**29a**,**b**,**l**,**n**,**p**) contain a *tert*-butoxycarbonyl (Boc) group, whereas the other three (**30a**,**n**,**p**) contain a benzyloxycarbonyl (Cbz) group. Interestingly, all of the compounds with Boc-Piz-Ala-Ala-AAsn and Cbz-Ala-Ala-AAsn peptide sequences were potent inhibitors of both SmAE and IrAE. We speculate that there is a hydrophobic pocket that is interacting with hydrophobic groups (Boc and Cbz) at P4 or P5. In particular, the Cbz-Ala-Ala-AAsn peptide sequence (**30a**,**n**,**p**) produced the best inhibitors of IrAE (IC_{50} = 0.14, 0.35, and 0.37 nM, respectively) herein reported. As the Boc-Ala-Ala-AAsn compounds were not as potent as those containing Cbz-Ala-Ala-AAsn, we conclude that the increased π-stacking of the aromatic residues in either the S4 or S5 pocket improves inhibitor binding.

To study the effect of P2 residue on inhibitor potency, we synthesized seven compounds with the Cbz-Ala-AA-AAsn (AA = Val, Ile, and Phe) peptide sequence. Compounds with valine (**31a**,**m**,**j**) or isoleucine (**32a**) residue at P2 were good to poor inhibitors, whereas those containing phenylalanine at P2 (**33e**,**j**,**k**) were extremely poor against SmAE and IrAE, confirming that both enzymes do not prefer large hydrophobic groups in the S2 position. With respect to the ethyl ester compounds **31a** and **32a** that differ only at P2, SmAE reacted with both equally, whereas inhibition of IrAE was 100-fold better with **32a**. This is a second example of divergent SAR between these enzymes and suggests that IrAE can accept larger aliphatic hydrophobic residues at S2. Previous studies with positional scanning substrate combinatorial libraries tested against SmAE[34] and IrAE[8] demonstrated that the preferred amino acids at P2 are Ala > Val > Ile > Phe and Ile > Ala > Val > Phe, respectively. Thus, our results are in good agreement with these data.

Inhibition of SmAE and IrAE with Aza-Peptidyl Epoxides. The IC_{50} values of eight aza-peptide epoxide inhibitors are listed in Table 2. Comparison of the three aza-peptidyl epoxide inhibitors with Pip-Ala-Ala-AAsn sequence showed that the ester analogue (**34q**) and the monosubstituted amide analogue (**34s**) were moderate inhibitors against SmAE with the IC_{50} values of 220 and 160 nM, respectively; however, the disubstituted amide derivative (**34r**) was best with an IC_{50} value of 72 nM. For IrAE, all compounds were moderate inhibitors, but unlike SmAE, IrAE favored the monosubstituted amide analogue (IC_{50} = 8.5 nM) to the ethyl ester analogue (IC_{50} = 30 nM) and the disubstituted amide analogue (IC_{50} = 12 nM). These results showed that the two enzymes differ in their S1′ or S2′ positions slightly. Replacement of the piperidine residue at P4 of compound **34s** (SmAE IC_{50} = 160 nM, IrAE IC_{50} = 8.5 nM) with a morpholine residue in compound **35s** (IC_{50} = 60 nM) resulted in an almost 3-fold increase in potency against SmAE, whereas it had almost no effect against IrAE (IC_{50} = 6 nM).

Table 3. Second-Order Rate Constants for Inhibition of Parasite AEs by Aza-Peptide Michael Acceptors and Epoxides[a]

	inhibitor	SmAE IC_{50} (nM)	SmAE k_{inact}/K_{iapp} or k_{ass} (M^{-1} s^{-1}) $\times 10^{-6}$	IrAE IC_{50} (nM)	IrAE k_{inact}/K_{iapp} or k_{ass} (M^{-1} s^{-1}) $\times 10^{-6}$
	Cbz-Ala-Ala-AAsn-CH=CH−COOEt[24]	31	0.16	4.5	0.64
37b	Piz-Ala-Ala-AAsn-CH=CH−COOBzl	250	0.046	13	0.17
29b	Boc-Piz-Ala-Ala-AAsn-CH=CH−COOBzl	45	0.023	7.5	0.67
38r	Piz-Ala-Ala-AAsn-EP(*S*,*S*)-$CON(Bzl)_2$	150	0.03	11	0.10
34r	Pip-Ala-Ala-AAsn-EP(*S*,*S*)-$CON(Bzl)_2$	72	0.037	12	0.45
36r	Boc-Piz-Ala-Ala-AAsn-EP(*S*,*S*)-$CON(Bzl)_2$	80	0.01	270	0.39
29l	Boc-Piz-Ala-Ala-AAsn-CH=CH−$CON(Bzl)_2$	45	0.04	30	0.35
28p	Mu-Ala-Ala-AAsn-CH=CH−$CON(CH_2$-1-naphthyl$)_2$	45	0.4	3.3	1.98
27p	Pip-Ala-Ala-AAsn-CH=CH−$CON(CH_2$-1-naphthyl$)_2$	57	0.27	8	0.77
30n	Cbz-Piz-Ala-Ala-AAsn-CH=CH−$CON(CH_3)CH_2$-1-naphthyl	70	0.084	0.35	1.79
37n	Piz-Ala-Ala-AAsn-CH=CH−$CON(CH_3)CH_2$-1-naphthyl	40	0.16	3.8	0.22

[a] AE zymogens from *Ixodes ricinus* and *Schistosoma mansoni* were expressed in *Pichia pastoris* and activated as described previously.[8,40] Inhibition assays were performed in 0.1 M citrate-phosphate, 4 mM DTT, pH 6.8, <1% DMSO for SmAE, or 0.1 M citrate-phosphate, 4 mM DTT, 0.1 M NaCl, pH 5.5 for IrAE, <1% DMSO, with a final concentration of 10 μM Cbz-Ala-Ala-AAsn-AMC (Cbz = $PhCH_2$−OCO−, AMC = 7-amino-4-methylcoumarin) as substrate. Second-order rate constants were determined by linear or nonlinear regression analysis using GRAPHPAD PRISM 3.0a, as described in the text.

To study the effect of stereochemistry on inhibitor potency, we synthesized two dibenzyl amide epoxide warheads with *S*,*S* and *cis* configurations and coupled them to the Mu-Ala-Ala-AAsn peptidyl precursor to form compounds **35r** and **35t**, respectively. Compound **35t** (*cis*) was inactive against both enzymes, whereas the *S*,*S* compound retained potency. Accordingly, we suggest that the *cis* stereochemistry of the epoxide unfavorably changes the orientation of the prime side substituents to cause a loss of inhibition. Piz-Ala-Ala-AAsn-EP(*S*,*S*)-$CON(Bzl)_2$ (**38r**) was a moderate inhibitor against both SmAE and IrAE with IC_{50} values of 150 and 11 nM, respectively. Replacement of the piperazine with *tert*-butoxycarbonyl piperazine (**36r**) increased the potency 2-fold against SmAE. On the other hand, the same replacement resulted in 24-fold decrease in potency against IrAE. Extension of the aza-peptides to the P5 position by introduction of Boc group resulted in increased potency in all compounds except this one example.

Finally, we synthesized five aza-peptidyl substrate analogue inhibitors such as Mu-Ala-AAsn-CH_2CH_2Ph; however, none of these compounds inhibited SmAE or IrAE (data not shown). The structures of these compounds can be found in the Supporting Information.

Second-Order Inhibition Rate Constants. Second-order inhibition rate constants for a selection of the better aza-peptide Michael acceptors and epoxides are presented in Table 3. We compared the rates of the new compounds with the gold standard inhibitor Cbz-Ala-Ala-AAsn-CH=CH−COOEt[24] (k_{inact}/K_{iapp} = 160 000 M^{-1} s^{-1} for SmAE and 640 000 M^{-1} s^{-1} for IrAE). In the case of SmAE, two compounds (**28p**, 400 000 M^{-1} s^{-1}, and **27p**, 270 000 M^{-1} s^{-1}) have higher rates than Cbz-Ala-Ala-AAsn-CH=CH−COOEt, and one compound (**37n**, 160 000 M^{-1} s^{-1}) has the same rate. All three compounds have Michael acceptors as a warhead and disubstituted amides with aromatic residues. In general, aza-peptide Michael acceptors inhibit SmAE more rapidly than epoxide analogues. For IrAE, three compounds (**29b**, 670 000 M^{-1} s^{-1}, **30n**, 1 790 000 M^{-1} s^{-1}, and **28p**, 1 980 000 M^{-1} s^{-1}) have higher rates than Cbz-Ala-Ala-AAsn-CH=CH−COOEt. Again, all compounds were aza-peptide Michael acceptors with aromatic residues in the P1′ position.

Mechanism of Inhibition. Aza-peptide Michael acceptors and epoxides are irreversible inhibitors of clan CD cysteine proteases.[24] The mechanism of inhibition involves

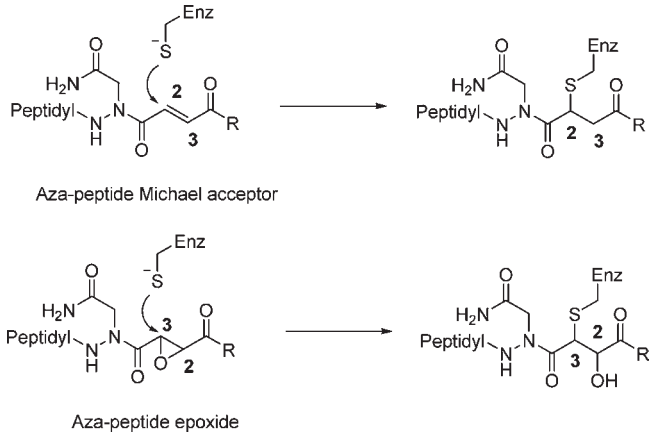


Figure 1. Mechanism of inhibition of cysteine proteases by Michael acceptor and epoxide inhibitors.

nucleophilic attack by the catalytic cysteine residue on the Michael acceptor double bond at C2 or epoxide at C3 forming a covalent bond and irreversibly inhibiting the enzyme (Figure 1).[37] The mechanism is supported by NMR studies,[23] X-ray crystal structures of aza-peptide Michael acceptor inhibitors bound to caspase-3 and caspase-8,[38] and crystal structures of aza-peptide epoxide inhibitors bound to caspase-3.[39] In view of these data, we suggest that, for AEs, the site of attack is the same in both warheads, that is, the carbon closest to the aza-peptide nitrogen atom (Figure 1).

Conclusion. We demonstrate that aza-peptide Michael acceptors and epoxides with the general structure Boc-AAsn-*trans*-CH=CHCOR, YCO-Ala-AAsn-*trans*-CH=CHCOR, YCO-Ala-Ala-AAsn-*trans*-CH=CHCOR, and YCO-Ala-Ala-AAsn-EP-COR are inhibitors of SmAE and IrAE. We have shown that tripeptides (YCO-Ala-Ala-AAsn-*trans*-CH=CHCOR) are favored over dipeptides (YCO-Ala-AAsn-*trans*-CH=CHCOR) that, in turn, are favored over simple AAsn derivatives (Boc-AAsn-*trans*-CH=CHCOR). Extension of the inhibitor structure to P5 resulted in the most potent compounds against IrAE with IC_{50} values as low as 0.14 nM and inhibition rates as high as 1 980 000 M^{-1} s^{-1}. Aza-peptide Michael acceptor inhibitors are more potent than aza-peptide epoxide inhibitors. We have also determined that the stereochemistry of the epoxide warhead is important; *S*,*S* is favored over *cis*. In addition, both enzymes prefer small

hydrophobic amino acids such as Val and Ile in the P2 position, whereas large hydrophobic amino acids such as Phe are not tolerated. The results and SAR generated here may facilitate the design of potent and selective AE inhibitors with antiparasite bioactivity.

Experimental Section

Material and Methods. Materials were obtained from Acros, Bachem Bioscience Inc., or Sigma Aldrich and used without further purification. The purity of each compound was confirmed by TLC, ^{1}H NMR, MS, and elemental analysis. Elemental analysis was carried out by Atlantic Microlab Inc., Norcross, GA. Elemental analysis revealed that the purity of all target compounds after purification was higher than 95%. Chemical shifts are reported in parts per million relative to an internal standard (trimethylsilane). TLC was performed on Sorbent Technologies (250 μm) silica gel plates. The ^{1}H NMR spectra were obtained on a Varian Mercury 400 MHz spectrometer. Electrospray ionization (ESI), fast atom bombardment (FAB), and high-resolution mass spectrometry were obtained using Micromass Quattro LC and VG Analytical 70-SE instruments. Elemental analysis was carried out by Atlantic Microlab Inc., Norcross, GA.

Determination of IC_{50} Values and Second-Order Inhibition Rates for Asparaginyl Endopeptidases. IC_{50} values were determined exactly as previously described.[23] For second-order rate inhibitions, the substrate Z-Ala-Ala-Asn-AMC and inhibitors were prepared as 10 and 20 mM stock solutions, respectively, in DMSO. Assays were performed at 25 °C and incorporated either activated recombinant *I. ricinus* legumain[8] or activated recombinant *S. mansoni* legumain.[40] An aliquot of (100 μL) *S. mansoni* legumain in assay buffer (0.1 M citrate-phosphate buffer at pH 6.8 containing 4 mM DTT) was added to 100 μL of inhibitor in assay buffer with 20 μM substrate and inhibitor present at 2, 1, 0.8, 0.6, 0.4, 0.2, and 0.1 μM (or DMSO alone) added as a 1 μL aliquot. The progress of inhibition was followed for 2−5 min, while the uninhibited activity was linear. An aliquot of (100 μL) *I. ricinus* legumain in assay buffer (0.1 M citrate-phosphate buffer at pH 5.5 containing 4 mM DTT with 0.1 M NaCl) was added to inhibitor in 100 μL assay buffer with inhibitor present at 1, 0.8, 0.5, 0.25, 0.125, 0.0625, 0.03125 μM and DMSO alone, as above. Release of free AMC was measured at emission and excitation wavelengths of 355 and 460 nm, respectively, in a Molecular Devices Flex-Station fluorometer. Measurements were taken every 1.52 s. The rate of inhibition (k_{obs}) was determined at each inhibitor concentration according to $[P] = v_o/k_{obs} \times [1 - \exp(-k_{obs} \times t)]$, where [P] is the concentration of product formed over time t and v_o is the initial rate, using nonlinear regression analysis (GRAPHPAD PRISM 3.0a). If the k_{obs} versus inhibitor concentration could reliably fit a two-step irreversible mechanism ($r^2 > 0.9$), the inhibition constant K_i and the inactivation constant k_{inact} were determined by nonlinear regression analysis according to $k_{obs} = k_{inact} \times [I]_o/([I]_o + K_I^* \times (1 + [S]_o/K_m))$, where $[I]_o$ and $[S]_o$ are the concentrations of inhibitor and substrate, respectively, and $K_I^* = K_i \times (1 = [S]_o/K_m)$. For those inhibitors with which k_{obs} was linear with increasing concentrations of inhibitor, linear regression analysis was used to obtain the association constant k_{ass} using $k_{obs} = k_{ass} \times [I]_o/(1 + [S]_o/K_m)$. Multiple determinations at each inhibitor concentration were performed.

General Procedure for the Synthesis of Capped Alanine Methyl Ester (YCO-Ala-OCH$_3$). Alanine methyl ester isocyanate, which was synthesized from alanine hydrochloride salt according to the procedure by Nowick et al.[31] was dissolved in CH_2Cl_2 and cooled to 0 °C. Y−H groups, which are commercially available except for benzyloxycarbonyl piperazine and *tert*-butoxycarbonyl piperazine, were added to the stirred solution of alanine methyl ester isocyanate. The reaction mixture was stirred vigorously for 16 h at room temperature. The solvent was then removed under vacuum.

To synthesize *tert*-butoxycarbonyl piperazine (Boc-Piz), a solution of di-*tert*-butyl dicarbonate (1 equiv) in dichloromethane was added slowly to a solution of piperazine (2 equiv) in dichloromethane at 0 °C. The reaction mixture was stirred at room temperature overnight. The dichloromethane solution was washed with saturated $NaHCO_3$ and water. The organic phase was dried with anhydrous Na_2SO_4 and evaporated. Purification by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent gave the product in 57% yield: ^{1}H NMR ($CDCl_3$) δ 1.42 (s, 9H, Boc), 2.00 (br s, 1H), 2.78 (m, 4H, piperazine), 3.36 (m, 4H, piperazine); MS (ESI) m/z 187.1 [$(M + 1)^+$].

To synthesize benzyloxycarbonyl piperazine (Cbz-Piz), monoprotection of piperazine was done with benzyl chloroformate (1 equiv) and Et_3N (1 equiv) in dichloromethane. The solvent was evaporated, and the crude product was purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent to obtain the product in 55% yield: ^{1}H NMR (DMSO-d_6) δ 2.33 (s, 1H, NH), 2.62 (m, 4H, piperazine), 3.30−3.36 (m, 4H, piperazine), 5.00 (s, 2H, Cbz), 7.15−7.22 (m, 5H, Ph); MS (ESI) m/z 221.0 [$(M + 1)^+$].

The piperidine derivative Pip-Ala-OCH$_3$ was prepared by adding piperidine to alanine isocyanate and purified by column chromatography on silica gel using 2:18:5 MeOH/CH_2Cl_2. EtOAc as the eluent: white solid, yield 83%; ^{1}H NMR ($CDCl_3$-*d*) δ 1.32 (d, 3H, Ala-CH_3), 1.48 (m, 6H, piperidine), 3.27 (m, 4H, piperidine), 3.67 (s, 3H, OCH_3), 4.41 (m, 1H, α-H), 5.05 (d, 1H, NH); MS (ESI) m/z 215.0 [$(M + 1)^+$].

The morpholine derivative Mu-Ala-OCH$_3$ was prepared by adding morpholine to alanine isocyanate and purified by column chromatography on silica gel using 10% MeOH/CH_2Cl_2 as the eluent: yellowish solid, yield 93%; ^{1}H NMR ($CDCl_3$-*d*) δ 1.29 (d, 3H, Ala-CH_3), 3.28 (m, 4H, morpholine), 3.58 (m, 4H, morpholine), 3.64 (s, 3H, OCH_3), 4.38 (m, 1H, α-H), 5.25 (s, 1H, NH); MS (ESI) m/z 217.0 [$(M + 1)^+$].

The *tert*-butoxycarbonyl piperazine derivative Boc-Piz-Ala-OCH$_3$ was prepared by adding *tert*-butoxycarbonyl piperazine to alanine isocyanate and purified by column chromatography on silica gel using 10% MeOH/CH_2Cl_2 as the eluent: white solid, yield 80%; ^{1}H NMR ($CDCl_3$-*d*) δ 1.37 (d, 3H, Ala-CH_3), 1.44 (s, 9H, Boc), 3.34−3.44 (m, 8H, piperazine), 3.72 (s, 3H, OCH_3), 4.47 (m, 1H, α-H), 5.08 (s, 1H, NH); MS (ESI) m/z 316.2 [$(M + 1)^+$].

