Aza-peptidyl Michael Acceptors. A New Class of Potent and Selective Inhibitors of Asparaginyl Endopeptidases (Legumains) from Evolutionarily Diverse Pathogens

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Aza-peptide Michael acceptors with the general structure of Cbz-Ala-Ala-AAsn-*trans*-CH═CHCOR are a new class of inhibitors specific for the asparaginyl endopeptidases (AE) (legumains). Structure–activity relationships (SARs) were characterized for a set of 31 aza-peptide Michael acceptors with AEs derived from three medically important parasites: the protist *Trichomonas vaginalis*, the hard tick *Ixodes ricinus,* and the flatworm *Schistosoma mansoni*. Despite arising from phylogenetically disparate organisms, all three AEs shared a remarkably similar SAR with lowest IC_{50} values extending into the picomolar range. The results suggest an evolutionary constraint on the topography of the prime side of the active site. SAR also revealed that esters in the P1′ position are more potent than disubstituted amides and that monosubstituted amides and alkyl derivatives show little or no inhibition. The preferred P1′ residues have aromatic substituents. Aza-asparaginyl Michael acceptors react with thiols, which provides insight into the mechanism of their inhibition of asparaginyl endopeptidases.

Introduction

Asparaginyl endopeptidases (AEs[a] or legumains (EC.3.4.22.34)) belong to the C13 family of clan CD cysteine proteases that also includes caspases, gingipains, clostripain, and separase. On the basis of homology modeling, all apparently share the same catalytic-site motif and a common scaffold within their catalytic domains.[1] The first AE for which a cDNA clone was derived was isolated from the parasitic bloodfluke *Schistosoma mansoni* in 1987.[2] However, it was not until 1993 that the protein was identified as a protease homologous to "legumains" that had just been characterized in plant seeds.[3] Subsequently, described in mammals,[4] AEs have been linked to osteoclast formation and bone resorption[5] and the processing of bacterial antigens.[6]

With the availability of genome sequence information, it has become clear that AEs and other clan CD enzymes form part of a much larger group of proteins containing a minimal "caspase-hemoglobinase fold" with members distributed in Archaea, bacteria, and Eukarya.[7] A simple BLASTp search of the NCBI nonredundant protein sequence database with an *S. mansoni* AE sequence (CAB71158) reveals many orthologous proteins, including those in organisms causing disease in humans and animals. In blood-feeding parasites, such as *Schistosoma*[8,9] and the hard tick *Ixodes ricinus*,[10] AEs are part of a proteolytic network incorporating clan AA (aspartic) and CA (cysteine) proteases that are associated with digestion of the blood meal (primarily hemoglobin and albumin) (Sojka, unpublished results). Given the specificity of AEs for Asn at P1,[11,12] one hypothesis as to their function in these parasites is the discrete processing and activation of clan AA and CA zymogens involved in alimentary degradation of host proteins.[13] The hypothesis has been substantiated in vitro with the finding that recombinant AE from both *S. mansoni* AE (SmAE) and *I. ricinus* (IrAE) can *trans*-process and activate a gut-associated *S. mansoni* cathepsin B zymogen to its mature catalytic form.[10,14] Cathepsin B is a major proteolytic activity in the gut of both organisms (Sojka, unpublished results).[14,15] Accordingly, if AE-mediated *trans*-activation is a key step by which proteolysis is regulated in vivo, inhibition of AE activity would represent a logical strategy to interfere with the parasite's ability to feed and, consequently, thrive. In the case of the *Ixodes* hard tick, the subsequent benefit would be a disruption or interruption of its position as a vector of various viral and bacterial diseases, not the least is Lyme disease.[16]

The synthetic AE inhibitors reported so far have only been tested against mammalian AEs.[17] These include aza-Asn halomethylketones (Cbz-Ala-Ala-AAsn-CH_2Cl, $k_{obs}/[I] = 139\,000$ M^{-1} s^{-1}, Cbz = benzyloxycarbonyl), Michael acceptors derived from Asn (Cbz-Ala-Ala-NHCH(CH_2CONH_2)CH═$CHCO_2$-CH_2CH═CH_2, $k_{obs}/[I]$ up to 766 M^{-1} s^{-1}),[18] and acyloxymethylketones ($k_{obs}/[I]$ from 769 up to 109 000 M^{-1} s^{-1}).

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[a] Abbreviations: AAsn, aza-asparagine; AAsp, aza-asparagine; Ac, acetyl; AcOH, acetic acid; AE, asparaginyl endopeptidase; AMC, 7-amino-4-methylcoumarin; Boc, *tert*-butoxycarbonyl; Bzl, benzyl, CH_2Ph; Cbz, benzyloxycarbonyl; $CDCl_3$, deuterated chloroform; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DCC, dicyclohexylcarbodiimide; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; DMSO-d_6, deuterated dimethyl sulfoxide-d_6; DTT, dithiothreitol; E-64c, *N*-(L-3-*trans*-carboxyoxiran-2-carbonyl)-L-leucyl-3-methylbutanamide; EDC, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride; EDTA, ethylenediaminetetraacetic acid; EP, epoxide; EtOAc, ethyl acetate; EtOH, ethanol; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; HOBt, *N*-hydroxybenzotriazole; *i*BCF, isobutyl chloroformate; *i*Bu, isobutyl; IrAE, *I. ricinus* asparaginyl endopeptidase; MeOH, methanol; NMM, *N*-methylmorpholine; OBzl, benzyloxy; Pipes, 1,4-piperazinediethanesulfonic acid; PhPr, 3-phenylpropanoyl; *p*NA, *p*-nitroanilide; SmAE, *S. mansoni* asparaginyl endopeptidase; *t*Bu, *tert*-butyl; TFA, trifluoroacetic acid; TvAE1, *Trichomonas vaginalis* asparaginyl endopeptidase.

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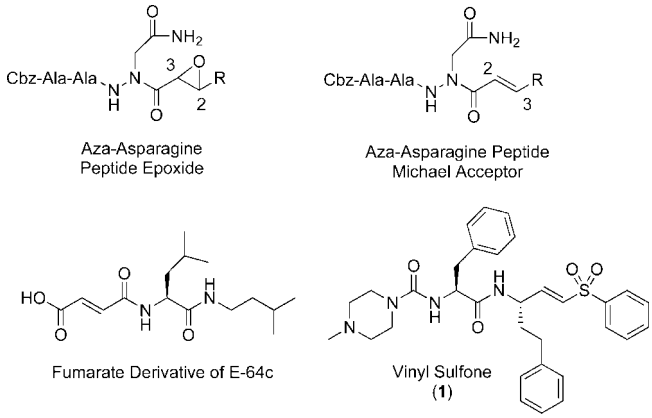


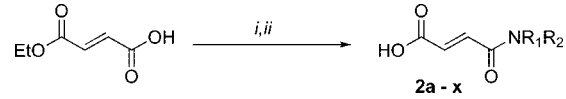
Figure 1. Design of aza-peptide Michael acceptors is derived from aza-peptidyl epoxides, the fumarate derivative of E-64c, and the C-terminal vinyl sulfone design. The aza-peptide Michael acceptors contain a nitrogen instead of the α-carbon at the P1 asparagine residue. Thus, the resulting aza-amino acid is referred to as aza-asparagine (AAsn). The numbering of epoxides and Michael acceptors is reversed. Thus, attack at C-2 of a Michael acceptor inhibitor would correspond to attack at C-3 of an epoxide inhibitor.

Aspartyl peptidyl fluoromethyl ketones designed specifically for caspase inhibition are also moderate inhibitors of mammalian AE.[19] In addition, fluorescein aspartyl peptidyl acyloxymethylketones have been used as efficient and selective labels of legumain in intact cells.[20] We recently reported the synthesis of aza-asparaginyl epoxide inhibitors specific for *S. mansoni* and mammalian AEs (Figure 1).[21] These epoxides were originally evaluated as inhibitors of caspases and other clan CD proteases.[22] In an effort to synthesize more potent inhibitors of AEs, we designed a new class of aza-peptidyl inhibitors by replacing the epoxide moiety with a double bond moiety to generate a Michael acceptor warhead (Figure 1).

Michael acceptors have previously been employed as warheads in inhibitors of several cysteine proteases.[23–25] The fumarate derivative (*trans*-HOOCCH═CHCO-Leu-NH$(CH_2)_2$-$CH(CH_3)_2$) of E-64c is one of the first Michael acceptor inhibitors reported and contains the fumarate warhead at the N-terminus of a peptidyl inhibitor (Figure 1).[26] The same fumarate inhibits clan CA cysteine proteases such as cathepsins B, H, and L but not clan CD proteases. Hanzlik et al. reported the first replacement of a carbonyl group of a cysteine protease substrate with a Michael acceptor moiety in order to trap the active site nucleophile of cysteine or serine proteases. They attached an α,β-unsaturated carbonyl moiety at the C-terminus of a peptide substrate.[23] This strategy was then applied in the design of peptidyl vinyl sulfones[27] and other α,β-unsaturated carbonyl derivatives (Figure 1).[28,29] The C-terminal Michael acceptor design has proven to be very successful, as two peptidyl Michael acceptor inhibitors, ruprintrivir and the homophenylalanine vinyl sulfone **1** (K11777, CRA-3316, or APC-3316),[30] are currently in clinical trials for rhinitis [31,32] and late-stage preclinical trials for Chagas' disease, respectively.[33]

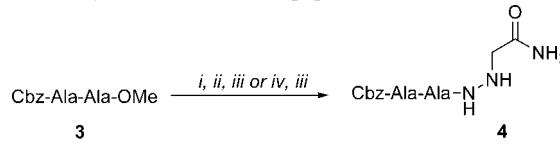
For this communication, we describe a new class of inhibitors of AEs by applying the Michael acceptor fumarate warhead of E-64c to our previous design of the potent and specific aza-peptide epoxides (Figure 1).[21] We report the design, synthesis, and evaluation of 31 aza-peptide Michael acceptors as potent and specific inhibitors of three AEs (SmAE, IrAE, and TvAE1) from medically important and phylogenetically divergent pathogens. TvAE1was one of two legumains originally identified in the sexually transmitted protist parasite, *Trichomonas vaginalis,* by Léon-Félix et al.,[34] but its functional expression in *Pichia pastoris* and characterization with the present inhibitors are described here for the first time. We also provide evidence for the mechanism of inhibition of AEs by aza-peptide Michael acceptors. In addition we have synthesized a biotinylated derivative of one of the better Michael acceptor inhibitors. A preliminary report of the aza-peptide Michael acceptor design has been published.[35]

Scheme 1. Synthesis of Fumarate Precursors[a]



[a] Reagents: (i) NMM, *i*BCF, CH_2Cl_2, HNR_1R_2; (ii) (1) NaOH, (2) HCl, MeOH. NMM = *N*-methylmorpholine. *i*BCF = isobutylchloroformate.

Scheme 2. Synthesis of the Aza-peptide Precursor[a]



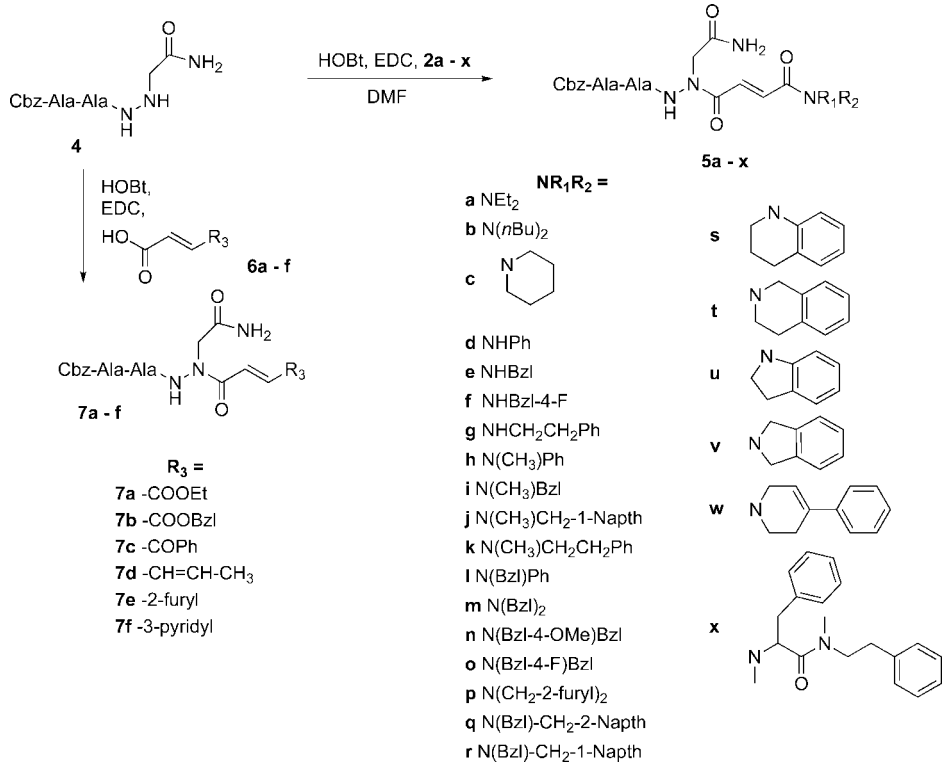
[a] Reagents: (i) H_2NNH_2, MeOH; (ii) $BrCH_2COOEt$, NMM, DMF; (iii) NH_3, cat. NaCN, MeOH, DMF; (iv) $HCl \cdot H_2NNHCH_2COOEt$, NMM, MeOH. Cbz = Ph-$CH_2$-OCO-. DMF = dimethylformamide. NMM = *N*-methylmorpholine.

Chemistry

The synthesis of the aza-peptide fumarate analogues is based on our previous synthesis of aza-peptide epoxide inhibitors[21] and involves coupling a peptidyl hydrazide to a fumarate or propenoic acid derivative. The fumaric acid monoamide precursors (**2a**−**x**) were prepared from monoethyl fumarate and the corresponding primary or secondary amines by standard mixed anhydride coupling using NMM and *i*BCF followed by deprotection of the ethyl ester in methanol using aqueous NaOH (Scheme 1). The various disubstituted aromatic amines were synthesized by reductive amination starting with aromatic aldehyde precursors and an aromatic primary amine. The monobenzyl fumarate derivative was formed from fumaric acid and benzyl alcohol using NMM and DCC as the coupling reagent.

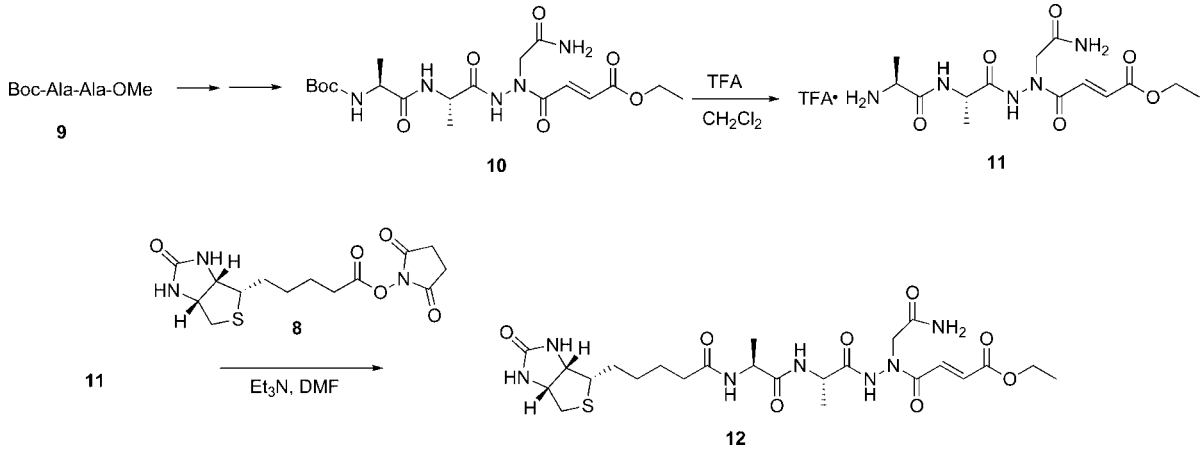
The preparation of the aza-peptidyl precursor is shown in Scheme 2. There are two possible routes to incorporate the aza moiety into the peptide backbone. The hydrazide can be prepared by conversion of the peptide methyl ester to the peptidyl hydrazide, followed by alkylation to introduce the asparagine side chain. Thus, we synthesized the asparaginyl side chain from Cbz-Ala-Ala-OMe (**3**) using excess hydrazine in methanol with subsequent alkylation of the hydrazide with ethyl bromoacetate and NMM followed by ammonolysis. We have previously used this route for the synthesis of the aza-peptidyl functionality with other amino acid analogues, such as aza-ornithine, aza-lysine, and aza-aspartate analogues.[22] A second approach is to directly add the aza-asparagine residue to the starting peptide Cbz-Ala-Ala-OMe (**3**) using a substituted hydrazide moiety. The reactivity of the hydrazide moiety depends on the nature of the substituent. An electron withdrawing substituent, such as an ester or amide, places a higher nucleophilic character on the primary hydrazide nitrogen. An electron donating substituent shifts the reactivity to the secondary nitrogen.[36,37] Therefore, we could also introduce the AAsn side chain by coupling the peptide precursor (**3**) to ethyl hydrazinoacetate in a one-step reaction. However, we did not observe an improvement in the overall yield. Subsequent

Scheme 3. Coupling of Fumarate and Propenoate Precursors to Cbz-Ala-Ala-NHNHCH$_2$CONH$_2$[a]



[a] HOBt = 1-hydroxybenzotriazole hydrate. EDC = 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride. DMF = dimethylformamide. Cbz = Ph-CH$_2$-OCO-.

Scheme 4. Preparation of a Biotinylated Michael Acceptor Inhibitor[a]



[a] Boc = *tert*-butyloxycarbonyl. TFA = trifluoroacetic acid. DMF = dimethylformamide.

conversion of the ethyl ester to the amide (**4**) by ammonolysis with catalytic amounts of NaCN followed the procedure described by Hogberg et al.[38]

The peptide hydrazide precursor was then coupled to a variety of substituted fumaric acid monoamides (**2a**−**x**), monoalkyl and monoaryl fumarate esters, or commercially available propenoic acid derivatives (**6a**−**f**) using HOBt and EDC. This completes the synthesis of the various aza-peptide fumaric acid or propenoic acid derivatives (**5a**−**x** and **7a**−**f**) (Scheme 3).

A possible side chain reaction that arose while planning the synthetic strategy was the intramolecular cyclization of the aza-asparagine's side chain with the Michael acceptor warhead. However, we speculated that the rigidity of the aza-peptide backbone probably prevents this intramolecular cyclization. Thus, we did not protect the Asn side chain, and as predicted, no cyclization was observed during the course of the synthesis. Niestroj et al. have previously reported the synthesis of aza-asparaginyl chloromethyl ketones.[18] In their synthetic approach, they employed a trityl protected amide on the Asn side chain.

