Design, Synthesis, and Evaluation of Aza-Peptide Epoxides as Selective and Potent Inhibitors of Caspases-1, -3, -6, and -8

Karen Ellis James,[†] Juliana L. Asgian,[†] Zhao Zhao Li,[†] Özlem Doğan Ekici,[†] John R. Rubin,[§] Jowita Mikolajczyk,[‡] Guy S. Salvesen,[‡] and James C. Powers*[,†]

School of Chemistry and Biochemistry and the Parker H. Petit Institute for Bioengineering and Bioscience, Georgia Institute of Technology, Atlanta, Georgia 30332-0400, Program in Apoptosis and Cell Death Research, The Burnham Institute, 10901 North Torrey Pines Road, La Jolla, California 92037, and Biophysics Research Division, The University of Michigan, Ann Arbor, Michigan

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Aza-peptide epoxides, a novel class of irreversible protease inhibitors, are specific for the clan CD cysteine proteases. Aza-peptide epoxides with an aza-Asp residue at P1 are excellent irreversible inhibitors of caspases-1, -3, -6, and -8 with second-order inhibition rates up to 1 910 000 M^{-1} s^{-1}. In general, the order of reactivity of aza-peptide epoxides is *S*,*S* > *R*,*R* > trans > cis. Interestingly, some of the *R*,*R* epoxides while being less potent are actually more selective than the *S*,*S* epoxides. Our aza-peptide epoxides designed for caspases are stable, potent, and specific inhibitors, as they show little to no inhibition of other proteases such as the aspartyl proteases porcine pepsin, human cathepsin D, plasmepsin 2 from *P. falciparum*, HIV-1 protease, and the secreted aspartic proteinase 2 (SAP-2) from *Candida albicans*; the serine proteases granzyme B and α-chymotrypsin; and the cysteine proteases cathepsin B and papain (clan CA), and legumain (clan CD).

Introduction

Cysteine proteases, which employ a nucleophilic thiol for peptide bond acylation, are involved in numerous important physiological processes, which are associated with a variety of disease states. Cysteine proteases have been classified in evolutionary families and clans by Rawlings and Barrett.[1] The most relevant structural classes of cysteine proteases are clan CA which contains papain, cathepsins, calpain, and clan CD which contains caspases, legumain, gingipain, clostripain, and separase.[2] One of the most noticeable differences between these two clans is their substrate specificity. The substrate specificity for clan CA of cysteine proteases is determined by the S2 pocket, while the substrate specificity for clan CD is determined by the S1 pocket.[3]

Caspases or *c*ysteinyl *asp*artate-specific prote*ase*s are a recently discovered family of cysteine endoproteases, which are involved in cytokine maturation and apoptosis.[4] One of the most striking features of this class of enzymes is their stringency for Asp at the P1 residue. Caspases have a specificity for at least the four amino acids to the amino terminal side of the cleavage site (P side). At present, there are 11 known homologous members of the caspase family in humans. The only other mammalian protease with specificity for Asp is the lymphocyte serine protease, granzyme B, whose physiologic role is to serve as a caspase activator. The roles and function of the individual caspases have been clarified over the past few years, and it is clear that they are recognized as novel therapeutic targets in drug discovery due to their roles in both apoptosis and inflammation.[5] Excessive apoptosis has been associated with a variety of disease states including ischemic injuries, acquired immunodeficiency syndrome (AIDS), osteoporosis, and neurodegenerative disorders such as amyotrophic lateral sclerosis (ALS), Huntington's disease, Alzheimer's disease, Parkinson's disease, and spinal muscular atrophy.[6−9] Therefore, selective and specific caspase inhibitors would be invaluable for elucidation of the roles of individual caspases in apoptosis and as potential therapeutics.

Numerous classes of inhibitors for cysteine proteases have been developed during the past decades.[10,11] Many of these classes of inhibitors have been applied recently to clan CD proteases, especially caspase-1 and caspase-3. Reversible inhibitors include aldehydes, ketones, and isatin sulfonamides.[12−15] The reversible inhibitors reported thus far, such as aldehydes, are potent caspase inhibitors and have considerable selectivity for individual caspases.[15−17] However, they have not been tested with other cysteine proteases and granzyme B. Only a few irreversible inhibitors have been investigated with caspases, and these include peptide halomethyl ketones (peptide-$COCH_2X$, X = Cl or F), diazomethyl ketones (peptide-CHN_2), and acyloxymethyl ketones (peptide-$COCH_2OCOR$). Irreversible inhibitors, such as fluoromethyl ketones and chloromethyl ketones, are claimed to be specific and are commonly used to determine the roles of the caspases in cell and animal models of apoptosis. Recently it has been shown that caspase-directed fluoromethyl ketones and chloromethyl ketones are not as specific for caspases as previously hoped, since they efficiently inhibit cathepsins, papain, and legumain.[18] Therefore, the therapeutic utility of fluoromethyl ketones and chloromethyl ketones may be limited due to nonspecific inhibition of caspases and other cysteine proteases such as cathepsins.

* Correspondence author. Tel: (404) 894-4038, Fax: (404) 894-2295, E-mail: james.powers@chemistry.gatech.edu.
[†] Georgia Institute of Technology.
[‡] The Burnham Institute.
[§] The University of Michigan.

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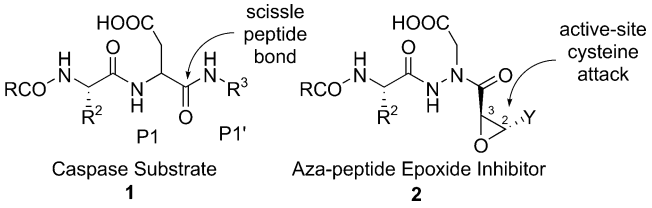

Figure 1. The design of aza-peptide epoxide inhibitors. We abbreviate the epoxide (C_2H_2O) as EP and aza-aspartic acid ($NHN(CH_2COOH)CO$) as AAsp, thus the above inhibitor (**2**) is designated RCO-AA_2-AAsp-EP-Y, where RCO can be peptidyl or acyl and Y can be simple alkyl groups (CH_2CH_2Ph), esters ($COOR_4$), amides ($CONHR_5$), or amino acids (CO-AA-R_6).

The epoxide moiety has previously been reported in a number of inhibitors for cysteine proteases and in a few serine protease inhibitor structures.[11] Epoxysuccinyl peptides are derivatives of the natural product E-64, which was first isolated from *Aspergillus japonicus*.[19] These irreversible inhibitors have been extensively studied due to their reactivity toward clan CA cysteine proteases such as calpain, papain, cruzain, and cathepsins.[20−22] However, E-64 derivatives are ineffective inhibitors of clan CD proteases, including caspases.[2] Epoxysuccinate derivatives are very useful in vitro and in vivo due to their stability, potent inhibitory activity, and permeability into cells and tissues.[11] Epoxide derivatives have been proposed as possible therapeutic agents and have been shown to have biological activity in a variety of animal disease models, such as muscular dystrophy,[23] ischemic neuronal death,[24,25] *Leishmania major* infection,[26] glomerulonephritis,[27,28] osteoclastic bone resorption,[29−31] and cancer.[32] One E-64 derivative, E-64d, prevents both calpain up-regulation and apoptosis in the lesion and penumbra following spinal cord injury in rats.[33,34]

In an effort to design and synthesize more specific and selective caspase inhibitors, our laboratory has recently reported a novel class of protease inhibitors, which we refer to as aza-peptide epoxides.[35,36] Aza-peptide epoxides were designed based on the structure of a good peptide substrate (**1**, Figure 1) with the placement of the carbonyl group of the epoxide moiety in a location identical to that of the carbonyl of the scissile bond in a substrate (**2**). Replacement of the α-carbon of the amino acid residue at P1 with a nitrogen results in the formation of an aza-amino acid residue. For example, in the design of the aza-peptide epoxide inhibitors for caspases, the α-carbon of the P1 Asp residue was converted to a nitrogen atom to obtain an aza-Asp residue (which will be abbreviated as AAsp).[37] This novel group of irreversible inhibitors was found to be highly specific for cysteine proteases of clan CD, particularly caspases. In this paper, we elaborate the aza-peptide epoxide structure in an effort to improve their potency and selectivity. In particular, we were interested in designing potent and selective aza-peptide epoxide inhibitors for caspases-1, -3, -6, and -8.