The benzyloxycarbonyl piperazine derivative Cbz-Piz-Ala-OCH$_3$ was prepared by adding benzyloxycarbonyl piperazine to alanine isocyanate and was purified by column chromatography on silica gel using 2:18:5 MeOH/CH_2Cl_2/EtOAc as the eluent: white solid, yield 72%; ^{1}H NMR ($CDCl_3$-*d*) δ 1.31 (d, 3H, Ala-CH_3), 3.34−3.36 (m, 4H, piperazine), 3.44−3.47 (m, 4H, piperazine), 3.65 (s, 3H, OCH_3), 4.39 (m, 1H, α-H), 5.07 (s, 2H, Cbz), 5.41 (d, 1H, NH), 7.23−7.29 (m, 5H, Ph); MS (ESI) m/z 350.2 [$(M + 1)^+$].

General Procedure for the Synthesis of Capped Dipeptide Methyl Ester (YCO-Ala-Ala-OCH$_3$). The methyl ester group of Y-Ala-OCH$_3$ was hydrolyzed in MeOH using 1 M aqueous NaOH (1.1 equiv) under standard deblocking conditions. The Y-Ala-OH derivative was then coupled to alanine methyl ester hydrochloride (HCl·H-Ala-OCH$_3$) using the DCC/HOBt coupling method. To a stirred solution of the Y-Ala-OH (1 equiv) in DMF at −15 °C was added HOBt (1.5 equiv). The hydrochloride salt of the alanine methyl ester was pretreated with NMM (1.5 equiv) at −15 °C in DMF prior to the addition. The reagent DCC (1.5 equiv) was added to the solution, and the reaction mixture was allowed to react for 16 h at room temperature. The DMF was evaporated, and the residue was redissolved in EtOAc. The organic layer was washed with 2% citric acid, saturated $NaHCO_3$, and saturated NaCl, dried over $MgSO_4$, and concentrated. Purification on a silica gel column with an appropriate eluent gave the product with yields of 60−89%.

Pip-Ala-OH was obtained by hydrolysis of Pip-Ala-OCH_3 in MeOH using 1 M aqueous NaOH (1.1 equiv): white solid, yield 85%; ^{1}H NMR (DMSO-d_6) δ 1.23 (d, 3H, J = 7.4 Hz, Ala-CH_3), 1.33−1.44 (m, 4H, piperidine), 1.49 (d, 2H, J = 5.0 Hz, piperidine), 3.24 (s, 4H, piperidine), 4.03 (q, 1H, J = 7.2 Hz, α-H), 6.50 (d, 1H, J = 7.3 Hz, NH).

After coupling Pip-Ala-OH with HCl·H-Ala-OCH_3, Pip-Ala-Ala-OCH_3 was purified by column chromatography on silica gel using 10% MeOH/CH_2Cl_2 as the eluent: white solid, yield 78%; ^{1}H NMR ($CDCl_3$-d) δ 1.37−1.40 (m, 6H, 2 × Ala-CH_3), 1.54−1.59 (m, 6H, piperidine), 3.31−3.34 (m, 4H, piperidine), 3.73 (s, 3H, OCH_3), 4.45−4.53 (m, 2H, 2 × α-H), 5.21 (s, 1H, NH), 7.12 (s, 1H, NH); MS (FAB) m/z 286.0 [$(M + 1)^+$].

Mu-Ala-OH was obtained by hydrolysis of Mu-Ala-OCH_3 in MeOH using 1 M aqueous NaOH (1.1 equiv): white solid, yield 89%; ^{1}H NMR (DMSO-d_6) δ 1.24 (d, 3H, J = 7.4 Hz, Ala-CH_3), 3.26 (t, 4H, morpholine), 3.50−3.52 (m, 4H, morpholine), 4.01−4.08 (m, 1H, α-H), 6.65 (d, 1H, J = 7.4 Hz, NH), 12.21 (br s, 1H, COOH); MS (FAB) m/z 202.9 [$(M + 1)^+$].

After coupling Mu-Ala-OH with HCl·H-Ala-OCH_3, Mu-Ala-Ala-OCH_3 was purified by column chromatography on silica gel using 10% MeOH/CH_2Cl_2 as the eluent: white solid, yield 60%; ^{1}H NMR ($CDCl_3$-d) δ 1.37 (d, 3H, J = 7.2 Hz, Ala-CH_3), 1.39 (d, 3H, Ala-CH_3), 3.34−3.36 (m, 4H, morpholine), 3.65−3.67 (m, 4H, morpholine), 3.73 (s, 3H, OCH_3), 4.45−4.53 (m, 2H, 2 × α-H), 5.32 (s, 1H, NH), 7.02 (d, 1H, J = 6.7 Hz, NH); MS (FAB) m/z 288.2 [$(M + 1)^+$].

Boc-Piz-Ala-OH was obtained by hydrolysis of Boc-Piz-Ala-OCH_3 in MeOH using 1 M aqueous NaOH (1.1 equiv): white solid, yield 94%; ^{1}H NMR (DMSO-d_6) δ 1.24 (d, 3H, J = 7.4 Hz, Ala-CH_3), 1.38 (s, 9H, Boc), 3.26 (s, 8H, piperazine), 3.97−4.10 (m, 1H, α-H), 6.70 (d, 1H, J = 7.3 Hz, NH).

After coupling Boc-Piz-Ala-OH with HCl·H-Ala-OCH_3, Boc-Piz-Ala-Ala-OCH_3 was purified by column chromatography on silica gel using 10% MeOH/CH_2Cl_2 as the eluent: white solid, yield 85%; ^{1}H NMR ($CDCl_3$-d) δ 1.36−1.39 (m, 6H, 2 × Ala-CH_3), 1.44 (s, 9H, Boc), 3.34−3.39 (m, 8H, piperazine), 3.72 (s, 3H, OCH_3), 4.44−4.54 (m, 2H, 2 × α-H), 5.38 (d, 1H, J = 7.3 Hz, NH), 7.09 (d, 1H, J = 7.2 Hz, NH).

Cbz-Piz-Ala-OH was obtained by hydrolysis of Cbz-Piz-Ala-OCH_3 in MeOH using 1 M aqueous NaOH (1.1 equiv): white solid, yield 87%; ^{1}H NMR (DMSO-d_6) δ 1.25 (d, 3H, Ala-CH_3), 3.32−3.34 (m, 8H, piperazine), 4.06 (m, 1H, α-H), 5.07 (s, 2H, Cbz), 6.72 (d, 1H, NH), 7.28−7.37 (m, 5H, Ph).

After coupling Cbz-Piz-Ala-OH with HCl·H-Ala-OCH_3, Cbz-Piz-Ala-Ala-OCH_3 was purified by column chromatography on silica gel using 10% MeOH/CH_2Cl_2 as the eluent: white solid, yield 89%; ^{1}H NMR ($CDCl_3$-d) δ 1.38 (m, 6H, Ala-CH_3), 3.38 (m, 4H, piperazine), 3.50 (m, 4H, piperazine), 3.72 (s, 3H, OCH_3), 4.46 (m, 1H, α-H), 5.12 (s, 2H, Cbz), 7.34 (m, 5H, Ph).

General Procedure for the Synthesis of Cbz-Capped Dipeptide Methyl Esters (Cbz-Ala-Val(Ile, Phe)-OCH_3). The Cbz-Ala-OH derivative was coupled to valine methyl ester (or isoleucine methyl ester or phenylalanine methyl ester) using the DCC and HOBt coupling method. To a stirred solution of the Cbz-Ala-OH (1 equiv) in DMF at −15 °C was added HOBt (1.5 equiv). The hydrochloride salt of the valine methyl ester was pretreated with NMM (1.5 equiv) at −15 °C in DMF prior to the addition. The reagent DCC (1.5 equiv) was added to the solution, and the reaction mixture was allowed to react for 16 h at room temperature. The DMF was evaporated, and the residue was redissolved in EtOAc. The organic layer was washed with 2% citric acid, saturated $NaHCO_3$, and saturated NaCl, dried over $MgSO_4$, and concentrated.

After coupling Cbz-Ala-OH with HCl·H-Val-OCH_3, Cbz-Ala-Val-OCH_3 was purified by column chromatography on silica gel using 10% MeOH/CH_2Cl_2 as the eluent: white solid, yield 85%; ^{1}H NMR ($CDCl_3$-d) δ 0.88 (m, 6H, 2 × Val-CH_3), 1.39 (d, 3H, Ala-CH_3), 2.16 (m, 1H, CH), 3.74 (s, 3H, OCH_3), 4,27 (m, 1H, α-H), 4.52 (m, 1H, α-H), 5.12 (s, 2H, Cbz), 5.36 (d, 1H, NH), 6.54 (d, 1H, NH), 7.36−7.32 (m, 5H, Ph); MS (ESI) m/z 337.1 [$(M + 1)^+$].

After coupling Cbz-Ala-OH with HCl·H-Ile-OCH_3, Cbz-Ala-Ile-OCH_3 was purified by column chromatography on silica gel using 1:1 EtOAc/hexane as the eluent: white solid, yield 89%; ^{1}H NMR ($CDCl_3$-d) δ 0.85−0.89 (m, 6H, 2 × Ile-CH_3), 1.08−1.19 (m, 2H, CH_2), 1.29−1.43 (m, 3H, Ala-CH_3), 1.86 (m, 1H, CH), 3.70 (s, 3H, OCH_3), 4.34 (q, 1H, α-H), 4.55 (q, 1H, α-H), 5.09 (m, 2H, Cbz), 5.63 (d, 1H, NH), 6.82 (d, 1H, NH), 7.26−7.36 (m, 5H, Ph).

After coupling Cbz-Ala-OH with HCl·H-Phe-OCH_3, Cbz-Ala-Phe-OCH_3 was purified by column chromatography on silica gel using 2:1 EtOAc/hexane as the eluent: white solid, yield 86%; ^{1}H NMR ($CDCl_3$-d) δ 1.32 (d, 3H, Ala-CH_3), 3.03−3.16 (m, 2H, CH_2Ph), 3.70 (s, 3H, OCH_3), 4.25 (q, 1H, α-H), 4.85 (q, 1H, α-H), 5.08 (m, 2H, Cbz), 5.38 (d, 1H, NH), 7.08 (d, 1H, NH), 7.21−7.34 (m, 10 H, 2 × Ph); MS (ESI) m/z 385.0 [$(M + 1)^+$].

Peptidyl Hydrazides. Anhydrous hydrazine (10 equiv) was added to a stirred solution of a peptidyl methyl ester (1 equiv) in MeOH, and the reaction mixture was stirred vigorously for 16 h at room temperature. Excess hydrazine and solvent were removed under vacuum, and the residue was washed with ether several times to give the peptidyl hydrazide as a white solid. Compounds were purified further on silica gel column using the eluent 10% MeOH/CH_2Cl_2 when needed.

Pip-Ala-NH-NH_2: white solid, yield 97%; ^{1}H NMR (DMSO-d_6) δ 1.15 (d, 3H, Ala-CH_3), 1.36−1.41 (m, 4H, piperidine), 1.46−1.49 (m, 2H, piperidine), 3.22−3.25 (m, 4H, piperidine), 4.06−4.12 (m, 3H, α-H and NH_2), 6.28 (d, 1H, NH), 8.96 (s, 1H, NH); MS (ESI) m/z 215.0 [$(M + 1)^+$].

Mu-Ala-NH-NH_2: white solid, yield 97%; ^{1}H NMR (DMSO-d_6) δ 1.16 (d, 3H, Ala-CH_3), 3.23−3.26 (m, 4H, morpholine), 3.49−3.52 (m, 4H, morpholine), 4.07−4.14 (m, 3H, α-H and NH_2), 6.45 (d, 1H, NH), 8.97 (s, 1H, NH); MS (ESI) m/z 217.0 [$(M + 1)^+$].

Cbz-Piz-Ala-NH-NH_2: white solid, yield 98%; ^{1}H NMR (DMSO-d_6) δ 1.16 (d, 3H, AlaCH_3), 3.30−3.34 (m, 8H, piperazine), 4.11 (m, 3H, α-H and NH_2), 5.07 (s, 2H, Cbz), 6.51 (d, 1H, NH), 7.34 (m, 5H, Ph), 8.95 (s, 1H, NH).

Pip-Ala-Ala-NH-NH_2: white solid, yield 91%; ^{1}H NMR (DMSO-d_6) δ 1.15−1.18 (m, 6H, 2 × Ala-CH_3), 1.38−1.40 (m, 4H, piperidine), 1.48−1.52 (m, 2H, piperidine), 3.25−3.27 (m, 4H, piperidine), 4.06 (m, 1H, α-H), 4.17−4.21 (m, 3H, α-H and NH_2), 6.40 (d, 1H, NH), 7.71 (d, 1H, NH), 9.01 (s, 1H, NH); MS (ESI) m/z 286.0 [$(M + 1)^+$].

Mu-Ala-Ala-NH-NH_2: white solid, yield 92%; ^{1}H NMR (DMSO-d_6) δ 1.15−1.18 (m, 6H, 2 × Ala-CH_3), 3.25−3.28 (m, 4H, morpholine), 3.51−3.53 (m, 4H, morpholine), 4.09 (q, 1H, J = 7.2 Hz, α-H), 4.16−4.23 (m, 3H, α-H and NH_2), 6.53 (d, 1H, J = 7.1 Hz, NH), 7.77 (d, 1H, J = 7.7 Hz, NH), 8.99 (s, 1H, NH).

Boc-Piz-Ala-Ala-NH-NH_2: white solid, yield 66%; ^{1}H NMR (DMSO-d_6) δ 1.15−1.19 (m, 6H, 2 × Ala-CH_3), 1.38 (s, 9H, Boc), 3.27 (s, 8H, piperazine), 4.05−4.12 (m, 1H, α-H), 4.18−4.19 (m, 3H, α-H and NH_2), 6.56 (d, 1H, J = 7.0 Hz, NH), 7.76 (d, 1H, J = 7.7 Hz, NH), 8.99 (s, 1H, NH).

Cbz-Piz-Ala-Ala-NH-NH_2: white solid, yield 98%; ^{1}H NMR (DMSO-d_6) δ 1.15−1.18 (m, 6H, 2 × Ala-CH_3), 3.45 (s, 8H, piperazine), 4.03−4.08 (m, 1H, α-H), 4.15−4.19 (m, 3H, α-H and NH_2), 5.06 (s, 2H, Cbz), 6.58 (d, 1H, NH), 7.29−7.35 (m, 5H, Ph), 7.78 (d, 1H, NH), 9.00 (s, 1H, NH).

Cbz-Ala-Val-NH-NH_2: white solid, yield 98%; ^{1}H NMR (DMSO-d_6) δ 0.78−0.81 (m, 6H, 2 × Val-CH_3), 1.15 (d, 3H, Ala-CH_3), 1.82 (m, 1H, Val-CH), 4.02−4.11 (m, 2H, 2 × α-H), 4.23 (s, 2H, NH_2), 4.99 (s, 2H, Cbz), 7.29−7.34 (m, 5H, Ph), 7.46 (d, 1H, NH), 7.68 (d, 1H, NH), 9.15 (s, 1H, NH).

Cbz-Ala-Ile-NH-NH_2: white solid, yield 98%; ^{1}H NMR (DMSO-d_6) δ 0.71−0.84 (m, 6H, 2 × Ala-CH_3), 0.97−1.05 (m, 1H, CH), 1.15 (d, 3H, CH_3), 1.38−1.44 (m, 1H, CH), 1.61−1.63 (m, 1H, CH), 4.05−4.13 (m, 2H, 2 × α-H), 4.23 (s, 2H, NH_2),

4.99 (m, 2H, Cbz), 7.29−7.36 (m, 5H, Ph), 7.45 (d, 1H, NH), 7.70 (d, 1H, NH), 9.16 (s, 1H, NH).

Cbz-Ala-Phe-NH-NH_2: white solid, yield 97%; ^{1}H NMR (DMSO-d_6) δ 1.08 (d, 3H, Ala-CH_3), 2.78−2.97 (m, 2H, CH_2Ph), 3.99 (q, 1H, α-H), 4.20 (br s, 2H, NH_2), 4.42 (q, 1H, α-H), 4.99 (m, 2H, Cbz), 7.14−7.39 (m, 11 H, 2 × Ph and NH), 7.92 (d, 1H, NH), 9.14 (s, 1H, NH); MS (ESI) m/z 385.2 [$(M + 1)^+$].

General Procedure for the Synthesis of Peptidyl-NH-NH-CH_2COOEt. The reagent *tert*-butyl bromoacetate (1.1 equiv) was added dropwise at −15 °C to a stirred solution of a peptidyl hydrazide (1 equiv) in DMF and NMM (1.1 equiv), and the mixture was allowed to react for 36 h at room temperature. The solvent was removed under vacuum, and the residue was purified on a silica gel column with the proper eluent.

Boc-NH-NH-CH_2COOEt was purified by column chromatography on silica gel using 15% MeOH/CH_2Cl_2 as the eluent: white solid, yield 90%; ^{1}H NMR (DMSO-d_6) δ 1.26 (t, 3H, NHCH$_2$COCH$_2$*CH*$_3$), 1.45 (m, 9H, Boc), 4.11 (s, 2H, N*CH*$_2$CO), 4.19 (q, 2H, NHCH$_2$CO*CH*$_2$CH$_3$); MS (ESI) m/z 219.2 [$(M + 1)^+$].

Pip-Ala-NH-NH-CH_2COOEt was purified by column chromatography on silica gel using 15% MeOH/CH_2Cl_2 as the eluent: yellow oil, yield 26%; ^{1}H NMR (DMSO-d_6) δ 0.78−0.84 (m, 3H, NHCH$_2$COCH$_2$*CH*$_3$), 1.13−1.19 (m, 3H, Ala-CH_3), 1.38−1.41 (m, 4H, piperidine), 1.47−1.50 (m, 2H, piperidine), 3.23−3.25 (m, 4H, piperidine), 3.45 (d, 2H, J = 4.4 Hz, NH*CH*$_2$CO-CH$_2$CH$_3$), 4.03−4.09 (m, 3H, α-H and NHCH$_2$CO*CH*$_2$CH$_3$), 5.09 (d, 1H, J = 5.2 Hz, NH), 6.32 (d, 1H, J = 7.5 Hz, NH), 9.16 (d, 1H, J = 5.4 Hz, NH); MS (ESI) m/z 301.0 [$(M + 1)^+$].

Mu-Ala-NH-NH-CH_2COOEt was purified by column chromatography on silica gel using 15% MeOH/CH_2Cl_2 as the eluent: yellow oil, yield 41%; ^{1}H NMR (DMSO-d_6) δ 0.78−0.84 (m, 3H, NHCH$_2$COCH$_2$*CH*$_3$), 1.14−1.18 (m, 3H, Ala-CH_3), 3.20−3.29 (m, 4H, morpholine), 3.45 (d, 2H, NH*CH*$_2$COCH$_2$CH$_3$), 3.49−3.55 (m, 4H, *CH*$_2$O*CH*$_2$ morpholine), 4.03−4.10 (m, 3H, α-H and NHCH$_2$CO*CH*$_2$CH$_3$), 5.10 (d, 1H, NH), 6.48 (d, 1H, NH), 9.23 (d, 1H, NH); MS (ESI) m/z 303.0 [$(M + 1)^+$].