The synthesis of the biotinylated inhibitor (**12**) is shown in Scheme 4. The Boc-protected aza-peptide Michael acceptor (**10**) was synthesized by hydrazinolysis of Boc-Ala-Ala-OMe (**9**) following the procedure in Scheme 1. We chose to replace the Cbz protecting group with the Boc protecting group, since Cbz deprotection requires catalytic hydrogenation, which could affect the double bond of the fumarate moiety. The Boc group can then be removed with dilute TFA. The *N*-hydroxysuccinimide ester of biotin (**8**) prepared by DCC coupling was reacted with the TFA salt of the aza-peptide Ala-Ala-AAsn-CH═CHCOOEt

Table 1. Inhibition of AEs from Three Parasitic Organisms by Aza-peptide Michael Acceptors

	inhibitor	IC_{50} (nM)[a] TvAE1	IrAE	SmAE	SmAE $k_{obs}/[I]$[b] (M^{-1} s^{-1})
7a	Cbz-Ala-Ala-AAsn-CH═CHCOOEt	4.5	4.5	31	17420
7b	Cbz-Ala-Ala-AAsn-CH═CHCOOBzl	5	<1	38	
5a	Cbz-Ala-Ala-AAsn-CH═CHCONEt$_2$	2000	2700	NI[c]	
5b	Cbz-Ala-Ala-AAsn-CH═CHCON(*n*Bu)$_2$	1000	100	550	
5c	Cbz-Ala-Ala-AAsn-CH═CHCO-Pip	500		1000	
5d	Cbz-Ala-Ala-AAsn-CH═CHCONHPh	20	10	700	
5e	Cbz-Ala-Ala-AAsn-CH═CHCONHBzl	10	200	1000	
5f	Cbz-Ala-Ala-AAsn-CH═CHCONHBzl-4-F	75	250	800	
5g	Cbz-Ala-Ala-AAsn-CH═CHCONHCH$_2$CH$_2$Ph	10	350	600	
5h	Cbz-Ala-Ala-AAsn-CH═CHCON(CH$_3$)Ph	5	1.8	60	
5i	Cbz-Ala-Ala-AAsn-CH═CHCON(CH$_3$)Bzl	0.55	<1	55	
5j	Cbz-Ala-Ala-AAsn-CH═CHCON(CH$_3$)CH$_2$-1-Napth	<1	5.5	35	16930
5k	Cbz-Ala-Ala-AAsn-CH═CHCON(CH$_3$)CH$_2$CH$_2$Ph	50		600	
5l	Cbz-Ala-Ala-AAsn-CH═CHCON(Bzl)Ph	<1	<1	70	
5m	Cbz-Ala-Ala-AAsn-CH═CHCON(Bzl)$_2$	10	450	45	5800
5n	Cbz-Ala-Ala-AAsn-CH═CHCON(Bzl-4-OMe)Bzl	2.3	12	90	
5o	Cbz-Ala-Ala-AAsn-CH═CHCON(Bzl-4-F)Bzl	<1	700	60	
5p	Cbz-Ala-Ala-AAsn-CH═CHCON(CH$_2$-2-furyl)$_2$	5000	15000	900	
5q	Cbz-Ala-Ala-AAsn-CH═CHCON(Bzl)-CH$_2$-2-Napth	6	8	70	6100
5r	Cbz-Ala-Ala-AAsn-CH═CHCON(Bzl)-CH$_2$-1-Napth	<1	6	62	6180
5s	Cbz-Ala-Ala-AAsn-CH═CHCO-tetrahydroquinoline	3.5	4	70	
5t	Cbz-Ala-Ala-AAsn-CH═CHCO-tetrahydroisoquinoline	10	25	200	
5u	Cbz-Ala-Ala-AAsn-CH═CHCO-indoline	50	18	70	
5v	Cbz-Ala-Ala-AAsn-CH═CHCO-isoindoline	20	4.5	300	
5w	Cbz-Ala-Ala-AAsn-CH═CHCO-(Py-4-Ph)	10	85	750	
5x	Cbz-Ala-Ala-AAsn-CH═CHCO-MePhe-N(CH$_3$)CH$_2$CH$_2$Ph[d]	80	300	750	
7c	Cbz-Ala-Ala-AAsn-CH═CHCOPh	≥10000	>20000	NI[c]	
7d	Cbz-Ala-Ala-AAsn-CH═CH-CH═CH-CH$_3$	8000		NI[c]	
7e	Cbz-Ala-Ala-AAsn-CH═CH-2-furyl		7500	>2000	
7f	Cbz-Ala-Ala-AAsn-CH═CH-3-Py	8000 to >10000	15000	>2000	
12	biotinyl-Ala-Ala-AAsn-CH═CHCOOEt	<10	2.8	10	

[a] AEs from *Trichomonas vaginalis*, *Ixodes ricinus*, and *Schistosoma mansoni* were expressed in *Pichia pastoris* and, if necessary, activated as described previously or herein.[53] Inhibition assays were performed in 0.1 M citrate phosphate, 4 mM DTT, pH 6.8, for SmAE and TvAE1, or pH 5.5 for IrAE, with a final concentration of 10 μM Cbz-Ala-Ala-Asn-AMC (Cbz = PhCH$_2$-OCO-; AMC = 7-amino-4-methylcoumarin) as substrate. [b] $k_{obs}/[I]$ values of the inhibitors were determined in 0.1 M citrate phosphate buffer, pH 6.8, containing 2 mM DTT and 20 μM Cbz-Ala-Ala-Asn-AMC substrate. The progress of inhibition was followed every 2 s for 30 min at 25 °C. The apparent $k_{obs}/[I]$ values were determined using nonlinear regression analysis (GraphPad Prism 3.X) and corrected for substrate ($1 + S/K_M$: $1 + {}^{20}/_{60} = 1.33$). [c] NI = no inhibition after 20 min of preincubation with inhibitor. [d] MePhe = *N*-methylphenylalanine. Cbz = Ph-CH$_2$-OCO-. AAsn = aza-Asn.

(**11**) in the presence of triethylamine to give the biotinylated aza-peptidyl Michael acceptor (**12**) in good yield.[39,40]

Results and Discussion

Synthetic Design. Aza-peptide active esters are known to act as acylating inhibitors of both serine and cysteine proteases.[41–44] Aza-peptide chloromethyl ketones and acyloxymethyl ketones have been observed to alkylate the active site cysteine of mammalian AE.[35] In our design, we have incorporated a Michael acceptor into an aza-peptide structure. We used the optimal substrate sequence Cbz-Ala-Ala-Asn-AMC (AMC = 7-amino-4-methylcoumarin) for AEs determined by Mathieu et al. as the basis for the peptide backbone of our Michael acceptor inhibitors.[11] Replacing the α-carbon of the P1 asparaginyl residue with a nitrogen provides a practical synthetic route for coupling peptide hydrazides to readily available fumaric acid derivatives or substituted propenoic acid analogues. Because of its planarity, the aza-peptide functionality changes the orientation of the scissile peptide bond and, therefore, restricts the conformation and rotational freedom of the original peptide bond. The fumaroyl carbonyl is now in the exact place as the scissile peptide carbonyl of an AE substrate. The P′ side of the inhibitor can be easily modified so that it is possible to scan for increased potency and specificity of interactions with the S′ subsites of the enzyme. Fumaric acid and propenoic acid derivatives are easily synthesized prior to coupling with the peptide hydrazide and, therefore, allowed us to perform an extensive SAR study with a variety of new inhibitor structures.

Inhibition of SmAE, TvAE1, and IrAE. The IC_{50} values for 31 aza-peptide Michael acceptors are reported in Table 1. In addition, we measured $k_{obs}/[I]$ values for the better inhibitors of SmAE. Strikingly, all three AEs show a similar SAR based on IC_{50} values across the inhibitor series tested. The more effective inhibitors inhibit SmAE in the mid to low nanomolar range, whereas for TvAE1 and IrAE the lowest IC_{50} values are less than 1 nM. The monobenzyl and monoethyl esters (**7a**, **7b**) are among the most potent inhibitors for all three AEs. Replacement of the ethoxycarbonyl group in (**7a**) or the benzyloxycarbonyl group in (**7b**) with another double bond (**7d**), a benzoyl group (**7c**), or aryl groups (**7e**, **7f**) effectively results in a loss of inhibition against all three enzymes. We attribute the loss of potency to a requirement for a second electron withdrawing group on the propenoyl functionality of the inhibitor. The ester derivatives such as (**7a**) and (**7b**) meet this requirement. The benzoyl derivative Cbz-Ala-Ala-AAsn-CH═CHCOPh (**7c**) also has an electron withdrawing group but does not inhibit any of the AEs, probably because of crowding or insufficient electronegativity.

The monobenzyl amide derivative (**5e**) is a weak inhibitor of all three AEs, even though it is a direct analogue of the potent benzyl ester derivative (**7b**). Furthermore, we found that all monosubstituted amides (**5e**, **5f**, **5g**) and the alkyl substituted propenoyl derivatives (**7d**, **7e**, **7f**) were weaker or extremely

Table 2. AE Inhibitors Reported in the Literature

inhibitor	mammalian AE $k_{obs}/[I]^a$ (M^{-1} s^{-1})	SmAE $k_{obs}/[I]$ (M^{-1} s^{-1})	SmAE IC_{50}(nM)	ref
Cbz-Ala-Ala-NHCH(CH_2CONH_2)-CH=$CHCO_2CH_2CH$=CH_2	776[b]			18
Cbz-Ala-Ala-AAsn-CH_2Cl	139088[b]			18
Cbz-Ala-Ala-AAsn-$CH_2OC(O)Ph$	13[b]			18
Cbz-Asn-$CH_2OC(O)$-Ph-2,5-dimethyl	109000[c]			17
Cbz-Ala-Ala-AAsn-(*S,S*)-EP-COOEt	43000[d]	17400[e]	53 ± 25[e]	22

[a] All inhibitors were assayed using Cbz-Ala-Ala-Asn-AMC (Cbz = $PhCH_2$-OCO-; AMC = 7-amino-4-methylcoumarin) as the substrate. [b] Inhibition was determined using pig AE. Inhibition buffer was 39.5 mM citric acid, 121 mM Na_2HPO_4, 1 mM EDTA, 0.1% (w/v) CHAPS, 0.015% Brij, and 1 mM DTT (dithiothreitol). [c] Inhibition was determined using human AE. Inhibitors were incubated in assay buffer (20 mM citric acid, 60 mM Na_2HPO_4, 1 mM EDTA, 0.1% (w/v) CHAPS, pH 5.8) at 25 °C with the substrate prior to addition of mammalian AE. [d] Inhibition was determined with pig kidney AE. Inhibition buffer was 39.5 mM citric acid, 121 mM Na_2HPO_4, pH 5.8, containing 1 mM EDTA, 1 mM TCEP (tris-(2-carboxyethyl)phosphine), and 0.01% CHAPS. [e] For detailed assay conditions, see Table 1.

poor inhibitors, respectively. We conclude that the hydrogen bonding network between the inhibitor and the active sites of the AEs is optimal with the additional carbonyl of the esters (**7a** and **7b**) next to the double bond and without the H-bond donor present in monosubstituted amides. TvAE1 on the other hand seems to be less affected by the additional H-bond donor of the monosubstituted amides (**5d**, **5e**, **5f**, **5g**) in contrast to IrAE and SmAE.

Disubstituted amide derivatives are much more effective than monosubstituted amide derivatives. In particular, the disubstituted amides with aromatic groups (**5h**, **5i**) give lower IC_{50} values than the alkyl derivatives (**5a** NEt_2, **5b** N(*n*Bu)$_2$, **5c** Pip). We speculate that increased π-stacking of the aromatic residues in either the S1′ or S2′ pocket improves binding of the inhibitor. Unexpectedly, however, the aromatic bisfurylmethyl Michael acceptor (**5p**) was a poor inhibitor.

The positioning and orientation of the phenyl or naphthyl rings play an important role in the inhibitors' potency for all three AEs. The *N*-methyl-*N*-phenyl analogue (**5h**) is as effective (TvAE1 IC_{50} = 5 nM, IrAE IC_{50} = 1.8 nM, SmAE IC_{50} = 60 nM) as the *N*-methyl-*N*-benzyl analogue (**5i**, TvAE1 IC_{50} = 0.55 nM, IrAE IC_{50} < 1 nM, SmAE IC_{50} = 55 nM). However, extending the alkyl spacer by one additional methylene group from the *N*-methyl-*N*-benzyl analogue to the *N*-methyl-*N*-phenethyl analogue (**5k**) decreases the inhibition potency against TvAE1 (IC_{50} = 50 nM) and SmAE (IC_{50} = 600 nM). This decreased potency is reversed, however, when the terminal *N*-methyl-*N*-phenyl groups of (**5k**) are replaced by an *N*,*N*-methyl-1-naphthylmethyl substituent (**5j**; TvAE1 IC_{50} <1 nM, IrAE IC_{50} = 5.5 nM, SmAE IC_{50} = 35 nM, $k_{obs}/[I]$ =16 930 M^{-1} s^{-1}). This analogue is one of three and four most effective inhibitors of SmAE and TvAE1, respectively. For SmAE, the dibenzyl amide (**5m**, SmAE IC_{50} = 45 nM, $k_{obs}/[I]$ = 5800) has the lowest IC_{50} of the aromatic disubstituted amides. In contrast, the active sites of IrAE and to some extent TvAE1 seem unable to accommodate the second methylene extended aromatic substituent of (**5m**). This is reflected in the IC_{50} values, which drop by 10- and 500-fold compared to (**5j**) for TvAE1 and IrAE, respectively. The less flexible *N*-benzyl-*N*-phenyl-amide derivative (**5l**), however, furnished excellent IC_{50} values against TvAE1 and IrAE with less than 1 nM for both enzymes. Compared to the unsubstituted dibenzyl analogue (**5m**), the substitution of one of the benzyl rings with an electron-donating methoxy (**5n**) or an electron-withdrawing fluorine (**5o**) did not significantly alter inhibition values for all three AEs except in the case of IrAE and (**5n**) for which a 10-fold smaller IC_{50} value was recorded.

The next step was to combine the potent effects of the naphthyl group and the benzyl substituent. Compared to dibenzylamide (**5m**) the *N*,*N*-benzyl-2-naphthylmethyl (**5q**) and the *N*,*N*-benzyl-1-naphthylmethyl analogue (**5r**) did not overall improve the IC_{50} for SmAE but increased the potency for TvAE1 and IrAE. Constraining the orientation and flexibility of the benzyl group led to the design of analogues such as quinoline (**5s**), isoquinoline (**5t**), indoline (**5u**), and isoindoline (**5v**). Both quinoline and indoline exhibited good potency but were less potent compared to the benzylamide (**5i**). The aromatic ring is much less flexible in the indoline and quinoline bicyclic system. The orientation of the aromatic ring in the isoindoline (**5v**) and isoquinoline (**5t**) compounds is also different from their indoline and quinoline parent compounds (**5u** and **5s**) because of the different location of the nitrogen. This altered orientation of the aromatic ring lowers their inhibitory potency by 3- to 6-fold for all three AEs. For IrAE and TvAE1, however, changing the indoline (**5u**) to the isoindoline (**5v**) resulted in slightly improved IC_{50} values, whereas the opposite was the case for SmAE. Additional studies showed that the 4-phenyltetrahydropyridine derivative (**5w**) and the amino acid derivative (**5x**) did not improve the effectiveness of the Michael acceptor warhead. The biotinylated analogue of (**7a**), biotin-Ala-Ala-AAsn-CH=CHCOOEt (**12**), remained an effective inhibitor of all three AEs.

In summary, the aza-peptide esters are more potent than their amide analogues. However, the difficulty of their preparation limits the variety of possible compounds. Also, the similarity of the SAR between these AEs arising from such phylogenetically disparate organisms is quite remarkable and suggests an evolutionary constraint on the structural topography on the prime side of the AE active site to perform such prescribed functions as protein processing and zymogen activation.

Table 2 presents inhibition data obtained with other AE inhibitors reported in the literature. Direct comparison with our results is not possible in most cases, as most of the reported second-order inhibition rate constants ($k_{obs}/[I]$) were obtained with mammalian AEs and not with SmAE. We have previously studied aza-peptide epoxide inhibitors with both pig kidney AE and SmAE.[21] In general the pig kidney enzyme was inhibited 2- to 3-fold faster than the SmAE, but this could be due to different subsite preferences of the two enzymes. Overall, aza-peptide Michael acceptors are very similar in reactivity to aza-peptide epoxides with SmAE. For example, the epoxide analogue (Cbz-Ala-Ala-AAsn-(*S,S*)-EP-COOEt, Table 2, $k_{obs}/[I]$ = 17 400 M^{-1} s^{-1}) inhibits SmAE with the same rate as the corresponding Michael acceptor monoethyl ester (**7a**, Table 1, $k_{obs}/[I]$ = 17 420 M^{-1} s^{-1}) but inhibits pig kidney AE more rapidly ($k_{obs}/[I]$ = 43 000 M^{-1} s^{-1}).

Aza-peptidyl Inhibitors and Clan CD Specificity. Representative aza-peptide Michael acceptor inhibitors displayed in Table 1 were further tested with other clan CD cysteine proteases including caspases-3, -6, and -8, clostripain, and gingipain K

Table 3. Inhibition of Various Clan CD and Clan CA Cysteine Proteases by AE Specific Aza-peptide Michael Acceptors

		k_{obs}/[I] (M^{-1} s^{-1})[a]					
	inhibitor Cbz-Ala-Ala-AAsn-R with R =	caspases -3, -6, -8[b]	calpain I[c]	papain[d]	cathepsin B[e]	gingipain K[f]	clostripain[g]
7a	-CH=CHCOOEt[h]	NI[i]	NI[i]	NI[i]	1	6	NI[i]
5i	-CH=CHCON(CH_3)Bzl	NI[i]	NI[i]	NI[i]	NI[i]	1	NI[i]
5m	-CH=CHCON(Bzl)$_2$	NI[i]	<1	1	NI[i]	2	NI[i]
5u	-CH=CHCO-indoline	NI[i]	NI[i]	NI[i]	NI[i]	1	NI[i]
5j	-CH=CHCON(CH_3)CH_2-1-Napth	NI[i]	NI[i]	NI[i]	NI[i]	6	NI[i]
5g	-CH=CHCONHCH_2CH_2Ph	NI[i]	NI[i]	12	NI[i]	NI[i]	NI[i]

[a] k_{obs} is the pseudo-first-order rate constant obtained from plots of ln v_t/v_0 vs time unless indicated otherwise. [b] Inhibition buffer was 40 mM Pipes, 200 mM NaCl, 0.2% (w/v) CHAPS, sucrose 20% (w/v), and 10 mM DTT (dithiothreitol), at pH 7.2, with Ac-Asp-Glu-Val-Asp-AMC as the substrate. [c] Inhibition buffer was 50 mM Hepes buffer (pH 7.5) containing 10 mM cysteine and 5 mM $CaCl_2$. [d] Inhibition buffer was 50 mM Hepes and 2.5 mM EDTA, at pH 7.5, and 25 μL of DTT (0.1 M). [e] Inhibition buffer was 0.1 M K_2HPO_4, 1.25 mM EDTA, 0.01% Brij, pH 6.0 buffer and at 23 °C. [f] Inhibition buffer was 0.2 M Tris-HCl, 0.1 M NaCl, 5 mM $CaCl_2$, 2 mM DTT at pH 8.0 with Suc-Ala-Phe-Lys-AMC as the substrate with gingipain K. [g] Inhibition buffer was 20 mM Tris-HCl, 10 mM $CaCl_2$, 0.005% Brij 35, 2 mM DTT at pH 7.6 with Cbz-Phe-Arg-AMC as the substrate. [h] Cbz = Ph-CH_2-OCO-. AAsn = aza-Asn. [i] NI = no inhibition.