Chemistry

Our preliminary communication reports the general method used to prepare aza-peptide epoxides.[35] The procedure involves coupling a substituted peptidyl hydrazide (**21**) with the corresponding oxirane-2-carboxylic acid derivative (**5a**−**m**) (Figure 2). Aza-peptide epoxides have two major components: a substituted peptide hydrazide moiety and an epoxide moiety. Peptidyl hydrazides (**20**) were prepared from mono-, di-, or tripeptide methyl esters by addition of excess hydrazine (yields of 47% to 97%). The aza-aspartic acid side chain was introduced by alkylation of **20** with *tert*-butyl bromoacetate in DMF to give the substituted peptidyl hydrazide (**21**, yields of 48% to 65%).

A variety of oxirane carboxylic acid derivatives which are substituted at the 3-position with alkyl groups (**5a** and **5b**), esters (**5c**−**e**), amides (**5f**−**i**), and amino acids (**5j**−**m**) were synthesized following the scheme in Figure 3. Using different epoxidation methods and starting materials we have obtained cis, trans, 2*R*,3*R*, and 2*S*,3*S* stereoisomers at the epoxide moiety.

trans-3-Phenethyloxirane-2-carboxylic acid (**5a**) was synthesized starting with 3-phenylpropionaldehyde (**3**) and malonic acid to form the α,β-unsaturated acid, which was then transformed into the ethyl ester **4**. The double bond was epoxidized using *tert*-butyl peroxide and *tert*-butyllithium,[38,39] and then the ethyl ester was hydrolyzed using KOH in methanol to yield **5a**. Epoxidation of 3-(4-chlorophenyl)-acrylic acid methyl ester using the same method followed by hydrolysis of the methyl ester using NaOH in methanol yielded *trans*-3-(4-chlorophenyl)-oxirane-2-carboxylic acid (**5b**).

Enantiomerically pure diethyl epoxysuccinate esters (**6,** 2*S*,3*S* and 2*R*,3*R*) were synthesized from diethyl D-(−) and L-(+)-tartrate, following the general method developed by Mori and Iwasawa.[40,41] The *trans*-oxirane-2,3-dicarboxylic acid diethyl ester (**6**, trans) was synthesized using a general procedure for the stereocontrolled epoxidation of α,β-unsaturated carbonyl compounds, which was similar to the method developed by Meth-Cohn.[38] The selective hydrolysis of one ester to yield the monoethyl epoxysuccinate (**7**) was accomplished using a previously described procedure.[42,43] Complete hydrolysis of both esters to give the oxirane-2,3-dicarboxylic acid (**8**, trans, 2*R*,3*R*, and 2*S*,3*S*) was accomplished by using 2 equiv of NaOH in methanol. The oxirane-2-carboxylic acid monoester derivatives **5d** (trans, 2*R*,3*R*, and 2*S*,3*S*) and **5e** (trans, 2*S*,3*S*) were obtained by addition of 1 equiv of benzyl alcohol or phenethyl alcohol to **8** using EDC and DMAP as coupling reagents. Amide derivatives of oxirane-2,3-dicarboxylic acid (**5f**−**m**) were obtained by condensing the corresponding amine or amino acid to **7**, followed by hydrolysis of the ethyl ester using 1.2 equiv of KOH in ethanol.[44]

To maximize the yield and simplify purification of the final aza-peptide epoxide products (**22**−**27 a**−**n**), different synthetic methods were used for coupling the substituted peptidyl moiety (**21**) with the epoxide derivatives (**5a**−**m**). The EDC/HOBt coupling method was used primarily for aza-peptide epoxides with shorter, less crowded peptide sequences such as Cbz-Val-$NHNHCH_2COO$-*t*Bu and PhPr-Val-Ala-$NHNHCH_2COO$-*t*Bu. Coupling of bulkier peptides, such as Cbz-Asp(O-*t*Bu)-Glu(O-*t*Bu)-Val-$NHNHCH_2COO$-*t*Bu and Cbz-Leu-Glu(O-*t*Bu)-Thr-$NHNHCH_2COO$-*t*Bu was accomplished using the mixed anhydride coupling method and an excess of the oxirane-2-carboxylic acid derivative. The pentafluorophenol method was used for coupling of Cbz-

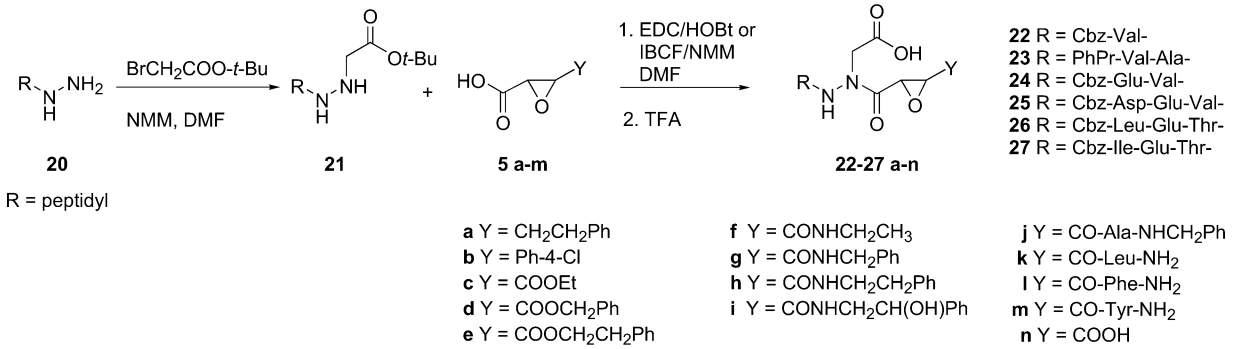

Figure 2. Synthesis of aza-peptide epoxide inhibitors. Abbreviations: DMF, *N*,*N*-dimethylformamide; EDC, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride; HOBt, 1-hydroxybenzotriazole; IBCF, isobutyl chloroformate; NMM, 4-methylmorpholine; TFA, trifluoroacetic acid.

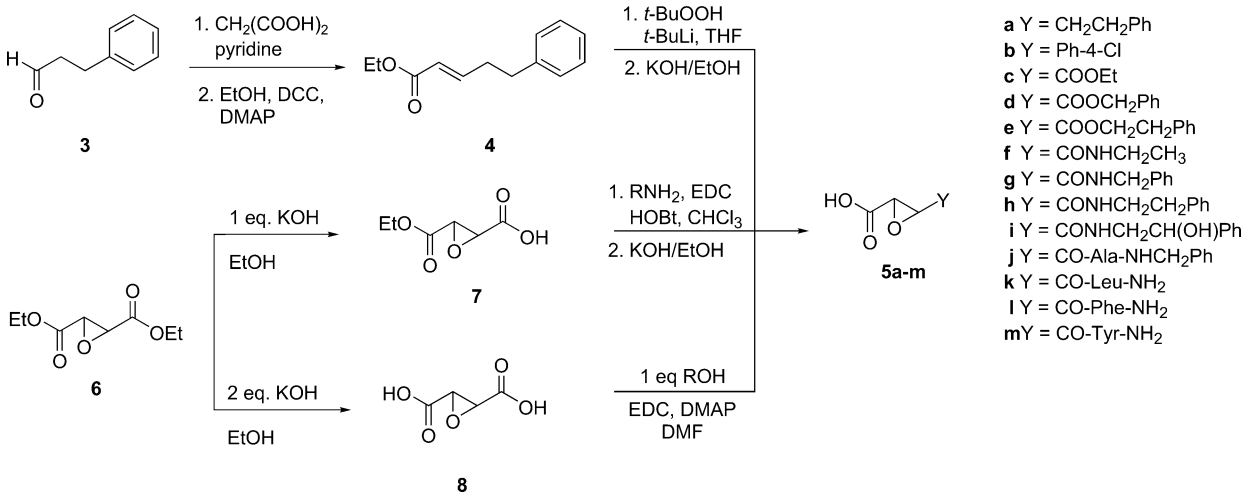

Figure 3. Synthesis of oxirane-2-carboxylic acid derivatives (**5a**−**m**). Abbreviations: DCC, 1,3-dicyclohexylcarbodiimide; DMAP, 4-(dimethylamino)pyridine; DMF, *N*,*N*-dimethylformamide; EDC, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride; HOBt, 1-hydroxybenzotriazole; THF, tetrahydrofuran.