Cbz-Piz-Ala-NH-NH-CH_2COOEt was purified by column chromatography on silica gel using 15% MeOH/CH_2Cl_2 as the eluent: white solid, yield 44%; ^{1}H NMR (DMSO-d_6) δ 1.14−1.18 (m, 6H, Ala-CH_3 and NHCH$_2$COCH$_2$*CH*$_3$), 3.30−3.33 (m, 8H, piperazine) 3.45 (d, 2H, NH*CH*$_2$COCH$_2$CH$_3$), 4.03−4.10 (m, 3H, α-H and NHCH$_2$CO*CH*$_2$CH$_3$), 5.07 (s, 2H, Cbz), 6.55 (d, 1H, NH), 7.29−7.36 (m, 5H, Ph), 9.23 (d, 1H, NH); MS (ESI) m/z 436.3 [$(M + 1)^+$].

Pip-Ala-Ala-NH-NH-CH_2COOEt was purified by column chromatography on silica gel using 15% MeOH/CH_2Cl_2 as the eluent: white solid, yield 20%; ^{1}H NMR (DMSO-d_6) δ 1.13−1.18 (m, 9H, 2 × Ala-CH_3 and NHCH$_2$COCH$_2$*CH*$_3$), 1.37−1.40 (m, 4H, piperidine), 1.48−1.52 (m, 2H, piperidine), 3.23−3.25 (m, 4H, piperidine), 3.44 (d, 2H, NH*CH*$_2$COCH$_2$CH$_3$), 4.04−4.09 (m, 3H, α-H and NHCH$_2$CO*CH*$_2$CH$_3$), 4.15 (m, 1H, α-H), 5.15 (m, 1H, NH), 6.40 (d, 1H, NH), 7.74 (d, 1H, NH), 9.32 (d, 1H, NH).

Mu-Ala-Ala-NH-NH-CH_2COOEt was purified by column chromatography on silica gel using 15% MeOH/CH_2Cl_2 as the eluent: white solid, yield 30%; ^{1}H NMR (DMSO-d_6) δ 0.78−0.84 (m, 6H, 2 × CH$_3$), 1.14−1.18 (m, 6H, 2 × CH$_3$), 3.25−3.32 (m, 4H, morpholine), 3.44 (d, 2H, J = 4.9 Hz, NH*CH*$_2$COCH$_2$CH$_3$), 3.50−3.53 (m, 4H, morpholine), 4.04−4.09 (m, 3H, α-H and NHCH$_2$CO*CH*$_2$CH$_3$), 4.18 (q, 1H, α-H), 5.15 (q, 1H, J = 5.0 Hz, NH), 6.53 (d, 1H, J = 7.2 Hz, NH), 7.81 (d, 1H, J = 7.5 Hz, NH), 9.29 (d, 1H, J = 5.9 Hz, NH).

Boc-Piz-Ala-Ala-NH-NH-CH_2COOEt was purified by column chromatography on silica gel using 15% MeOH/CH_2Cl_2 as the eluent: white solid, yield 50%; ^{1}H NMR (DMSO-d_6) δ 1.26 (t, 3H, NHCH$_2$COCH$_2$*CH*$_3$), 1.34−1.37 (m, 6H, 2 × Ala-CH_3), 1.45 (s, 9H, Boc), 3.37−3.42 (m, 10H, piperazine and NH-*CH*$_2$COCH$_2$CH$_3$), 4.15−4.22 (m, 2H, NHCH$_2$CO*CH*$_2$CH$_3$), 4.39 (m, 1H, α-H), 4.46 (m, 1H, α-H and NHCH$_2$CO*CH*$_2$CH$_3$), 5.54 (d, 1H, NH), 7.35 (d, 1H, NH), 8.89 (s, 1H, NH).

Cbz-Piz-Ala-Ala-NH-NH-CH_2COOEt was purified by column chromatography on silica gel using 15% MeOH/CH_2Cl_2 as the eluent: white solid, yield 57%; ^{1}H NMR (DMSO-d_6) δ 1.12−1.18 (m, 9H, 2 × Ala-CH_3 and NHCH$_2$COCH$_2$*CH*$_3$), 3.32−3.34 (m, 8H, piperazine), 3.44 (d, 2H, NH*CH*$_2$COCH$_2$CH$_3$), 4.03−4.10 (m, 4H, 2 × α-H and NHCH$_2$CO*CH*$_2$CH$_3$), 5.04 (s, 2H, Cbz), 6.59 (d, 1H, NH), 7.29−7.35 (m, 5H, Ph), 7.81 (d, 1H, NH), 9.29 (d, 1H, NH).

Cbz-Ala-Val-NH-NH-CH_2COOEt was purified by column chromatography on silica gel using 15% MeOH/CH_2Cl_2 as the eluent: white solid, yield 64%; ^{1}H NMR (DMSO-d_6) δ 0.78−0.84 (m, 6H, 2 × CH$_3$), 1.14−1.18 (m, 6H, 2 × CH$_3$), 1.84 (m, 1H, CH), 3.48 (d, 2H, NH*CH*$_2$COCH$_2$CH$_3$), 4.02−4.08 (m, 4H, 2 × α-H and NHCH$_2$CO*CH*$_2$CH$_3$), 4.99 (m, 2H, Cbz), 7.28−7.34 (m, 5H, Ph), 7.46 (d, 1H, NH), 7.70 (d, 1H, NH).

Cbz-Ala-Ile-NH-NH-CH_2COOEt was purified by column chromatography on silica gel using 15% MeOH/CH_2Cl_2 as the eluent: white solid, yield 55%; ^{1}H NMR (DMSO-d_6) δ 0.72−0.82 (m, 6H, 2 × CH$_3$), 0.97 (m, 1H, CH), 1.14 (m, 6H, 2 × CH$_3$), 1.35 (m, 1H, CH), 1.60 (m, 1H, CH), 3.38 (s, 2H, NH*CH*$_2$CO), 4.03−4.19 (m, 4H, CO*CH*$_2$CH$_3$ and 2 × α-H), 4.99 (m, 2H, Cbz), 7.29−7.34 (m, 5H, Ph), 7.46 (d, 1H, NH), 7.66 (d, 1H, NH), 9.44 (s, 1H, NH).

Cbz-Ala-Phe-NH-NH-CH_2COOEt was purified by column chromatography on silica gel using 10% MeOH/CH_2Cl_2 as the eluent: white solid, yield 53%; ^{1}H NMR (DMSO-d_6) δ 1.09 (d, 3H, Ala-CH_3), 1.15−1.19 (t, 3H, NHCH$_2$COCH$_2$*CH*$_3$), 2.71 (s, 1H, NH), 2.76−2.92 (m, 2H, *CH*$_2$Ph), 3.33−3.39 (m, 2H, NH*CH*$_2$COCH$_2$CH$_3$), 3.98−4.08 (m, 3H, α-H and NHCH$_2$CO*CH*$_2$CH$_3$), 4.21 (s, 1H, NH), 4.43 (q, 1H, α-H), 4.99 (m, 2H, Cbz), 7.15−7.41 (m, 10H, 2 × Ph), 7.93 (d, 1H, NH), 9.13 (s, 1H, NH).

General Procedure for the Synthesis of Peptidyl-NH-NH-CH_2CONH$_2$. The peptidyl-NH-NHCH$_2$COOEt (1 equiv) was dissolved in a 7 N solution (100 equiv) of NH_3 in methanol and a small amount of DMF and allowed to stir in an ice bath. To this solution was added a catalytic amount of NaCN (0.1 equiv). The flask was closed with a rubber septum and allowed to stir at 4 °C for 5 days. The solvent was evaporated, and the product was purified by column chromatography on a silica gel using the proper eluent.

Boc-NH-NH-CH_2CONH$_2$ was purified by column chromatography on silica gel using 15% MeOH/CH_2Cl_2 as the eluent: white solid, yield 80%; ^{1}H NMR (DMSO-d_6) δ 1.36 (s, 9H, Boc), 3.16 (s, 2H, N*CH*$_2$CO), 4.96 (d, 1H, NH), 7.16 (s, 1H, NH), 7.38 (s, 1H, NH), 8.24 (s, 1H, NH); MS (ESI) m/z 189.9 [$(M + 1)^+$].

Pip-Ala-NH-NH-CH_2CONH$_2$ was purified by column chromatography on silica gel using 15% MeOH/CH_2Cl_2 as the eluent: white solid, yield 86%; ^{1}H NMR (DMSO-d_6) δ 1.15 (d, 3H, Ala-CH_3), 1.38−1.41 (m, 4H, piperidine), 1.47−1.50 (m, 2H, piperidine), 3.17 (d, 2H, N*CH*$_2$CO), 3.23−3.26 (m, 4H, piperidine), 4.04 (q, 1H, α-H), 5.13 (d, 1H, NH), 5.36 (d, 1H, NH), 7.10 (s, 1H, NH), 7.46 (s, 1H, NH), 9.18 (d, 1H, NH); MS (ESI) m/z 272.0 [$(M + 1)^+$].

Mu-Ala-NH-NH-CH_2CONH$_2$ was purified by column chromatography on silica gel using 15% MeOH/CH_2Cl_2 as the eluent: white solid, yield 53%; ^{1}H NMR (DMSO-d_6) δ 1.16 (d, 3H, J = 7.1 Hz, Ala-CH_3), 3.18 (d, 2H, J = 3.5 Hz, N*CH*$_2$CO), 3.25−3.26 (m, 4H, morpholine), 3.50−3.52 (m, 4H, morpholine), 4.02−4.09 (m, 1H, α-H), 5.14 (d, 1H, J = 3.9 Hz, NH), 6.52 (d, 1H, J = 7.4 Hz, NH), 7.12 (s, 1H, NH), 7.46 (s, 1H, NH), 9.23 (d, 1H, J = 3.8 Hz, NH); MS (ESI) m/z 274.0 [$(M + 1)^+$].

Cbz-Piz-Ala-NH-NH-CH_2CONH$_2$ was purified by column chromatography on silica gel using 15% MeOH/CH_2Cl_2 as the eluent: white solid, yield 62%; ^{1}H NMR (DMSO-d_6) δ 1.16 (d, 3H, Ala-CH_3), 3.18 (d, 2H, N*CH*$_2$CO), 3.31−3.33 (m, 8H, piperazine), 4.05 (m, 1H, α-H), 5.07 (s, 2H, Cbz), 5.15 (d, 1H, NH), 6.58 (d, 1H, NH), 7.11 (s, 1H, NH), 7.30−7.38 (m, 5H, Ph), 7.45 (s, 1H, NH), 7.85 (d, 1H, NH), 9.23 (d, 1H, NH).

Pip-Ala-Ala-NH-NH-CH_2CONH_2 was purified by column chromatography on silica gel using 15% MeOH/CH_2Cl_2 as the eluent: white solid, yield 58%; ^{1}H NMR (DMSO-d_6) δ 1.15−1.18 (m, 6H, 2 × Ala-CH_3), 1.40 (s, 4H, piperidine), 1.48−1.51 (m, 2H, piperidine), 3.17 (d, 2H, J = 3.6 Hz, NCH_2CO), 3.21−3.27 (m, 4H, piperidine), 4.05 (q, 1H, J = 6.9 Hz, α-H), 4.14 (q, 1H, J = 7.0 Hz, α-H), 5.19 (d, 1H, J = 4.1 Hz, NH), 6.40 (d, 1H, J = 6.9 Hz, NH), 7.11 (s, 1H, NH), 7.42 (s, 1H, NH), 7.79 (d, 1H, J = 7.5 Hz, NH), 9.29 (d, 1H, J = 3.8 Hz, NH); MS (ESI) m/z 343.1 [(M + 1)$^+$].

Mu-Ala-Ala-NH-NH-CH_2CONH_2 was purified by column chromatography on silica gel using 15% MeOH/CH_2Cl_2 as the eluent: white solid, yield 48%; ^{1}H NMR (DMSO-d_6) δ 1.16−1.19 (m, 6H, 2 × Ala-CH_3), 3.17 (d, 2H, J = 4.1 Hz, NCH_2CO), 3.26−3.27 (m, 4H, morpholine), 3.50−3.53 (m, 4H, morpholine), 4.04−4.18 (m, 2H, 2 × α-H), 5.19 (d, 1H, J = 4.2 Hz, NH), 6.54 (d, 1H, J = 7.0 Hz, NH), 7.12 (s, 1H, NH), 7.42 (s, 1H, NH), 7.86 (d, 1H, J = 7.5 Hz, NH), 9.27 (d, 1H, J = 4.3 Hz, NH).

Boc-Piz-Ala-Ala-NH-NH-CH_2CONH_2 was purified by column chromatography on silica gel using 15% MeOH/CH_2Cl_2 as the eluent: white solid, yield 67%; ^{1}H NMR (DMSO-d_6) δ 1.16−1.19 (m, 6H, 2 × Ala-CH_3), 1.39 (s, 9H, Boc), 3.17 (d, 2H, J = 2.9 Hz, NCH_2CO), 3.27−3.33 (m, 8H, piperazine), 4.03−4.14 (m, 2H, 2 × α-H), 5.19 (d, 1H, NH), 6.57 (d, 1H, J = 6.9 Hz, NH), 7.11 (s, 1H, NH), 7.41 (s, 1H, NH), 7.85 (d, 1H, J = 7.5 Hz, NH), 9.26 (d, 1H, J = 3.2 Hz, NH).

Cbz-Piz-Ala-Ala-NH-NH-CH_2CONH_2 was purified by column chromatography on silica gel using 15% MeOH/CH_2Cl_2 as the eluent: white solid, yield 34%; ^{1}H NMR (DMSO-d_6) δ 1.15−1.19 (m, 6H, 2 × Ala-CH_3), 3.17 (d, 2H, J = 3.7 Hz, NCH_2CO), 3.32−3.35 (m, 8H, piperazine), 4.06−4.14 (m, 2H, 2 × α-H), 5.07 (s, 2H, Cbz), 5.20 (d, 1H, NH), 6.60 (d, 1H, J = 6.9 Hz, NH), 7.12 (s, 1H, NH), 7.23−7.35 (m, 5H, Ph), 7.41 (s, 1H, NH), 7.87 (d, 1H, J = 7.5 Hz, NH), 9.27 (d, 1H, J = 4.0 Hz, NH).

Cbz-Ala-Val-NH-NH-CH_2CONH_2 was purified by column chromatography on silica gel using 15% MeOH/CH_2Cl_2 as the eluent: white solid, yield 41%; ^{1}H NMR (DMSO-d_6) δ 0.78−0.81 (m, 6H, 2 × Val-CH_3), 1.16 (d, 3H, Ala-CH_3), 1.85 (m, 1H, CH), 3.40 (d, 2H, NHCH_2CO), 4.09−4.13 (m, 2H, 2 × α-H), 4.99 (m, 2H, Cbz), 5.27 (d, 1H, NH), 7.13 (s, 1H, NH), 7.28−7.32 (m, 5H, Ph), 7.45 (s, 1H, NH), 7.73 (d, 1H, NH), 9.46 (d, 1H, NH).

Cbz-Ala-Ile-NH-NH-CH_2CONH_2 was purified by column chromatography on silica gel using 15% MeOH/CH_2Cl_2 as the eluent: white solid, yield 25%; ^{1}H NMR (DMSO-d_6) δ 0.76−0.79 (m, 6H, 2 × Ile-CH_3), 1.02 (m, 1H, CH), 1.14 (d, 3H, Ala-CH_3), 1.40 (m, 1H, CH), 1.63 (m, 1H, CH), 3.18 (d, 2H, NHCH_2CO), 4.00−4.10 (m, 2H, 2 × α-H), 4.99 (m, 2H, Cbz), 5.29 (d, 1H, NH), 7.15−7.46 (m, 7H, Ph and 2 × NH), 7.77 (d, 1H, NH), 9.47 (d, 1H, NH).

Cbz-Ala-Phe-NH-NH-CH_2CONH_2 was purified by column chromatography on silica gel using 15% MeOH/CH_2Cl_2 as the eluent: white solid, yield 64%; ^{1}H NMR (DMSO-d_6) δ 1.10 (d, 3H, Ala-CH_3), 2.80−2.89 (m, 2H, CH_2Ph), 3.13 (d, 2H, NHCH_2CO), 4.00 (q, 1H, α-H), 4.38 (q, 1H, α-H), 4.99 (m, 2H, Cbz), 5.21 (d, 1H, NH), 7.12−7.40 (m, 12H, 2 × Ph and 2 × NH), 7.93 (s, 1H, NH), 7.97 (s, 1H, NH).

General Procedure for the Synthesis of Fumaric Acid Monoamides by the Mixed Anhydride Coupling Method. Coupling of the amine precursors to monoethyl fumarate was accomplished using the mixed anhydride coupling method. To a solution of the monoethyl fumarate (1 equiv) in CH_2Cl_2 at −20 °C was added *N*-methylmorpholine (NMM, 1 equiv) followed by isobutyl chloroformate (iBCF, 1 equiv). After the reaction mixture was allowed to stir for 30 min, the amine (1 equiv) was added to the mixture. Hydrochloride salts of the amine were pretreated with NMM (1 equiv) at −20 °C in CH_2Cl_2 prior to addition. After 30 min, the reaction mixture was allowed to stir overnight at room temperature. The methylene chloride was evaporated, and the residue was redissolved in ethyl acetate and washed with 2% citric acid, saturated $NaHCO_3$, and saturated NaCl, dried over $MgSO_4$, and concentrated. The product was purified by column chromatography as needed.

***trans*-3-Benzyloxycarbonylpropenoic Acid or Monobenzyl Fumarate (2b, HOOCCH=CHCOOBzl).** Equimolar amounts of fumaric acid and benzyl alcohol were dissolved in anhydrous DMF. The reagent NMM (1 equiv) was added at 0 °C followed by EDC after 15 min. The reaction mixture was stirred overnight at room temperature. The DMF was evaporated, and the crude residue was redissolved in EtOAc. The product was extracted with saturated aqueous $NaHCO_3$. The aqueous layer was then acidified with 1 N HCl to pH 2. The product was extracted with EtOAc, and the organic layer was washed with water and dried with $MgSO_4$. The solvent was evaporated, and purification by column chromatography using 5% MeOH/CH_2Cl_2 as the eluent gave a white powder (51% yield): ^{1}H NMR (DMSO-d_6) δ 5.21 (s, 2H, CH=CH−COOCH_2Ph), 6.73 (m, 2H, CH=CH−COOCH_2Ph), 7.29−7.43 (m, 5H, Ph); MS (ESI) m/z 207 [(M + 1)$^+$].