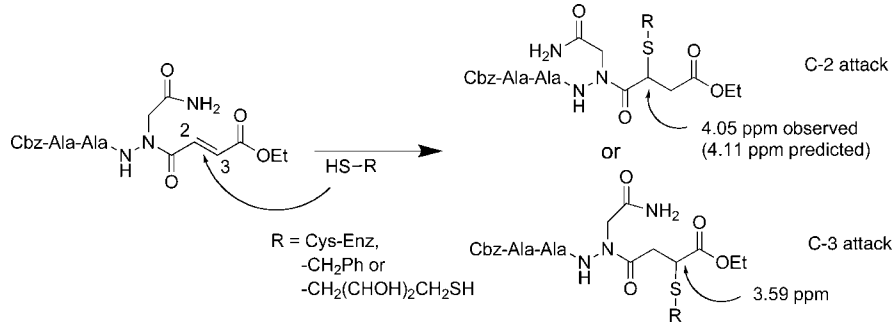


Figure 2. Two possible sites for the thioalkylation of aza-peptide Michael acceptor inhibitors with the enzyme cysteine residue, benzyl thiol, or DTT.

Table 4. Rates of Degradation and Half-Lives of Various Inhibitors in the Presence of DTT

		pH 5.8		pH 6.8	
	inhibitor, R = Cbz-Ala-Ala-AAsn	k_{obs} (s^{-1})[a]	$t_{1/2}$ (min)	k_{obs} (s^{-1})[a]	$t_{1/2}$ (min)
7a	R-CH=CHCOOEt	6.06×10^{-4}	19	3.10×10^{-3}	3.7
7b	R-CH=CHCOOBzl			4.60×10^{-3}	2.5
5i	R-CH=CHCON(CH_3)Bzl	3.07×10^{-5}	376	5.78×10^{-4}	20
5m	R-CH=CHCON(Bzl)$_2$			6.73×10^{-4}	17
7d	R-CH=CH-CH=CH-CH_3	no reaction		no reaction	

[a] An aliquot (15 μL) of 10 mM aza-peptidyl Michael acceptors in DMSO (dimethyl sulfoxide) was added to buffer (485 μL, 0.1 M citrate phosphate, pH 5.8 or 6.8) containing 3.4 mM DTT (dithiothreitol), and the reaction of the double bond of the inhibitor with DTT was monitored spectrophotometrically at 250 nm over time. The k_{obs} was obtained from pseudo-first-order rate plots of ln(A_t/A_0) versus time, and the half-lives were derived from $t_{1/2} = \ln 2/k_{obs}$.

(Table 3). Caspases require Asp at P1 of the substrate and prefer tri- and tetrapeptides over dipeptides.[45] Clostripain and gingipains require peptides with Arg, Lys, or Orn at P1.[46] It is, therefore, not surprising that the present AE inhibitors neither inhibit the caspases nor clostripains, whereas compounds (**7a**) and (**5j**) inhibit gingipain at low rates (k_{obs}/[I] <6 M^{-1} s^{-1}). Thus, the present data confirm the unique P1 specificity of each family member of the clan CD cysteine proteases, which facilitates the design of selective inhibitors.

Aza-peptidyl Michael Acceptors Essentially Do Not React with Clan CA Cysteine Proteases. The aza-peptide Michael acceptors were also tested with the clan CA cysteine proteases calpain I, papain, and cathepsin B and showed little to no inhibition after 30 min of incubation (Table 3). The fastest rate measured was for compound (**5g**) with papain at 12 M^{-1} s^{-1}. Otherwise, the ethyl ester derivative (**7a**) was essentially unreactive with cathepsin B (<1 M^{-1} s^{-1}). A similar rate was also observed with (**5m**) and calpain I (<1 M^{-1} s^{-1}). Unlike clan CD proteases, the specificity of clan CA enzymes is strongly influenced by the S2 subsite. We hypothesize that the lack of inhibition of clan CA proteases by the present inhibitor series arises from the rigid nature of the aza-peptide moiety, which most likely prevents effective binding of the warhead close to the enzyme's catalytic residues. Consistent with the current data, our previously reported aza-peptide epoxide inhibitors of AEs[21] and caspases[22] also did not inhibit clan CA proteases.

Mechanism of Inhibition. The mechanism of inhibition of cysteine proteases by Michael acceptors involves a nucleophilic attack of the catalytic cysteine residue on the β-carbon of the Michael acceptor double bond.[47] A covalent bond forms, and the inhibitor is irreversibly bound to the enzyme. With the present inhibitors, nucleophilic attack of the active site cysteine thiol could occur at either carbon (C-2 or C-3) of the double bond (Figure 2). With the related aza-peptidyl epoxide inhibitors also shown in Figure 1, X-ray crystal structures of caspase-1 inhibited by PhPr-Val-Ala-AAsp-EP-$COOCH_2$Ph and PhPr-Val-Ala-AAsp-EP-CH_2CH_2Ph revealed that the attack of the cysteine thiol occurred at the C-2 carbon of the epoxide moiety, although this is not based on a refined structure.[48] In contrast, more recent refined X-ray studies of aza-peptide epoxide inhibitors that were specifically designed to inhibit caspase-3 demonstrated that the cysteine thiol attack occurs at the C-3 carbon of the epoxide moiety.[49] It should be noted that attack on the C-3 of the epoxide corresponds to attack at C-2 of the Michael acceptor inhibitor due to different number schemes (Figure 1).

In order to investigate the point of attack of the active site cysteine thiol, we studied the reactivity of the aza-peptidyl Michael acceptors with the small thiols DTT and benzylmercaptan using ^{1}H NMR and UV spectroscopy (Table 4). We predicted C-2 thioalkylation due to the difference in potency between the ester, amide, and alkyl aza-peptidyl Michael acceptor inhibitors. Upon incubation of various Michael acceptor inhibitors with DTT in the SmAE/TvAE1 assay buffer, we observed the disappearance of the Michael acceptor double bond over time. The potent inhibitor (**7a**) reacted with excess DTT in assay buffer at pH 6.8 and room temperature with a first-order rate constant of 3.10×10^{-3} s^{-1} ($t_{1/2} = 3.7$ min; Table 4). Michael acceptors with a terminal amide rather than an ester react with DTT at much slower rates. Thus, the rate constants for reaction of (**5i**) and (**5m**) with DTT were 5.78×10^{-4} s^{-1} ($t_{1/2} = 20$ min) and 6.73×10^{-4} s^{-1} ($t_{1/2} = 17$ min), respectively. Overall, the order of reactivity of the various Michael acceptors with differing terminal substituents was esters > amides > alkyls (Table 4). Freidig et al. (1999) also reported the reaction rates of ethyl acrylate and acrylamide with glutathione. Ethyl acrylate reacts 85 times more quickly than acrylamide with second-order rate constants of 0.66 and 7.8×10^{-3} M^{-1} s^{-1}, respectively.[50] Acrylates are more reactive toward nucleophilic attack than acrylamides because of differences in the electron withdrawing properties of esters and amides. Our thiol reactivity data in combination with the results by Freidig et al. suggest that simple thiols react at C-2 (Michael addition to an acrylate) rather than at C-3 (Michael addition to an acrylamide). This suggests that the enzyme is also likely to attack C-2 of the Michael addition inhibitor.

In order to obtain additional evidence for the site of alkylation, we studied the products in a model system by proton NMR spectroscopy. We chose benzyl thiol as the model nucleophile and studied its reaction with the Michael acceptor (**7a**). We followed the reaction of **7a** with benzylmercaptan in DMSO-d_6 by ^{1}H NMR for 48 h and observed the expected gradual disappearance of the vinyl proton at 6.59 ppm together with a simultaneous increase of a new signal at 4.05 ppm. The NMR additivity rules[51] as used by ChemDraw were applied in estimating the chemical shifts of the two possible thioalkylation products. The estimated chemical shift for a C-2 thioalkylation of the Michael acceptor double bond by benzylmercaptan predicts the shift of the proton on the C-2 carbon of the thioether product at 4.11 ppm. A C-3 thioalkylation would result in an estimated chemical shift of 3.59 ppm (Figure 2). Unfortunately, the new signal coincides with the α-H of the inhibitor peptide backbone, which scrambles the multiplicity of the peak. However, the chemical shift of the new signal closely corresponds to the predicted shift for a C-2 thioalkylation. Similar results have been obtained with Michael acceptor inhibitors of caspases.[35] Thus, the data from the NMR study also suggest that the C-2 carbon is the site of attack on the Michael acceptor.

Several Michael acceptor inhibitors specific for caspase-3 and caspase-8 have recently been analyzed by X-ray crystallography.[52] This Michael acceptor warhead is identical to the warhead of the AE inhibitors used here and was found to be thioalkylated by the active site cysteine thiol at the C-2 carbon of the reactive moiety. Thus, we conclude that it is likely that AEs are also alkylated at the C-2 carbon, which corresponds to the C-3 carbon of aza-peptide epoxide inhibitors. Thus, the site of alkylation in both aza-peptide epoxides and aza-peptide Michael acceptor inhibitors is located at approximately the same distance from the aza-peptide nitrogen atom.

Factors Influencing Reactivity and Half-Life of Aza-peptide Michael Acceptors. Aza-peptide Michael acceptors react with DTT in the assay buffer at variable rates depending on pH and the nature of the substituents on the double bond (Table 4). Lowering the pH of the assay decreases the rate of reactivity dramatically. For example, the ethyl ester has a half-life of 3.7 min in the presence of DTT at pH 6.8. At pH 5.8, the half-life increases to 19 min. With the amide derivatives, the effect of pH on the stability is more pronounced. Thus, the half-life of compound (**5i**) increases from 20 to 376 min when lowering the pH from 6.8 to 5.8. In a previous study with caspase-specific, tetrapeptidyl, aza-aspartyl Michael acceptors,[35] we found that the half-life of the caspase-specific ethyl ester analogue in the presence of DTT was 10 min at pH 7.2. At the same pH, the *N*-methylbenzylamide had a half-life of 116 min. Accordingly, we suspect that the length of the peptidyl inhibitor and the size of the side chains have an effect on the degradation rate. All of the above influencing factors, therefore, need to be taken into account when evaluating inhibitor potency.

Conclusions

Aza-peptidyl Michael acceptors are a recently discovered class of inhibitors of AEs.[35] The present report describes a unique and comprehensive set of comparative analyses for three AEs from disparate pathogenic organisms. On the basis of their similar SAR, the data suggest that the topography on the prime side of the active site is evolutionarily constrained, perhaps contributing to the maintenance of such critical functions as protein/peptide (hormone) processing and zymogen activation. The inhibitors are both potent and selective toward AEs among other clan CD proteases and clan CA cysteine proteases. The SAR study of the P1′ specificity discovered that AEs prefer aromatic residues and that the orientation and extension of such residues into the S1′ subsite play an important role. In addition, the strength of the electron withdrawing group on the P1′ position of the double bond controls the potency of the inhibitor. We determined that the inhibitor warhead reacts with thiols and that the progress of this decay reaction is dependent on both pH and the electron-withdrawing nature of the P1′ substituent. Mechanistic and NMR studies with simple thiols indicate that the most likely reaction mechanism involves attack of the active site cysteine on the carbon (C-2) immediately next to the scissile bond. X-ray analysis of Michael acceptor inhibitors to the active sites of caspase-3 and caspase-8 also show C-2 thioalkylation.[52]

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. Chemical shifts are reported in ppm 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.

Experiments. AE Assays. The zymogen forms of SmAE and IrAE were expressed in *Pichia pastoris* as previously described.[12,53] For expression of TvAE1 in *Pichia, T. vaginalis* (G3 strain) genomic DNA was kindly provided by Dr. Kirkwood Land, Department of Biological Sciences, University of the Pacific, Stockton, CA, and was used as a template to PCR-amplify the putative TvAE1 zymogen. The start position of the zymogen was estimated by amino acid sequence alignments with the *S. mansoni*

and human AEs using the deposited TvAE1 sequence (GenBank accession number AY326446).[34] PCR primers were forward, 5′→3′ ATA*CTGCAG*CACAGCTCGCAAGGTGTGATAG that contained a *Pst*I restriction endonuclease site (italicized) and two additional nucleotides (underlined) to preserve the in-frame translation, and reverse, 5′→3′ TAT*GCGGCCGC*TTAGCAGATGGCATCAATAGCAGC that contained a *Not*I endonuclease site (italicized) and a translation termination codon (underlined). PCR amplification, cloning into the expression vector pPICZα B, and expression under methanol induction were as described.[53,54] Induction medium was lyophilized and stored indefinitely at −20 °C. To autoactivate recombinant SmAE and TvAE1, lyophilized induction medium (50–100 mg) was reconstituted in 1.5 mL of 0.5 M sodium acetate, 4 mM DTT, pH 4.5, and left to stand at 37 °C for 3 h to overnight. Autoactivation was not required for IrAE produced in *P. pastoris*.[12] AE activity in the induction medium was measured with the substrate Cbz-Ala-Ala-Asn-AMC as described.[10,53] In a black 96-well microtiter plate, 50 μL of activated enzyme was added to an equal volume of 0.1 M citrate phosphate, 4 mM DTT, pH 6.8 for SmAE and TvAE1 or pH 5.5 for IrAE. Aza-peptidyl inhibitors were added as 1 μL aliquots (serial water dilutions of a 10 mM DMSO stock solution) to give a final concentration range of between 2.0 and 0.0002 μM and allowed to incubate at room temperature for 20 min. Then, 100 μL of the same buffer containing 20 μM Cbz-Ala-Ala-Asn-AMC was added and the reaction allowed to proceed for 20 min. Plotting the relative fluorescence units (RFU)/min against the inhibitor concentration (μM) permitted calculation of the IC_{50} value.

Second-order inhibition rates were determined for SmAE with some of the more reactive inhibitors. Zymogen SmAE was autoactivated as described above. Inhibitor (1 μL) at six concentrations (to yield 0−1 μM final concentrations) was spotted into a 96-well black microtiter plate. Then, 180 μL of 0.1 M citrate phosphate, 2 mM DTT, pH 6.8, containing 20 μM Cbz-Ala-Ala-Asn-AMC was added. Activated AE (20 μL) was added to the mix and the progress of inhibition followed every 2 s for 30 min at 25 °C (Molecular Devices Flex Station fluorometer in injection mode; excitation at 355 nm, emission at 460 nm). The k_{inact}/K_i values were determined using nonlinear regression analysis (GraphPad Prism 3.X) and corrected for substrate ($1 + S/K_M$: $1 + {}^{20}/_{60} = 1.33$).

Cathepsin B and Papain Assays. The incubation method was used to measure the irreversible inhibition of papain and cathepsin B. For cathepsin B, 30 μL of a stock inhibitor solution (1.19 g/mL) was added to 300 μL of 0.1 M potassium phosphate, 1.25 mM EDTA, 0.01% Brij 35, pH 6.0. Then, 30 μL of a freshly prepared cathepsin B solution (approximate concentration of 6.98×10^{-3} μg/μL) in the same buffer with 1 mM DTT was added. Aliquots (50 μL) of the inhibition mixture were withdrawn at various time intervals and added to 200 μL of the above buffer containing 499 μM Cbz-Phe-Arg-AMC. The release of AMC was measured at room temperature in a Tecan Spectra Fluor microplate reader ($\lambda_{ex} = 360$ nm, $\lambda_{em} = 465$ nm). Pseudo-first-order inactivation rate constants were obtained from plots of ln v_t/v_o versus time, where v_o is the rate of hydrolysis of fluorogenic substrate and v_t the rate of hydrolysis of substrate in presence of the inhibitor.

For papain, a similar incubation method was used with 50 mM Hepes, 2.5 mM DTT, 2.5 mM EDTA, pH 7.5, and approximately 0.29 mg/mL papain in the buffer. The substrate used was Cbz-Phe-Arg-*p*NA at 53.7 μM in the same buffer. The release of *p*-nitroanilide was measured at 405 nm and room temperature with a Molecular Devices Thermomax microplate reader.

Caspase-3, -6, and -8 Assays. Assays using the fluorogenic substrate Ac-Asp-Glu-Val-Asp-AMC ($\lambda_{ex} = 360$ nm, $\lambda_{em} = 465$ nm) were carried out in a Tecan Spectra Fluor microplate reader at 37 °C. Inhibition rates were determined by the progress curve method. The concentration of the caspase-3 stock solution was 2 nM in the assay buffer. Assay buffer consists of a 1:1 mixture of 40 mM Pipes, 200 mM NaCl, 0.2% (w/v) CHAPS, and 20% (w/v) sucrose to 20 mM DTT solution in H_2O at pH 7.2. The concentration of the substrate stock solution was 2 mM in DMSO. The enzyme was preactivated for 10 min at 37 °C in the assay buffer. The standard 100 μL reaction was started by adding 40 μL of assay buffer, 5 μL of various amounts of inhibitor (stock solution concentrations varied from 5×10^{-3} to 4.84×10^{-7} M in DMSO), and 5 μL of substrate in DMSO (100 μM final concentration) at 37 °C. Enzyme stock solution (50 μL of 2 nM; final concentration of 1 nM) was added to the mixture after 1 min, and measurements were started immediately for 20 min at 37 °C. Inhibition experiments were repeated in duplicate, and standard deviations were determined.

Caspase-6 kinetic assays were performed using the same conditions and the same substrate (Ac-Asp-Glu-Val-Asp-AMC, 2 mM stock solution in DMSO). The enzyme stock solution was 10 nM (final concentration in the well, 5 nM) in the assay buffer. The inhibitor stock solution concentrations varied from 5×10^{-3} to 2.42×10^{-6} M in DMSO.

Caspase-8 kinetic assays were performed using the same conditions and the same substrate (Ac-Asp-Glu-Val-Asp-AMC, 2 mM stock solution in DMSO). The enzyme stock solution was 100 nM (final concentration in the well, 50 nM) in the assay buffer. The inhibitor stock solution concentrations varied from 5×10^{-3} to 2.42×10^{-6} M in DMSO.

K_M values for Ac-Asp-Glu-Val-Asp-AMC with caspase-3 ($K_M = 9.7$ μM), caspase-6 ($K_M = 236.35$ μM), and caspase-8 ($K_M = 6.79$ μM) were determined (G. Salvesen, personal communication). The k_2 values are 11.3-fold higher than the apparent rate for caspase-3 because [S] was 100 mM and $K_M = 9.7$ μM. The k_2 values are 1.4-fold higher than the apparent rate for caspase-6 because [S] was 100 mM and $K_M = 236.35$ μM. The k_2 values are 15.7-fold higher than the apparent rate for caspase-8 because [S] was 100 mM and $K_M = 6.79$ μM.

Gingipain K and Clostripain Assays. Clostripain was purchased from Sigma Chemical Co. (St. Louis, MO) as a solid which was dissolved in an activation solution of 8 mM DTT at a concentration of 5.962 μM and stored at −20 °C prior to use. The inhibition of clostripain began with the addition of 25 μL of stock inhibitor solution (concentration varies by inhibitor) in DMSO to a solution of 250 μL of 20 mM Tris-HCl, 10 mM $CaCl_2$, 0.005% Brij 35, 2 mM DTT buffer at pH 7.6 (clostripain buffer), and 5 μL of the stock enzyme solution. Aliquots (25 μL) of this incubation mixture were taken at various time points and added to a solution containing 100 μL of the clostripain buffer and 5 μL of Cbz-Phe-Arg-AMC substrate solution (139 μM) in DMSO. The enzymatic activity was monitored by following the change in fluorescence at 465 nm. All data obtained were processed by pseudo-first-order kinetics.