Leu-Glu(O-*t*Bu)-Thr-NHNHCH$_2$COO-*t*Bu with the monoethyl epoxysuccinate.[41] The final step of aza-peptide epoxides synthesis involved hydrolysis of the *tert*-butyl protecting group on the aza-Asp, Asp, and Glu residues using TFA at 0 °C (**22**−**28**, yields 59% to 85%). In addition, hydrolysis of the ethyl ester moiety of **22c** with KOH yielded an aza-peptide epoxide with an acid moiety at the P′ position (**22n**).

Results and Discussion

Aza-Peptide Epoxide Inhibitor Design. The aza-peptide epoxide inhibitor structure was designed to mimic a peptide substrate from the N-terminus of the peptide up to the scissile bond (Figure 1). The electrophilic epoxide moiety was then placed in the vicinity of the active site cysteine residue and can covalently inhibit the enzyme. Aza-peptide epoxides also have the advantage of being easily extendable in the P′ direction allowing additional interactions with the S′ subsites of the caspases.

In the design of the first aza-peptide epoxide caspase inhibitors, we utilized peptide sequences derived from natural caspase substrate cleavage sites or obtained by peptide mapping of caspases with libraries of AMC substrates.[45] The VAD sequence for caspase-1 is derived from the cleavage sequence in proIL-1β.[46] The DEVD and LETD sequences are optimal sequences for caspase-3 and caspases-8, respectively, and were determined using a positional scanning synthetic combinatorial library.[47,48] The IETD sequence for caspases-6 and -8 is the cleavage sequence of a natural caspase-8 substrate, the caspase-3 proenzyme.[45] The EVD sequence was chosen to represent a truncated caspase-3 inhibitor.

Aza-peptide epoxides with an aza-Asp residue at P1 are excellent irreversible inhibitors of various caspases (Table 1). We report second-order inhibition rates (k_2 values) with caspases-1, -3, -6, and -8, and the observed k_2 values ranged up to 1 910 000 M^{-1} s^{-1}. In general, the caspase-1 sequence Val-Ala-AAsp was most reactive with caspase-1, the caspase-3 sequence Asp-Glu-Val-AAsp was most reactive with caspase-3, the sequence Ile-Glu-Thr-AAsp was most reactive with caspase-6, and the caspase-8 sequence Leu-Glu-Thr-AAsp was most reactive with caspase-8. In the table, we abbreviate the aza-aspartic acid as AAsp and the epoxide as EP (the nomenclature is discussed in Figure 1). To understand the specificity and selectivity of the inhibitors at the P′ position, we have changed the substituents on the epoxide moiety (**2**) from simple alkyl groups (Y = CH_2-CH_2Ph) to esters (Y = $COOR_4$), amides (Y = $CONHR_5$), or amino acids (Y = CO-AA-R_6). We sought to obtain increased selectivity by utilizing interactions with the S′ subsites of the various caspases.

Stereochemistry. The epoxide moiety in aza-peptide epoxides has two chiral centers, and the stereochemistry at these centers plays an important role in the potency

Table 1. Inhibition of Caspases-1, -3, -6, and -8 by Aza-Peptide Epoxides[a]

			k_2 ($M^{-1}s^{-1}$)			
	inhibitor	EP	caspase-1	caspase-3	caspase-6	caspase-8
22c	Cbz-Val-AAsp-EP-COOEt	S,S	ND[b]	3650 ± 570	350 ± 80	1260 ± 350
22n	Cbz-Val-AAsp-EP-COOH	S,S	ND	1110 ± 340	90 ± 20	370 ± 80
22a	Cbz-Val-AAsp-EP-CH_2CH_2Ph	trans	ND	310 ± 5	44 ± 9	180 ± 1
22b	Cbz-Val-AAsp-EP-Ph-4-Cl	trans	ND	145 ± 18	NI	NI
23a	PhPr-Val-Ala-AAsp-EP-CH_2CH_2Ph	trans	300 ± 140	ND	ND	ND
23c	PhPr-Val-Ala-AAsp-EP-COOEt	*S,S*	32200 ± 14500	1040 ± 80	75 ± 15	1570 ± 230
		R,R	6290 ± 1500	NI	NI	NI
23d	PhPr-Val-Ala-AAsp-EP-$COOCH_2Ph$	trans	18100 ± 2600	680 ± 140	ND	ND
		S,S	52100 ± 5300	1470 ± 40	165 ± 30	1560 ± 110
		R,R	1860 ± 300	NI	15	185
23e	PhPr-Val-Ala-AAsp-EP-$COOCH_2CH_2Ph$	*S,S*	56600 ± 9300	2110 ± 50	240 ± 14	3780 ± 630
23f	PhPr-Val-Ala-AAsp-EP-$CONHCH_2CH_3$	*S,S*	ND	1910 ± 370	142 ± 14	6050 ± 860
23g	PhPr-Val-Ala-AAsp-EP-$CONHCH_2Ph$	*S,S*	65900 ± 9150	1610 ± 5	155 ± 3	9360 ± 1570
		R,R	1630 ± 522	NI	NI	NI
23h	PhPr-Val-Ala-AAsp-EP-$CONHCH_2CH_2Ph$	*S,S*	17100 ± 2250	1110 ± 100	130 ± 10	10100 ± 1890
		R,R	950 ± 50	NI	NI	NI
23i	PhPr-Val-Ala-AAsp-EP-$CONHCH_2CH(OH)Ph$	*S,S*	12200 ± 20	510 ± 100	63 ± 6	4090 ± 630
		R,R	400 ± 20	NI	NI	NI
23j	PhPr-Val-Ala-AAsp-EP-CO-Ala-$NHCH_2Ph$	*S,S*	13400	260 ± 20	25 ± 5	140 ± 60
		R,R	270 ± 70	NI	NI	NI
23k	PhPr-Val-Ala-AAsp-EP-CO-Leu-NH_2	*S,S*	26100 ± 2560	420 ± 6	71 ± 15	315 ± 40
23l	PhPr-Val-Ala-AAsp-EP-CO-Phe-NH_2	*S,S*	32700 ± 9930	625 ± 160	65 ± 1	390 ± 130
		R,R	330	NI	NI	NI
23m	PhPr-Val-Ala-AAsp-EP-CO-Tyr-NH_2	*S,S*	3250 ± 2330	87 ± 4	11	125
24c	Cbz-Glu-Val-AAsp-EP-COOEt	*S,S*	58500	50500 ± 2080	4180	195000 ± 24300
24l	Cbz-Glu-Val-AAsp-EP-CO-Phe-NH_2	*R,R*	1040 ± 450	470 ± 80	25 ± 5	ND
24h	Cbz-Glu-Val-AAsp-EP-$CONHCH_2CH_2Ph$	*S,S*	41100 ± 440	27300 ± 5400	3130 ± 270	72700 ± 15000
25c	Cbz-Asp-Glu-Val-AAsp-EP-COOEt	*S,S*	11800 ± 1200	1070000 ± 155000	5440 ± 140	95500 ± 13000
		R,R	4320 ± 830	464000 ± 186000	475	785 ± 120
25l	Cbz-Asp-Glu-Val-AAsp-EP-CO-Phe-NH_2	*S,S*	9250 ± 2800	722000 ± 341000	6140 ± 530	29600 ± 4700
25g	Cbz-Asp-Glu-Val-AAsp-EP-$CONHCH_2Ph$	*S,S*	25400 ± 4010	1090000 ± 95000	6000 ± 390	84400 ± 21600
25d	Cbz-Asp-Glu-Val-AAsp-EP-$COOCH_2Ph$	*S,S*	54700 ± 16000	1910000 ± 209000	12700 ± 2070	188000 ± 52000
26c	Cbz-Leu-Glu-Thr-AAsp-EP-COOEt	*S,S*	25700	3210 ± 310	8620	61200 ± 6000
		R,R	19600	3250 ± 160	4390	34700 ± 6600
26d	Cbz-Leu-Glu-Thr-AAsp-EP-$COOCH_2Ph$	*S,S*	43500 ± 2100	3520 ± 600	10300 ± 690	72700 ± 18000
26h	Cbz-Leu-Glu-Thr-AAsp-EP-$CONHCH_2CH_2Ph$	*S,S*	ND	2550 ± 30	10000 ± 690	53300 ± 2200
26j	Cbz-Leu-Glu-Thr-AAsp-EP-CO-Ala-$NHCH_2Ph$	*S,S*	ND	1280 ± 80	6660 ± 2200	44800 ± 2800
27c	Cbz-Ile-Glu-Thr-AAsp-EP-COOEt	*S,S*	12400	4080 ± 360	45800 ± 4200	52800 ± 11000
		R,R	13300 ± 3700	2630 ± 360	34000 ± 1100	8800 ± 2700
27d	Cbz-Ile-Glu-Thr-AAsp-EP-$COOCH_2Ph$	*S,S*	45800	9500 ± 960	86200 ± 6600	58500 ± 4900
		R,R	13000 ± 980	3110 ± 120	45400 ± 1600	4560 ± 470
27g	Cbz-Ile-Glu-Thr-AAsp-EP-$CONHCH_2Ph$	*R,R*	18100 ± 4100	6500 ± 240	60100 ± 2000	6500 ± 1100
27j	Cbz-Ile-Glu-Thr-AAsp-EP-CO-Ala-$NHCH_2Ph$	*S,S*	ND	3050	38200 ± 2900	56000 ± 12000