***trans*-3-Cyclopropylcarbamoylpropenoic Acid Ethyl Ester (EtOOCCH=CHCONH−Cyclopropyl)** was obtained by mixed anhydride coupling of equimolar amounts of monoethyl fumarate and cyclopropylamine. Purification by column chromatography using 2:1 hexane/EtOAc as the eluent gave a white powder (84% yield): ^{1}H NMR ($CDCl_3$) δ 0.56−0.59 (m, 2H, CH_2), 0.80−0.82 (m, 2H, CH_2), 1.28 (t, 3H, CH_3), 2.81−2.84 (m, 1H, CH), 4.20 (q, 2H, CH_2CH_3), 6.77−6.81 (d, 2H, CH=CHCON and NH), 6.92 (d, 1H, J = 15.5 Hz, CH=CHCON).

***trans*-3-Cyclopropylcarbamoylpropenoic Acid (2c, HOOCCH=CHCONH−Cyclopropyl).** EtOOCCH=CHCONH−cyclopropyl was hydrolyzed in EtOH using NaOH (1 M aqueous, 1.1 equiv) under standard deblocking conditions to give a white solid (91% yield): ^{1}H NMR (DMSO-d_6) δ 0.41−0.45 (m, 2H, CH_2), 0.64−0.69 (m, 2H, CH_2), 2.69−2.76 (m, 1H, CH), 6.47 (d, 1H, J = 15.5 Hz, CH=CHCON), 6.80 (d, 1H, J = 15.5 Hz, CH=CHCON), 8.53 (d, 1H, NH).

***trans*-3-Cyclohexylcarbamoylpropenoic Acid Ethyl Ester (EtOOCCH=CHCONH−cyclohexyl)** was obtained by mixed anhydride coupling of equimolar amounts of monoethyl fumarate and cyclohexylamine. Purification by column chromatography using 2:1 EtOAc/hexane as the eluent gave a white powder (63% yield).

***trans*-3-Cyclohexylcarbamoylpropenoic Acid (2d, HOOCCH=CHCONH−cyclohexyl).** EtOOCCH=CHCONH−cyclohexyl was hydrolyzed in EtOH using NaOH (1 M aqueous, 1.1 equiv) under standard deblocking conditions to give a white solid (81% yield): ^{1}H NMR (DMSO-d_6) δ 0.82−0.90 (m, 2H, CH_2), 1.07−1.39 (m, 4H, 2 × CH_2), 1.59−1.65 (m, 5H, 2 × CH_2 and CH), 2.98 (t, 2H, J = 6.1 Hz, NHCH_2), 6.47 (d, 1H, J = 15.6, CH=CHCON), 6.93 (d, 1H, J = 15.6, CH=CHCON), 8.43 (t, 1H, J = 5.5 Hz, NH).

***trans*-3-(2-Furyl)carbamoylpropenoic Acid Ethyl Ester (EtOOCCH=CHCONHCH$_2$-2-furyl)** was obtained by mixed anhydride coupling of equimolar amounts of monoethyl fumarate and 2-furylamine. Purification by column chromatography using 5% MeOH/CH_2Cl_2 as the eluent gave a yellow oil (84% yield): ^{1}H NMR ($CDCl_3$) δ 1.26 (t, 3H, CH_3CH_2), 4.14−4.19 (m, 2H, CH_3CH_2), 4.50 (d, 2H, NHCH_2), 6.22−6.23 (d, 1H, furyl), 6.28−6.29 (d, 1H, furyl), 6.81 (d, 1H, J = 15.5 Hz, CH=CHCON), 6.92 (t, 1H, NH), 7.00 (d, 1H, J = 15.5 Hz, CH=CHCON), 7.32 (s, 1H, furyl).

***trans*-3-(2-Furyl)carbamoylpropenoic Acid (2e, HOOCCH=CHCONHCH$_2$-2-furyl).** EtOOCCH=CHCONHCH$_2$-2-furyl was hydrolyzed in EtOH using NaOH (1 M aqueous, 1.1 equiv) under standard deblocking conditions to give a white solid (79% yield): ^{1}H NMR (DMSO-d_6) δ 4.38 (d, 2H, NHCH_2), 6.29−6.30 (d, 1H, J = 7.8 Hz, furyl), 6.40−6.41 (d, 1H, furyl), 6.53−6.57 (d, 1H, J = 15.5 Hz, CH=CHCON), 6.95 (d, 1H, J = 15.6 Hz, CH=CHCON), 7.60 (s, 1H, furyl), 8.96 (t, 1H, J = 5.5 Hz, NH).

***trans*-3-Benzylcarbamoylpropenoic Acid Ethyl Ester (EtOOCCH=CHCONHBzl)** was obtained by mixed anhydride coupling of equimolar amounts of monoethyl fumarate and benzylamine. Purification by column chromatography using 5% MeOH/CH_2Cl_2 gave a white powder (81% yield): ^{1}H NMR (DMSO-d_6) δ 1.20−1.24 (t, 3H, J = 14.4 Hz, CH_2CH_3), 4.14−4.19 (q, 2H, J = 7.1 Hz, CH_2CH_3), 4.37 (d, 2H, J = 5.9 Hz, NCH_2Ph), 6.59 (d, 1H, J = 15.2 Hz, CH=CHCON), 7.05 (d, 1H, J = 15.2 Hz, CH=CHCON), 7.22−7.34 (m, 5H, Ph), 9.04 (s, 1H, NH).

***trans*-3-Benzylcarbamoylpropenoic Acid (2f, HOOCCH=CHCONHBzl).** EtOOCCH=CHCONHBzl was hydrolyzed in EtOH using NaOH (1 M aqueous, 1.1 equiv) under standard deblocking conditions to give a white solid (73% yield): ^{1}H NMR (DMSO-d_6) δ 4.37 (d, 2H, J = 5.9 Hz, NCH_2Ph), 6.52−6.56 (d, 1H, J = 15.2 Hz, CH=CHCON), 6.95−6.99 (d, 1H, J = 15.2 Hz, CH=CHCON), 7.23−7.33 (m, 5H, Ph), 8.98 (t, 1H, J = 5.6 Hz, NH); MS (ESI) m/z 206 [$(M + 1)^+$].

***trans*-3-Phenylethylcarbamoylpropenoic Acid Ethyl Ester (EtOOCCH=CHCONHCH$_2$CH$_2$Ph)** was obtained by a mixed anhydride coupling of equimolar amounts of monoethyl fumarate and phenethylamine to give a clear colorless syrup (78% yield): ^{1}H NMR (DMSO-d_6) δ 2.74 (t, 2H, J = 7.3 Hz, NCH$_2CH_2$Ph), 3.33−3.43 (m, 2H, NCH_2CH$_2$Ph), 6.56 (d, 1H, J = 15.6 Hz, CH=CHCON), 6.97 (d, 1H, J = 15.6 Hz, CH=CHCON), 7.15−7.28 (m, 5H, Ph), 8.63 (t, 1H, J = 5.4 Hz, NH).

***trans*-3-Phenylethylcarbamoylpropenoic Acid (2g, HOOCCH=CHCONHCH$_2$CH$_2$Ph).** EtOOCCH=CHCONHCH$_2$CH$_2$Ph was hydrolyzed in EtOH using NaOH (1 M aqueous, 1.1 equiv) under standard deblocking conditions to give a clear, colorless syrup (81% yield): ^{1}H NMR (DMSO-d_6) δ 3.54 (t, 2H, N-CH$_2$-CH_2-Ph), 3.61 (t, 2H, N-CH_2-CH$_2$-Ph), 6.45z (d, 1H, J = 15.2 Hz, CH=CHCON), 7.00 (d, 1H, J = 15.6 Hz, CH=CHCON), 7.14−7.31 (m, 5H, Ph).

***trans*-3-Phenylpropylcarbamoylpropenoic Acid Ethyl Ester (EtOOCCH=CHCONHCH$_2$CH$_2$CH$_2$Ph)** was obtained by mixed anhydride coupling of equimolar amounts of monoethyl fumarate and 3-phenyl-1-propylamine. Purification by column chromatography using 1:1 EtOAc/hexane gave a white powder (80% yield).

***trans*-3-Phenylpropylcarbamoylpropenoic Acid (2h, HOOCCH=CHCONHCH$_2$CH$_2$CH$_2$Ph).** EtOOCCH=CHCONHCH$_2$CH$_2$CH$_2$Ph was hydrolyzed in EtOH using NaOH (1 M aqueous, 1.1 equiv) under standard deblocking conditions to give a white powder (81% yield): ^{1}H NMR (DMSO-d_6) δ 1.68−1.76 (m, 2H, NH-CH$_2$-CH_2-CH$_2$-Ph), 2.49−2.59 (m, 2H, NH-CH$_2$−CH$_2$-CH_2-Ph), 3.12−3.17 (m, 2H, NH-CH_2-CH$_2$-CH$_2$-Ph), 6.49 (d, 1H, J = 15.6 Hz, CH=CHCON), 6.92 (d, 1H, J = 15.6 Hz, CH=CHCON), 7.15−7.28 (m, 5H, Ph), 8.51 (t, 1H, J = 5.5 Hz, NH).

***trans*-3-(3,4-Dimethoxybenzylcarbamoyl)propenoic Acid Ethyl Ester (EtOOCCH=CH−CONHCH$_2$Ph-3,4-OCH$_3$)** was obtained by mixed anhydride coupling of equimolar amounts of monoethyl fumarate and 3,4-dimethoxyphenylmethylamine. Purification by column chromatography using 5% MeOH/CH_2Cl_2 gave a white powder (69% yield): ^{1}H NMR (DMSO-d_6) δ 1.22 (t, 3H, CH_3), 3.70−3.72 (d, 6H, 2 × OCH_3), 4.16 (q, 2H, CH_2CH_3), 4.29 (d, 2H, NHCH_2), 6.57−6.61 (d, 1H, J = 15.6 Hz, CH=CHCON), 6.76−6.79 (d, 1H, Ph), 6.87−6.89 (d, 2H, Ph), 7.04 (d, 1H, J = 15.6 Hz, CH=CHCON), 8.92 (t, 1H, NH).

***trans*-3-(3,4-Dimethoxybenzylcarbamoyl)propenoic Acid (2i, HOOCCH=CH−CONHCH$_2$Ph-3,4-OCH$_3$).** EtOOCCH=CH−CONHCH$_2$Ph-3,4-OCH_3 was hydrolyzed in EtOH using NaOH (1 M aqueous, 1.1 equiv) under standard deblocking conditions to give a white powder (72% yield).

***trans*-3-(3,4-Dihydro-2*H*-quinolin-1-ylcarbonyl)propenoic Acid Ethyl Ester (EtOOCCH=CHCO-tetrahydroquinoline)** was obtained by a mixed anhydride coupling of equimolar amounts of monoethyl fumarate and 1,2,3,4-tetrahydroquinoline to give a brown syrup (83% yield): ^{1}H NMR ($CDCl_3$) δ 1.29 (t, 3H, CH_3CH$_2$OC), 1.99−2.02 (m, 2H, N-CH$_2$-CH_2-CH$_2$), 2.73−2.76 (t, 1H, N-CH$_2$-CH$_2$-CH_2), 3.86−3.98 (t, 1H, N-CH_2-CH$_2$-CH$_2$), 4.24−4.30 (q, 2H, CH_3CH_2OOC), 6.80 (d, 1H, J = 14.8 Hz, CH=CHCON), 7.18−7.22 (m, 4H, quinoline), 7.46 (d, 1H, J = 14.8 Hz, CH=CHCON).

***trans*-3-(3,4-Dihydro-2*H*-quinolin-1-ylcarbonyl)propenoic Acid (2j, HOOCCH=CHCO-tetrahydroquinoline).** EtOOCCH=CHCO-tetrahydroquinoline was hydrolyzed in EtOH using NaOH (1 M aqueous, 1.1 equiv) under standard deblocking conditions to give a clear syrup, which was recrystallized using cold EtOAc to give a yellow powder (78% yield): ^{1}H NMR (DMSO-d_6) δ 1.84−1.91 (m, 2H, N-CH$_2$-CH_2-CH$_2$), 2.68−2.71 (t, 1H, N-CH$_2$-CH$_2$-CH_2), 3.71−3.75 (t, 1H, N-CH_2-CH$_2$-CH$_2$), 6.58 (d, 1H, J = 15.6 Hz, CH=CHCON), 7.06−7.24 (m, 5H, quinoline and CH=CHCON).

***trans*-3-(3,4-Dihydro-2*H*-quinolin-1-ylcarbonyl)propenoic Acid Ethyl Ester (EtOOCCH=CHCO-tetrahydroisoquinoline)** was obtained by mixed anhydride coupling of equimolar amounts of monoethyl fumarate and 1,2,3,4-tetrahydroisoquinoline to give a brown syrup (83% yield): ^{1}H NMR ($CDCl_3$) δ 1.29 (t, 3H, CH_3CH$_2$OC), 1.99−2.02 (m, 2H, N-CH$_2$-CH_2-CH$_2$), 2.73−2.76 (t, 1H, N-CH$_2$-CH$_2$-CH_2), 3.86−3.98 (t, 1H, N-CH_2-CH$_2$-CH$_2$), 4.24−4.30 (q, 2H, CH_3CH_2OOC), 6.80 (d, 1H, J = 14.8 Hz, CH=CHCON), 7.20 (m, 4H, quinoline), 7.46 (d, 1H, J = 14.8 Hz, CH=CHCON).

***trans*-3-(3,4-Dihydro-2*H*-quinolin-1-ylcarbonyl)propenoic Acid (2k, HOOCCH=CHCO-tetrahydroisoquinoline).** EtOOCCH=CHCO-tetrahydroquinoline was hydrolyzed in EtOH using NaOH (1 M aqueous, 1.1 equiv) under standard deblocking conditions to give a clear syrup, which was recrystallized using cold EtOAc to give a yellow powder (68% yield): ^{1}H NMR (DMSO-d_6) δ 1.2.78−2.87 (m, 2H, $CH_2NCH_2CH_2$), 3.71−3.78 (m, 2H, $CH_2NCH_2CH_2$), 4.66−4.76 (d, 2H, CH_2-NCH$_2$CH$_2$CH$_2$), 6.52 (d, 1H, J = 15.6 Hz, CH=CHCON), 7.16−7.21 (m, 4H, quinoline), 7.43−7.49 (d, 1H, J = 15.6 Hz, CH=CHCON).

***trans*-3-Dibenzylcarbamoylpropenoic Acid Ethyl Ester (EtOOCCH=CHCON(Bzl)$_2$)** was obtained by mixed anhydride coupling of equimolar amounts of monoethyl fumarate and dibenzylamine to give a clear, pink syrup (87% yield).

***trans*-3-Dibenzylcarbamoylpropenoic Acid (2l, HOOCCH=CHCON(Bzl)$_2$).** EtOOCCH=CHCON(Bzl)$_2$ was hydrolyzed in EtOH using NaOH (1 M aqueous, 1.1 equiv) under standard deblocking conditions to give a white powder (91% yield): ^{1}H NMR (DMSO-d_6) δ 4.57 (s, 2H, NCH_2Ph), 4.65 (s, 2H, NCH_2Ph), 6.61−6.65 (d, 1H, J = 15.2 Hz, CH=CHCON), 7.15−7.17 (d, 1H, J = 15.2 Hz, CH=CHCON), 7.25−7.50 (m, 10H, 2 × Ph).

***trans*-3-(1-Naphthylmethylcarbamoyl)propenoic Acid Ethyl Ester (EtOOCCH=CH−CONHCH$_2$-1-Napth)** was obtained by mixed anhydride coupling of equimolar amounts of monoethyl fumarate and 1-naphthylmethylamine. Purification by column chromatography using 2:1 hexane/EtOAc to give white powder (86% yield): ^{1}H NMR ($CDCl_3$) δ 1.20 (t, 3H, CH_3), 3.99 (q, 2H, CH_2), 4.93 (d, 2H, CH_2-naphth), 6.84 (d, 1H, CH=CHCON), 6.90 (d, 1H, CH=CHCON), 7.38−7.44 (m, 2H, naphthyl), 7.47−7.54 (m, 2H, naphthyl), 7.79 (d, 1H, naphthyl), 7.84 (d, 1H, naphthyl), 7.95 (d, 1H, naphthyl).

***trans*-3-(1-Naphthylmethylcarbamoyl)propenoic Acid (2m, HOOCCH=CH−CONHCH$_2$-1-Napth).** EtOOCCH=CHCO-NHCH$_2$-1-Napth was hydrolyzed in EtOH using NaOH (1 M aqueous, 1.1 equiv) under standard deblocking conditions to give a white solid (76% yield): ^{1}H NMR (DMSO-d_6) δ 4.82 (d, 2H, J = 5.5 Hz, CH_2-naphthyl), 6.58 (d, 1H, J = 15.6 Hz, CH=CHCON), 6.93 (d, 1H, J = 15.6 Hz, CH=CHCON), 7.40−7.57 (m, 4H, naphthyl), 7.84−7.86 (m, 1H, naphthyl), 7.94 (d, 1H, J = 7.7 Hz, naphthyl), 8.03 (d, 1H, J = 8.2 Hz, naphthyl), 9.00 (t, 1H, J = 5.4 Hz, NH).

***trans*-3-(*N*-Methyl-1-naphthylmethylcarbamoyl)propenoic Acid Ethyl Ester (EtOOCCH=CHCON(CH_3)CH_2-1-Naphthyl)** was obtained by mixed anhydride coupling of equimolar amounts of monoethyl fumarate and *N*-methyl-1-naphthylmethylamine hydrochloride. Purification by column chromatography using 1:1 EtOAc/hexane gave a white powder (62% yield): ^{1}H NMR ($CDCl_3$) δ 1.29 (t, 3H, CH_3), 3.01 (s, 3H, CH_3), 5.11 (s, 2H, CH_2), 6.89 (d, 1H, J = 15.2 Hz, *CH*=CHCON), 7.40−7.52 (m, 5H, CH=*CH*CON and naphthyl), 7.78−7.89 (m, 3H, naphthyl).

***trans*-3-(*N*-Methyl-1-naphthylmethylcarbamoyl)propenoic Acid (2n, HOOCCH=CHCON(CH_3)CH_2-1-Naphthyl).** EtOOCCH=CHCON(CH_3)CH_2-1-Naphthyl was hydrolyzed in EtOH using NaOH (1 M aqueous, 1.1 equiv) under standard deblocking conditions to give a white solid (13% yield): ^{1}H NMR (DMSO-d_6) δ 3.01 (s, 3H, CH_3), 5.01 (s, 2H, CH_2), 6.63 (d, 1H, J = 15.2 Hz, *CH*=CHCON), 7.19 (d, 1H, J = 15.2 Hz, CH=*CH*CON), 7.37−7.60 (m, 4H, naphthyl), 7.85−8.01 (m, 3H, naphthyl).