Gingipain K stock solution was obtained from J. Potempa (University of Georgia, Athens, GA) in a buffer containing 20 mM Bis-Tris, 150 mM NaCl, 5 mM $CaCl_2$, 0.02% NaN_3, at pH 8.0, at a concentration of 9 μM. The solution was stored at −20 °C prior to use. Before the enzyme was used, an aliquot (1 μL) of the stock enzyme was diluted to a concentration of 4.61 nM in 1.951 mL of 0.2 M Tris-HCl, 0.1 M NaCl, 5 mM $CaCl_2$, 2 mM DTT at pH 8.0 (gingipain K buffer) and kept at 0 °C. This solution was used only for 1 day, as freezing the enzyme at this concentration destroyed all activity. The inhibition of gingipain K began with the addition of 25 μL of stock inhibitor solution (concentration varies by inhibitor) in DMSO to 244 μL of the diluted enzyme solution (4.61 nM) in gingipain K buffer warmed to room temperature. Aliquots (20 μL) of this inhibition mixture were taken at various time points and added to a solution containing 100 μL of the gingipain K buffer and 5 μL of Suc-Ala-Phe-Lys-AMC·TFA as the substrate (910 μM stock) in DMSO. Activity was monitored by following the change in fluorescence at 465 nm. The data for gingipain K were processed by pseudo-first-order kinetics.

Stability Studies of Aza-peptidyl Michael Acceptors. An aliquot (15 μL) of 10 mM aza-peptidyl Michael acceptors in DMSO was added to AE buffer (485 μL, pH 5.8 or 6.8) containing DTT (3.4 mM) and the reaction of the double bond of the inhibitor with DTT monitored spectrophotometrically at 250 nm over time. The k_{obs} was obtained from pseudo-first-order rate plots of $\ln(A_t/A_0)$ versus time and the half-lives were derived from $t_{1/2} = \ln 2/k_{obs}$.

^{1}H NMR Study. The progress of thioalkylation of Cbz-Ala-Ala-AAsn-CH═CHCOOEt (**7a**) by benzylmercaptan was followed by ^{1}H NMR on a 400 MHz Varian spectrometer in DMSO-d_6 over a period of 48 h. All solutions were purged with argon and a 10 equiv excess of benzylmercaptan over the peptidyl inhibitor was used. A spectrum was taken every 5 h.

Cbz-Ala-Ala-AAsn-CH═CHCOOEt (7a). Time 0 h. ^{1}H NMR (DMSO-d_6): 1.15–1.3 (m, 9H, CH_3), 3.3 (m, 2H, NCH_2CO), 4.05 (m, 1H, α-H), 4.1–4.2 (q, 2H, OCH_2CH_3), 4.3 (m, 1H, α-H), 5.0 (m, 2H, Cbz), 6.55–6.65 (d, 1H, CH═CH), 7.5 (s, 1H, NH), 8.15 (d, 1H, NH). Benzylmercaptan signals are dominant in the aromatic region and are therefore not included.

Time 48 h. ^{1}H NMR (DMSO-d_6): 1.15–1.3 (m, 9H, CH_3), 3.3 (m, 2H, NCH_2CO), 4.05 (m, 2H, α-H and CH-S), 4.1–4.2 (q, 2H, OCH_2CH_3), 4.3 (m, 1H, α-H), 5.0 (m, 2H, Cbz), 7.6 (s, 1H, NH), 8.15 (d, 1H, NH). Benzylmercaptan signals are dominant in the aromatic region and are therefore not included. Changes in the spectrum are as follows: The vinyl proton at 6.55–6.65 ppm has disappeared. A new peak has formed at 4.05 ppm.

General Procedure for the Synthesis of Aza-peptide Michael Acceptors: HOBt/EDC Coupling Method. To a stirred solution of the peptidyl hydrazide precursor (**4**, 1 equiv) and the fumaric acid or propenoic acid precursor (**2a**−**x** or **6a**−**f**, 2 equiv), HOBt (2 equiv) and EDC (2 equiv) were added. 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 $NaHCO_3$, saturated NaCl, dried over $MgSO_4$, and concentrated. Chromatography on a silica gel column using 10% MeOH/CH_2Cl_2 as the eluent afforded the aza-peptidyl fumarate or acrylate derivatives. MS and ^{1}H NMR ($CDCl_3$ or DMSO-d_6) data were consistent with the proposed structures.

General Procedure for the Synthesis of Fumaric Acid Monoamides: 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 (*i*BCF, 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 mixture was stirred for 4 h at room temperature. The DMF was evaporated, and the residue was washed and purified using the same procedure as described above for the EDC/HOBt coupling method. MS and ^{1}H NMR (DMSO-d_6 or $CDCl_3$) results were consistent with the proposed structures.

Benzyl-(2-naphthylmethyl)amine (Bzl(2-Napth-CH_2)NH). Reductive Amination Procedure. Benzylamine (1 equiv) was dissolved in absolute ethanol. A solution of 2-naphthaldehyde (1 equiv) in absolute ethanol was added dropwise via an addition funnel to the benzylamine solution while stirring. The mixture was heated at reflux for 2 h. Sodium borohydride (2.1 equiv) was added to the mixture. The mixture was heated at reflux for 2 h. The solvent was removed under reduced pressure. The residue was dissolved in dichloromethane. The solution was washed with aqueous base (NaOH, 1 M) and dried ($MgSO_4$). The solvent was removed under reduced pressure to give a clear, colorless oil (90% yield). ^{1}H NMR ($CDCl_3$): 2.37 (s, 1H, CH_2NHCH_2), 3.84 (s, 2H, NHCH_2PH), 3.97 (s, 2H, Napth-CH_2NH), 7.23–7.50 (m, 8H, Ph, Napth), 7.76–7.81 (m, 4H, Napth).

Bis-(2-furylmethyl)amine ((2-furyl-CH_2)$_2$NH). The amine was prepared from 2-furylaldehyde and 2-fufurylamine following the reductive amination procedure (89% yield). ^{1}H NMR ($CDCl_3$): 3.84 (s, 4H, CH_2NHCH_2), 6.29–6.33 (2 dd, 4H, furyl), 7.37–7.38 (dd, 2H, furyl).

Benzyl-(4-methoxybenzyl)amine (Bzl(Bzl-4-OMe)NH). The amine was prepared prepared from *p*-anisaldehyde and benzylamine following the reductive amination procedure (98% yield). ^{1}H NMR ($CDCl_3$): 2.16 (s, 1H, CH_2NHCH_2), 3.74 (s, 3H, CH_3O), 3.78–3.86 (m, 4H, CH_2NHCH_2), 6.83–6.97 (m, 2H, MeO-*Ph*), 7.24–7.33 (m, 7H, Ph).

Benzyl-(4-fluorobenzyl)amine (Bzl(4-F-Bzl)NH). The amine was prepared from 4-fluorobenzylamine and benzaldehyde following the reductive amination procedure (89% yield). ^{1}H NMR ($CDCl_3$): 2.05 (s, 1H, CH_2NHCH_2), 3.77–3.79 (d, 4H, CH_2NHCH_2), 6.96–7.02 (m, 4H, F-Ph), 7.19–7.23 (m, 5H, Ph).

Benzyl-(1-naphthylmethyl)amine (Bzl(1-Napth-CH_2)NH). The amine was prepared from 1-naphthaldehyde and benzylamine following the reductive amination procedure (97% yield). ^{1}H NMR ($CDCl_3$): 2.03 (s, 1H, CH_2NHCH_2), 3.91 (s, 2H, NHCH_2Ph), 4.24 (s, 2H, Napth-CH_2NH), 7.22–7.51 (m, 10H, Ph, Napth), 7.71–7.85 (m, 2H, Napth).

***N*-Benzyloxycarbonylalanylalanyl Hydrazide (3, Cbz-Ala-Ala-NHNH$_2$).** **3** was synthesized from Cbz-Ala-Ala-OMe by hydrazinolysis. Anhydrous hydrazine (10 equiv) was added to a solution of Cbz-Ala-Ala-OMe (1 equiv) in MeOH at room temperature, and the resulting mixture was then stirred at room temperature for 16 h. Excess hydrazine and solvent were removed by evaporation. The resulting residue was washed with ethanol and ether to give Cbz-Ala-Ala-NHNH$_2$ as a white solid (57% yield). ^{1}H NMR (DMSO-d_6): 1.1–1.3 (d, CH_3), 4.0–4.1 (m, 1H, α-H), 4.1–4.3 (m, 2H, α-H and NH), 5.05 (s, 2H, Cbz), 7.3–7.4 (m, 5H, Ph), 7.5 (d, 1H, NH), 7.9 (d, 1H, NH), 9.05 (s, 1H, NH).

***N*^{1}-(*N*-Benzyloxycarbonylalanylalanyl)-*N*^{2}-ethoxycarbonylmethylhydrazine (Cbz-Ala-Ala-NHNHCH$_2$COOEt).** Ethyl bromoacetate (1.1 equiv) was added dropwise to a stirred solution of Cbz-Ala-Ala-NHNH$_2$ (1 equiv) and NMM (1.1 equiv) in DMF that was cooled to −10 °C. The resulting solution was stirred for 30 min at −10 °C, after which the mixture was allowed to react at room temperature for 36 h. The DMF was evaporated, and the residue was purified on a silica gel column using 1:9 MeOH/CH_2Cl_2 as the eluting solvent system to give the ethyl ester as a white solid (yield 36%). ^{1}H NMR (DMSO-d_6): 1.18 (t, 9H, CH_3), 3.5 (d, 2H, NCH_2COOEt), 4.0–4.15 (m, 3H, α-H and OCH_2CH_3), 4.2 (m, 1H, α-H), 5.03 (m, 2H, Cbz), 5.18 (m, 1H, NH), 7.22–7.40 (m, 5H, Ph), 7.4–7.5 (d, 1H, NH), 7.9 (m, 1H, NH), 9.35 (m, 1H, NH). MS (FAB) *m*/*z* 395 [(M + 1)$^+$].

***N*^{1}-(*N*-Benzyloxycarbonylalanylalanyl)-*N*^{2}-carbamoylmethylhydrazine (4, Cbz-Ala-Ala-NHNHCH$_2$CONH$_2$).** The ethyl ester Cbz-Ala-Ala-NHNHCH$_2$COOEt (1 equiv) was dissolved in a 9 M solution (100 equiv) of NH_3 in methanol and a small amount of DMF and allowed to stir on an ice bath. To this solution was added catalytic NaCN (0.1 equiv). The flask was closed with a rubber septum and allowed to stir at 0 °C for 3 days. The solvent was evaporated and the product was precipitated with 1:9 MeOH/CH_2Cl_2 and methanol to yield a white solid (68% yield). ^{1}H NMR (DMSO-d_6): 1.18 (d, 6H, CH_3), 3.2 (d, 2H, NCH_2CONH_2), 4.0–4.12 (m, 1H, α-H), 4.2 (m, 1H, α-H), 5.03 (m, 2H, Cbz), 5.22 (m, 1H, NH), 7.18 (d, 1H, NH), 7.3–7.5 (m, 6H, Ph and NH), 8.0 (m, 1H, NH), 9.38 (m, 1H, NH). MS (FAB) *m*/*z* 366 [(M + 1)$^+$]. HRMS (FAB) calculated for $C_{16}H_{24}N_5O_5$: 366.177 74. Observed *m*/*z* 366.176 65.

***N*^{2}-(*N*-Benzyloxycarbonylalanylalanyl)-*N*^{1}-carbamoylmethyl-*N*^{1}-*trans*-(3-ethoxycarbonylpropenoyl)hydrazine (7a, Cbz-Ala-Ala-AAsn-CH═CHCOOEt). 7a** was synthesized using the EDC/HOBt coupling method, purified by chromatography on a silica gel column using 1:13 MeOH/CH_2Cl_2 as the eluent, and then recrystallized from EtOAc/hexane; white solid, yield 33%. ^{1}H NMR (DMSO-d_6): 1.15–1.3 (m, 9H, CH_3), 3.3 (m, 2H, NCH_2CO), 4.05 (m, 1H, α-H), 4.1–4.2 (q, 2H, OCH_2CH_3), 4.3 (m, 1H, α-H), 5.0 (m, 2H, Cbz), 6.55–6.65 (d, 1H, CH═CH), 7.1–7.2 (m, 2H, NH and CH═CH), 7.2–7.4 (m, 5H, Ph), 7.5 (s, 1H, NH), 8.15 (d, 1H, NH). HRMS (FAB) calculated for $C_{22}H_{30}N_5O_8$: 492.209 44. Observed *m*/*z* 492.205 65.

***trans*-3-Benzyloxycarbonylpropenoic Acid or Monobenzyl Fumarate (HOOCCH═CHCOOBzl).** Equimolar amounts of fumaric acid and benzyl alcohol were dissolved in anhydrous DMF. NMM (1 equiv) was added at 0 °C followed by EDC after 15 min. The mixture was stirred overnight at room temperature. 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 ($MgSO_4$). The solvent was evaporated and the crude residue was subjected to column chromatography (MeOH/CH_2Cl_2) to give a white powder (51% yield). ^{1}H NMR (DMSO-d_6): 5.21 (s, 2H, CH═CH-COOCH_2Ph), 6.73 (s, 2H, CH═CH-COOCH_2Ph), 7.29–7.43 (m, 5H, Ph). MS (ESI) m/z 207 [(M + 1)$^+$].

***N*^{2}-(*N*-Benzyloxycarbonylalanylalanyl)-*N*^{1}-*trans*-(3-benzyloxycarbonylpropenoyl)-*N*^{1}-carbamoylmethylhydrazine (7b, Cbz-Ala-Ala-AAsn-CH═CHCOOBzl).** This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography on silica gel using 10% MeOH/CH_2Cl_2 as the eluent and then recrystallized from EtOAc/hexane to give a white powder (20% yield). ^{1}H NMR ($(CD_3)_2CO$): 1.37 (m, 6H, 2 × Ala-CH_3), 2.88 (s, 2H, NCH_2CO), 4.21–4.24 (m, 1H, α-H), 4.50 (m, 1H, α-H), 5.10 (m, 2H, Cbz), 5.24 (s, 2H, O-CH_2-Ph), 6.58–6.33 (m, 2H, NH and CH═CH), 6.74–6.88 (d, 1H, CH═CH), 7.31–7.44 (m, 11H, 2 × Ph and NH), 7.76 (s, 1H, NH), 8.16 (d, 1H, NH), 9.89 (s, 1H, NH). MS (ESI) m/z 554 [(M + 1)$^+$]. HRMS (ESI) calculated for $C_{27}H_{32}N_5O_8$: 554.2219. Observed m/z 554.225 088.

***trans*-3-Diethylcarbamoylpropenoic Acid Ethyl Ester (EtOOCCH═CHCONEt$_2$).** This compound was obtained by mixed anhydride coupling of equimolar amounts of monoethyl fumarate and diethylamine. The crude product was recrystallized from hexane/EtOAc to give a white powder (95% yield). ^{1}H NMR ($CDCl_3$): 1.17–1.21 (t, 3H, N-CH_2CH_3), 1.22–1.24 (t, 3H, N-CH_2CH_3), 1.32 (t, 3H, CH_3CH_2O), 3.40–3.47 (m, 4H, 2 × N-CH_2), 4.24–4.26 (q, 2H, CH_3CH_2O), 6.78–6.82 (d, 1H, J = 14.8 Hz, CH═CHCON), 7.31–7.35 (d, 1H, J = 14.8 Hz, CH═CHCON).

***trans*-3-Diethylcarbamoylpropenoic Acid (2a, HOOCCH═CHCONEt$_2$).** EtOOCCH═CHCONEt$_2$ was hydrolyzed in MeOH using NaOH (1 M aqueous, 1.1 equiv) under standard deblocking conditions to give a white crystalline solid (53% yield).

***N*^{2}-(*N*-Benzyloxycarbonylalanylalanyl)-*N*^{1}-carbamoylmethyl-*N*^{1}-*trans*-(3-diethylcarbamoylpropenoyl)hydrazine (5a, Cbz-Ala-Ala-AAsn-CH═CHCONEt$_2$).** This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography on silica gel using 10% MeOH/CH_2Cl_2 as the eluent and then recrystallized from EtOAc/hexane to give a white powder (11% yield). ^{1}H NMR (DMSO-d_6): 1.02–1.05 (t, 3H, NCH_2CH_3), 1.08–1.11 (t, 3H, NCH_2CH_3), 1.17–1.19 (d, 2H, Ala-CH_3), 1.24–1.26 (d, 2H, Ala-CH_3), 3.20–3.32 (d, 6H, NCH_2CO and 2 × N-CH_2), 4.02–4.07 (m, 1H, α-H), 4.27–4.31 (m, 1H, α-H), 4.99 (m, 2H, Cbz), 7.00–7.04 (d, 1H, CH═CHCON), 7.18 (s, 1H, NH), 7.33 (m, 6H, Ph and CH═CHCON), 7.39 (d, 1H, NH), 8.20 (d, 1H, NH), 10.68 (s, 1H, NH). MS (ESI) m/z 519 [(M + 1)$^+$]. HRMS (ESI) calculated for $C_{24}H_{35}N_6O_7$: 519.2608. Observed m/z 519.2657.

***trans*-3-Dibutylcarbamoylpropenoic Acid Ethyl Ester (EtOOCCH═CHCON(*n*Bu)$_2$).** This compound was obtained by mixed anhydride coupling of equimolar amounts of monoethyl fumarate and dibutylamine. The crude product was recrystallized from hexane/EtOAc to give a white powder (95% yield). ^{1}H NMR ($CDCl_3$): 0.95 (m, 6H, 2 × nBu-CH_3), 1.32 (m, 7H, 2 × $CH_2CH_2CH_2CH_3$ and CH_3CH_2O), 1.51–1.59 (m, 4H, 2 × $CH_2CH_2CH_2CH_3$), 3.33 (t, 2H, N-CH_2), 3.39 (t, 2H, N-CH_2), 4.24 (q, 2H, CH_3CH_2O), 6.76–6.81 (d, 1H, J = 15.6 Hz, CH═CHCON), 7.33–7.36 (d, 1H, J = 15.2 Hz, CH═CHCON).

***trans*-3-Dibutylcarbamoylpropenoic Acid (2b, HOOCCH═CHCON(*n*Bu)$_2$).** EtOOCCH═CHCON(nBu)$_2$ was hydrolyzed in MeOH using NaOH (1 M aqueous, 1.1 equiv) under standard deblocking conditions to give a clear, colorless syrup (80% yield). ^{1}H NMR ($CDCl_3$): 0.95 (m, 6H, 2 × nBu-CH_3), 1.30–1.37 (m, 4H, 2 × $CH_2CH_2CH_2CH_3$), 1.53–1.59 (m, 4H, 2 × CH_2CH_2-CH_2CH_3), 3.33 (t, 2H, N-CH_2), 3.39 (t, 2H, N-CH_2), 6.79–6.83 (d, 1H, J = 15.6 Hz, CH═CHCON), 7.33–7.36 (d, 1H, J = 15.2 Hz, CH═CHCON).