[a] Inhibition rates were determined by the progress curve method. For caspase-1, the buffer was 100 mM HEPES, 0.5 mM EDTA, glycerol 20% (v/v), 5 mM DTT, at pH 7.5, and the substrate was Ac-YVAD-AMC. For caspases-3, -6, and -8, the buffer was 50 mM HEPES, 100 mM NaCl, 0.1% (w/v) CHAPS, sucrose 10% (w/v), 10 mM DTT, at pH 7.4, and the substrate was Cbz-DEVD-AFC or Cbz-DEVD-AMC. [b] ND = not determined, PhPr = $PhCH_2CH_2CO$, NI = no inhibition, EP = epoxide (C_2H_2O), AAsp = aza-Asp, Cbz = $PhCH_2CO$.

of the inhibitor. The more potent form of the natural cysteine protease inhibitor E-64 possesses the *S,S* stereochemistry at the epoxide moiety, while the *R,R* isomer is less reactive toward cysteine proteases. In general, the order of reactivity of aza-peptide epoxides was *S,S* > *R,R* > trans > cis (Table 1).[35] In comparing the rates of inhibition of caspase-1 by the *S,S*/*R,R* pairs (PhPr-Val-Ala-AAsp-EP-Y), we observed that the S,S isomer was more reactive by a factor of 5 to 100. With caspase-3, a comparison of the *S,S*/*R,R* pair for Cbz-Asp-Glu-Val-AAsp-EP-COOEt (**25c**) gave a ratio of 2. With caspase-6, comparison of the *S,S*/*R,R* pairs (Cbz-Ile-Glu-Thr-AAsp-EP-Y) gave ratios of 1 to 2. With caspase-8, comparison of the *S,S*/*R,R* pairs (Cbz-Leu-Glu-Thr-AAsp-EP-Y and Cbz-Ile-Glu-Thr-AAsp-EP-Y) gave ratios of 2 to 13. Thus, it appears that the epoxide stereochemistry was less significant with caspases-3, -6, and -8 than with caspase-1. Interestingly, some of the *R,R* epoxides while being less potent are actually more selective than the *S,S* epoxides. Almost all of the caspase-1 aza-peptide epoxides with *R,R* stereochemistry (PhPr-Val-Ala-AAsp-(2*R*,3*R*)-EP-Y, **23**) reacted only with caspase-1, although at slow rates, and showed no inhibition of caspases-3, -6, and -8 (Table 1). The *R,R* isomer of **25c** (Cbz-Asp-Glu-Val-AAsp-EP-COOEt) was almost as reactive an inhibitor with caspase-3 as the *S,S* isomer, but was much more selective and was 105-fold, 975-fold, and 590-fold more reactive with caspase-3 than with caspase-1, -6, and -8, respectively. In contrast, the *S,S* isomer of the caspase-3 inhibitor **25c** only showed selective ratios of 90-fold, 195-fold, and 11-fold with caspase-1, -6, and -8, respectively. The *R,R* isomers of **27c** (Cbz-Ile-Glu-Thr-AAsp-EP-COOEt) and **27d** (Cbz-Ile-Glu-Thr-AAsp-EP-$COOCH_2Ph$) are very selective caspase-6 inhibitors, being 4-fold and 10-fold more reactive with caspase-6 than caspase-8, while the respective *S,S* isomers are equally, if not more potent with caspase-8 than caspase-6.

Caspase-1. The caspase-1 cleavage sequence for proIL-1β is YVAD and X-ray studies with inhibitors demonstrate that the S4 subsite is a hydrophobic pocket.[46] The best caspase-1 inhibitor was **23g** (PhPr-Val-Ala-AAsp-EP-$CONHCH_2Ph$, $k_2 = 65\,900\ M^{-1}\ s^{-1}$) which has a phenylpropanoyl group at P4 which was designed to fit the S4 subsite of caspase-1. The most specific inhibitor, while still being quite potent with caspase-1, was PhPr-Val-Ala-AAsp-(*S,S*)-EP-CO-Phe-NH_2 (**23l**, $k_2 = 32\,700\ M^{-1}\ s^{-1}$) which was 50-fold, 500-fold, and 80-fold more reactive with caspase-1 than with caspases-3, -6, and -8, respectively. Similarly, other aza-peptide inhibitors (**23j**−**m**) which contain an amino acid moiety at P1′ are also highly selective for caspase-1.

With caspase-1, the order of reactivity of the epoxides with differing Y groups (Figure 1) is $CONHCH_2Ph$ > $COOCH_2CH_2Ph \geq COOCH_2Ph$ > CO-Phe-$NH_2 \geq$ COO-Et > CO-Leu-NH_2 > $CONHCH_2CH_2Ph$ > CO-Ala-$NHCH_2Ph \geq CONHCH_2CH(OH)Ph$ > CO-Tyr-NH_2 > CH_2CH_2Ph. The VAD benzyl amide (**23g** *S,S*) is almost four times more potent than the phenethyl amide (**23h** *S,S*) with caspase-1, while both remain equally as reactive with caspases-3, -6, and -8. However, the corresponding benzyl and phenethyl esters (**23d** *S,S* and **23e** *S,S*) are equally potent with caspase-1. It appears that hydrogen bonding with the amides has a considerable effect on their reactivity. Not only did extending the side chain of the amide at the P′ position by one CH_2 group from benzyl (**23g** *S,S*) to phenethyl (**23h** *S,S*) decrease its potency 4-fold, but introducing a hydroxyl group (**23i** *S,S*) in order to facilitate hydrogen bonding also decreased its potency. Interestingly, the amides (such as **23h** *S,S*) are quite reactive with caspase-1 ($k_2 = 17\,100\ M^{-1}\ s^{-1}$) and have considerable activity with caspase-8 ($k_2 = 10\,100\ M^{-1}\ s^{-1}$). In contrast, the esters (such as **23d** *S,S*) are also reactive toward caspase-1, but show much less reactivity toward the other caspases.