***trans*-3-(*N*-Benzyl-1-naphthylmethylcarbamoyl)propenoic Acid Ethyl Ester (EtOOCCH=CHCON(Bzl)-CH_2-1-Naphthyl)** was obtained by mixed anhydride coupling of equimolar amounts of monoethyl fumarate and *N*-benzyl-1-naphthylmethylamine. Purification by column chromatography using 1:1 EtOAc/hexane gave a yellow oil (89% yield): ^{1}H NMR ($CDCl_3$) δ 1.31 (t, 3H, CH_3CH_2), 4.23 (q, 2H, CH_3CH_2), 4.55−4.69 (d, 2H, N−CH_2), 4.71−4.81 (d, 2H, N−CH_2), 6.93 (d, 1H, J = 14.4 Hz, *CH*=CHCON), 7.17 (d, 1H, J = 14.4 Hz, CH=*CH*CON), 7.23−7.57 (m, 10H, naphthyl and Ph), 7.85 (m, 2H, naphthyl).

***trans*-3-(*N*-Benzyl-1-naphthylmethylcarbamoyl)propenoic Acid (2o, HOOCCH=CHCON(Bzl)-CH_2-1-Naphthyl).** EtOOCCH=CHCON(Bzl)-1-CH_2-Napth was hydrolyzed in EtOH using NaOH (1 M aqueous, 1.1 equiv) under standard deblocking conditions to give a white solid after recrystallization from cold EtOAc (77% yield): ^{1}H NMR (DMSO-d_6) δ 4.82 (d, 2H, N−CH_2), 5.01 (d, 2H, N−CH_2), 6.63 (d, 1H, J = 15.2 Hz, *CH*=CHCON), 7.19 (d, 1H, J = 15.2 Hz, CH=*CH*CON), 7.37−7.60 (m, 9H, naphthyl and Ph), 7.85−8.01 (m, 3H, naphthyl).

***trans*-3-(Di-1-naphthylmethylcarbamoyl)propenoic Acid Ethyl Ester (EtOOCCH=CHCON(CH_2-1-Naphthyl)$_2$)** was obtained by a mixed anhydride coupling of equimolar amounts of monoethyl fumarate and *N,N*-di(1-naphthylmethyl)amine. Purification by column chromatography using 2:1 hexane/EtOAC gave white solid (40% yield): ^{1}H NMR (DMSO-d_6) δ 1.10 (t, 3H, J = 7.2 Hz, CH_3), 4.04 (q, 2H, J = 7.1 Hz, CH_2), 5.22 (s, 4H, N-1-*CH*$_2$-Napth), 6.75 (d, 1H, J = 15.2 Hz, *CH*=CHCON), 7.19 (d, 1H, J = 15.2 Hz, CH=*CH*CON), 7.17−8.11 (m, 14H, N(1-CH_2-*Napth*)$_2$).

***trans*-3-(Di-1-naphthylmethylcarbamoyl)propenoic Acid (2p, HOOCCH=CHCON(CH_2-1-Naphthyl)$_2$).** EtOOCCH=CHCON(CH_2-1-Napth)$_2$) was hydrolyzed in EtOH using NaOH (1 M aqueous, 1.1 equiv) under standard deblocking conditions to give a white solid (69% yield): ^{1}H NMR (DMSO-d_6) δ 5.21 (s, 4H, N-1-*CH*$_2$-Napth), 6.61 (d, 1H, J = 15.2 Hz, *CH*=CHCON), 6.70 (d, 1H, J = 15.2 Hz, CH=*CH*CON), 7.19−8.10 (m, 14H, N(1-CH_2-*Napth*)$_2$).

(2*S*,3*S*)-Oxirane-2,3-dicarboxylic Acid Monoethyl Esters (4q, Monoethyl Epoxysuccinates, HOOC-EP-COOEt). The diethyl *trans*-epoxysuccinates were synthesized stereoselectively by a procedure adapted from one described previously by Mori and Iwasawa.[41] One ethyl ester functional group was hydrolyzed using 1 M KOH (1 equiv) in EtOH at 0 °C: ^{1}H NMR (DMSO-d_6) δ 1.19 (t, 3H, OCH_2CH_3), 3.55 (d, 1H, epoxy CH), 3.64 (d, 1H, epoxy CH), 4.15 (q, 2H, OCH_2CH_3).

***cis*-Oxirane-2,3-dicarboxylic Acid Monoethyl Ester (Monoethyl Epoxysuccinate, HOOC-EP-COOEt).** The diethyl ester *cis*-epoxysuccinate was synthesized using the procedure described by Meth-Cohn.[42] One ethyl ester functional group was selectively hydrolyzed as described by Rich and Schaschke:[43,44] ^{1}H NMR ($CDCl_3$) δ 1.27−1.36 (m, 3H, OCH_2CH_3), 3.75−3.81 (m, 2H, epoxy CH), 4.25−4.37 (m, 2H, OCH_2CH_3).

General Procedure for Coupling of the Mono Ethyl Ester Epoxysuccinates to Amines. The method used was the mixed anhydride coupling method. The monoethyl epoxysuccinate (1 equiv) was dissolved in CH_2Cl_2 and cooled to −20 °C. To the reaction mixture were added NMM (3 equiv) and then iBCF (3 equiv). The reaction mixture was stirred at −20 °C for 15−20 min, and then the amine (3 equiv) was added. The reaction mixture was then stirred at −20 °C for 1 h and then at room temperature overnight. The solvent was removed, and the crude product was dissolved in EtOAc. The organic layer was then washed with 2% citric acid, saturated $NaHCO_3$, and brine. The product was purified with column chromatography as needed. Hydrolysis of the ethyl ester with 1 M NaOH (1.5 equiv) in EtOH gave the desired amides.

(2*S*,3*S*)-2-Carboxylic acid-3-(dibenzylcarbamoyl)oxirane (4r, HOOC-EP-CON(Bzl)$_2$) was obtained by mixed anhydride coupling of the monoethyl epoxysuccinate and dibenzylamine to give a clear oil and which was then hydrolyzed under basic conditions to the corresponding acid: ^{1}H NMR (DMSO-d_6) δ 3.51 (d, 1H, epoxy CH), 4.03 (d, 1H, epoxy CH), 4.50 (dd, 2H, NCH_2Ph), 4.70 (dd, 2H, NCH_2Ph), 7.18−7.36 (m, 10H, 2 × Ph).

(2*S*,3*S*)-2-Carboxylic acid-3-(1-naphthylmethylcarbamoyl)-oxirane (4s, HOOC-EP-CONH−CH_2-1-Naphthyl) was obtained by a mixed anhydride coupling of the monoethyl epoxysuccinate and 1-naphthylmethylamine to give a yellow powder, which was then hydrolyzed under basic conditions to the corresponding acid: ^{1}H NMR (acetone-d_6) δ 3.59 (d, 1H, epoxy CH), 3.82 (d, 1H, epoxy CH), 5.21−5.36 (m, 4H, N(1-CH_2-Napth)$_2$), 7.37−7.56 (m, 8H, N(1-CH_2-*naphthyl*)$_2$), 7.84−7.96 (m, 5H, N(1-CH_2-*naphthyl*)$_2$), 8.15−8.18 (m, 1H, N(1-CH_2-*naphthyl*)$_2$).

***cis*-2-Carboxylic acid-3-(dibenzylcarbamoyl)oxirane (6t, HO-OC-EP-CON(Bzl)$_2$)** was obtained by a mixed anhydride coupling of the monoethyl epoxysuccinate and dibenzylamine to give a clear oil, which was then hydrolyzed under basic conditions to the corresponding acid: ^{1}H NMR (DMSO-d_6) δ 3.79 (d, 1H, epoxy CH), 4.13 (d, 1H, epoxy CH), 4.23−4.63 (dd, 4H, NCH_2Ph), 7.16 (d, 2H, Ph), 7.24−7.37 (m, 8H, Ph).

General Procedure for the Synthesis of Aza-Peptide Michael Acceptors and Aza-Peptide Epoxides by the HOBt/EDC Coupling Method. To a stirred solution of the fumaric acid or epoxide precursor (1.5 equiv) in DMF at −10 °C were added HOBt (1.5 equiv), the peptidyl hydrazide precursor (1 equiv), and EDC (1.5 equiv). The mixture was allowed to react for 16 h at room temperature. The DMF was evaporated, and the residue was redissolved in EtOAc. The organic layer was washed with 2% citric acid, saturated $NaHCO_3$, and saturated NaCl, dried over $MgSO_4$, and concentrated. Column chromatography on silica gel afforded the aza-peptide Michael acceptor and aza-peptide epoxide derivatives.

***N*^{2}-*tert*-Butoxycarbonyl-*N*^{1}-carbamoylmethyl-*N*^{1}-*trans*-(3-ethoxycarbonylpropenoyl)hydrazine (23a, Boc-AAsn-CH=CH−COOEt).** This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (47% yield): ^{1}H NMR (DMSO-d_6) δ 1.19 (t, 3H, CH_3), 1.39 (s, 9H, Boc), 3.59 (d, 1H, CH), 4.17 (q, 2H, CH_2), 4.43 (d, 1H, CH), 6.59 (d, 1H, *CH*=CHCON), 7.19−7.25 (m, 3H, CH=*CH*CON and NH), 7.43 (s, 1H, NH), 9.93 (s, 1H, NH); MS (FAB) m/z 316.1 [(M + 1)$^+$]. Anal. ($C_{13}H_{21}N_3O_6 \cdot 0.15CH_2Cl_2 \cdot 0.2H_2O$) C, H, N.

***N*^{2}-*tert*-Butoxycarbonyl-*N*^{1}-carbamoylmethyl-*N*^{1}-*trans*-(3-cyclohexylcarbamoylpropenoyl)hydrazine (23d, Boc-AAsn-CH=CH-NHCH$_2$-cyclohexyl).** This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (24% yield): ^{1}H NMR (DMSO-d_6) δ 0.84−0.89 (m, 2H, cyclohexyl), 1.12−1.19 (m, 4H, cyclohexyl), 1.39 (s, 9H, Boc),

1.42−1.64 (m, 5H, cyclohexyl), 2.98 (t, 2H, CH_2), 3.57 (s, 1H, CH), 4.38 (s, 1H, CH), 6.89 (d, 1H, *CH*=CHCON), 7.08 (d, 1H, J = 15.2 Hz, CH=*CH*CON), 7.17 (s, 1H, NH), 7.41 (s, 1H, NH), 8.43 (t, 1H, J = 5.5 Hz, NH), 9.88 (s, 1H, NH); MS (FAB) m/z 383.2 [$(M + 1)^+$]. Anal. ($C_{18}H_{30}N_4O_5 \cdot 0.15H_2O$) C, H, N.

***N*²-*tert*-Butoxycarbonyl-*N*¹-carbamoylmethyl-*N*¹-*trans*-(3-benzylcarbamoylpropenoyl)hydrazine (23f, Boc-AAsn-CH=CH−CONHBzl).** This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (44% yield): ^{1}H NMR (DMSO-d_6) δ 0.84−0.89 (m, 2H, cyclohexyl), 1.12−1.19 (m, 4H, cyclohexyl), 1.39 (s, 9H, Boc), 3.59 (s, 1H, CH), 4.35−4.41 (m, 3H, CH and CH_2), 6.92−6.96 (d, 1H, J = 15.2 Hz, *CH*=CHCON), 7.14−7.33 (m, 7H, Ph and CH=*CH*CON and NH), 7.41 (s, 1H, NH), 8.98 (t, 1H, J = 5.4 Hz, NH), 9.89 (s, 1H, NH); MS (FAB) m/z 377.2 [$(M + 1)^+$]. Anal. ($C_{18}H_{24}N_4O_5$) C, H, N.

***N*²-*tert*-Butoxycarbonyl-*N*¹-carbamoylmethyl-*N*¹-*trans*-(3-phenylpropylcarbamoylpropenoyl)hydrazine (23h, Boc-AAsn-CH=CH−CONHCH₂CH₂CH₂Ph).** This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (53% yield): ^{1}H NMR (DMSO-d_6) δ 1.39 (s, 9H, Boc), 1.67−1.74 (m, 2H, NHCH$_2$*CH*$_2$CH$_2$Ph), 2.55−2.58 (t, 2H, NHCH$_2$CH$_2$-*CH*$_2$Ph), 3.11−3.16 (m, 2H, NH*CH*$_2$CH$_2$CH$_2$Ph), 3.59 (s, 1H, CH), 4.41 (s, 1H, NH), 6.87−6.91 (d, 1H, J = 15.2 Hz, *CH*=CHCON), 7.08−7.28 (m, 7H, Ph and CH=*CH*CON and NH), 7.41 (s, 1H, NH), 8.51 (t, 1H, J = 5.5 Hz, NH), 9.89 (s, 1H, NH); MS (FAB) m/z 405.3 [$(M + 1)^+$]. Anal. ($C_{20}H_{28}N_4O_5 \cdot$ 1.2hexane) C, H, N.

***N*²-(*N*-Piperidinylcarbonylalanyl)-*N*¹-carbamoylmethyl-*N*¹-*trans*-(3-(3,4-dihydro-1*H*-isoquinolin-2-ylcarbonyl)propenoyl)hydrazine (24k, Pip-Ala-AAsn-CH=CHCO-tetrahydroisoquinoline).** This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (41% yield): ^{1}H NMR (DMSO-d_6) δ 1.23 (d, 3H, CH_3), 1.37 (s, 4H, piperidine), 1.48 (2H, piperidine), 1.74−1.77 (m, 2H, N−CH$_2$-*CH*$_2$-CH$_2$), 2.79−2.87 (m, 2H, N−CH$_2$-CH$_2$-*CH*$_2$), 3.23 (m, 4H, piperidine), 3.60 (s, 2H, NCH$_2$CO), 3.72−3.75 (m, 2H, N-*CH*$_2$-CH$_2$), 4.12 (m, 1H, α-H), 6.55 (d, 1H, NH), 7.06−7.37 (m, 7H, isoquinoline and *CH*=CHCON and NH and CH=*CH*CON), 7.57 (s, 1H, NH), 10.53 (s, 1H, NH); HRMS (FAB) calcd for $C_{22}H_{30}N_5O_8$ 485.2507, observed m/z 485.2579. Anal. ($C_{24}H_{32}N_6O_5 \cdot 1.3H_2O$) C, H, N.

***N*²-(*N*-Morpholinylcarbonylalanyl)-*N*¹-carbamoylmethyl-N¹-*trans*-(3-cyclopropylcarbamoylpropenoyl)hydrazine (25c, Mu-Ala-AAsn-CH=CH−CONH-cyclopropyl).** This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (67% yield): ^{1}H NMR (DMSO-d_6) δ 0.40−0.43 (m, 2H, CH_2), 0.63−0.68 (m, 2H, CH_2), 1.24 (d, 3H, CH_3), 2.69−2.73 (m, 1H, CH), 3.28−3.29 (m, 4H, morpholine), 3.33 (s, 2H, N*CH*$_2$CO), 3.51−3.53 (m, 4H, morpholine), 4.11−4.18 (m, 1H, α-H), 6.71−6.77 (m, 2H, NH and *CH*=CHCON), 7.03−7.07 (d, 1H, CH=*CH*CON), 7.17 (s, 1H, NH), 7.53(s, 1H, NH), 8.47 (d, 1H, NH), 10.58 (s, 1H, NH); HRMS (FAB) calcd for $C_{17}H_{27}N_6O_6$ 411.1987, observed m/z 411.1981. Anal. ($C_{17}H_{26}N_6O_6 \cdot 1H_2O$) C, H, N.

***N*²-(*N*-Morpholinocarbonylalanyl)-*N*¹-carbamoylmethyl-*N*¹-*trans*-(3-(2-furylmethyl)carbamoylpropenoyl)hydrazine (25e, Mu-Ala-AAsn-CH=CH−CONHCH₂-2-furyl).** This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a yellow powder (48% yield): ^{1}H NMR (DMSO-d_6) δ 1.24 (d, 3H, CH_3), 3.27−3.28 (m, 4H, morpholine), 3.34 (s, 2H, N*CH*$_2$CO), 3.50−3.52 (m, 4H, morpholine), 4.11−4.18 (m, 1H, α-H), 4.34 (d, 2H, NH*CH*$_2$), 6.24−6.25 (d, 1H, furyl), 6.37−6.38 (d, 1H, furyl), 6.71 (d, 1H, NH), 6.84−6.88 (d, 1H, *CH*=CHCON), 7.09−7.13 (d, 1H, CH=*CH*CON), 7.18 (s, 1H, NH), 7.54−7.56 (d, 2H, NH and furyl), 8.86 (t, 1H, NH), 10.59 (s, 1H, NH); HRMS (FAB) calcd for $C_{19}H_{27}N_6O_7$ 451.1936, observed m/z 451.2037. Anal. ($C_{19}H_{26}N_6O_7 \cdot 1.3H_2O$) C, H, N.

***N*²-(*N*-Morpholinocarbonylalanyl)-*N*¹-carbamoylmethyl-*N*¹-*trans*-(3-(3,4-dimethoxybenzyl)carbamoylpropenoyl)hydrazine (25i, Mu-Ala-AAsn-CH=CH−CONHCH₂Ph-3,4-OCH₃).** This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (14% yield): ^{1}H NMR (DMSO-d_6) δ 1.24 (d, 3H, CH_3), 3.25−3.26 (m, 4H, morpholine), 3.32 (s, 2H, N*CH*$_2$CO), 3.49−3.51 (m, 4H, morpholine), 3.70−3.71 (d, 6H, 2 × OCH_3), 4.11−4.18 (m, 1H, α-H), 4.28 (d, 2H, NH*CH*$_2$), 6.70−6.72 (d, 1H, NH), 6.74−6.76 (d, 1H, *CH*=CHCON), 6.86−6.91 (m, 3H, Ph and CH=*CH*CON), 7.10−7.18 (m, 2H, Ph and NH), 7.53 (s, 1H, NH), 8.82 (t, 1H, NH), 10.59 (s, 1H, NH); HRMS (FAB) calcd for $C_{23}H_{33}N_6O_8$ 521.2354, observed m/z 521.2431. Anal. ($C_{23}H_{32}N_6O_8 \cdot 0.1$EtOAc$\cdot 1H_2O$) C, H, N.

***N*²-(*N*-Morpholinocarbonylalanyl)-*N*¹-carbamoylmethyl-*N*¹-*trans*-(3-phenylpropylcarbamoylpropenoyl)hydrazine (25h, Mu-Ala-AAsn-CH=CH−CONHCH₂CH₂CH₂Ph).** This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (41% yield): ^{1}H NMR (DMSO-d_6) δ 1.24 (d, 3H, CH_3), 1.67−1.75 (m, 2H, NHCH$_2$*CH*$_2$CH$_2$Ph), 2.55−2.59 (t, 2H, NHCH$_2$CH$_2$*CH*$_2$Ph), 3.09−3.18 (m, 2H, NH*CH*$_2$CH$_2$CH$_2$Ph), 3.26−3.28 (m, 4H, morpholine), 3.33 (s, 2H, N*CH*$_2$CO), 3.50−3.52 (m, 4H, morpholine), 4.11−4.18 (m, 1H, α-H), 6.71 (d, 1H, NH), 6.84−6.88 (d, 1H, *CH*=CHCON), 7.09−7.14 (d, 1H, CH=*CH*CON), 7.15−7.28 (m, 6H, Ph and NH), 7.53 (s, 1H, NH), 8.45 (t, 1H, NH), 10.58 (s, 1H, NH); HRMS (FAB) calcd for $C_{23}H_{33}N_6O_6$ 489.2462, observed m/z 489.2486. Anal. ($C_{23}H_{32}N_6O_6 \cdot 0.5H_2O$) C, H, N.