***N*^{2}-(*N*-Benzyloxycarbonylalanylalanyl)-*N*^{1}-carbamoylmethyl-*N*^{1}-*trans*-(3-dibutylcarbamoylpropenoyl)hydrazine (5b, Cbz-Ala-Ala-AAsn-CH═CHCON(*n*Bu)$_2$).** This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography on silica gel using 10% MeOH/CH_2Cl_2 as the eluent and then recrystallized from EtOAc/hexane to give a white powder (15% yield). ^{1}H NMR (DMSO-d_6): 0.95 (m, 6H, 2 × nBu-CH_3), 1.11–1.27 (m, 6H, 2 × Ala-CH_3), 1.30–1.37 (m, 4H, 2 × $CH_2CH_2CH_2CH_3$), 1.53–1.59 (m, 4H, 2 × $CH_2CH_2CH_2CH_3$), 3.20–3.32 (d, 6H, NCH_2CO and 2 × N-CH_2), 4.00–4.01 (m, 1H, α-H), 4.25–4.30 (m, 1H, α-H), 4.99 (m, 2H, Cbz), 7.00–7.04 (d, 1H, CH═CHCON), 7.21 (s, 1H, NH), 7.33 (m, 6H, Ph and CH═CHCON), 7.42 (d, 1H, NH), 8.20 (d, 1H, NH), 10.73 (s, 1H, NH). MS (FAB) m/z 575 [(M + 1)$^+$]. HRMS (FAB) calculated for $C_{28}H_{43}N_6O_7$: 575.320 52. Observed m/z 575.319 32.

***trans*-3-(1-Piperidylcarbonyl)propenoic Acid Ethyl Ester (EtOOCCH═CHCO-Pip).** This compound was obtained by mixed anhydride coupling of equimolar amounts of monoethyl fumarate and piperidine to give a clear syrup (99% yield).

***trans*-3-(1-Piperidylcarbonyl)propenoic Acid (2c, HOOCCH═CHCO-Pip).** EtOOCCH═CHCO-Pip was hydrolyzed in MeOH using NaOH (1 M aqueous, 1.1 equiv) under standard deblocking conditions to give a clear, colorless syrup which was recrystallized overnight from hexane/EtOAc to give a white powder (27% yield). ^{1}H NMR (DMSO-d_6): 1.46–1.60 (m, 6H, 3 × piperidine CH_2), 3.45–3.49 (m, 4H, CH_2-N-CH_2), 6.41–6.44 (d, 1H, J = 15.2 Hz, CH═CHCON), 7.33–7.37 (d, 1H, J = 15.2 Hz, CH═CHCON).

***N*^{2}-(*N*-Benzyloxycarbonylalanylalanyl)-*N*^{1}-carbamoylmethyl-*N*^{1}-*trans*-(3-(1-piperidylcarbonyl)propenoyl)hydrazine (5c, Cbz-Ala-Ala-AAsn-CH═CHCO-Pip).** This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography on silica gel using 10% MeOH/CH_2Cl_2 as the eluent and recrystallized from hexane/EtOAc to give a white powder (10% yield). ^{1}H NMR ($(CD_3)CO$): 1.28 (m, 2H, piperidine-CH_2), 1.37 (d, 3H, Ala-CH_3), 1.41 (d, 3H, Ala-CH_3), 1.52–1.67 (m, 4H, 2 × piperidine-CH_2), 2.80 (s, 2H, NCH_2CO), 3.55 (m, 4H, CH_2-N-CH_2), 4.24 (m, 1H, α-H), 4.50 (m, 1H, α-H), 5.10 (m, 2H, Cbz), 7.11–7.15 (d, 1H, J = 14.8 Hz, CH═CHCON), 7.30–7.44 (m, 7H, CH═CHCON and Ph and NH), 7.77 (s, 1H, NH), 9.82 (s, 1H, NH). MS (ESI) m/z 531 [(M + 1)$^+$]. HRMS (ESI) calculated for $C_{25}H_{35}N_6O_7$: 531.2532. Observed m/z 531.256 723.

***trans*-3-Phenylcarbamoylpropenoic Acid Ethyl Ester (EtOOCCH═CHCONHPh).** This compound was obtained by mixed anhydride coupling of equimolar amounts of monoethyl fumarate and aniline to give a white solid (59% yield). ^{1}H NMR ($CDCl_3$): 1.34 (t, 3H, CH_3CH_2), 4.29 (q, 2H, CH_3CH_2), 6.93–6.97 (d, 1H, J = 15.2 Hz, CH═CHCON), 7.10–7.14 (d, 1H, J = 15.2 Hz, CH═CHCON), 7.15 (t, 1H, Ph), 7.34 (t, 2H, Ph), 7.61 (d, 2H, Ph), 7.85 (s, 1H, NH).

***trans*-3-Phenylcarbamoylpropenoic Acid (2d, HOOCCH═CHCONHPh).** EtOOCCH═CHCONHPh was hydrolyzed in MeOH 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): 6.61–6.65 (d, 1H, J = 15.2 Hz, CH═CHCON), 7.02–7.14 (m, 1H, CH═CHCON), 7.14–7.31 (m, 2H, Ph), 7.32 (t, 2H, Ph), 7.66 (d, 2H, Ph), 10.47 (s, 1H, NH).

***N*^{2}-(*N*-Benzyloxycarbonylalanylalanyl)-*N*^{1}-carbamoylmethyl-*N*^{1}-*trans*-(3-phenylcarbamoylpropenoyl)hydrazine (5d, Cbz-Ala-Ala-AAsn-CH═CHCONHPh).** This compound was obtained using the HOBt/EDC coupling method with minimal $NaHCO_3$ washing during the workup. The product was isolated as a yellow solid without chromatography by recrystallization from 10% MeOH/CH_2Cl_2 (21% yield). ^{1}H NMR (DMSO-d_6): 1.18–1.27 (m, 6H, 2 × Ala-CH_3), 3.20–3.32 (d, 2H, NCH_2CO), 4.04–4.08 (m, 1H, α-H), 4.28–4.31 (m, 1H, α-H), 4.99 (m, 2H, Cbz), 7.05–7.42 (m, 11H, 2 × Ph, NH CH═CHCON and CH═CHCON), 7.63–7.65 (d, 2H, Ph), 7.52 (s, 1H, NH), 8.15 (d, 1H, NH), 10.42 (s, 1H, NH), 10.72 (s, 1H, NH). MS (ESI) m/z 539 [(M + 1)$^+$]. HRMS (ESI) calculated for $C_{26}H_{31}N_6O_7$: 539.2208. Observed m/z 539.2254.

***trans*-3-Benzylcarbamoylpropenoic Acid Ethyl Ester (EtOOCCH═CHCONHBzl).** This compound was obtained by mixed anhydride coupling of equimolar amounts of monoethyl fumarate and benzylamine to give a white powder (72% yield).

***trans*-3-Benzylcarbamoylpropenoic Acid (2e, HOOCCH═CHCONHBzl).** EtOOCCH═CHCONHBzl was hydrolyzed in MeOH 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): 4.37 (d, 2H, N-CH_2-Ph), 6.52–6.56 (d, 1H, J = 15.2 Hz, CH═CHCON), 6.94–6.98 (d, 1H, J = 15.6 Hz, CH═CHCON), 7.14–7.31 (m, 5H, Ph), 8.97 (t, 1H, NH).

***N*^{2}-(*N*-Benzyloxycarbonylalanylalanyl)-*N*^{1}-carbamoylmethyl-*N*^{1}-*trans*-(3-benzylcarbamoylpropenoyl)hydrazine (5e, Cbz-Ala-Ala-AAsn-CH═CHCONHBzl).** This compound was obtained using the HOBt/EDC coupling method with minimal washing during the workup and purified by recrystallization from ice-cold EtOAc without column chromatography (18% yield). ^{1}H NMR (DMSO-d_6): 1.17–1.26 (m, 6H, 2 × Ala-CH_3), 3.30–3.32 (d, 2H, NCH_2CO), 4.02–4.07 (m, 1H, α-H), 4.27–4.32 (m, 1H, α-H), 4.35–4.36 (d, 2H, CH_2Ph), 4.99 (m, 2H, Cbz), 6.89–6.92 (d, 1H, J = 14.2 Hz, CH═CHCON), 7.06–7.10 (d, 1H, J = 14.8 Hz, CH═CHCON), 7.16–7.41 (m, 11H, 2 × Ph and NH), 7.42 (d, 1H, NH), 8.16 (d, 1H, NH), 8.94 (t, 1H, NH), 10.72 (s, 1H, NH). MS (ESI) m/z 553 [(M + 1)$^+$]. HRMS (ESI) calculated for $C_{27}H_{33}N_6O_7$: 553.2397. Observed m/z 553.2411.

***trans*-3-(4-Fluorobenzylcarbamoyl)propenoic Acid Ethyl Ester (EtOOCCH═CHCONH-Bzl-4-F).** This compound was obtained by mixed anhydride coupling of equimolar amounts of monoethyl fumarate and 4-fluorobenzylamine to give a pink solid (66% yield). ^{1}H NMR ($CDCl_3$): 1.32 (t, 3H, CH_2CH_3), 4.21 (q, 2H, CH_2CH_3), 4.47 (d, 2H, N-CH_2-Ph), 6.52–6.56 (d, 1H, J = 15.2 Hz, CH═CHCON), 6.94–6.98 (d, 1H, J = 15.6 Hz, CH═CHCON), 6.99–7.04 (m, 2H, Ph), 7.20–7.27 (m, 2H, Ph).

***trans*-3-(4-Fluorobenzylcarbamoyl)propenoic Acid (2f, HOOCCH═CHCONH-Bzl-4-F).** EtOOCCH═CHCONH-Bzl-4-F was hydrolyzed in MeOH using NaOH (1 M aqueous, 1.1 equiv) under standard deblocking conditions to give a clear, colorless syrup (45% yield). ^{1}H NMR (DMSO-d_6): 4.34–4.35 (d, 2H, N-CH_2-Ph), 6.52–6.55 (d, 1H, J = 15.2 Hz, CH═CHCON), 6.93–6.94 (d, 1H, J = 15.6 Hz, CH═CHCON), 7.14–7.16 (t, 2H, Ph), 7.27–7.31 (t, 2H, Ph), 8.99 (t, 1H, NH).

***N*^{2}-(*N*-Benzyloxycarbonylalanylalanyl)-*N*^{1}-carbamoylmethyl-*N*^{1}-*trans*-(3-(4-fluorobenzyl)carbamoylpropenoyl)hydrazine (5f, Cbz-Ala-Ala-AAsn-CH═CHCONH-Bzl-4-F).** This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent to give a pink powder (8% yield). ^{1}H NMR (DMSO-d_6): 1.17–1.26 (m, 6H, 2 × Ala-CH_3), 3.30–3.32 (d, 2H, NCH_2CO), 4.02–4.07 (m, 1H, α-H), 4.27–4.35 (m, 3H, α-H and CH_2PhF), 4.99 (m, 2H, Cbz), 6.88–6.92 (d, 1H, J = 16 Hz, CH═CHCON), 7.06–7.32 (m, 11H, CH═CHCON and NH and 2 × Ph), 7.43 (d, 1H, NH), 7.53 (s, 1H, NH), 8.19 (d, 1H, NH), 8.97 (t, 1H, NH), 10.72 (s, 1H, NH). MS (ESI) m/z 571 [(M + 1)$^+$]. HRMS (ESI) calculated for $C_{27}H_{32}N_6O_7F$: 571.2323. Observed m/z 571.231 651.

***trans*-3-Phenethylcarbamoylpropenoic Acid Ethyl Ester (EtOOCCH═CHCONHCH$_2$CH$_2$Ph).** This compound was obtained by mixed anhydride coupling of equimolar amounts of monoethyl fumarate and phenethylamine to give a clear colorless syrup (78% yield).

***trans*-3-Phenethylcarbamoylpropenoic Acid (2g, HOOCCH═CHCONHCH$_2$CH$_2$Ph).** EtOOCCH═CHCONHCH$_2$CH$_2$Ph was hydrolyzed in MeOH 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): 3.54 (t, 2H, N-CH_2-CH_2-Ph), 3.61 (t, 2H, N-CH_2-CH_2-Ph) 6.43–6.47 (d, 1H, J = 15.2 Hz, CH═CHCON), 6.98–7.02 (d, 1H, J = 15.6 Hz, CH═CHCON), 7.14–7.31 (m, 5H, Ph).

***N*^{2}-(*N*-Benzyloxycarbonylalanylalanyl)-*N*^{1}-carbamoylmethyl-*N*^{1}-*trans*-(3-phenethylcarbamoylpropenoyl)hydrazine (5g, Cbz-Ala-Ala-AAsn-CH═CHCONHCH$_2$CH$_2$Ph).** This compound was obtained using the HOBt/EDC coupling method with minimal washing during the workup and purified by column chromatography on silica gel using 10% MeOH/CH_2Cl_2 as the eluent and then washed with EtOAc to give a white powder (12% yield). ^{1}H NMR (DMSO-d_6): 1.18–1.27 (m, 6H, 2 × Ala-CH_3), 2.74 (m, 2H, N-$CH_2$$CH_2$Ph), 3.20–3.32 (d, 2H, NCH_2CO), 3.52–3.58 (m, 2H, N-$CH_2$$CH_2$Ph), 4.04–4.08 (m, 1H, α-H), 4.28–4.31 (m, 1H, α-H), 4.99 (m, 2H, Cbz), 6.86–6.89 (d, 1H, J = 14.4 Hz, CH═CHCON), 7.04–7.08 (d, 1H, J = 14.8 Hz, CH═CHCON), 7.16–7.41 (m, 11H, 2 × Ph and NH), 7.50 (d, 1H, NH), 8.16 (d, 1H, NH), 8.53 (s, 1H, NH), 10.72 (s, 1H, NH). MS (ESI) m/z 567 [(M + 1)$^+$]. HRMS (ESI) calculated for $C_{28}H_{35}N_6O_7$: 567.2596. Observed m/z 567.256 723.

***trans*-*N*-Methylphenylcarbamoylpropenoic Acid Ethyl Ester (EtOOCCH═CHCON(CH$_3$)Ph).** This compound was obtained by mixed anhydride coupling of equimolar amounts of monoethyl fumarate and *N*-methylaniline to give a white solid (99% yield). ^{1}H NMR ($CDCl_3$): 1.25 (t, 3H, CH_3CH_2), 3.39 (s, 3H, N-CH_3), 4.17 (q, 2H, CH_3CH_2), 6.93–6.97 (d, 1H, J = 15.2 Hz, CH═CHCON), 7.10–7.14 (d, 1H, J = 15.2 Hz, CH═CHCON), 7.15 (t, 1H, Ph), 7.34 (t, 2H, Ph), 7.61 (d, 2H, Ph).

***trans*-*N*-Methylphenylcarbamoylpropenoic Acid (2h, HOOCCH═CHCON(CH$_3$)Ph).** EtOOCCH═CHCON(CH$_3$)Ph was hydrolyzed in MeOH using NaOH (1 M aqueous, 1.1 equiv) under standard deblocking conditions to give a white solid (29% yield). ^{1}H NMR (DMSO-d_6): 3.14 (s, 3H, N-CH_3), 6.50–6.54 (d, 1H, J = 15.2 Hz, CH═CHCON), 6.60–6.64 (d, 1H, J = 15.2 Hz, CH═CHCON), 7.32 (t, 2H, Ph), 7.40 (d, 1H, Ph), 7.47 (d, 1H, Ph).

***N*^{2}-(*N*-Benzyloxycarbonylalanylalanyl)-*N*^{1}-carbamoylmethyl-*N*^{1}-*trans*-(3-(methylphenylcarbamoyl)propenoyl)hydrazine (5h, Cbz-Ala-Ala-AAsn-CH═CHCON(CH$_3$)Ph).** This compound was obtained using the HOBt/EDC coupling method. The product was isolated by chromatography with 10% MeOH/CH_2Cl_2 as the eluent and recrystallized from hexane/EtOAc to give a white powder (34% yield). ^{1}H NMR (DMSO-d_6): 1.18–1.27 (m, 6H, 2 × Ala-CH_3), 3.20–3.32 (m, 5H, NCH_2CO and N-CH_3), 4.02–4.07 (m, 1H, α-H), 4.30 (m, 1H, α-H), 4.99 (m, 2H, Cbz), 6.56 (d, 1H, CH═CHCON), 7.06–7.10 (d, 1H, CH═CHCON), 7.14 (s, 1H, NH), 7.29–7.46 (m, 11H, 2 × Ph, NH), 8.14 (d, 1H, NH), 10.65 (s, 1H, NH). MS (ESI) m/z 553 [(M + 1)$^+$]. HRMS (ESI) calculated for $C_{27}H_{33}N_6O_7$: 553.2356. Observed m/z 553.2411.

***trans*-3-Benzylmethylcarbamoylpropenoic Acid Ethyl Ester (EtOOCCH═CHCON(CH$_3$)Bzl).** This compound was obtained by mixed anhydride coupling of equimolar amounts of monoethyl fumarate and *N*-methylbenzylamine to give a clear, pink syrup (76% yield).

***trans*-3-Benzylmethylcarbamoylpropenoic Acid (2i, HOOCCH═CHCON(CH$_3$)Bzl).** EtOOCCH═CHCON(CH$_3$)Bzl was hydrolyzed in MeOH using NaOH (1 M aqueous, 1.1 equiv) under standard deblocking conditions to give a clear, colorless syrup (77% yield). ^{1}H NMR ($CDCl_3$): 3.03 (s, 3H, N-CH_3), 4.62–4.67 (d, 2H, N-CH_2-Ph), 6.83–6.87 (d, 1H, J = 16 Hz, CH═CHCON), 7.15–7.17 (d, 1H, J = 8 Hz, CH═CHCON), 7.25–7.50 (m, 5H, Ph).

***N*^{2}-(*N*-Benzyloxycarbonylalanylalanyl)-*N*^{1}-carbamoylmethyl-*N*^{1}-*trans*-(3-benzylmethylcarbamoylpropenoyl)hydrazine (5i, Cbz-Ala-Ala-AAsn-CH═CHCON(CH$_3$)Bzl).** This compound was synthesized using the HOBt/EDC coupling method and purified by column chromatography on silica gel using 10% MeOH/CH_2Cl_2 as the eluent and then recrystallized from EtOAc/hexane to give a white powder (54% yield). ^{1}H NMR (DMSO-d_6): 1.18–1.27 (m, 6H, 2 × Ala-CH_3), 2.89 (s, 3H, N-CH_3), 3.20–3.32 (d, 2H, NCH_2CO), 4.04–4.08 (m, 1H, α-H), 4.28–4.31 (m, 1H, α-H), 4.57 (m, 2H, N-CH_2-Ph), 4.99 (m, 2H, Cbz), 7.05–7.41 (m, 13H, CH═CHCON and CH═CHCON and 2 × Ph and NH), 7.49 (d, 1H, NH), 8.16 (d, 1H, NH), 10.72 (s, 1H, NH). MS (ESI) m/z 567 [(M + 1)$^+$]. HRMS (ESI) calculated for $C_{28}H_{35}N_6O_7$: 567.2604. Observed m/z 567.256 723.