Caspase-3. The most reactive caspase-3 inhibitor was the benzyl ester **25d** with the DEVD sequence. We attempted to reduce the tetrapeptide DEVD sequence to the tripeptide EVD sequence in order to more readily synthesize inhibitor structures and quickly determine the best P′ moiety for caspase-3. However, we quickly learned that the P4 Asp was essential for caspase-3 selectivity and potency, as the tripeptide aza-peptide epoxides (Cbz-Glu-Val-AAsp, **24c** and **24h**) turned out to be better inhibitors for both caspase-8 and caspase-1 than caspase-3. These tripeptide inhibitors (**24c** and **24h**) could actually be used as general caspase-1, caspase-3, and caspase-8 inhibitors. Most of the caspase-3 inhibitors (DEVD and EVD sequences, **24c**−**25d**) while working best with caspase-3 (10^4−$10^6\ M^{-1}\ s^{-1}$), still decently inhibit caspase-8 (10^4−$10^5\ M^{-1}\ s^{-1}$) and caspase-1 (up to $58\,500\ M^{-1}\ s^{-1}$), but are much less reactive toward caspase-6 (up to $12\,700\ M^{-1}\ s^{-1}$). The addition of the amino acid Asp residue at the P4 position (going from **24c** to **25c**) doubled the rate of inhibition of caspase-3 and decreased by half the rate of inhibition of caspase-8. It has been previously shown that caspase-3 requires charged amino acids at P4, while caspase-8 likes branched, aliphatic side chains at P4.[45]

With caspase-3, the order of reactivity of the epoxides with differing Y groups (Figure 1) was $COOCH_2Ph$ > $CONHCH_2Ph \geq$ COOEt > CO-Phe-NH_2. Substitution of the ethyl ester (**25c**) with a benzyl ester (**25d**) on the epoxide at the P′ position doubled the inhibition rate for caspase-3 to give the most potent inhibitor with a k_2 value of $1\,910\,000\ M^{-1}\ s^{-1}$. However, the selectivity was not improved as the inhibition rates for this compound with the other caspases also increased. Our caspase-3 inhibitors are equally if not more potent than chloromethyl ketone inhibitors such as Cbz-DEVD-CMK which has a k_2/K_1 value of $1\,000\,000\ M^{-1}\ s^{-1}$.[18] More importantly, the aza-peptide epoxides are much more selective (see section on specificity).

Caspase-6 and Caspase-8. The most reactive caspase-6 inhibitor was the benzyl ester **27d** Cbz-Ile-Glu-Thr-AAsp-(*S,S*)-EP-$COOCH_2Ph$ ($k_2 = 86\,200\ M^{-1}\ s^{-1}$). Unfortunately, this inhibitor **27d** was also quite potent with caspase-1 ($k_2 = 45\,800\ M^{-1}\ s^{-1}$) and caspase-8 ($k_2 = 58\,500\ M^{-1}\ s^{-1}$). However, changing the stereochemistry from *S,S* to *R,R* with inhibitor **27d** ($k_2 = 45\,400\ M^{-1}\ s^{-1}$) increases the selectivity toward caspase-6 almost 10-fold over caspase-8 and 3.5-fold over caspase-1. With caspase-6, the order of reactivity of the epoxides with differing Y groups (Figure 1) was $COOCH_2Ph$ > $CONHCH_2Ph$ > COOEt > CO-Ala-$NHCH_2Ph$, similar to caspase-3.

Caspase-6 and caspase-8 have similar substrate specificities. At the P4 position, caspase-8 prefers leucine > valine > aspartic acid, while caspase-6 prefers valine > threonine > leucine.[49] However, caspase-6 prefers Ile over Leu at P4 while caspase-8 prefers Leu over Ile.[47] This explains the 9-fold difference in potency of the benzyl amide Cbz-Ile-Glu-Thr-AAsp-EP-$CONHCH_2Ph$ (**27g** *S,S*) with caspase-6 ($k_2 = 60\,100\ M^{-1}\ s^{-1}$) versus caspase-8 ($k_2 = 6500\ M^{-1}\ s^{-1}$). However, the IETD benzyl and ethyl esters **27c** (*S,S*) and **27d** (*S,S*) are not as selective and equally inhibit caspases-6 and -8.

The tripeptide inhibitor **24c** (Cbz-Glu-Val-AAsp-EP-COOEt) with *S,S* stereochemistry, which was designed for caspase-3, was the most potent inhibitor of caspase-8 ($k_2 = 195\,000\ M^{-1}\ s^{-1}$) and was 3-fold, 4-fold, and 45-fold more reactive with caspase-8 than caspases-1, -3, and -6, respectively. This compound (**24c**) was equally as potent an inhibitor of caspase-1 as **23e**, which was actually based on the caspase-1 sequence. Interestingly, the inhibitors based on the caspase-3 sequences DEVD and EVD were more potent ($73\,000$−$195\,000\ M^{-1}\ s^{-1}$) than the inhibitors with the LETD sequence ($35\,000$−$73\,000\ M^{-1}\ s^{-1}$), which were actually designed for caspase-8. However, the inhibitors based on LETD or IETD sequences, while working best with caspases-6 and -8 ($34\,000$−$86\,000\ M^{-1}\ s^{-1}$), were still potent inhibitors of caspase-1 ($12\,000$−$45\,000\ M^{-1}\ s^{-1}$), but were much less reactive with caspase-3 (1200−$9500\ M^{-1}\ s^{-1}$). With caspase-8 sequences, the order of reactivity of the epoxides with differing Y groups (Figure 1) was $COOCH_2Ph$ > COOEt > $CONHCH_2CH_2Ph$ > CO-Ala-$NHCH_2Ph$. Based on the LETD sequence, the best caspase-8 inhibitor was **26d** (Cbz-Leu-Glu-Thr-AAsp-EP-$COOCH_2Ph$, $k_2 = 72\,700\ M^{-1}\ s^{-1}$), which also was a potent inhibitor of caspase-1 ($k_2 = 43\,500\ M^{-1}\ s^{-1}$).

Caspase and Clan CD Selectivity. As expected, overall the most potent inhibitors had the preferred substrate sequence for the target caspase. The most potent caspase-1 inhibitors, such as PhPr-Val-Ala-AAsp-

Table 2. Inhibition of Non-Caspase Proteases by Caspase-Specific Aza-Peptide Epoxides

		k_{obs}/[I] (M^{-1} s^{-1})					
	inhibitor	papain[a]	cathepsin B[b]	chymotrypsin[c]	granzyme B[d]	legumain[e]	aspartyl proteases[f]
23a	PhPr-Val-Ala-AAsp-*trans*-EP-CH_2CH_2Ph	NI[g]	NI	NI	ND	NI	ND
23c	PhPr-Val-Ala-AAsp-(*S*,*S*)-EP-COOEt	ND	ND	ND	ND	ND	NI
23d	PhPr-Val-Ala-AAsp-*trans*-EP-$COOCH_2Ph$	<10	<10	NI	ND	21	ND
23d	PhPr-Val-Ala-AAsp-(*S*,*S*)-EP-$COOCH_2Ph$	ND	<10	ND	<0.3	ND	NI
24c	Cbz-Glu-Val-AAsp-(*S*,*S*)-EP-COOEt	ND	ND	ND	ND	86	NI
25c	Cbz-Asp-Glu-Val-AAsp-(*S*,*S*)-EP-COOEt	NI	NI	NI	<0.1	46	ND
25c	Cbz-Asp-Glu-Val-AAsp-(*R*,*R*)-EP-COOEt	NI	NI	NI	<0.1	NI	NI
26c	Cbz-Leu-Glu-Thr-AAsp-(*S*,*S*)-EP-COOEt	NI	NI	NI	NI	36	NI
26c	Cbz-Leu-Glu-Thr-AAsp-(*R*,*R*)-EP-COOEt	NI	NI	NI	ND	15	ND
27c	Cbz-Ile-Glu-Thr-AAsp-(*S*,*S*)-EP-COOEt	ND	NI	ND	0.65[h]	ND	NI