***N*²-(*N*-Morpholinocarbonylalanyl)-*N*¹-carbamoylmethyl-*N*¹-*trans*-(3-(3,4-dihydro-1*H*-isoquinolin-2-ylcarbonyl)propenoyl)-hydrazine (25k, Mu-Ala-AAsn-CH=CHCO-tetrahydroisoquinoline).** This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (27% yield): ^{1}H NMR (DMSO-d_6) δ 1.23 (d, 3H, J = 7.1 Hz, Ala-CH_3), 2.78−2.86 (m, 2H, N−CH$_2$-*CH*$_2$-CH$_2$), 3.22−3.34 (m, 6H, morpholine and N*CH*$_2$CO), 3.47−3.58 (m, 6H, N−CH$_2$-CH$_2$-*CH*$_2$ and morpholine), 3.71−3.73 (m, 2H, N-*CH*$_2$-CH$_2$), 4.11−4.15 (m, 1H, α-H), 6.69 (d, 1H, J = 6.6 Hz, NH), 7.05−7.39 (m, 7H, *CH*=CHCON and CH=*CH*CON and isoquinoline and NH), 7.55 (s, 1H, NH), 10.58 (s, 1H, NH); HRMS (FAB) calcd for $C_{23}H_{31}N_6O_6$ 485.2507, observed m/z 485.2579. Anal. ($C_{23}H_{30}N_6O_6 \cdot 1.4H_2O$) C, H, N.

***N*²-(4-(Benzyloxycarbonyl)piperazin-1-ylcarbonylalanyl)-*N*¹-carbamoylmethyl-*N*¹-*trans*-(3-(3,4-dihydro-2*H*-quinolin-1-ylcarbonyl)-propenoyl)hydrazine (26j, Cbz-Piz-Ala-AAsn-CH=CHCO-tetrahydroquinoline).** This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a yellow powder (29% yield): ^{1}H NMR (DMSO-d_6) δ 1.27 (d, 3H, J = 7.0 Hz, Ala-CH_3), 1.84−1.89 (m, 2H, N-CH$_2$-*CH*$_2$-CH$_2$), 2.67−2.71 (m, 2H, N−CH$_2$-CH$_2$-*CH*$_2$), 3.30−3.33 (m, 10H, piperazine and N*CH*$_2$CO), 3.70−3.76 (m, 2H, N-*CH*$_2$-CH$_2$), 4.13−4.20 (m, 1H, α-H), 5.07 (s, 2H, Cbz), 6.79 (d, 1H, J = 6.6 Hz, NH), 7.05−7.37 (m, 12H, Ph, quinoline, NH, *CH*=CHCON and CH=*CH*CON), 7.53 (s, 1H, NH), 10.60 (s, 1H, NH); HRMS (FAB) calcd for $C_{31}H_{38}N_7O_7$ 620.2827, observed m/z 620.2891. Anal. ($C_{31}H_{37}N_7O_7 \cdot 0.9H_2O$) C, H, N.

N^2-(4-(Benzyloxycarbonyl)piperazin-1-ylcarbonylalanyl)-N^1-carbamoylmethyl-N^1-*trans*-(3-(3,4-dihydro-1*H*-isoquinolin-2-ylcarbonyl)propenoyl)hydrazine (26k, Cbz-Piz-Ala-AAsn-CH=CHCO-tetrahydroisoquinoline). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (46% yield): ^{1}H NMR (DMSO-d_6) δ 1.23 (d, 3H, J = 7.0 Hz, CH_3), 2.75−2.87 (m, 2H, N-CH_2-CH_2-CH_2), 3.30−3.33 (m, 10H, piperazine and NCH_2CO), 3.69−3.77 (m, 2H, N-CH_2-CH_2), 4.10−4.15 (m, 1H, α-H), 4.64−4.74 (m, 2H, NCH_2C), 5.07 (s, 2H, Cbz), 6.76 (d, 1H, J = 6.5 Hz, NH), 7.05−7.39 (m, 12H, Ph, isoquinoline, NH, *CH*=CHCON and CH=*CH*CON), 7.55 (s, 1H, NH), 10.57 (s, 1H, NH); HRMS (FAB) calcd for $C_{31}H_{38}N_7O_7$ 620.2827, observed m/z 620.2879. Anal. ($C_{31}H_{37}N_7O_7 \cdot 1H_2O \cdot 0.5$EtOAc) C, H, N.

N^2-(*N*-Piperidinocarbonylalanylalanyl)-N^1-carbamoylmethyl-N^1-*trans*-(3-ethoxycarbonylpropenoyl)hydrazine (27a, Pip-Ala-Ala-AAsn-CH=CH−COOEt). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (35% yield): ^{1}H NMR (DMSO-d_6) δ 1.17−1.25 (m, 9H, 2 × Ala-CH_3 and $COCH_2CH_3$), 1.39 (s, 4H, piperidine), 1.48−1.52 (m, 2H, piperidine), 3.25 (s, 4H, piperidine), 3.32 (s, 2H, NCH_2CO), 4.09−4.19 (m, 3H, α-H and $COCH_2CH_3$), 4.22−4.26 (m, 1H, α-H), 6.35 (d, 1H, J = 6.7 Hz, NH), 6.59 (d, 1H, J = 15.5 Hz, *CH*=CHCON), 7.18−7.20 (m, 2H, CH=*CH*CON and NH), 7.51 (s, 1H, NH), 8.04 (br s, 1H, NH), 10.75 (s, 1H, NH); HRMS (FAB) calcd for $C_{20}H_{33}N_6O_7$ 469.2405, observed m/z 469.2426. Anal. ($C_{20}H_{32}N_6O_7 \cdot 0.9H_2O$) C, H, N.

N^2-(*N*-Piperidinocarbonylalanylalanyl)-N^1-carbamoylmethyl-N^1-*trans*-(3-phenylethylcarbamoylpropenoyl)hydrazine (27g, Pip-Ala-Ala-AAsn-CH=CH−CONHCH$_2$CH$_2$Ph). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (48% yield): ^{1}H NMR (DMSO-d_6) δ 1.19−1.28 (m, 6H, 2 × CH_3), 1.39 (s, 4H, piperidine), 1.50 (m, 2H, piperidine), 2.75 (t, 2H, CH_2), 3.25−3.28 (m, 4H, piperidine), 3.34 (s, 2H, NCH_2CO), 3.35−3.40 (m, 2H, CH_2), 4.13 (m, 1H, α-H), 4.27 (m, 1H, α-H), 6.41 (d, 1H, NH), 6.83−6.87 (d, 1H, *CH*=CHCON), 7.03−7.07 (d, 1H, CH=*CH*CON), 7.18−7.31 (m, 6H, Ph and NH), 7.52 (s, 1H, NH), 8.07 (s, 1H, NH), 8.56 (t, 1H, NH), 10.69 (br s, 1H, NH); HRMS (FAB) calcd for $C_{26}H_{38}N_7O_6$ 544.2878, observed m/z 544.3001. Anal. ($C_{26}H_{37}N_7O_6 \cdot 0.1H_2O$) C, H, N.

N^2-(*N*-Piperidinocarbonylalanylalanyl)-N^1-carbamoylmethyl-N^1-*trans*-(3-dibenzylcarbamoylpropenoyl)hydrazine (27l, Pip-Ala-Ala-AAsn-CH=CH−CON(Bzl)$_2$). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (52% yield): ^{1}H NMR (DMSO-d_6) δ 1.18−1.27 (m, 6H, 2 × Ala-CH_3), 1.37 (s, 4H, piperidine), 1.46−1.49 (m, 2H, piperidine), 3.23−3.25 (m, 4H, piperidine), 3.33 (s, 2H, NCH_2CO), 4.11−4.16 (m, 1H, α-H), 4.24−4.30 (m, 1H, α-H), 4.56−4.63 (d, 4H, J = 27.4 Hz, 2 × CH_2Ph), 6.38 (d, 1H, J = 7.2 Hz, NH), 7.14−7.37 (m, 13H, 2 × Ph and *CH*=CHCON and CH=*CH*CON and NH), 7.49 (s, 1H, NH), 8.05 (s, 1H, NH), 10.72 (s, 1H, NH); HRMS (FAB) calcd for $C_{32}H_{42}N_7O_6$ 620.3191, observed m/z 620.3143. Anal. ($C_{32}H_{41}N_7O_6 \cdot 1.1H_2O$) C, H, N.

N^2-(*N*-Piperidinocarbonylalanylalanyl)-N^1-*trans*-(3-benzyl-1-naphthylmethylcarbamoylpropenoyl)-N^1-carbamoylmethylhydrazine (27o, Pip-Ala-Ala-AAsn-CH=CH−CON(Bzl)-CH$_2$-1-naphthyl). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (27% yield): ^{1}H NMR (DMSO-d_6) δ 1.20 (d, 3H, Ala-CH_3), 1.27 (d, 3H, Ala-CH_3), 1.37 (s, 4H, piperidine), 1.48 (m, 2H, piperidine), 3.24 (s, 4H, piperidine), 3.33 (s, 2H, NCH_2CO), 4.13 (m, 1H, α-H), 4.28 (m, 1H, α-H), 4.65−4.67 (m, 2H, CH_2Ph), 5.06−5.17 (m, 2H, CH_2Naph) 6.39 (t, 1H, NH), 7.12−7.56 (m, 13H, Ph and Naph and *CH*=CHCON and CH=*CH*CON and 2 × NH) 7.84−8.05 (m, 4H, Naph and NH), 10.72 (s, 1H, NH); HRMS (FAB) calcd for $C_{36}H_{44}N_7O_6$ 670.3348, observed m/z 670.3374. Anal. ($C_{36}H_{43}N_7O_6 \cdot 1.2H_2O$) C, H, N.

N^2-(*N*-Piperidinocarbonylalanylalanyl)-N^1-carboxymethyl-N^1-*trans*-(3-(di-1-naphthylmethylcarbamoyl)propenoyl)hydrazine (27p, Pip-Ala-Ala-AAsn-CH=CH−CON(CH$_2$-1-naphthyl)$_2$). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (63% yield): ^{1}H NMR (DMSO-d_6) δ 1.20 (d, 3H, J = 6.8 Hz, Ala-CH_3), 1.28 (d, 3H, J = 6.9 Hz, Ala-CH_3), 1.35 (s, 4H, piperidine), 1.42−1.46 (m, 2H, piperidine), 3.23 (s, 4H, piperidine), 3.33 (s, 2H, NCH_2CO), 4.06−4.20 (m, 1H, α-H), 4.22−4.34 (m, 1H, α-H), 5.21 (s, 4H, 2 × N-CH_2-naphthyl), 6.38 (d, 1H, J = 7.6 Hz, NH), 7.13−7.56 (m, 12H, naphthyl and CH=*CH*CON and *CH*=CHCON and 2 × NH), 7.82−8.10 (m, 7H, naphthyl and NH), 10.72 (s, 1H, NH); HRMS (FAB) calcd for $C_{40}H_{46}N_7O_6$ 720.3568, observed m/z 720.350409. Anal. ($C_{40}H_{45}N_7O_6 \cdot 1.3H_2O$) C, H, N.

N^2-(*N*-Morpholinocarbonylalanylalanyl)-N^1-carbamoylmethyl-N^1-*trans*-(3-ethoxycarbonylpropenoyl)hydrazine (28a, Mu-Ala-Ala-AAsn-CH=CH−COOEt). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 15% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (36% yield): ^{1}H NMR (DMSO-d_6) δ 1.17−1.25 (m, 9H, 2 × Ala-CH_3 and $COCH_2CH_3$), 3.24−3.26 (m, 4H, morpholine), 3.32 (s, 2H, NCH_2CO), 3.50−3.52 (m, 4H, morpholine), 4.09−4.18 (m, 3H, α-H and $COCH_2CH_3$), 4.20−4.25 (m, 1H, α-H), 6.50 (d, 1H, J = 7.3 Hz, NH), 6.59 (d, 1H, J = 15.5 Hz, *CH*=CHCON), 7.17−7.20 (m, 2H, CH=*CH*CON and NH), 7.51 (s, 1H, NH), 8.08 (s, 1H, NH), 10.73 (s, 1H, NH); HRMS (FAB) calcd for $C_{19}H_{31}N_6O_8$ 471.2227, observed m/z 471.219789. Anal. ($C_{19}H_{30}N_6O_8 \cdot 1H_2O$) C, H, N.

N^2-(*N*-Morpholinocarbonylalanylalanyl)-N^1-carbamoylmethyl-N^1-*trans*-(3-phenylpropylcarbamoylpropenoyl)hydrazine (28h, Mu-Ala-Ala-AAsn-CH=CH−CONHCH$_2$CH$_2$CH$_2$Ph). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (32% yield): ^{1}H NMR (DMSO-d_6) δ 1.18 (d, 3H, J = 7.2 Hz, Ala-CH_3), 1.25 (d, 3H, J = 7.1 Hz, Ala-CH_3), 1.68−1.75 (m, 2H, NHCH$_2$*CH*$_2$CH$_2$Ph), 2.55−2.59 (t, 2H, NHCH$_2$CH$_2$*CH*$_2$Ph), 3.11−3.16 (m, 2H, NH*CH*$_2$-CH$_2$CH$_2$Ph), 3.25−3.29 (m, 4H, morpholine), 3.35 (s, 2H, NCH_2CO), 3.49−3.51 (m, 4H, morpholine), 4.10−4.17 (m, 1H, α-H), 4.22−4.28 (m, 1H, α-H), 6.53 (d, 1H, J = 7.3 Hz, NH), 6.87 (d, 1H, J = 15.2 Hz, *CH*=CHCON), 7.05 (d, 1H, J = 15.2 Hz, CH=*CH*CON), 7.14−7.28 (m, 6H, Ph and NH), 7.50 (s, 1H, NH), 8.10 (s, 1H, NH), 8.49 (t, 1H, J = 5.5 Hz, NH), 10.67 (s, 1H, NH); HRMS (FAB) calcd for $C_{26}H_{38}N_7O_7$ 560.2834, observed m/z 560.2817. Anal. ($C_{26}H_{37}N_7O_7 \cdot 0.4H_2O$) C, H, N.

N^2-(*N*-Morpholinocarbonylalanylalanyl)-N^1-carbamoylmethyl-N^1-*trans*-(3-(3,4-dihydro-1*H*-isoquinolin-2-ylcarbonyl)propenoyl)-hydrazine (28k, Mu-Ala-Ala-AAsn-CH=CHCO-tetrahydroisoquinoline). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (29% yield): ^{1}H NMR (DMSO-d_6) δ 1.16−1.23 (m, 6H, 2 × Ala-CH_3), 2.79−2.85 (m, 2H, N-CH_2-CH_2-CH_2), 3.25−3.35 (m, 8H, morpholine and NCH_2CO and N-CH_2-CH_2-CH_2), 3.50 (m, 4H, morpholine), 3.72−3.75 (m, 2H, N-CH_2-CH_2), 4.12 (m, 1H, α-H), 4.25 (m, 1H, α-H), 4.65−4.75

(m, 2H, N-CH_2-C), 6.51 (d, 1H, J = 7.2 Hz, NH), 7.04−7.42 (m, 7H, isoquinoline, CH=CHCON, CH=CHCON and NH), 7.53 (s, 1H, NH), 8.08 (s, 1H, NH), 10.68 (s, 1H, NH); HRMS (FAB) calcd for $C_{26}H_{36}N_7O_7$ 558.2676, observed m/z 558.2660. Anal. ($C_{26}H_{35}N_7O_7 \cdot 1.8H_2O$) C, H, N.

N^2-(N-Morpholinocarbonylalanylalanyl)-N^1-carboxymethyl-N^1-*trans*-(3-(di-1-naphthylmethylcarbamoyl)propenoyl)hydrazine (28p, Mu-Ala-Ala-AAsn-CH=CH−CON(CH_2-1-naphthyl)$_2$). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white solid (40% yield): ^{1}H NMR (DMSO-d_6) δ 1.21 (d, 3H, J = 7.1 Hz, Ala-CH_3), 1.29 (d, 3H, J = 7.1 Hz, Ala-CH_3), 3.21−3.26 (m, 4H, morpholine), 3.29 (s, 2H, NCH_2CO), 3.47−3.49 (m, 4H, morpholine), 4.13−4.20 (m, 1H, α-H), 4.25−4.32 (m, 1H, α-H), 5.20 (s, 4H, 2 x N−CH_2-naphthyl), 6.52 (d, 1H, J = 7.4 Hz, NH), 7.12−7.55 (m, 12H, naphthyl and CH=CHCON and CH=CHCON and NH_2), 7.82−8.10 (m, 7H, naphthyl and NH), 10.71 (s, 1H, NH); HRMS (FAB) calcd for $C_{39}H_{44}N_7O_7$ 722.3340, observed m/z 722.329674. Anal. ($C_{39}H_{43}N_7O_7 \cdot 1.6H_2O$) C, H, N.

N^2-(N-Piperazinocarbonylalanylalanyl)-N^1-carbamoylmethyl-N^1-*trans*-(3-ethoxycarbonylpropenoyl)hydrazine (37a, Piz-Ala-Ala-AAsn-CH=CH−COOEt). This compound was obtained by the removal of the *tert*-butyl protecting group of Boc-Piz-Ala-Ala-AAsn-CH=CH−COOEt with trifluoroacetic acid/methylene chloride (1:1) for 3 h at room temperature. The volatiles were evaporated, and the TFA salt was washed several times with diethyl ether to give a white solid (98% yield): ^{1}H NMR (DMSO-d_6) δ 1.18−1.25 (m, 9H, 2 × Ala-CH_3 and $COCH_2CH_3$), 3.04 (s, 4H, piperazine), 3.36−3.50 (m, 6H, piperazine and NCH_2CO), 4.13−4.26 (m, 4H, 2 × α-H and $COCH_2CH_3$), 6.59 (d, 1H, J = 15.4 Hz, CH=CHCON), 6.75 (d, 1H, J = 7.0 Hz, NH), 7.17−7.21 (m, 2H, CH=CHCON and NH), 7.53 (s, 1H, NH), 8.12 (s, 1H, NH), 8.78 (s, 1H, NH), 10.75 (s, 1H, NH); HRMS (FAB) calcd for $C_{19}H_{32}N_7O_7$ 470.2358, observed m/z 470.2386. Anal. ($C_{19}H_{31}N_7O_7 \cdot 1.2TFA \cdot 1.5H_2O$) C, H, N.