***trans*-3-(Methyl-1-naphthylmethylcarbamoyl)propenoic Acid Ethyl Ester (EtOOCCH═CHCON(CH$_3$)CH$_2$-1-Napth).** This compound was obtained by mixed anhydride coupling of equimolar amounts of monoethyl fumarate and *N*-methyl-1-naphthylmethylamine hydrochloride to give a clear, colorless syrup (99% yield). ^{1}H NMR ($CDCl_3$): 1.31 (t, 3H, CH_3CH_2), 3.03 (s, 3H, N-CH_3) 4.29 (q, 2H, CH_3CH_2), 5.30 (s, 2H, N-CH_2-naphthyl), 6.92 (d, 1H, J =

15.2 Hz, *CH*=CHCON), 7.10–7.52 (m, 5H, naphthyl and CH=*CH*CON), 7.81–7.91 (m, 2H, naphthyl), 7.85 (d, 1H, naphthyl).

***trans*-3-(Methyl-1-naphthylmethylcarbamoyl)propenoic Acid (2j, HOOCCH=CHCON(CH_3)CH_2-1-Napth).** EtOOCCH=CHCON(CH_3)CH_2-1-Napth was hydrolyzed in MeOH using NaOH (1 M aqueous, 1.1 equiv) under standard deblocking conditions to give a white solid after recrystallization from cold EtOAc (13% yield). ^{1}H NMR (DMSO-d_6): 3.01 (s, 3H, CH_3), 5.01 (s, 2H, CH_2), 6.61–6.65 (d, 1H, J = 15.2 Hz, *CH*=CHCON), 7.17–7.21 (d, 1H, CH=*CH*CON), 7.37–7.60 (m, 4H, naphthyl), 7.85–8.01 (m, 3H, naphthyl).

***N*^{2}-(*N*-Benzyloxycarbonylalanylalanyl)-*N*^{1}-carbamoylmethyl-*N*^{1}-*trans*-(3-(methyl-1-naphthylmethylcarbamoyl)propenoyl)hydrazine (5j, Cbz-Ala-Ala-AAsn-CH=CHCON(CH_3)CH_2-1-Napth).** This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography on silica gel using 10% MeOH/CH_2Cl_2 as the eluent and then recrystallized from EtOAc/hexane to give a yellow powder (31% yield). ^{1}H NMR (DMSO-d_6): 1.18–1.27 (m, 6H, 2 × Ala-CH_3), 3.10 (s, 3H, N-CH_3), 3.20–3.32 (d, 2H, NCH_2CO), 4.04–4.08 (m, 1H, α-H), 4.28–4.31 (m, 1H, α-H), 4.99 (m, 4H, Cbz and N-CH_2-naphthyl), 7.07–7.61 (m, 12H, naphthyl and Ph CH=*CH*CON and *CH*=CHCON and NH), 7.85–8.10 (m, 3H, naphthyl), 8.15 (d, 1H, NH), 10.42 (s, 1H, NH), 10.72 (s, 1H, NH). MS (ESI) *m*/*z* 617 [(M + 1)$^+$]. HRMS (ESI) calculated for $C_{32}H_{37}N_6O_7$: 617.265. Observed *m*/*z* 617.2724.

***trans*-3-(Methylphenethylcarbamoyl)propenoic Acid Ethyl Ester (EtOOCCH=CHCON(CH_3)CH_2CH_2Ph).** This compound was obtained by mixed anhydride coupling of equimolar amounts of monoethyl fumarate and *N*-methylphenethylamine to give a clear colorless syrup (64% yield).

***trans*-3-(Methylphenethylcarbamoyl)propenoic Acid (2k, HOOCCH=CHCON(CH_3)CH_2CH_2Ph).** EtOOCCH=CHCON(CH_3)-CH_2CH_2Ph was hydrolyzed in MeOH 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): 2.82 (s, 3H, N-CH_3), 3.54 (t, 2H, N-CH_2-*CH_2*-Ph), 3.61 (t, 2H, N-*CH_2*-CH_2-Ph), 6.43–6.47 (d, 1H, J = 15.2 Hz, *CH*=CHCON), 6.98–6.7.02 (d, 1H, J = 15.6 Hz, CH=*CH*CON), 7.14–7.31 (m, 5H, Ph).

***N*^{2}-(*N*-Benzyloxycarbonylalanylalanyl)-*N*^{1}-carbamoylmethyl-*N*^{1}-*trans*-(3-(methylphenethylcarbamoyl)propenoyl)hydrazine (5k, Cbz-Ala-Ala-AAsn-CH=CHCON(CH_3)CH_2CH_2Ph).** This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography on silica gel using 10% MeOH/CH_2Cl_2 as the eluent and then recrystallized from EtOAc/hexane to give a white powder (28% yield). ^{1}H NMR (DMSO-d_6): 1.18–1.27 (m, 6H, 2 × Ala-CH_3), 2.74–2.82 (m, 2H, N-CH_2*CH_2*Ph), 2.86 (s, 3H, N-CH_3), 3.20–3.32 (d, 2H, NCH_2CO), 3.52–3.58 (m, 2H, N-*CH_2CH_2*Ph), 4.04–4.08 (m, 1H, α-H), 4.28–4.31 (m, 1H, α-H), 4.99 (m, 2H, Cbz), 6.86–6.89 (d, 1H, J = 14.4 Hz, *CH*=CHCON), 7.04–7.08 (d, 1H, J = 14.8 Hz, CH=*CH*CON), 7.16–7.41 (m, 11H, 2 × Ph and NH), 7.50 (d, 1H, NH), 8.16 (d, 1H, NH), 10.72 (s, 1H, NH). MS (ESI) *m*/*z* 581 [(M + 1)$^+$]. HRMS (ESI) calculated for $C_{29}H_{37}N_6O_7$: 581.2717. Observed *m*/*z* 581.272 373.

***trans*-3-Phenylbenzylcarbamoylpropenoic Acid Ethyl Ester (EtOOCCH=CHCON(Bzl)Ph).** This compound was obtained by mixed anhydride coupling of equimolar amounts of monoethyl fumarate and phenylbenzylamine to give an orange oil (87% yield).

***trans*-3-Phenylbenzylcarbamoylpropenoic Acid (2l, HOOCCH=CHCON(Bzl)Ph).** EtOOCCH=CHCON(Bzl)Ph was hydrolyzed in MeOH using NaOH (1 M aqueous, 1.1 equiv) under standard deblocking conditions to give a clear, colorless syrup (74% yield). ^{1}H NMR (DMSO-d_6): 4.97 (s, 2H, N-*CH_2*-Ph), 6.61–6.62 (d, 1H, *CH*=CHCON), 7.15–7.43 (m, 11H, CH=*CH*CON and 2 × Ph).

***N*^{2}-(*N*-Benzyloxycarbonylalanylalanyl)-*N*^{1}-carbamoylmethyl-*N*^{1}-*trans*-(3-phenylbenzylcarbamoylpropenoyl)hydrazine (5l, Cbz-Ala-Ala-AAsn-CH=CHCON(Bzl)Ph).** This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography on silica gel using 10% MeOH/CH_2Cl_2 as the eluent to give a white powder (14% yield). ^{1}H NMR (DMSO-d_6): 1.20–1.21 (d, 3H, Ala-CH_3), 1.26 (d, 3H, Ala-CH_3), 3.31 (s, 2H, NCH_2CO), 4.04–4.08 (m, 1H, α-H), 4.28–4.31 (m, 1H, α-H), 4.96–4.99 (d, 4H, N-*CH_2*-Ph and Cbz), 6.57–6.61 (d, 1H, J = 15.2 Hz, *CH*=CHCON), 7.14–7.45 (m, 18H, CH=*CH*CON and 3 × Ph and 2 × NH), 8.16 (d, 1H, NH), 10.71 (s, 1H, NH). MS (ESI) *m*/*z* 629 [(M + 1)$^+$]. HRMS (ESI) calculated for $C_{33}H_{37}N_6O_7$: 629.2691. Observed *m*/*z* 629.272 373.

***trans*-3-Dibenzylcarbamoylpropenoic Acid Ethyl Ester (EtOOCCH=CHCON(Bzl)$_2$).** This compound 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 (2m, HOOCCH=CHCON(Bzl)$_2$).** EtOOCCH=CHCON(Bzl)$_2$ was hydrolyzed in MeOH using NaOH (1 M aqueous, 1.1 equiv) under standard deblocking conditions to give a clear, colorless syrup which was recrystallized overnight from hexane/EtOAc (30% yield). ^{1}H NMR (DMSO-d_6): 4.57 (s, 2H, N-*CH_2*-Ph), 4.65 (s, 2H, N-*CH_2*-Ph), 6.59–6.63 (d, 1H, J = 15.2 Hz, *CH*=CHCON), 7.15–7.17 (d, 1H, J = 8 Hz, CH=*CH*CON), 7.25–7.50 (m, 10H, 2 × Ph).

***N*^{2}-(*N*-Benzyloxycarbonylalanylalanyl)-*N*^{1}-carbamoylmethyl-*N*^{1}-*trans*-(3-dibenzylcarbamoylpropenoyl)hydrazine (5m, Cbz-Ala-Ala-AAsn-CH=CHCON(Bzl)$_2$).** This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography on silica gel using 10% MeOH/CH_2Cl_2 as the eluent to give a white powder (34% yield). ^{1}H NMR (DMSO-d_6): 1.19 (d, 3H, Ala-CH_3), 1.26 (d, 3H, Ala-CH_3), 3.31 (s, 2H, NCH_2CO), 4.04–4.08 (m, 1H, α-H), 4.28–4.31 (m, 1H, α-H), 4.56 (s, 2H, N-*CH_2*-Ph), 4.63 (s, 2H, N-*CH_2*-Ph), 5.00 (m, 2H, Cbz), 7.13–7.41 (m, 17H, *CH*=CHCON and CH=*CH*CON and 3 × Ph and NH), 7.48 (s, 1H, NH), 8.14 (d, 1H, NH), 10.71 (s, 1H, NH). MS (ESI) *m*/*z* 643 [(M + 1)$^+$]. HRMS (ESI) calculated for $C_{34}H_{39}N_6O_7$: 643.2843. Observed *m*/*z* 643.288 023.

***trans*-3-(Benzyl-4-methoxybenzylcarbamoyl)propenoic Acid Ethyl Ester (EtOOCCH=CHCON(Bzl-4-OMe)Bzl).** This compound was obtained by mixed anhydride coupling of equimolar amounts of monoethyl fumarate and 4-methoxybenzylbenzylamine to give a clear colorless syrup (91% yield).

***trans*-3-(Benzyl-4-methoxybenzylcarbamoyl)propenoic Acid (2n,HOOCCH=CHCON(Bzl-4-OMe)Bzl).** EtOOCCH=CHCON(Bzl-4-OMe)Bzl was hydrolyzed in MeOH using NaOH (1 M aqueous, 1.1 equiv) under standard deblocking conditions to give a clear, colorless syrup (44% yield). ^{1}H NMR (DMSO-d_6): 3.73 (s, 3H, OCH_3), 4.50–4.54 (d, 2H, N-*CH_2*-Ph), 4.56–4.61 (d, 2H, N-*CH_2*-Ph), 6.59–6.63 (d, 1H, J = 15.2 Hz, *CH*=CHCON), 6.86–6.92 (2 × d, 2H, Ph), 7.07–7.09 (d, 1H, J = 8 Hz, CH=*CH*CON), 7.14–7.39 (m, 7H, 2 × Ph).

***N*^{2}-(*N*-Benzyloxycarbonylalanylalanyl)-*N*^{1}-*trans*-(3-benzyl-(4-methoxybenzyl)carbamoyl)propenoyl-*N*^{1}-carbamoylmethylhydrazine (5n, Cbz-Ala-Ala-AAsn-CH=CHCON(Bzl-4-OMe)Bzl).** This compound was obtained using the HOBt/EDC coupling method and purified by recrystallization from 10% MeOH/CH_2Cl_2 to give a yellow powder (9% yield). ^{1}H NMR (DMSO-d_6): 1.19–1.20 (d, 3H, Ala-CH_3), 1.26–1.28 (d, 3H, Ala-CH_3), 3.32 (s, 2H, NCH_2CO), 3.72 (s, 3H, OCH_3), 4.04–4.09 (m, 1H, α-H), 4.28–4.32 (m, 1H, α-H), 4.48–4.54 (d, 2H, N-*CH_2*-Ph), 4.54–4.59 (d, 2H, N-*CH_2*-Ph), 5.00 (m, 2H, Cbz), 6.84–6.91 (2 × d, 2H, Ph), 7.06–7.08 (d, 1H, J = 8.8 Hz, CH=CHCON), 7.12–7.42 (m, 14H, CH=*CH*CON and 3 × Ph and NH), 7.50 (s, 1H, NH), 8.16–8.17 (d, 1H, NH), 10.71 (s, 1H, NH). MS (ESI) *m*/*z* 673 [(M + 1)$^+$]. HRMS (ESI) calculated for $C_{35}H_{41}N_6O_8$: 673.3001. Observed *m*/*z* 673.298 588. Anal. Calcd for $C_{35}H_{40}N_6O_8 \cdot 0.5CH_2Cl_2$: C, 59.61; H, 5.78; N, 11.75. Found: C, 59.76; H, 5.71; N, 11.54.

***trans*-3-Benzyl(4-fluorobenzyl)carbamoylpropenoic Acid Ethyl Ester (EtOOCCH=CHCON(Bzl-4-F)Bzl).** This compound was obtained by mixed anhydride coupling of equimolar amounts of monoethyl fumarate and 4-fluorobenzylbenzylamine to give a clear, colorless syrup (95% yield).

***trans*-3-Benzyl(4-fluorobenzyl)carbamoylpropenoic Acid (2o, HOOCCH═CHCON(Bzl-4-F)Bzl).** EtOOCCH═CHCON(Bzl-4-F)Bzl was hydrolyzed in MeOH using NaOH (1 M aqueous, 1.1 equiv) under standard deblocking conditions to give a clear, colorless syrup (44% yield). ^{1}H NMR (DMSO-d_6): 4.50–4.54 (d, 2H, N-CH_2-Ph), 4.56–4.61 (d, 2H, N-CH_2-Ph), 6.59–6.63 (d, 1H, J = 15.2 Hz, *CH*═CHCON), 6.99–7.44 (m, 11H, CH═*CH*CON and *CH*═CHCON and 2 × Ph).

***N*^{2}-(*N*-Benzyloxycarbonylalanylalanyl)-*N*^{1}-*trans*-(3-benzyl(4-fluorobenzyl)carbamoylpropenoyl)-*N*^{1}-carbamoylmethylhydrazine (5o, Cbz-Ala-Ala-AAsn-CH═CH-CON(Bzl-4-F)Bzl).** 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 hexane/EtOAc gave a white powder (11% yield). ^{1}H NMR (DMSO-d_6): 1.19–1.20 (d, 3H, Ala-CH_3), 1.26–1.28 (d, 3H, Ala-CH_3), 3.32 (s, 2H, NCH_2CO), 4.05–4.09 (m, 1H, α-H), 4.28–4.32 (m, 1H, α-H), 4.35 (s, 2H, CH_2Ph), 4.62–4.64 (d, 2H, N-CH_2-Ph), 5.00 (m, 2H, Cbz), 7.09–7.34 (m, 17H, *CH*═CHCON and CH═*CH*CON and 3 × Ph and NH), 7.40–7.42 (d, 1H, NH), 7.50 (s, 1H, NH), 8.15–8.16 (d, 1H, NH), 10.71 (s, 1H, NH). MS (ESI) *m*/*z* 661 [(M + 1)$^+$]. HRMS (ESI) calculated for $C_{34}H_{38}N_6O_7F$: 661.2781. Observed *m*/*z* 661.278 601.

***trans*-3-(Bis-(2-furylmethyl)carbamoyl)propenoic Acid Ethyl Ester (EtOOCCH═CHCON(CH_2-2-furyl)$_2$).** This compound was obtained by mixed anhydride coupling of equimolar amounts of monoethyl fumarate and bis-2-furylmethylamine to give a brown syrup (83% yield). ^{1}H NMR ($CDCl_3$): 1.26 (t, 3H, CH_3CH_2), 2.94 (d, 1H, J = 3.2 Hz, furyl), 3.47 (t, 1H, furyl), 3.64 (d, 1H, N-CH_2), 3.97 (d, 1H, N-CH_2), 4.12 (q, 2H, CH_3CH_2), 4.36 (d, 1H, N-CH_2), 4.71 (d, 1H, N-CH_2), 5.25 (d, 1H, furyl), 6.26–6.33 (m, 3H, furyl), 6.51–6.53 (d, 1H, J = 15.2 Hz, *CH*═CHCON), 7.37 (s, 1H, CH═*CH*CON).

***trans*-3-(Bis-(2-furylmethyl)carbamoyl)propenoic Acid (2p, HOOCCH═CHCON(CH_2-2-furyl)$_2$).** EtOOCCH═CHCON(CH_2-2-furyl)$_2$ was hydrolyzed in MeOH using NaOH (1 M aqueous, 1.1 equiv) under standard deblocking conditions to give a brown solid (91% yield). ^{1}H NMR (DMSO-d_6): 2.73 (d, 1H, J = 3.6 Hz, furyl), 3.13 (t, 1H, furyl), 3.49 (d, 1H, N-CH_2), 4.03 (d, 1H, N-CH_2), 4.35–4.49 (dd, 2H, N-CH_2), 5.17 (d, 1H, furyl), 6.30–6.40 (m, 3H, furyl), 6.63–6.64 (d, 1H, J = 15.2 Hz, *CH*═CHCON), 7.60 (s, 1H, CH═*CH*CON).

***N*^{2}-(*N*-Benzyloxycarbonylalanylalanyl)-*N*^{1}-carbamoylmethyl-*N*^{1}-*trans*-(3-bis-(2-furylmethyl)carbamoylpropenoyl)hydrazine (5p, Cbz-Ala-Ala-AAsn-CH═CHCON(CH_2-2-furyl)$_2$).** This compound was obtained using the HOBt/EDC coupling method. The product was isolated by chromatography with 10% MeOH/CH_2Cl_2 as the eluent and recrystallized from hexane/EtOAc to give a white powder (34% yield). ^{1}H NMR (DMSO-d_6): 1.18–1.27 (m, 6H, 2 × Ala-CH_3), 2.92 (d, 1H, furyl), 3.28–3.38 (m, 4H, N-CH_2 and NCH_2CO and furyl), 3.47–3.50 (d, 1H, N-CH_2), 3.99–4.07 (m, 2H, α-H and N-CH_2), 4.30–4.49 (m, 2H, α-H and N-CH_2), 4.99 (m, 2H, Cbz), 6.02 (s, 1H, NH), 6.11 (s, 1H, NH), 6.31(s, 1H, *CH*═CHCON), 6.39 (s, 1H, CH═*CH*CON), 6.55 (t, 1H, furyl), 7.12 (s, 1H, NH), 7.32 (m, 5H, Ph), 7.41–7.59 (m, 3H, furyl), 8.21 (d, 1H, NH), 10.58 (s, 1H, NH). MS (ESI) *m*/*z* 623 [(M + 1)$^+$]. HRMS (ESI) calculated for $C_{30}H_{35}N_6O_9$: 623.2486. Observed *m*/*z* 623.2466.