[a] For papain, the buffer was 50 mM HEPES containing 2.5 mM DTT and 2.5 mM EDTA at pH 7.5, and the substrate was Cbz-Phe-Arg-*p*NA. [b] For cathepsin B, the buffer was 0.1 M potassium phosphate containing 1.25 mM EDTA, 0.01% Brij 35, at pH 6.0, and the substrate was Cbz-Arg-Arg-AMC. [c] For chymotrypsin, the buffer was 0.1 M HEPES containing 0.5 M NaCl at pH 7.5, and the substrate was Suc-Ala-Ala-Pro-Phe-*p*NA. [d] For granzyme B, the buffer was phosphate-buffered saline at pH 7.3, containing 1.4 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, and 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, and the substrate was Cbz-Ile-Glu-Thr-Asp-*p*NA. [e] For legumain, the buffer was 39.5 mM citric acid containing 121 mM Na_2HPO_4, 1 mM EDTA, 1 mM TCEP and 0.01% CHAPS at pH 5.8, with Cbz-Ala-Ala-Asn-AMC as the substrate. [f] Inhibitors were assayed with the following aspartyl proteases: porcine pepsin, human cathepsin D, plasmepsin 2 (from *P. falciparum*), the secreted aspartic proteinase 2 (SAP-2) from *Candida albicans*, and the HIV-1 protease. The following buffers were used: 0.05 M sodium acetate, 0.15 M NaCl, and 1 mM DTT for HIV-1 protease; 0.1 M sodium formate at pH 4.5 for plasmepsin-2 and SAP-2; and 0.1 M sodium formate at pH 3.5 for pepsin and cathepsin D. [g] ND = not determined, PhPr = $PhCH_2CH_2CO$, NI = no inhibition, EP = epoxide (C_2H_2O), AAsp = aza-Asp, Cbz = $PhCH_2CO$. [h] An IC_{50} value of 105 μM was also obtained.

(*S*,*S*)-EP-$CONHCH_2Ph$ (**23g**), have the optimal caspase-1 peptide recognition sequence VAD. The most potent caspase-3 inhibitors, such as Cbz-Asp-Glu-Val-AAsp-(*S*,*S*)-EP-$COOCH_2Ph$ (**25d**), have the optimal caspase-3 peptide recognition sequence DEVD. In addition, the most potent caspase-6 inhibitors, such as Cbz-Ile-Glu-Thr-AAsp-(*S*,*S*)-EP-$COOCH_2Ph$ (**27d**), have the optimal caspase-6 peptide recognition sequence IETD. The only exception was caspase-8, where the caspase-3 tripeptide **24c** (Cbz-Glu-Val-AAsp-(*S*,*S*)-EP-COOEt) with EVD sequence was better than the caspase-8 peptide recognition sequence LETD (**26d**, Cbz-Leu-Glu-Thr-AAsp-(*S*,*S*)-EP-$COOCH_2Ph$).

In general, the inhibitors designed for each caspase do inhibit other caspases somewhat, just not as potently as the one for which they were designed. The most specific, although not the most potent, inhibitor for caspase-1 was PhPr-Val-Ala-AAsp-(*S*,*S*)-EP-CO-Phe-NH_2 (**23l**, k_2 = 32 700 M^{-1} s^{-1}) which was 52-fold, 504-fold, and 84-fold more reactive with caspase-1 than caspase-3, caspase-6, and caspase-8, respectively. The most specific and still quite reactive inhibitor for caspase-3 was Cbz-Asp-Glu-Val-AAsp-(*R*,*R*)-EP-COOEt (**25c**, k_2 = 464 000 M^{-1} s^{-1}) which was 107-fold, 977-fold, and 591-fold more reactive with caspase-3 than caspase-1, caspase-6, and caspase-8, respectively. The most specific inhibitor for caspase-6 was Cbz-Ile-Glu-Thr-AAsp-(*R*,*R*)-EP-$COOCH_2Ph$ (**27d**, k_2 = 45 400 M^{-1} s^{-1}, a moderate inhibitor) which was 3.4-fold, 14.5-fold, and 10-fold more reactive with caspase-6 than caspase-1, caspase-3, and caspase-8, respectively. The most specific for caspase-8 was Cbz-Leu-Glu-Thr-AAsp-(*S*,*S*)-EP-COOEt (**26c**, k_2 = 61 200 M^{-1} s^{-1}, a moderate inhibitor) which was 2.3-fold, 19-fold, and 7-fold more reactive with caspase-8 than caspase-1, caspase-3, and caspase-6, respectively.

The inhibitors designed for caspase-1 are specific for caspase-1 as they only poorly inhibit caspase-3, -6, and -8. Unfortunately, when one obtained amazingly high inhibition rates with caspase-3 (**25d**), the inhibitor also showed potent inhibition of the other enzymes. To obtain specificity, one might have to give up some potency.

To show that aza-peptide epoxides designed for caspases do not react with other clan CD enzymes, some of our caspase inhibitors were also tested with the clan CD enzyme legumain by W. Carter and Dr. Barrett at the Babraham Institute, UK. The aza-peptide epoxides designed for caspases were specific for caspases as the k_{obs}/[I] values with legumain (Table 2) ranged from 15 to 86 M^{-1} s^{-1} for inhibitors **23d** (PhPr-Val-Ala-AAsp-*trans*-EP-$COOCH_2Ph$), **24c** (Cbz-Glu-Val-AAsp-(*S*,*S*)-EP-COOEt), **25c** (Cbz-Asp-Glu-Val-AAsp-(*S*,*S*)-EP-COOEt), and **26c** (Cbz-Leu-Glu-Thr-AAsp-EP-COOEt, both isomers).[35] More importantly, two inhibitors, **23a** (PhPr-Val-Ala-AAsp-*trans*-EP-CH_2CH_2Ph) and **25c** (Cbz-Asp-Glu-Val-AAsp-(*R*,*R*)-EP-COOEt), showed no reactivity with legumain at all as more than 90% of the legumain activity remained after a 10 min incubation period (inhibitor concentrations ranged from 100 μM to 100 nM). Aza-peptide epoxides, such as Cbz-Ala-Ala-AAsn-(*S*,*S*)-EP-COOEt, which were designed with sequences specific for legumain, were potent legumain inhibitors with second-order rate constants (k_{obs}/[I] values) up to 43 000 M^{-1} s^{-1}.[35,36] However, aza-peptide epoxides with amides on the P′ epoxide moiety (Y, Figure 1), that were synthesized with sequences specific for legumain (such as Cbz-Ala-Ala-AAsn-(*S*,*S*)-EP-$CONHCH_2Ph$), show absolutely no inhibition of legumain.[36] Interestingly, amides, which were easier to synthesize and should be chemically more stable than esters, were equally as effective inhibitors with the caspases. Therefore, since legumain does not tolerate monosubstituted amides, caspase inhibitors with monosubstituted amides on the epoxide moiety will have increased specificity for caspases over legumain.

We were hoping that a dipeptide aza-peptide epoxide such as **22** would be a fairly potent caspase inhibitor, but more importantly a general caspase inhibitor. Unfortunately, this inhibitor was a weak inhibitor (<3650 M^{-1} s^{-1}) and still worked best with caspase-3, suggesting that the caspases do require at least tripeptides for binding recognition. In addition, replacement of the ester on the epoxide moiety with an acid (**22n**) also decreased the potency of the inhibitor.