N^2-(N-Piperazinocarbonylalanylalanyl)-N^1-*trans*-(3-benzyloxycarbonylpropenoyl)-N^1-carbamoylmethylhydrazine (37b, Piz-Ala-Ala-AAsn-CH=CH−COOBzl). This compound was obtained by the removal of the *tert*-butyl protecting group of Boc-Piz-Ala-Ala-AAsn-CH=CH−COOBzl with trifluoroacetic acid/methylene chloride (1:1) for 3 h at room temperature. The volatiles were evaporated, and the TFA salt was washed several times with diethyl ether to give a white solid (98% yield): ^{1}H NMR (DMSO-d_6) δ 1.18 (s, 6H, 2 × Ala-CH_3), 3.03 (s, 4H, piperazine), 3.37−3.49 (m, 6H, piperazine and NCH_2CO), 4.08−4.23 (m, 1H, α-H), 4.37−4.52 (m, 1H, α-H), 5.19 (s, 2H, OCH_2Ph), 6.64−6.74 (m, 2H, CH=CHCON and NH), 7.20−7.37 (m, 7H, Ph and CH=CHCON and NH), 7.52 (s, 1H, NH), 8.11 (s, 1H, NH), 8.85 (s, 1H, NH), 10.76 (s, 1H, NH); HRMS (FAB) calcd for $C_{24}H_{34}N_7O_7$ 532.2520, observed m/z 532.2505. Anal. ($C_{24}H_{33}N_7O_7 \cdot 1.05TFA \cdot 1.5H_2O$) C, H, N.

N^2-(N-Piperazinocarbonylalanylalanyl)-N^1-carbamoylmethyl-N^1-*trans*-(3-dibenzylcarbamoylpropenoyl)hydrazine (37l, Piz-Ala-Ala-AAsn-CH=CH−CON(Bzl)$_2$). This compound was obtained by the removal of the *tert*-butyl protecting group of Boc-Piz-Ala-Ala-AAsn-CH=CH−CON(Bzl)$_2$ with trifluoroacetic acid/methylene chloride (1:1) for 3 h at room temperature. The volatiles were evaporated, and the TFA salt was washed several times with diethyl ether to give a white solid (99% yield): ^{1}H NMR (DMSO-d_6) δ 1.19−1.27 (m, 6H, 2 × Ala-CH_3), 3.03 (s, 4H, piperazine), 3.49−3.61 (m, 6H, piperazine and NCH_2-CO), 4.11−4.27 (m, 1H, α-H), 4.23−4.30 (m, 1H, α-H), 4.56 (s, 2H, NCH_2Ph), 4.64 (s, 2H, NCH_2Ph), 6.76 (d, 1H, J = 7.4 Hz, NH), 7.14−7.35 (m, 13H, 2 × Ph and CH=CHCON and CH=CHCON and NH), 7.50 (s, 1H, NH), 8.13 (s, 1H, NH), 8.81 (s, 1H, NH), 10.71 (s, 1H, NH); HRMS (FAB) calcd for $C_{31}H_{41}N_8O_6$ 621.3179, observed m/z 621.314358. Anal. ($C_{31}H_{40}N_8O_6 \cdot 1.7TFA \cdot 1.1H_2O$) C, H, N.

N^2-(N-Piperazinocarbonylalanylalanyl)-N^1-carbamoylmethyl-N^1-*trans*-(3-(methyl-1-naphthylmethylcarbamoyl)propenoyl)hydrazine (37n, Piz-Ala-Ala-AAsn-CH=CH−CON(CH_3)CH_2-1-naphthyl). This compound was obtained by the removal of the *tert*-butyl protecting group of Boc-Piz-Ala-Ala-AAsn-CH=CH−CON-(CH_3)CH_2-1-naphthyl with trifluoroacetic acid/methylene chloride (1:1) for 3 h at room temperature. The volatiles were evaporated, and the TFA salt was washed several times with diethyl ether to give a white solid (99% yield): ^{1}H NMR (DMSO-d_6) δ 1.19−1.28 (m, 6H, 2 × Ala-CH_3), 2.98−3.03 (m, 7H, piperazine and N−CH_3), 3.50−3.51 (m, 6H, piperazine and NCH_2CO), 4.13−4.29 (m, 2H, 2 × α-H), 5.04−5.09 (m, 2H, N-CH_2-naphthyl), 5.21 (s, 1H, NH), 6.76 (d, 1H, J = 6.9 Hz, NH), 7.07−7.61 (m, 8H, naphthyl and CH=CHCON and CH=CHCON and 2 × NH), 7.86−8.13 (m, 3H, naphthyl), 8.83 (s, 1H, NH), 10.71 (s, 1H, NH); HRMS (FAB) calcd for $C_{29}H_{39}N_8O_6$ 595.2987, observed m/z 595.2947. Anal. ($C_{29}H_{38}N_8O_6 \cdot 1.4TFA \cdot 2H_2O$) C, H, N.

N^2-(N-Piperazinocarbonylalanylalanyl)-N^1-carboxymethyl-N^1-*trans*-(3-(di-1-naphthylmethylcarbamoyl)propenoyl)hydrazine (37p, Piz-Ala-Ala-AAsn-CH=CH−CON(CH_2-1-naphthyl)$_2$). This compound was obtained by the removal of the *tert*-butyl protecting group of Boc-Piz-Ala-Ala-AAsn-CH=CH−CON-(CH_2-1-naphthyl)$_2$ with trifluoroacetic acid/methylene chloride (1:1) for 3 h at room temperature. The volatiles were evaporated, and the TFA salt was washed several times with diethyl ether to give a white solid (93% yield): ^{1}H NMR (DMSO-d_6) δ 1.21 (d, 3H, Ala-CH_3), 1.29 (d, 3H, Ala-CH_3), 1.37 (s, 9H, Boc), 3.03 (s, 4H, piperazine), 3.50−3.73 (m, 6H, piperazine and NCH_2CO), 4.14−4.19 (m, 1H, α-H), 4.25−4.31 (m, 1H, α-H), 5.21 (s, 4H, 2 × N-CH_2-naphthyl), 6.77 (d, 1H, J = 7.3 Hz, NH), 7.13−7.56 (m, 12H, naphthyl and CH=CHCON and CH=CHCON and 2 × NH), 7.82−8.14 (m, 7H, naphthyl and NH), 8.82 (s, 1H, NH), 10.73 (s, 1H, NH); HRMS (FAB) calcd for $C_{39}H_{45}N_8O_6$ 721.3384, observed m/z 721.3379. Anal. ($C_{39}H_{44}N_8O_6 \cdot 1.6TFA \cdot 1H_2O$) C, H, N.

N^2-(4-(*tert*-Butoxycarbonyl)piperazin-1-yl carbonylalanylalanyl)-N^1-carbamoylmethyl-N^1-*trans*-(3-ethoxycarbonylpropenoyl)-hydrazine (29a, Boc-Piz-Ala-Ala-AAsn-CH=CH−COOEt). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (34% yield): ^{1}H NMR (DMSO-d_6) δ 1.17−1.25 (m, 9H, 2 x Ala-CH_3 and $COCH_2CH_3$), 1.38 (s, 9H, Boc), 3.26 (s, 8H, piperazine), 3.32 (s, 2H, NCH_2CO), 4.08−4.26 (m, 4H, 2 × α-H and $COCH_2CH_3$), 6.54 (d, 1H, J = 7.1 Hz, NH), 6.59 (d, 1H, J = 15.7 Hz, CH=CHCON), 7.17−7.20 (m, 2H, CH=CHCON and NH), 7.51 (s, 1H, NH), 8.08 (s, 1H, NH), 10.72 (s, 1H, NH); HRMS (FAB) calcd for $C_{24}H_{40}N_7O_9$ 570.2882, observed m/z 570.2906. Anal. ($C_{24}H_{39}N_7O_9 \cdot 0.9H_2O$) C, H, N.

N^2-(4-(*tert*-Butoxycarbonyl)piperazin-1-yl carbonylalanylalanyl)-N^1-*trans*-(3-benzyloxycarbonylpropenoyl)-N^1-carbamoylmethylhydrazine (29b, Boc-Piz-Ala-Ala-AAsn-CH=CH−CO-OBzl). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (25% yield): ^{1}H NMR (DMSO-d_6) δ 1.17 (d, 6H, J = 7.1 Hz, 2 × CH_3), 1.38 (s, 9H, Boc), 3.25 (s, 8H, piperazine), 3.33 (s, 2H, NCH_2CO), 4.07−4.13 (m, 1H, α-H), 4.19−4.24 (m, 1H, α-H), 5.19 (s, 2H, O-CH_2-Ph), 6.54 (d, 1H, J = 7.1 Hz, NH), 6.65 (d, 1H, J = 15.3 Hz, CH=CHCON), 7.22−7.39 (m, 7H, Ph and CH=CHCON and NH), 7.52 (s, 1H, NH), 8.09 (s, 1H, NH), 10.45 (s, 1H, NH); HRMS (FAB) calcd for $C_{29}H_{42}N_7O_9$ 632.6771, observed m/z 632.6753. Anal. ($C_{29}H_{41}N_7O_9 \cdot 0.9H_2O$) C, H, N.

N^2-(4-(*tert*-Butoxycarbonyl)piperazin-1-yl carbonylalanylalanyl)-N^1-carbamoylmethyl-N^1-*trans*-(3-dibenzylcarbamoylpropenoyl)-hydrazine (29l, Boc-Piz-Ala-Ala-AAsn-CH=CH−CON(Bzl)$_2$). This

compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (50% yield): ^{1}H NMR (DMSO-d_6) δ 1.19 (d, 3H, J = 7.2 Hz, Ala-CH_3), 1.26 (d, 3H, J = 7.1 Hz, Ala-CH_3), 1.38 (s, 9H, Boc), 3.25 (s, 8H, piperazine), 3.32 (s, 2H, NCH_2CO), 4.11−4.18 (m, 1H, α-H), 4.23−4.30 (m, 1H, α-H), 4.56 (s, 2H, N−CH_2-Ph), 4.63 (s, 2H, N-CH_2-Ph), 6.55 (d, 1H, J = 7.4 Hz, NH), 7.14−7.37 (m, 13H, 2 × Ph and CH=CHCON and CH=CHCON and NH), 7.82−8.10 (m, 7H, naphthyl and NH), 7.48 (s, 1H, NH), 8.09 (s, 1H, NH), 10.69 (s, 1H, NH); HRMS (FAB) calcd for $C_{36}H_{49}N_8O_8$ 721.3668, observed m/z 721.3660. Anal. ($C_{36}H_{48}N_8O_8 \cdot 1.5H_2O$) C, H, N.

N^2-(4-(*tert*-Butoxycarbonyl)piperazin-1-ylcarbonylalanylalanyl)-N^1-carbamoylmethyl-N^1-*trans*-(3-(methyl-1-naphthylmethylcarbamoyl)propenoyl)hydrazine (29n, Boc-Piz-Ala-Ala-AAsn-CH=CH−CON(CH_3)CH_2-1-naphthyl). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (29% yield): ^{1}H NMR (DMSO-d_6) δ 1.18−1.26 (m, 6H, 2 × CH_3), 1.37 (s, 9H, Boc), 2.99 (d, 3H, J = 10.5 Hz, N−CH_3), 3.26 (s, 8H, piperazine), 3.34 (s, 2H, NCH_2CO), 4.10−4.16 (m, 1H, α-H), 4.23−4.29 (m, 1H, α-H), 4.99−5.10 (m, 2H, N-CH_2-naphthyl), 5.21 (s, 1H, NH), 6.58 (d, 1H, J = 7.2 Hz, NH), 7.07−7.61 (m, 8H, naphthyl and CH=CHCON and CH=CHCON and NH_2), 7.85−8.10 (m, 3H, naphthyl), 8.12 (s, 1H, NH), 10.42 (s, 1H, NH), 10.72 (s, 1H, NH); HRMS (FAB) calcd for $C_{34}H_{47}N_8O_8$ 695.3511, observed m/z 695.3559. Anal. ($C_{34}H_{46}N_8O_8 \cdot 1.4H_2O$) C, H, N.

N^2-(4-(*tert*-Butoxycarbonyl)piperazin-1-ylcarbonylalanylalanyl)-N^1-carboxymethyl-N^1-*trans*-(3-di-1-CH_2-naphthylcarbamoylacryloyl)hydrazine (29p, Boc-Piz-Ala-Ala-AAsn-CH=CH−CON(CH_2-1-naphthyl)$_2$). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (25% yield): ^{1}H NMR (DMSO-d_6) δ 1.20 (d, 3H, CH_3), 1.28 (d, 3H, CH_3), 1.37 (s, 9H, Boc), 3.25 (s, 8H, piperazine), 3.33 (s, 2H, NCH_2CO), 4.15 (m, 1H, α-H), 4.25 (m, 1H, α-H), 5.20 (s, 4H, 2 × N-CH_2-naphthyl), 6.57 (d, 1H, NH), 7.11−7.56 (m, 12H, naphthyl and CH=CHCON and CH=CHCON and NH_2), 7.82−8.10 (m, 7H, naphthyl and NH), 10.43 (s, 1H, NH); HRMS (FAB) calcd for $C_{44}H_{53}N_8O_8$ 821.4025, observed m/z 821.3981. Anal. ($C_{44}H_{52}N_8O_8 \cdot 1.1H_2O$) C, H, N.

N^2-(4-(Benzyloxycarbonyl)piperazin-1-ylcarbonylalanylalanyl)-N^1-carbamoylmethyl-N^1-*trans*-(3-ethoxycarbonylpropenoyl)hydrazine (30a, Cbz-Piz-Ala-Ala-AAsn-CH=CH−COOEt). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 15% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (41% yield): ^{1}H NMR (DMSO-d_6) δ 1.16−1.28 (m, 9H, 2 × Ala-CH_3 and $COCH_2CH_3$), 3.31−3.34 (m, 10H, piperazine and NCH_2CO), 4.09−4.18 (m, 3H, α-H and $COCH_2CH_3$), 4.20−4.29 (m, 1H, α-H), 5.07 (s, 2H, Cbz), 6.55−6.61 (m, 2H, NH and CH=CHCON), 7.18−7.38 (m, 7H, Ph and NH and CH=CHCON), 7.50 (s, 1H, NH), 8.09 (d, 1H, J = 5.4 Hz, NH), 10.72 (s, 1H, NH); HRMS (FAB) calcd for $C_{27}H_{38}N_7O_9$ 604.2726, observed m/z 604.2777. Anal. ($C_{27}H_{37}N_7O_9 \cdot 0.9H_2O$) C, H, N.

N^2-(4-(Benzyloxycarbonyl)piperazin-1-ylcarbonylalanylalanyl)-N^1-carbamoylmethyl-N^1-*trans*-(3-(methyl-1-naphthylmethylcarbamoyl)propenoyl)hydrazine (30n, Cbz-Piz-Ala-Ala-AAsn-CH=CH−CON(CH_3)CH_2-1-naphthyl). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (38% yield): ^{1}H NMR (DMSO-d_6) δ 1.15−1.28 (m, 6H, 2 × CH_3), 2.99 (d, 3H, J = 9.0 Hz, N−CH_3), 3.32 (s, 8H, piperazine), 3.34 (s, 2H, NCH_2CO), 4.07−4.20 (m, 1H, α-H), 4.21−4.32 (m, 1H, α-H), 4.97−5.12 (m, 4H, Cbz and N-CH_2-naphthyl), 5.20 (s, 1H, NH), 6.60 (t, 1H, J = 7.7 Hz, NH), 7.07−7.61 (m, 12H, naphthyl and CH=CHCON and CH=CHCON and NH_2), 7.85−8.10 (m, 4H, naphthyl), 10.43 (s, 1H, NH); HRMS (FAB) calcd for $C_{37}H_{45}N_8O_8$ 729.3355, observed m/z 729.3376. Anal. ($C_{37}H_{44}N_8O_8 \cdot 1.3H_2O$) C, H, N.

N^2-(4-(Benzyloxycarbonyl)piperazin-1-ylcarbonylalanylalanyl)-N^1-carboxymethyl-N^1-*trans*-(3-(di-1-naphthylmethylcarbamoyl)-propenoyl)hydrazine (30p, Cbz-Piz-Ala-Ala-AAsn-CH=CH−CON(CH_2-1-naphthyl)$_2$). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (39% yield): ^{1}H NMR (DMSO-d_6) δ 1.20 (d, 3H, Ala-CH_3), 1.28 (d, 3H, Ala-CH_3), 3.31 (m, 8H, piperazine), 3.34 (s, 2H, NCH_2CO), 4.16 (m, 1H, α-H), 4.27 (m, 1H, α-H), 5.06 (s, 2H, Cbz), 5.20 (s, 4H, N-CH_2-Ph and N-CH_2-naphthyl), 6.59 (d, 1H, NH), 7.12−7.54 (m, 17H, naphthyl and Ph and CH=CHCON and CH=CHCON and NH_2), 7.82−8.13 (m, 5H, naphthyl and NH), 10.44 (s, 1H, NH); HRMS (FAB) calcd for $C_{47}H_{51}N_8O_8$ 855.3838, observed m/z 855.382438. Anal. ($C_{47}H_{50}N_8O_8 \cdot 1.2H_2O$) C, H, N.

N^2-(*N*-Benzyloxycarbonylalanylvalyl)-N^1-carbamoylmethyl-N^1-*trans*-(3-ethoxycarbonylpropenoyl)hydrazine (31a, Cbz-Ala-Val-AAsn-CH=CH−COOEt). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (25% yield): ^{1}H NMR (DMSO-d_6) δ 0.84−0.85 (d, 6H, J = 6.2 Hz, 2×Val-CH_3), 1.15−1.21 (m, 6H, Ala-CH_3 and $COCH_2CH_3$), 1.97 (s, 1H, CH), 3.32 (s, 2H, NHCH_2CO), 4.08−4.17 (m, 4H, 2 × α-H and $COCH_2CH_3$), 4.95−5.02 (m, 2H, Cbz), 6.59 (d, 1H, J = 15.6 Hz, CH=CHCON), 7.18−7.44 (m, 7H, Ph and CH=CHCON and NH), 7.50 (s, 1H, NH), 7.92 (s, 1H, NH), 10.90 (s, 1H, NH); HRMS (FAB) calcd for $C_{24}H_{34}N_5O_8$ 520.2456, observed m/z 520.24019. Anal. ($C_{24}H_{33}N_5O_8 \cdot 1.9CH_2Cl_2$) C, H, N.