***trans*-3-(Benzyl-2-naphthylmethylcarbamoyl)propenoic Acid Ethyl Ester (EtOOCCH═CHCON(Bzl)-CH_2-2-Napth).** This compound was obtained by mixed anhydride coupling of equimolar amounts of monoethyl fumarate and benzyl-2-naphthylmethylamine to give a clear colorless syrup (87% yield). ^{1}H NMR ($CDCl_3$): 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), 6.95–6.91 (d, 1H, J = 14.4 Hz, *CH*═CHCON), 7.16–7.18 (d, 1H, CH═*CH*CON), 7.23–7.57 (m, 10H, naphthyl and Ph), 7.85 (m, 2H, naphthyl).

***trans*-3-(Benzyl-2-naphthylmethylcarbamoyl)propenoic Acid (2q, HOOCCH═CHCON(Bzl)-CH_2-2-Napth).** EtOOCCH═CHCON(Bzl)-2-CH_2-Napth was hydrolyzed in MeOH using NaOH (1 M aqueous, 1.1 equiv) under standard deblocking conditions to give a white solid after recrystallization from cold EtOAc (13% yield). ^{1}H NMR (DMSO-d_6): 4.82 (d, 2H, N-CH_2), 5.01 (d, 2H, N-CH_2), 6.61–6.65 (d, 1H, J = 15.2 Hz, *CH*═CHCON), 7.17–7.21 (d, 1H, CH═*CH*CON), 7.37–7.60 (m, 9H, naphthyl and Ph), 7.85–8.01 (m, 3H, naphthyl).

***N*^{2}-(*N*-Benzyloxycarbonylalanylalanyl)-*N*^{1}-*trans*-(3-benzyl-2-naphthylmethylcarbamoylpropenoyl)-*N*^{1}-carbamoylmethylhydrazine (5q, Cbz-Ala-Ala-AAsn-CH═CHCON(Bzl)-CH_2-2-Napth).** This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography on silica gel using 10% MeOH/CH_2Cl_2 as the eluent and then recrystallized from EtOAc/hexane to give a yellow powder (11% yield). ^{1}H NMR (DMSO-d_6): 1.18–1.28 (m, 6H, 2 × Ala-CH_3), 3.29–3.32 (d, 2H, NCH_2CO), 4.04–4.08 (m, 1H, α-H), 4.28–4.31 (m, 1H, α-H), 4.63–4.69 (d, 2H, N-CH_2), 4.73–4.80 (d, 2H, N-CH_2), 4.99 (m, 2H, Cbz), 7.14–7.48 (m, 18H, naphthyl and 2 × Ph and CH═*CH*CON and *CH*═CHCON and 2 × NH), 7.84–7.90 (m, 3H, naphthyl), 8.15 (d, 1H, NH), 10.72 (s, 1H, NH). MS (ESI) *m*/*z* 693 [(M + 1)$^+$]. HRMS (ESI) calculated for $C_{38}H_{41}N_6O_7$: 693.2998. Observed *m*/*z* 693.303 673.

***trans*-3-(Benzyl-1-naphthylmethylcarbamoyl)propenoic Acid Ethyl Ester (EtOOCCH═CHCON(Bzl)-CH_2-1-Napth).** This compound was obtained by mixed anhydride coupling of equimolar amounts of monoethyl fumarate and benzyl-1-naphthylmethylamine to give a yellow oil (79% yield). ^{1}H NMR ($CDCl_3$): 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), 6.95–6.91 (d, 1H, J = 14.4 Hz, *CH*═CHCON), 7.16–7.18 (d, 1H, CH═*CH*CON), 7.23–7.57 (m, 10H, naphthyl and Ph), 7.85 (m, 2H, naphthyl).

***trans*-3-(Benzyl-1-naphthylmethylcarbamoyl)propenoic Acid (2r, HOOCCH═CHCON(Bzl)-CH_2-1-Napth).** EtOOCCH═CHCON(Bzl)-1-CH_2-Napth was hydrolyzed in MeOH using NaOH (1 M aqueous, 1.1 equiv) under standard deblocking conditions to give a white solid after recrystallization from cold EtOAc (62% yield). ^{1}H NMR (DMSO-d_6): 4.82 (d, 2H, N-CH_2), 5.01 (d, 2H, N-CH_2), 6.61–6.65 (d, 1H, J = 15.2 Hz, *CH*═CHCON), 7.17–7.21 (d, 1H, CH═*CH*CON), 7.37–7.60 (m, 9H, naphthyl and Ph), 7.85–8.01 (m, 3H, naphthyl).

***N*^{2}-(*N*-Benzyloxycarbonylalanylalanyl)-*N*^{1}-*trans*-(3-benzyl-1-naphthylmethylcarbamoylpropenoyl)-*N*^{1}-carbamoylmethylhydrazine (5r, Cbz-Ala-Ala-AAsn-CH═CHCON(Bzl)-CH_2-1-Napth).** This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography on silica gel using 10% MeOH/CH_2Cl_2 as the eluent and then recrystallized from EtOAc/hexane to give a white powder (17% yield). ^{1}H NMR (DMSO-d_6): 1.19–1.29 (m, 6H, 2 × Ala-CH_3), 3.30–3.32 (d, 2H, NCH_2CO), 4.04–4.08 (m, 1H, α-H), 4.29–4.33 (m, 1H, α-H), 4.64–4.67 (d, 2H, N-CH_2), 4.99 (m, 2H, Cbz), 5.06–5.17 (d, 2H, N-CH_2), 7.14–7.48 (m, 18H, naphthyl and 2 × Ph and CH═*CH*CON and *CH*═CHCON and 2 × NH), 7.84–7.90 (m, 3H, naphthyl), 8.15 (d, 1H, NH), 10.72 (s, 1H, NH). MS (ESI) *m*/*z* 693 [(M + 1)$^+$].

***trans*-3-(3,4-Dihydro-2*H*-quinolin-1-ylcarbonyl)propenoic Acid Ethyl Ester (EtOOCCH═CHCO-tetrahydroquinoline).** This compound was obtained by 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 ($CDCl_3$): 1.29 (t, 3H, CH_3CH_2OC), 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), 6.78–6.82 (dd, 1H, J = 14.8 Hz, *CH*═CHCON), 7.18–7.22 (m, 4H, quinoline), 7.44–7.48 (d, 1H, J = 14.8 Hz, CH═*CH*CON).

***trans*-3-(3,4-Dihydro-2*H*-quinolin-1-ylcarbonyl)propenoic Acid (2s, HOOCCH═CHCO-tetrahydroquinoline).** EtOOCCH═CHCO-tetrahydroquinoline was hydrolyzed in MeOH 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).

N^2-(*N*-Benzyloxycarbonylalanylalanyl)-N^1-carbamoylmethyl-N^1-*trans*-(3-(3,4-dihydro-2*H*-quinolin-1-ylcarbonyl)propenoyl)hydrazine (5s, Cbz-Ala-Ala-AAsn-CH=CHCO-tetrahydroquinoline). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography on silica gel using 10% MeOH/CH_2Cl_2 as the eluent and then recrystallized from EtOAc/hexane to give a yellow powder (28% yield). ^{1}H NMR (DMSO-d_6): 1.18–1.27 (m, 6H, 2 × Ala-CH_3), 1.87 (m, 2H, N-CH_2-*CH_2*-CH_2), 2.70 (t, 2H, N-CH_2-CH_2-*CH_2*), 3.29–3.32 (d, 2H, NCH_2CO), 3.73 (m, 2H, N-*CH_2*-CH_2), 4.02–4.06 (m, 1H, α-H), 4.31 (m, 1H, α-H), 4.99 (m, 2H, Cbz), 7.02–7.07 (dd, 2H, J = 14.8 Hz, *CH*=CHCON), 7.15–7.41 (m, 11H, quinoline and Ph and NH and CH=*CH*CON), 7.49 (s, 1H, NH), 8.16 (d, 1H, NH), 10.73 (s, 1H, NH). MS (ESI) m/z 579 [$(M + 1)^+$]. HRMS (ESI) calculated for $C_{29}H_{35}N_6O_{7:}$ 579.2525. Observed m/z 579.2567.

***trans*-3-(3,4-Dihydro-2*H*-quinolin-1-ylcarbonyl)propenoic Acid Ethyl Ester (EtOOCCH=CHCO-tetrahydroisoquinoline).** This compound 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 ($CDCl_3$): 1.29 (t, 3H, *CH_3*CH_2OC), 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_3*CH_2*OOC), 6.78–6.82 (dd, 1H, J = 14.8 Hz, *CH*=CHCON), 7.18–7.22 (m, 4H, quinoline), 7.44–7.48 (d, 1H, J = 14.8 Hz, CH=*CH*CON).

***trans*-3-(3,4-Dihydro-1*H*-1soquinolin-2-ylcarbonyl)propenoic Acid (2t, HOOCCH=CHCO-tetrahydroisoquinoline).** EtOOC-CH=CHCO-tetrahydroquinoline was hydrolyzed in MeOH 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).

N^2-(*N*-Benzyloxycarbonylalanylalanyl)-N^1-carbamoylmethyl-N^1-*trans*-(3-(3,4-dihydro-1*H*-quinolin-2-ylcarbonyl)propenoyl)hydrazine (5t, Cbz-Ala-Ala-AAsn-CH=CHCO-tetrahydroisoquinoline). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography on silica gel using 10% MeOH/CH_2Cl_2 as the eluent and then recrystallized from EtOAc/hexane to give a yellow powder (28% yield). ^{1}H NMR (DMSO-d_6): 1.18–1.27 (m, 6H, 2 × Ala-CH_3), 1.87 (m, 2H, N-CH_2-*CH_2*-CH_2), 2.70 (t, 2H, N-CH_2-CH_2-*CH_2*), 3.29–3.32 (d, 2H, NCH_2CO), 3.73 (m, 2H, N-*CH_2*-CH_2), 4.02–4.06 (m, 1H, α-H), 4.31 (m, 1H, α-H), 4.99 (m, 2H, Cbz), 7.02–7.07 (dd, 2H, J = 14.8 Hz, *CH*=CHCON), 7.15–7.41 (m, 11H, isoquinoline and Ph and NH and CH=*CH*CON), 7.49 (s, 1H, NH), 8.16 (d, 1H, NH), 10.73 (s, 1H, NH). MS (ESI) m/z 579 [$(M + 1)^+$]. HRMS (ESI) calculated for $C_{29}H_{35}N_6O_{7:}$ 579.2525. Observed m/z 579.2567.

***trans*-3-(2,3-Dihydroindol-1-ylcarbonyl)propenoic Acid Ethyl Ester (EtOOCCH=CHCO-indoline).** This compound was obtained by mixed anhydride coupling of equimolar amounts of monoethyl fumarate and indoline to give a bright-yellow solid (84% yield).

***trans*-3-(2,3-Dihydroindol-1-ylcarbonyl)propenoic Acid (2u, HOOC-CH=CHCO-indoline).** EtOOC-CH=CHCO-indoline was hydrolyzed in MeOH using NaOH (1 M aqueous, 1.1 equiv) under standard deblocking conditions to give a clear syrup, which was washed several times with CH_2Cl_2 to give a bright-yellow solid (40% yield). ^{1}H NMR (DMSO-d_6): 3.16 (t, 2H, N-CH_2-*CH_2*), 4.27 (t, 1H, N-*CH_2*-CH_2), 6.64–6.67 (dd, 1H, J = 15.2 Hz *CH*=CHCON), 7.03 (t, 1H, indoline-H), 7.17 (t, 1H, indoline-H), 7.24–7.26 (d, 1H, J = 7.2 Hz, indoline-H), 7.28–7.32 (d, 1H, J = 15.2 Hz, CH=*CH*CON), 8.11–8.13 (d, 1H, J = 8 Hz, indoline-H).

N^2-(*N*-Benzyloxycarbonylalanylalanyl)-N^1-carbamoylmethyl-N^1-*trans*-(3-(2,3-dihydroindol-1-ylcarbonyl)propenoyl)hydrazine (5u, Cbz-Ala-Ala-AAsn-CH=CHCO-indoline). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography on silica gel using 10% MeOH/CH_2Cl_2 as the eluent and then recrystallized from EtOAc/hexane to give a bright-yellow, flaky powder (15% yield). ^{1}H NMR (DMSO-d_6): 1.18–1.19 (d, 3H, Ala-CH_3), 1.27–1.28 (d, 3H, Ala-CH_3), 3.15 (t, 2H, N-CH_2*CH_2*), 3.20–3.31 (d, 2H, NCH_2CO), 4.02–4.06 (m, 1H, α-H), 4.06 (t, 2H, N-*CH_2*-CH_2), 4.31 (m, 1H, α-H), 4.99 (m, 2H, Cbz), 7.02 (t, 1H, indoline-H), 7.13–7.41 (m, 10H, indoline and Ph and NH and *CH*=CHCON and CH=*CH*CON), 7.52 (s, 1H, NH), 8.13 (d, 1H, NH), 8.15–8.16 (d, 1H, indoline-H), 10.76 (s, 1H, NH). MS (ESI) m/z 565 [$(M + 1)^+$]. HRMS (ESI) calculated for $C_{28}H_{33}N_6O_{7:}$ 565.2416. Observed m/z 565.241 073.

***trans*-3-(1,3-Dihydroisoindol-2-ylcarbonyl)propenoic Acid Ethyl Ester (EtOOCCH=CHCO-isoindoline).** This compound was obtained by mixed anhydride coupling of equimolar amounts of monoethyl fumarate and isoindoline to give a brown solid (86% yield).

***trans*-3-(1,3-Dihydroisoindol-2-ylcarbonyl)propenoic Acid (2v, HOOCCH=CHCO-isoindoline).** EtOOCCH=CHCO-isoindoline was then hydrolyzed in MeOH using NaOH (1 M aqueous, 1.1 equiv) under standard deblocking conditions to give a white solid after recrystallization with cold EtOAc (37% yield). ^{1}H NMR (DMSO-d_6): 4.73 (s, 2H, NCH_2), 5.02 (s, 2H, NCH_2), 6.58–6.62 (d, 1H, J = 15.2 Hz, *CH*=CHCON), 7.25 (m, 5H, isoindoline and CH=*CH*CON).

N^2-(*N*-Benzyloxycarbonylalanylalanyl)-N^1-carbamoylmethyl-N^1-*trans*-(3-(1,3-dihydroisoindol-2-ylcarbonyl)propenoyl)hydrazine (5v, Cbz-Ala-Ala-AAsn-CH=CHCO-isoindoline). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography on silica gel using 10% MeOH/CH_2Cl_2 as the eluent and then recrystallized from EtOAc/hexane to give a bright-yellow, flaky powder (15% yield). ^{1}H NMR (DMSO-d_6): 1.18–1.19 (d, 3H, Ala-CH_3), 1.27–1.28 (d, 3H, Ala-CH_3), 3.20–3.31 (d, 2H, NCH_2CO), 4.04–4.07 (m, 1H, α-H), 4.29–4.33 (m, 1H, α-H), 4.72 (s, 2H, NCH_2), 4.99 (m, 2H, Cbz and NCH_2), 7.11–7.32 (m, 11H, indoline and Ph and NH and *CH*=CHCON and CH=*CH*CON), 7.39 (d, 1H, NH), 7.52 (s, 1H, NH), 8.14 (d, 1H, NH), 10.76 (s, 1H, NH). MS (ESI) m/z 565 [$(M + 1)^+$]. HRMS (ESI) calculated for $C_{28}H_{33}N_6O_{7:}$ 565.2414. Observed m/z 565.241 073.

***trans*-3-(4-Phenyl-5,6-dihydro-2*H*-pyridin-1-ylcarbonyl)propenoic Acid Ethyl Ester (EtOOCCH=CHCO-(Py-4-Ph)).** This compound was obtained by mixed anhydride coupling of equimolar amounts of monoethyl fumarate and 4-phenyl-1,2,3,6-tetrahydropyridine hydrochloride to give a pink solid (80% yield). ^{1}H NMR ($CDCl_3$): 1.32 (t, 3H, CH_2*CH_3*), 2.64 (s, 2H, pyridyl-CH_2), 3.78–3.81 (t, 1H, pyridyl-CH_2), 3.90–3.93 (t, 1H, pyridyl-CH_2), 4.24–4.33 (m, 4H, *CH_2*CH_3 and pyridyl-CH_2), 6.02–6.09 (d, 1H, pyridyl-CH=), 6.78–6.82 (d, 1H, J = 15.6 Hz, *CH*=CHCON), 6.99–7.49 (m, 6H, Ph and CH=*CH*CON).

***trans*-3-(4-Phenyl-5,6-dihydro-2*H*-pyridin-1-ylcarbonyl)propenoic Acid (2w, HOOCCH=CHCO-(Py-4-Ph)).** EtOOCCH=CH-CO-(4-Ph-Py) was hydrolyzed in MeOH using NaOH (1 M aqueous, 1.1 equiv) under standard deblocking conditions to give a white solid (59% yield). ^{1}H NMR (DMSO-d_6): 2.57 (s, 2H, pyridyl-CH_2), 3.73–3.76 (t, 2H, pyridyl-CH_2), 4.17–4.26 (d, 2H, pyridyl-CH_2), 6.14–6.17 (d, 1H, pyridyl CH=), 7.23–7.27 (t, 1H, Ph), 7.31–7.35 (t, 2H, Ph), 7.39–7.48 (m, 3H, *CH*=CHCON and Ph).

N^2-(*N*-Benzyloxycarbonylalanylalanyl)-N^1-carbamoylmethyl-N^1-*trans*-(3-(4-phenyl-5,6-dihydro-2*H*-pyridin-1-ylcarbonyl)propenoyl)hydrazine (5w, Cbz-Ala-Ala-AAsn-CH=CHCO-(Py-4-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 to give a white solid (11% yield). ^{1}H NMR (DMSO-d_6): 1.15–1.26 (m, 6H, 2 × Ala-CH_3), 2.56 (s, 2H, pyridyl-CH_2), 3.30–3.32 (d, 2H, NCH_2CO), 3.74 (t, 2H, pyridyl-CH_2), 4.02–4.07 (m, 1H, α-H), 4.17 (s, 2H, pyridyl-CH_2), 4.27–4.35 (m, 1H, α-H), 4.99 (m, 2H, Cbz), 6.16 (s, 1H, pyridyl-CH=), 7.02–7.06 (d, 1H, J = 15.2 Hz, *CH*=CHCON), 7.19 (s, 1H, NH), 7.23–7.42 (m, 11H, CH=*CH*CON and 2 × Ph), 7.51 (d, 1H, NH), 8.14–8.15 (d, 1H, NH), 10.72 (s, 1H, NH). MS (ESI) m/z 605 [$(M + 1)^+$]. HRMS (ESI) calculated for $C_{31}H_{37}N_6O_{7:}$ 605.2757. Observed m/z 605.272 373.

***tert*-Butyloxycarbonyl-*N*-methylphenylalanine Methylphenethylamide (Boc-MePhe-N(CH_3)(CH_2)$_2$Ph).** This compound was obtained by mixed anhydride coupling of equimolar amounts of

Boc-MePhe-OH and *N*-methylphenethylamine. The product was purified by column chromatography using hexane/EtOAc (1:1) as the eluent to give a colorless oil (89% yield). ^{1}H NMR ($CDCl_3$): 1.14–1.35 (m, 9H, *t*Bu), 2.73–2.98 (m, 10H, Phe-CH_2 and CH_2Ph and 2 × Me), 3.40–3.51 (m, 2H, N-CH_2), 5.20 (m, 1H, α-H), 7.10–7.33 (m, 10H, 2 × Ph).