Similarly, the inhibitory potency decreased when the tetrapeptide caspase-3 inhibitor Cbz-Asp-Glu-Val-AAsp (**25**) was compared to the tripeptide Cbz-Glu-Val-AAsp (**24**); however, this tripeptide inhibitor remained quite reactive with multiple caspases.

Stability. Aza-peptide epoxides are potent inhibitors and exhibit considerable stability in buffer solutions. The inhibitor Cbz-Leu-Glu-Thr-AAsp-(*S*,*S*)-EP-$COOCH_2$-Ph (**26d**, 488 nM) was incubated in buffer containing DTT (50 mM HEPES, 100 mM NaCl, 0.1% (w/v) CHAPS, sucrose 10% (w/v), 10 mM DTT, at pH 7.4) for 32 min, substrate (Ac-DEVD-AFC, 100 μM) and enzyme (caspase-6, 10 nM) were added, and the reaction was monitored. The inhibitor was equally as potent as when not incubated with DTT. The inhibitors were also stable when dissolved in DMSO and stored in the freezer for six months. Freshly prepared inhibitor stock solutions of **26c** (Cbz-Leu-Glu-Thr-AAsp-(*R*,*R*)-EP-COOEt) and **27c** (Cbz-Ile-Glu-Thr-AAsp-(*S*,*S*)-EP-COOEt) in DMSO had k_2 values of 3250 and 4080 M^{-1} s^{-1} with caspase-3, respectively (Table 1). Stock solutions (in DMSO) of the same inhibitors removed from the freezer after six months of storage had similar rates with k_2 values of 3000 and 3600 M^{-1} s^{-1} with caspase-3 for **26c** and **27c**, respectively. Aza-peptide epoxides designed for dipeptidyl peptidase I (DPPI) have a half-life ($t_{1/2}$) of 350 min at pH 7 and 7.5, while vinyl sulfone inhibitors are less stable ($t_{1/2} \sim 50$ min). For example, the half-life of Nva-AHph-EP-COOEt was 7-fold and 9-fold higher than the half-life of Nva-Hph-VS-Ph at pH 7 and 7.5, respectively (unpublished results). Aza-peptides with blocked N-terminals are much more stable than their unblocked dipeptide derivatives. One advantage of epoxysuccinyl peptide inhibitors, reported in the literature, is their stability under physiological conditions toward simple thiols.[42] At physiological conditions, E-64 does not react with 100 mM cysteine nor does it inactivate lactate dehydrogenase, a nonproteolytic thiol-dependent enzyme.[22]

Specificity With Non-Clan CD Proteases. The aza-peptide epoxides designed for caspases show little to no inhibition of the clan CA cysteine proteases papain and cathepsin B, the serine proteases granzyme B and chymotrypsin, and various aspartyl proteases (Table 2).[35] The inhibitors PhPr-Val-Ala-AAsp-EP-CH_2CH_2Ph (**23a**), Cbz-Asp-Glu-Val-AAsp-EP-COOEt (**25c**, both isomers), and Cbz-Leu-Glu-Thr-AAsp-EP-COOEt (**26c**, both isomers) at 208 μM showed no inhibition of papain and cathepsin B after 80 min of incubation. The inhibitor PhPr-Val-Ala-AAsp-*trans*-EP-$COOCH_2$Ph (**23d**, 208 μM) had a second-order rate constant (k_{obs}/[I]) of <10 M^{-1} s^{-1} with papain and cathepsin B after 80 min of incubation.

Our caspase inhibitors do not inhibit clan CA cysteine proteases, but we also wanted to determine if aza-peptide epoxides designed with sequences specific for clan CA enzymes were reactive. Examples of these compounds are Ac-Leu-ALeu-EP-COOEt, Cbz-Phe-ALeu-EP-COOEt, Cbz-Leu-AAbu-EP-COOEt, Cbz-Leu-AHph-EP-COOEt, and Cbz-Leu-Leu-ALeu-EP-COOEt. Inhibition constants (k_{obs}/[I]) were less than or equal to 20 M^{-1} s^{-1} (unpublished results) with papain, cathepsin B, and calpain. The aza-peptide epoxide Suc-Np2-ALeu-*trans*-EP-COOEt (where Np2 = 2-naphthyl-alanyl) was designed for cathepsin F and tested by Dr. Brömme at the Mount Sinai School of Medicine with cathepsins F, K, L, and S. Time dependent inhibition was not observed, and the inhibition ranges were greater than 100 μM for cathepsins K and L, and between 50 and 100 μM for cathepsins F and S. Clearly, this aza-peptide epoxide design is specific for clan CD cysteine proteases.

The aza-peptide epoxide Cbz-Leu-Leu-ALeu-EP-COOEt was actually designed to inhibit the proteasome, a threonine protease.[50] This compound, tested by Dr. Orlowski at Mount Sinai School of Medicine, showed no inhibition of the proteasome.

It was also important to test for specificity against serine proteases such as chymotrypsin and granzyme B (Table 2). We tested a few compounds with the serine protease α-chymotrypsin. The inhibitors tested were PhPr-Val-Ala-AAsp-EP-CH_2CH_2Ph (**23a**), PhPr-Val-Ala-AAsp-*trans*-EP-$COOCH_2$Ph (**23d**), Cbz-Asp-Glu-Val-AAsp-EP-COOEt (**25c**, both isomers), and Cbz-Leu-Glu-Thr-AAsp-EP-COOEt (**26c**, both isomers). No inhibition of chymotrypsin was observed after 90 min of incubation with an inhibitor concentration of 223 μM (unpublished results). The protease granzyme B is thus far the only major mammalian serine protease which cleaves substrates that contain a P1 Asp. Since it can also activate caspases and trigger apoptosis, it was essential to test our inhibitors with this enzyme. The inhibitors PhPr-Val-Ala-AAsp-(*S*,*S*)-EP-$COOCH_2$Ph (**23d**), Cbz-Asp-Glu-Val-AAsp-EP-COOEt (**25c**, both isomers), Cbz-Leu-Glu-Thr-AAsp-(*S*,*S*)-EP-COOEt (**26c**), and Cbz-Ile-Glu-Thr-AAsp-(*S*,*S*)-EP-COOEt (**27c**) were tested with granzyme B by Z. Wang and Dr. Froelich at Northwestern. The inhibitor Cbz-Leu-Glu-Thr-AAsp-(*S*,*S*)-EP-COOEt (**26c**, 500 μM) was totally inactive after incubation with enzyme for 1 h. With PhPr-Val-Ala-AAsp-(*S*,*S*)-EP-$COOCH_2$Ph (**23d**), some inhibition was seen over a period of 4 h (k_{obs}/[I] < 0.3 M^{-1} s^{-1}). Even less inhibition was observed with Cbz-Asp-Glu-Val-AAsp-EP-COOEt (**25c**, both isomers, k_{obs}/[I] < 0.1 M^{-1} s^{-1}). The aza-peptide epoxide Cbz-Ile-Glu-Thr-AAsp-(*S*,*S*)-EP-COOEt (**27c**) did show some inhibition but was a very weak competitive inhibitor (IC_{50} = 105 μM). The activity of the enzyme dropped quickly in the presence of the inhibitor and remained almost constant with time. Time-dependent inhibition (if any) was not easily observed (k_{obs}/[I] = 0.65 M^{-1} s^{-1}). Coincidently, the best and preferred substrate sequence for granzyme B is Ile-Glu-Pro-Asp, with granzyme B almost exclusively preferring isoleucine at P4.[47]

Dr. B. Dunn, B. Beyer, and J. Clemente at the Univerity of Florida tested some of our most potent compounds with a variety of aspartyl proteases, including porcine pepsin, human cathepsin D, plasmepsin 2 from *P. falciparum*, HIV-1 protease, and the secreted aspartic proteinase 2 (SAP-2) from *Candida albicans*. The inhibitors tested were PhPr-Val-Ala-AAsp-(*S*,*S*)-EP-COOEt (**23c**), PhPr-Val-Ala-AAsp-(*S*,*S*)-EP-$COOCH_2$Ph (**23d**), Cbz-Glu-Val-AAsp-(*S*,*S*)-EP-COOEt (**24c**), Cbz-Asp-Glu-Val-AAsp-(*R*,*R*)-EP-COOEt (**25c**), Cbz-Leu-Glu-Thr-AAsp-(*S*,*S*)-EP-COOEt (**26c**), and Cbz-Ile-Glu-Thr-AAsp-(*S*,*S*)-EP-COOEt (**27c**). After incubation of aza-peptide epoxides with enzyme for 20 min (30 min incubation for HIV-1), no inhibition of more than 20% was observed (Table 2).