N^2-(Benzyloxycarbonylalanylvalyl)-N^1-carbamoylmethyl-N^1-*trans*-(3-(1-naphthylmethylcarbamoyl)propenoyl)hydrazine (31m, Cbz-Ala-Val-AAsn-CH=CH−CONHCH_2-1-naphthyl). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (11% yield): ^{1}H NMR (DMSO-d_6) δ 0.85 (s, 6H, 2 × Val-CH_3), 1.17 (d, 3H, J = 6.8 Hz, Ala-CH_3), 2.00 (s, 1H, CH), 3.32 (s, 2H, NHCH_2CO), 4.11−4.22 (m, 2H, 2 × α-H), 4.81 (d, 2H, J = 4.7 Hz, NHCH_2-Naph), 4.99 (s, 2H, Cbz), 6.93 (d, 1H, J = 15.2 Hz, CH=CHCON), 7.13−7.54 (m, 12H, Ph and Naph and CH=CHCON and NH), 7.73−8.02 (m, 3H, Naph and NH), 8.96 (s, 1H, NH), 10.85 (s, 1H, NH); HRMS (FAB) calcd for $C_{33}H_{39}N_6O_7$ 631.2875, observed m/z 631.2885. Anal. ($C_{33}H_{38}N_6O_7 \cdot 1.4H_2O$) C, H, N.

N^2-(*N*-Benzyloxycarbonylalanylvalyl)-N^1-carbamoylmethyl-N^1-*trans*-(3-(3,4-dihydro-2*H*-quinolin-1-ylcarbonyl)propenoyl)hydrazine (31j, Cbz-Ala-Val-AAsn-CH=CH−CO-tetrahydroquinoline). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a yellowish solid (36% yield): ^{1}H NMR (DMSO-d_6) δ 0.86−0.88 (d, 6H, J = 6.4 Hz, 2 × CH_3), 1.79 (d, 3H, J = 7.0 Hz, Ala-CH_3), 1.83−1.89 (m, 2H, N-CH_2-CH_2-CH_2), 2.00 (s, 1H, CH), 2.67−2.70 (t, 2H, J = 6.4 Hz, N-CH_2-CH_2-CH_2), 3.32 (s, 2H, NHCH_2CO), 3.70−3.73 (m, 2H, N-CH_2-CH_2−CH_2), 4.07−4.16 (m, 1H, α-H), 4.21−4.25 (m, 1H, α-H), 4.96−5.03 (m, 2H, Cbz), 7.07−7.44 (m, 13H, Ph, quinoline, CH=CHCON, CH=CHCON and NH), 7.49 (s, 1H, NH), 7.93 (s, 1H, NH), 10.88 (s, 1H, NH); HRMS (FAB) calcd for $C_{31}H_{39}N_6O_7$ 607.2875, observed m/z 607.2910. Anal. ($C_{31}H_{38}N_6O_7 \cdot 0.3H_2O$) C, H, N.

N^2-(*N*-Benzyloxycarbonylalanylisoleucyl)-N^1-carbamoylmethyl-N^1-*trans*-(3-ethoxycarbonylpropenoyl)hydrazine (32a, Cbz-Ala-Ile-AAsn-CH=CH−COOEt). This compound was obtained using

the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white solid (23% yield): ^{1}H NMR (DMSO-d_6) δ 0.79−0.82 (m, 6H, 2 × Ile-CH_3), 1.15−1.24 (m, 7H, CH and Ala-CH_3 and $COCH_2CH_3$), 1.39−1.46 (m, 1H, CH), 1.70−1.76 (m, 1H, CH), 3.32 (s, 2H, $NHCH_2CO$), 4.06−4.24 (m, 4H, 2 x α-H and $COCH_2CH_3$), 4.99 (s, 2H, Cbz), 6.60 (d, 1H, J = 15.5 Hz, CH = CHCON), 7.19−7.36 (m, 7H, Ph and CH=CHCON and NH), 7.49 (s, 1H, NH), 7.94 (s, 1H, NH), 10.90 (s, 1H, NH); HRMS (FAB) calcd for $C_{25}H_{36}N_5O_8$ 534.2558, observed m/z 534.2600. Anal. ($C_{25}H_{35}N_5O_8 \cdot 0.8H_2O$) C, H, N.

N^2-(*N*-Benzyloxycarbonylalanylphenylalanyl)-N^1-carbamoylmethyl-N^1-*trans*-(3-(2-furyl)carbamoylpropenoyl)hydrazine (33e, Cbz-Ala-Phe-AAsn-CH=CH−CONHCH$_2$-2-furyl). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a yellowish solid (7% yield): ^{1}H NMR (DMSO-d_6) δ 1.11 (d, 3H, Ala-CH_3), 2.81−2.98 (m, 2H, CH_2Ph), 3.32 (d, 2H, $NHCH_2CO$), 4.01 (m, 1H, α-H), 4.34 (d, 2H, CH_2-furyl), 4.53 (m, 1H, α-H), 4.96−4.99 (m, 2H, Cbz), 6.24 (d, 1H, furyl CH), 6.36 (t, 1H, furyl CH), 6.87−6.91 (d, 1H, CH=CHCON), 7.09−7.13 (d, 1H, CH=CHCON), 7.18−7.34 (m, 11H, 2 × Ph and NH), 7.43 (s, 1H, NH), 7.55 (d, 1H, furyl CH), 8.19 (s, 1H, NH), 8.91 (t, 1H, NH), 10.89 (s, 1H, NH); HRMS (FAB) calcd for $C_{31}H_{35}N_6O_8$ 619.2511, observed m/z 619.2575. Anal. ($C_{31}H_{34}N_6O_8 \cdot 0.75H_2O$) C, H, N.

N^2-(*N*-Benzyloxycarbonylalanylphenylalanyl)-N^1-carbamoylmethyl-N^1-*trans*-(3-(3,4-dihydro-2*H*-quinolin-1-ylcarbonyl)propenoyl)hydrazine (33j, Cbz-Ala-Phe-AAsn-CH=CH−CO-tetrahydroquinoline). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a yellowish solid (27% yield): ^{1}H NMR (DMSO-d_6) δ 1.12 (d, 3H, J = 6.8 Hz, Ala-CH_3), 1.84−1.89 (m, 2H, N-CH_2-CH_2-CH_2), 2.67−2.70 (t, 2H, J=6.5 Hz, N-CH_2-CH_2-CH_2), 2.87−3.01 (m, 2H, CH_2Ph), 3.32 (s, 2H, NH-CH_2CO), 3.70−3.71 (m, 2H, N-CH_2-CH_2-CH_2), 3.99−4.06 (m, 1H, α-H), 4.55−4.60 (m, 1H, α-H), 4.94−5.02 (m, 2H, Cbz), 7.05−7.34 (m, 17H, 2 × Ph, quinoline, CH=CHCON, CH=CHCON and NH), 7.48 (s, 1H, NH), 8.18 (s, 1H, NH), 10.92 (s, 1H, NH); HRMS (FAB) calcd for $C_{35}H_{39}N_6O_7$ 655.2875, observed m/z 655.2923. Anal. ($C_{35}H_{38}N_6O_7 \cdot 0.4H_2O$) C, H, N.

N^2-(*N*-Benzyloxycarbonylalanylphenylalanyl)-N^1-carbamoylmethyl-N^1-*trans*-(3-(3,4-dihydro-2*H*-isoquinolin-1-ylcarbonyl)propenoyl)hydrazine (33k, Cbz-Ala-Phe-AAsn-CH=CH−CO-tetrahydroisoquinoline). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (11% yield): ^{1}H NMR (DMSO-d_6) δ 1.10 (d, 3H, J = 6.0 Hz, Ala-CH_3), 2.76−2.98 (m, 4H, CH_2Ph and N-CH_2-CH_2-CH_2), 3.35 (s, 2H, $NHCH_2CO$), 3.66−3.76 (m, 2H, N-CH_2-CH_2), 3.98−4.04 (m, 1H, α-H), 4.50−4.58 (m, 1H, α-H), 4.65−4.73 (m, 2H, N-CH_2-C), 4.94−5.02 (m, 2H, Cbz), 7.00−7.33 (m, 12H, Ph, isoquinoline, CH=CHCON, CH=CHCON and NH), 7.51 (s, 1H, NH), 8.18 (s, 1H, NH), 10.89 (s, 1H, NH); HRMS (FAB) calcd for $C_{35}H_{39}N_6O_7$ 655.2875, observed m/z 655.2893. Anal. ($C_{35}H_{38}N_6O_7 \cdot$ 0.75EtOAc) C, H, N.

(2*S*,3*S*)-3-(N^2-(*N*-Piperidinocarbonylalanylalanyl)-N^1-carbamoylmethylhydrazinocarbonyl)oxirane-2-carboxylic Acid Ethyl Ester (34q, Pip-Ala-Ala-AAsn-EP(*S*,*S*)-COOEt). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (3% yield): ^{1}H NMR (DMSO-d_6) δ 1.12−1.20 (m, 9H, 2 × Ala-CH_3 and $COCH_2CH_3$), 1.33−1.44 (m, 4H, piperidine), 1.46−1.50 (m, 2H, piperidine), 3.19−3.29 (m, 4H, piperidine), 3.32 (s, 2H, NCH_2CO), 3.49 (s, 1H, epoxy CH), 3.67 (s, 1H, epoxy CH), 4.05−4.28 (m, 4H, 2 × α-H and $COCH_2CH_3$), 6.41 (d, 1H, J = 6.2 Hz, NH), 7.23 (s, 1H, NH), 7.51 (s, 1H, NH), 8.06 (s, 1H, NH), 10.72 (s, 1H, NH); HRMS (FAB) calcd for $C_{20}H_{33}N_6O_8$ 485.2354, observed m/z 485.2363. ($C_{20}H_{32}N_6O_8 \cdot 1.2H_2O$) C, H, N.

(2*S*,3*S*)-2-(Dibenzylcarbamoyl)-3-(N^2-(*N*-piperidinocarbonylalanylalanyl)-N^1-carbamoylmethylhydrazinocarbonyl)oxirane (34r, Pip-Ala-Ala-AAsn-EP(*S*,*S*)-CON(Bzl)$_2$). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (29% yield): ^{1}H NMR (DMSO-d_6) δ 1.17−1.19 (m, 6H, 2 × Ala-CH_3), 1.34−1.43 (m, 4H, piperidine), 1.47−1.51 (m, 2H, piperidine), 3.22−3.29 (m, 4H, piperidine), 3.32 (s, 2H, NCH_2CO), 3.80 (s, 1H, epoxy CH), 4.05−4.12 (m, 1H, α-H), 4.23−4.74 (m, 6H, α-H and epoxy CH and 2 × N-CH_2-Ph), 6.41 (d, 1H, J = 6.0 Hz, NH), 7.21−7.39 (m, 11H, 2 × Ph and NH) 7.47 (s, 1H, NH) 8.09 (s, 1H, NH), 10.68 (s, 1H, NH); HRMS (FAB) calcd for $C_{32}H_{42}N_7O_7$ 636.3140, observed m/z 636.3129. Anal. ($C_{32}H_{41}N_7O_7 \cdot 1.1H_2O$) C, H, N.

(2*S*,3*S*)-2-(1-Naphthylmethylcarbamoyl)-3-(N^2-(*N*-piperidinocarbonylalanylalanyl)-N^1-carbamoylmethylhydrazinocarbonyl)oxirane (34s, Pip-Ala-Ala-AAsn-EP(*S*,*S*)-CONHCH$_2$-1-naphthyl). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a yellowish solid (47% yield): ^{1}H NMR (DMSO-d_6) δ 1.14−1.20 (m, 6H, 2 × Ala-CH_3), 1.33−1.42 (m, 4H, piperidine), 1.46−1.50 (m, 2H, piperidine), 3.22−3.26 (m, 4H, piperidine), 3.32 (s, 2H, NCH_2CO), 3.52 (s, 1H, epoxy CH), 4.10−4.20 (m, 3H, 2 x α-H and epoxy CH), 4.73−4.78 (m, 2H, N-CH_2-naphthyl), 6.36 (d, 1H, J = 7.2 Hz, NH), 7.21 (s, 1H, NH), 7.43−7.58 (m, 4H, naphthyl), 7.45−7.56 (m, 4H, naphthyl), 7.84−8.03 (m, 4H, naphthyl and NH), 9.00 (s, 1H, NH), 10.77 (s, 1H, NH); HRMS (FAB) calcd for $C_{29}H_{38}N_7O_7$ 596.2827, observed m/z 596.2847. Anal. ($C_{29}H_{37}N_7O_7 \cdot 1.4H_2O$) C, H, N.

(2*S*,3*S*)-2-(1-Naphthylmethylcarbamoyl)-3-(N^2-(*N*-morpholinocarbonylalanylalanyl)-N^1-carbamoylmethylhydrazinocarbonyl)oxirane (35s, Mu-Ala-Ala-AAsn-EP(*S*,*S*)-CONHCH$_2$-1-naphthyl). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (22% yield): ^{1}H NMR (DMSO-d_6) δ 1.15−1.21 (m, 6H, 2 × Ala-CH_3), 3.23−3.29 (m, 4H, morpholine), 3.34 (s, 2H, NCH_2CO), 3.44−3.55 (m, 4H, morpholine), 4.12−4.20 (m, 4H, 2 × α-H and 2 × epoxy CH), 4.70−4.82 (m, 2H, N-CH_2-naphthyl), 6.52 (d, 1H, J = 6.9 Hz, NH), 7.21 (s, 1H, NH), 7.43−7.58 (m, 4H, naphthyl), 7.84−8.10 (m, 4H, naphthyl and NH), 9.00 (s, 1H, NH), 10.77 (s, 1H, NH); HRMS (FAB) calcd for $C_{28}H_{36}N_7O_8$ 598.2620, observed m/z 598.2656. Anal. ($C_{28}H_{35}N_7O_8 \cdot 1.7H_2O$) C, H, N.

(2*S*,3*S*)-2-(Dibenzylcarbamoyl)-3-(N^2-(*N*-morpholinocarbonylalanylalanyl)-N^1-carbamoylmethylhydrazinocarbonyl)oxirane (35r, Mu-Ala-Ala-AAsn-EP(*S*,*S*)-CON(Bzl)$_2$). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (19% yield): ^{1}H NMR (DMSO-d_6) δ 1.13−1.25 (m, 6H, 2 × Ala-CH_3), 3.21−3.27 (m, 4H, morpholine), 3.34 (s, 2H, NCH_2CO), 3.42−3.57 (m, 4H, morpholine), 3.80−4.25 (m, 4H, 2 × α-H, 2 × epoxy CH), 4.55−4.78 (m, 4H, 2 × N-CH_2-Ph), 6.55 (d, 1H, NH), 7.22−7.47 (m, 12H, 2 × Ph and 2 × NH), 8.15 (s, 1H, NH), 10.66 (s, 1H, NH); HRMS (FAB) calcd for $C_{31}H_{40}N_7O_8$ 638.2938, observed m/z 638.2937. Anal. ($C_{31}H_{39}N_7O_8 \cdot 1.4H_2O$) C, H, N.

***cis*-2-(Dibenzylcarbamoyl)-3-(N^2-(*N*-morpholinocarbonylalanylalanyl)-N^1-carbamoylmethylhydrazinocarbonyl)oxirane (35t, Mu-Ala-Ala-AAsn-EP(*cis*)-CON(Bzl)$_2$).** This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder

(13% yield): ^{1}H NMR (DMSO-d_6) δ 1.16−1.25 (m, 6H, 2 × Ala-CH_3), 3.20−3.28 (m, 4H, morpholine), 3.33 (s, 2H, NCH_2CO), 3.46−3.53 (m, 4H, morpholine), 4.04−4.25 (m, 4H, 2 × α-H, 2 × epoxy CH), 4.37−4.73 (m, 4H, 2 × N-CH_2-Ph), 6.53 (d, 1H, J=7.3 Hz, NH), 7.15−7.41 (m, 12H, 2 × Ph and 2 × NH), 8.16 (d, 1H, J= 5.9 Hz, NH), 10.87 (s, 1H, NH); HRMS (FAB) calcd for $C_{31}H_{40}N_7O_8$ 638.2938, observed m/z 638.2943. Anal. ($C_{31}H_{39}N_7O_8 \cdot 1.5H_2O$) C, H, N.

(2*S*,3*S*)-2-(Dibenzylcarbamoyl)-3-(*N*^{2}-(*N*-piperazinocarbonylalanylalanyl)-*N*^{1}-carbamoylmethylhydrazinocarbonyl)oxirane (38r, Piz-Ala-Ala-AAsn-EP(*S*,*S*)-CON(Bzl)$_2$). This compound was obtained by the removal of the *tert*-butyl protecting group of Boc-Piz-Ala-Ala-AAsn-EP(*S*,*S*)-CON(Bzl)$_2$ with trifluoroacetic acid/methylene chloride (1:1) for 3 h at room temperature. The volatiles were evaporated, and the TFA salt was washed several times with diethyl ether to give a white solid (91% yield): ^{1}H NMR (DMSO-d_6) δ 1.18−1.21 (m, 6H, 2 × Ala-CH_3), 3.03 (s, 4H, piperazine), 3.37−3.51 (m, 6H, piperazine and NCH_2CO), 3.83 (s, 1H, epoxide CH), 4.10−4.33 (m, 3H, 2 × α-H and epoxide CH), 4.53−4.57 (m, 4H, 2 × NCH_2Ph), 6.76 (d, 1H, J = 7.0 Hz, NH), 7.21−7.39 (m, 11H, 2 × Ph and NH), 7.47 (s, 1H, NH), 8.18 (s, 1H, NH), 8.71 (s, 1H, NH), 10.62 (s, 1H, NH); HRMS (FAB) calcd for $C_{31}H_{41}N_8O_7$ 637.3093, observed m/z 637.3032. Anal. ($C_{31}H_{40}N_8O_7 \cdot 1.1TFA \cdot 2H_2O$) C, H, N.

(2*S*,3*S*)-2-(Dibenzylcarbamoyl)-3-(*N*^{2}-(4-(*tert*-butoxycarbonyl)-piperazin-1-yl carbonylalanylalanyl)-*N*^{1}-carbamoylmethylhydrazinocarbonyl)oxirane (36r, Boc-Piz-Ala-Ala-AAsn-EP(*S*,*S*)-CON(Bzl)$_2$). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (22% yield): ^{1}H NMR (DMSO-d_6) δ 1.18 (s, 6H, 2 × Ala-CH_3), 1.38 (s, 9H, Boc), 3.25−3.31 (m, 10H, piperazine and NCH_2CO), 3.81 (s, 1H, epoxide CH), 4.08−4.30 (m, 3H, 2 × α-H and epoxide CH), 4.57 (s, 2H, NCH_2Ph), 4.70 (s, 2H, NCH_2Ph), 6.57 (d, 1H, J = 7.6 Hz, NH), 7.17−7.36 (m, 11H, 2 × Ph and NH), 7.46 (s, 1H, NH), 8.14 (s, 1H, NH), 10.67 (s, 1H, NH); HRMS (FAB) calcd for $C_{36}H_{49}N_8O_9$ 737.3617, observed m/z 737.3630. Anal. ($C_{36}H_{48}N_8O_9 \cdot 1.4H_2O$) C, H, N.

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Supporting Information Available: Synthesis of aza-peptide substrate analogues (figure), inhibition data (table), and synthetic details. This material is available free of charge via the Internet at http://pubs.acs.org.

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