***N*-Methylphenylalanine Methylphenethylamide Hydrochloride (MePhe-N(CH_3)(CH_2)$_2$Ph).** Boc-MePhe-N(CH_3)(CH_2)$_2$Ph was hydrolyzed under standard deblocking conditions using 12 equiv of HCl in EtOAc (4 N) to give a white powder (100% yield).

***N*-(3-Ethoxycarbonylpropenoyl)-*N*-methylphenylalanine Methylphenethylamide (EtOOCCH═CH-MePhe-N(CH_3)(CH_2)$_2$-Ph).** This compound was obtained by mixed anhydride coupling of equimolar amounts of monoethyl fumarate and MePhe-N(CH_3)(CH_2)$_2$Ph to give a clear colorless syrup (52% yield). ^{1}H NMR ($CDCl_3$): 1.20–1.25 (t, 3H, CH_3CH_2O), 2.73–2.98 (m, 10H, Phe-CH_2 and CH_2Ph and 2 × Me), 3.40–3.51 (m, 2H, N-CH_2), 4.19–4.34 (q, 2H, CH_3CH_2O), 5.20 (m, 1H, α-H), 6.63–5.78 (2 × t, 1H,*CH*═CHCON), 6.80–6.99 (m, 1H, CH═*CH*CON), 7.10–7.33 (m, 10H, 2 × Ph).

***N*-Fumaroyl-*N*-methylphenylalanine Methylphenethylamide (2x, HOOCCH═CH-MePhe-N(CH_3)(CH_2)$_2$Ph).** (HOOCCH═CH-MePhe-N(CH_3)(CH_2)$_2$Ph) was hydrolyzed in MeOH using NaOH (1 M aqueous, 1.1 equiv) under standard deblocking conditions to give a clear, colorless syrup (75% yield).

***N*^{2}-(*N*-Benzyloxycarbonylalanylalanyl)-*N*^{1}-carbamoylmethyl-*N*^{1}-*trans*-3-(*N*-methyl-*N*-(1-(*N*-methyl-*N*-phenethylcarbamoyl)phenylethyl)carbamoylpropenoyl)hydrazine (5x, Cbz-Ala-Ala-AAsn-CH═CHCO-MePhe-N(CH_3)(CH_2)$_2$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 hexane/EtOAc gave a yellow powder (11% yield). ^{1}H NMR (DMSO-d_6): 1.19–1.20 (d, 3H, Ala-CH_3), 1.26–1.28 (d, 3H, Ala-CH_3), 2.61–2.95 (m, 10H, Phe-CH_2 and CH_2Ph and 2 × N-Me), 3.32 (s, 2H, NCH_2CO), 3.62 (m, 2H, N-CH_2), 4.05–4.09 (m, 1H, α-H), 4.28–4.32 (m, 1H, α-H), 4.99 (m, 2H, Cbz), 5.52 (m, 1H, α-H), 6.93–7.32 (m, 18H, *CH*═CHCON and CH═*CH*CON and 3 × Ph and NH), 7.40–7.42 (d, 1H, NH), 7.50 (s, 1H, NH), 8.15–8.16 (d, 1H, NH), 10.71 (s, 1H, NH). MS (ESI) *m*/*z* 607 [(M − HN(CH_3)CH_2CH_2Ph + 1, 100%)$^+$]. HRMS (ESI) calculated for $C_{39}H_{48}N_7O_8$: 742.3575. Observed *m*/*z* 742.3564.

***trans*-*N*^{2}-(*N*-Benzyloxycarbonylalanylalanyl)-*N*^{1}-carbamoylmethyl-*N*^{1}-*trans*-(3-benzoylpropenoyl)hydrazine (7c, Cbz-Ala-Ala-AAsn-CH═CHCOPh).** This compound was obtained using the HOBt/EDC coupling method starting from the peptide precursor Cbz-Ala-Ala-NHNHCH$_2$CONH$_2$ and commercially available *trans*-3-benzoylpropenoic acid. The workup omitted the $NaHCO_3$ washings. The crude product was purified by column chromatography on silica gel using 10% MeOH/CH_2Cl_2 as the eluent and then washed with EtOAc to give a white powder (39% yield). ^{1}H NMR (DMSO-d_6): 1.18–1.27 (m, 6H, 2 × Ala-CH_3), 3.20–3.32 (d, 2H, NCH_2CO), 3.98–4.05 (m, 1H, α-H), 4.26–4.29 (m, 1H, α-H), 4.99 (m, 2H, Cbz), 7.14–7.21 (m, 2H, *CH*═CHCOPh and NH), 7.32–7.35 (m, 5H, Ph), 7.54 (t, 2H, Ph), 7.69 (t, 1H, Ph), 7.76–7.80 (d, 1H, J = 15.6 Hz, CH═*CH*COPh), 7.97–7.99 (d, 2H, Ph), 8.14 (d, 1H, NH), 10.77 (s, 1H, NH). MS (ESI) *m*/*z* 524 [(M + 1)$^+$]. HRMS (ESI) calculated for $C_{26}H_{30}N_5O_7$: 524.2056. Observed *m*/*z* 524.2145.

***N*^{2}-(*N*-Benzyloxycarbonylalanylalanyl)-*N*^{1}-carbamoylmethyl-*N*^{1}-*trans*-*trans*-hexa-2,4-dienoylhydrazine (7d, Cbz-Ala-Ala-AAsn-CH═CHCH═CHCH$_3$).** This compound was synthesized by coupling the peptide precursor Cbz-Ala-Ala-NHNHCH$_2$CONH$_2$ and commercially available 2,4-hexadienoic acid using HOBt/EDC and was purified by column chromatography on silica gel using 10% MeOH/CH_2Cl_2 as the eluent and then recrystallized from CH_2Cl_2/hexane to give a white powder (14% yield). ^{1}H NMR (DMSO-d_6): 1.18–1.24 (m, 6H, 2 × Ala-CH_3), 1.79 (d, 3H, CH_3-CH═CH-), 3.30–3.32 (d, 2H, NCH_2CO), 4.06 (m, 1H, α-H), 4.25 (m, 1H, α-H), 4.98 (m, 2H, Cbz), 6.19 (m, 3H, CH_3-*CH*═*CH*-*CH*), 7.21 (m, 2H, NH and CH═*CH*-CO), 7.33 (m, 5H, Ph), 7.5 (d, 1H, NH), 8.19 (d, 1H, NH), 10.53 (s, 1H, NH). MS (ESI) *m*/*z* 460 [(M + 1)$^+$]. HRMS (ESI) calculated for $C_{22}H_{30}N_5O_6$: 460.2157. Observed *m*/*z* 460.219 609.

***N*^{2}-(*N*-Benzyloxycarbonylalanylalanyl)-*N*^{1}-carbamoylmethyl-*N*^{1}-*trans*-(3-(2-furyl)propenoyl)hydrazine (7e, Cbz-Ala-Ala-AAsn-CH═CH-2-furyl).** This compound was obtained using the HOBt/EDC coupling method starting with the peptide precursor Cbz-Ala-Ala-NHNHCH$_2$CONH$_2$ and commercially available 3-(2-furyl)propenoic acid and purified by column chromatography on silica gel using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization from hexane/EtOAc gave a white powder (23% yield). ^{1}H NMR ($(CD_3)_2CO$): 1.37 (d, 3H, Ala-CH_3), 1.44 (d, 3H, Ala-CH_3), 2.88 (s, 2H, NCH_2CO), 4.24 (m, 1H, α-H), 4.52 (m, 1H, α-H), 5.10 (m, 2H, Cbz), 6.41 (m, 1H, furyl-H), 6.55 (m, 1H, furyl-H), 6.68 (m, 1H, *CH*═CHCON), 6.88 (s, 2H, NH_2), 7.31–7.36 (m, 5H, Ph), 7.39–7.43 (d, 1H, J = 15.6 Hz, CH═*CH*CON), 7.48 (s, 1H, NH), 7.65 (s, 1H, furyl-H), 7.81 (s, 1H, NH), 9.83 (s, 1H, NH). MS (ESI) *m*/*z* 486 [(M + 1)$^+$]. HRMS (ESI) calculated for $C_{23}H_{28}N_5O_7$: 486.1957. Observed *m*/*z* 486.198 874.

***N*^{2}-(*N*-Benzyloxycarbonylalanylalanyl)-*N*^{1}-carbamoylmethyl-*N*^{1}-*trans*-(3-(3-pyridyl)propenoyl)hydrazine (7f, Cbz-Ala-Ala-AAsn-CH═CH-3-Py).** This compound was obtained using the HOBt/EDC coupling method starting from the peptide precursor Cbz-Ala-Ala-NHNHCH$_2$CONH$_2$ and commercially available 3-(3-pyridyl) propenoic acid. Upon completion of the reaction saturated $NaHCO_3$ was added, and the volatiles were evaporated. Without further workup, the crude product was chromatographed on silica gel using 10−20% MeOH/CH_2Cl_2 as the eluent and recrystallized from hexane/EtOAc to give a white powder (7% yield). ^{1}H NMR ($(CD_3)_2CO$): 1.37 (d, 3H, Ala-CH_3), 1.44 (d, 3H, Ala-CH_3), 2.91 (s, 2H, NCH_2CO), 4.24 (m, 1H, α-H), 4.42 (m, 1H, α-H), 5.10 (m, 2H, Cbz), 6.53 (m, 1H, pyridine-H), 6.68 (d, 1H, *CH*═CHCON), 7.31–7.36 (m, 5H, Ph), 7.59–7.64 (m, 1H, NH and CH═*CH*CON), 7.98 (s, 1H, pyridine-H), 8.32 (s, 1H, NH), 7.53 (s, 1H, pyridine-H), 8.90 (s, 1H, pyridine-H), 10.08 (s, 1H, NH). MS (ESI) *m*/*z* 497 [(M + 1)$^+$]. HRMS (ESI) calculated for $C_{24}H_{29}N_6O_6$: 497.2078. Observed *m*/*z* 497.2149.

***N*-*tert*-Butyloxycarbonylalanylalanyl Hydrazide (Boc-Ala-Ala-NHNH$_2$).** This compound was synthesized from Boc-Ala-Ala-OMe by hydrazinolysis. Anhydrous hydrazine (10 equiv) was added to a solution of Boc-Ala-Ala-OMe (1 equiv) in MeOH at room temperature, and the resulting mixture was then stirred at room temperature for 16 h. Excess hydrazine and solvent were removed by evaporation. The resulting residue was washed with ethanol and ether to give Boc-Ala-Ala-NHNH$_2$ as a white solid (95% yield). ^{1}H NMR (DMSO-d_6): 1.1–1.3 (d, 6H, CH_3), 1.36 (s, 9H, Boc), 4.0–4.1 (m, 1H, α-H), 4.1–4.3 (m, 2H, α-H and NH), 7.5 (d, 1H, NH), 7.9 (d, 1H, NH), 9.05 (s, 1H, NH).

***N*^{1}-(*N*-*tert*-Butyloxycarbonylalanylalanyl)-*N*^{2}-ethoxycarbonylmethylhydrazine (Boc-Ala-Ala-NHNHCH$_2$COOEt).** Ethyl bromoacetate (1.1 equiv) was added dropwise to a stirred solution of Boc-Ala-Ala-NHNH$_2$ (1 equiv) and NMM (1.1 equiv) in DMF that was cooled to −10 °C. The resulting solution was stirred for 30 min at −10 °C, after which the mixture was allowed to react at room temperature for 36 h. The DMF was evaporated, and the residue was purified on a silica gel column using 1:9 to 2:8 MeOH/CH_2Cl_2 as the eluting solvent system to give the ethyl ester as a white solid (yield 34%). ^{1}H NMR (DMSO-d_6): 1.18 (t, 9H, CH_3), 1.41 (s, 9H, Boc), 3.5 (d, 2H, NCH_2COOEt), 4.0–4.15 (m, 3H, α-H and OCH_2CH_3), 4.2 (m, 1H, α-H), 5.18 (m, 1H, NH), 7.22–7.40 (m, 5H, Ph), 7.4–7.5 (d, 1H, NH), 7.9 (m, 1H, NH), 9.35 (m, 1H, NH).

***N*^{1}-(*N*-*tert*-Butyloxycarbonylalanylalanyl)-*N*^{2}-carbamoylmethylhydrazine (Boc-Ala-Ala-NHNHCH$_2$CONH$_2$).** The ethyl ester Boc-Ala-Ala-NHNHCH$_2$COOEt (1 equiv) was dissolved in a 9 M solution (100 equiv) of NH_3 in methanol and a small amount of DMF and allowed to stir on an ice bath. To this solution was added catalytic NaCN (0.1 equiv). The flask was closed with a rubber septum and allowed to stir at 0 °C for 5 days. The solvent was evaporated and the crude product was purified by column chromatography (1:9 MeOH/CH_2Cl_2) to yield a white solid (53% yield). ^{1}H NMR (DMSO-d_6): 1.15 (2d, 6H, CH_3), 1.36 (s, 9H, Boc),

3.3 (d, 2H, NCH_2CONH$_2$), 3.9–4.0 (m, 1H, α-H), 4.1–4.2 (m, 1H, α-H), 5.22 (m, 1H, NH), 6.93 (d, 1H, NH), 7.1 (s, 1H, NH), 7.4 (s, 1H, NH), 7.85 (d, 1H, NH), 9.3 (s, 1H, NH).

***N*^{2}-(*N-tert*-Butyloxycarbonylalanylalanyl)-*N*^{1}-carbamoylmethyl-*N*^{1}-*trans*-(3-ethoxycarbonylpropenoyl)hydrazine (10, Boc-Ala-Ala-AAsn-CH=CHCOOEt).** This compound was synthesized using the EDC/HOBt coupling method, purified by chromatography on a silica gel column using 1:9 MeOH/CH_2Cl_2 as the eluent, and then recrystallized from EtOAc/hexane to give a white solid, yield 17%. ^{1}H NMR (DMSO-d_6): 1.14–1.16 (d, 3H, Ala-CH_3), 1.21–1.24 (t, 3H, OCH$_2$$CH_3$), 1.24–1.26 (d, 3H, Ala-CH_3), 1.36 (s, 9H, Boc), 3.33 (m, 2H, NCH_2CO), 3.96 (m, 1H, α-H), 4.07–4.11 (q, 2H, OCH_2CH$_3$), 4.26–4.29 (m, 1H, α-H), 6.56–6.60 (d, 1H, J = 15.6 Hz, CH=CH,), 7.16–7.20 (m, 2H, NH and CH=CH), 7.51 (s, 1H, NH), 8.03–8.04 (d, 1H, NH), 10.78 (s, 1H, NH).

***N*^{2}-(Alanylalanyl)-*N*^{1}-carbamoylmethyl-*N*^{1}-*trans*-(3-ethoxycarbonylpropenoyl)hydrazine Trifluoroacetate Salt (11, TFA·Ala-Ala-AAsn-CH=CHCOOEt).** Boc-Ala-Ala-AAsn-CH=CHCOOEt was deblocked with trifluoroacetic acid/methylene chloride (1:5) for 3 h at room temperature. The volatiles were evaporated, and the crude TFA salt was washed several times with to give a white solid, yield 99%. ^{1}H NMR (DMSO-d_6): 1.14–1.16 (d, 3H, Ala-CH_3), 1.21–1.24 (t, 3H, OCH$_2$$CH_3$), 1.24–1.26 (d, 3H, Ala-CH_3), 3.41 (m, 2H, NCH_2CO), 3.83 (m, 1H, α-H), 4.14–4.19 (q, 2H, OCH_2CH$_3$), 4.38–4.39 (m, 1H, α-H), 6.63–6.67 (d, 1H, J = 15.6 Hz, CH=CH,), 7.16–7.23 (m, 2H, NH and CH=CH), 7.54 (s, 1H, NH), 8.07 (s, 2H, 2 × NH), 10.97 (s, 1H, NH).

Biotin *N*-Hydroxysuccinimide (8, Biotin-OSu). This compound was prepared by dissolving biotin (1 equiv) in DMF at 80 °C. Upon cooling to room temperature *N*-hydroxysuccinimde (1.3 equiv) was added together with DCC (1 equiv). The mixture was stirred for 12 h at room temperature. Dicyclohexylurea was filtered off, and the solution was evaporated to dryness. The residue was taken up in boiling isopropanol, and the resulting suspension was cooled to room temperature. The solid was filtered and characterized, yield 72%. ^{1}H NMR (DMSO-d_6): 1.31–1.55 (m, 4H, biotin-CH_2CH_2), 1.55–1.69 (m, 2H, biotin-CH_2), 2.54–2.57 (d, 1H, CHS), 2.63–2.70 (t, 2H, biotin-CH_2CO), 2.78–2.90 (m, 7H, CH_2S and Su-CH_2CH_2, and CHS), 4.1–4.2(m, 1H, biotin-CH), 4.25–4.31 (m, 1H, biotin-CH), 6.35 (s, 1H, biotin-NH), 6.41 (s, 1H, biotin-NH).

***N*^{2}-(*N*-Biotinylalanylalanyl)-*N*^{1}-carbamoylmethyl-*N*^{1}-*trans*-(3-ethoxycarbonylpropenoyl)hydrazine (12, Biotin-Ala-Ala-AAsn-CH=CHCOOEt).** To a solution of TFA·Ala-Ala-AAsn-CH=CHCOOEt (1 equiv) in DMF, triethylamine (1 equiv) and biotin *N*-hydroxysuccinimide (1.5 equiv) in DMF were added. The mixture was stirred overnight at room temperature. The solvent was evaporated, and the crude residue was washed several times with cold methanol to give biotin-Ala-Ala-AAsn-CH=CHCOOEt as a white solid, yield 31%. ^{1}H NMR (DMSO-d_6): 1.14–1.16 (d, 3H, Ala-CH_3), 1.19–1.23 (t, 3H, OCH$_2$$CH_3$), 1.23–1.24 (d, 3H, Ala-CH_3), 1.27–1.28 (m, 2H, biotin-CH_2), 1.45–1.64 (m, 4H, biotin-CH_2CH_2), 2.06–2.10 (t, 2H, biotin-CH_2CO), 2.54–2.57 (d, 1H, CHS), 2.78–2.82 (dd, 1H, CH_2S, J = 5.2 and 12.4 Hz), 3.05–3.10 (m, 1H, CH_2S), 3.31 (s, 2H, NCH_2CO), 4.08–4.13 (m, 2H, biotin-CH and α-H), 4.13–4.19 (q, 2H, OCH_2CH$_3$), 4.23–4.30 (m, 1H, biotin-CH and α-H), 6.35 (s, 1H, biotin-NH), 6.41 (s, 1H, biotin-NH), 6.57–6.61 (d, 1H, J = 15.2 Hz, CH=CH,), 7.16–7.23 (m, 2H, NH and CH=CH), 7.51 (s, 1H, NH), 7.92 (d, 1H, NH), 8.15–8.16 (d, 1H, NH), 10.76 (s, 1H, NH). MS (ESI) *m*/*z* 584 [(M + 1)$^+$]. HRMS (ESI) calculated for $C_{24}H_{38}N_7O_8S$: 584.2447. Observed *m*/*z* 584.249 71.

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Supporting Information Available: Results from elemental analysis of target componds. This material is available free of charge via the Internet at http://pubs.acs.org.

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