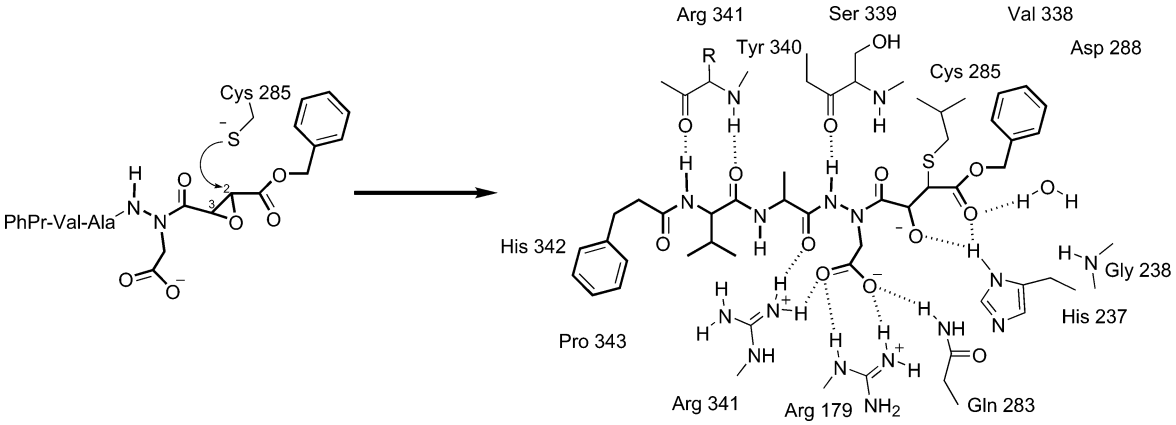

Figure 4. Mechanism of inhibition of caspase-1 by the aza-peptide epoxide inhibitor PhPr-Val-Ala-AAsp-*trans*-EP-$COOCH_2Ph$ (**23d**).

In summary, aza-peptide epoxide inhibitors are highly specific for clan CD cysteine proteases. Effectively, they are noninhibitors of serine proteases, clan CA cysteine proteases, threonine, and aspartate proteases. In contrast, other widely used caspase inhibitors are not specific at all. For example, Cbz-DEVD-CMK reacts potently with caspase-3 ($k_2/K_1 = 1\ 000\ 000\ M^{-1}\ s^{-1}$), but also is quite reactive with cathepsin V ($355\ 000\ M^{-1}\ s^{-1}$), cathepsin L ($36\ 000\ M^{-1}\ s^{-1}$), cathepsin B ($5200\ M^{-1}\ s^{-1}$), cathepsin S ($28\ 100\ M^{-1}\ s^{-1}$), papain ($1750\ M^{-1}\ s^{-1}$), and legumain ($660\ M^{-1}\ s^{-1}$).[18] Aza-peptide epoxides are more selective than halomethyl ketones as recent experiments have demonstrated that biotinylated Cbz-VAD-FMK and Ac-YVAD-CMK can label cathepsin B and cathepsin H.[51,52]

Binding Mode and Mechanism. The X-ray structures of caspase-1 inhibited by the peptide aldehydes Ac-Asp-Glu-Val-Asp-H[53] and Ac-Tyr-Val-Ala-Asp-H,[54] caspase-3 inhibited by Ac-Asp-Val-Ala-Asp-FMK[55] and Ac-Asp-Glu-Val-Asp-H,[56] and caspase-8 inhibited by Cbz-Asp-Glu-Val-Asp-H,[57] Cbz-Glu-Val-Asp-DCBMK,[58] and Ac-Ile-Glu-Thr-Asp-H[59] have been determined. X-ray crystal structures of caspase-1 inhibited by two aza-peptide epoxides PhPr-Val-Ala-AAsp-EP-$COOCH_2Ph$ and PhPr-Val-Ala-AAsp-EP-CH_2CH_2Ph have also been determined (Ron Rubin, unpublished results). Figure 4 shows a schematic drawing of the mechanism and interactions observed in the X-ray structure of PhPr-Val-Ala-AAsp-EP-$COOCH_2Ph$ with caspase-1. Nucleophilic attack by the active site Cys 285 of caspase-1 occurs at the C-2 position of the epoxide (Figure 4), resulting in a thioester linkage between the enzyme and the inhibitor. It is clear that the active site cysteine can attack functional groups quite distant from the side chain of the P1 aza-amino acid residue. We had expected the site of nucleophilic attack to be the C-3 position of the epoxide ring. Usually in transition-state inhibitors, such as peptide aldehydes, the active site cysteine adds to the aldehyde carbonyl group that is equivalent to the scissile peptide carbonyl group in a substrate.

Aza-peptide epoxide inhibitors containing the epoxysuccinate moiety are more potent inhibitors than derivatives which have an aryl alkyl group attached to the epoxide ring, such as Cbz-Val-AAsp-EP-CH_2CH_2Ph. The inhibitors Cbz-Val-AAsp-EP-COOEt (**22c**) and Cbz-Val-AAsp-EP-COOH (**22n**) were 10-fold more reactive than the alkyl derivatives Cbz-Val-AAsp-EP-CH_2CH_2-Ph (**22a**) and Cbz-Val-AAsp-EP-Ph-4-Cl (**22b**) with caspase-3. We propose that the derivatives with two carboxyl derivatives attached to the epoxide are more potent due to an electronegative effect of the additional carbonyl group.

The selectivity of aza-peptide epoxides for clan CD cysteine proteases is a novel feature of these inhibitors. We propose that our aza-peptide epoxides cannot bind in a suitable orientation for effective irreversible inhibition in the active sites of papain and other clan CA cysteine proteases, as a result of the inability of the fairly rigid aza-peptide epoxysuccinate moiety to bind properly near the enzyme's catalytic residues.

The enzymes in clan CD and clan CA have varying active site topologies, such that the caspase substrate-binding region is quite open and shallow with several binding pockets, while papain has a canyon-like binding site. In most caspase inhibitor complexes, the peptide chain of the inhibitor makes antiparallel β-sheet interactions with several residues in the active site of the caspases. The more open active site in the caspases coupled with the strict specificity for a P1 Asp residue allows the aza-Asp side chain of the aza-peptide epoxide to bind in the S1 pocket. This positions the epoxide moiety in close proximity to His 237 and the nucleophilic Cys 285 where covalent bond formation can occur. However, in papain, specificity is determined by interaction with the S2 subsite and a loop structure on the wall of the canyon, which contains the active site histidine, restricts possible binding modes of inhibitors. Papain family enzymes form parallel β-sheet interactions between the enzyme and the inhibitor and use an Asn residue to orientate the histidine properly in the active site. The caspases only have a catalytic dyad, but it is hypothesized that some caspases do use the backbone of residue 177 to create a catalytic triad.[60] The distance between the catalytic histidine and cysteine in caspases is longer which permits rotation of the histidine, and caspases only use one hydrogen bond to stabilize the oxyanion hole versus two hydrogen bonds in papain. These active site differences may contribute to the difference in specificity and reactivity observed for aza-peptide epoxides with caspases and clan CA enzymes.

One of the most noticeable differences between E-64 and aza-peptide epoxides is their binding mode in the enzyme's active site. Generally, it has been observed

that E-64 and its derivatives bind in the reverse peptide mode in the active site of clan CA proteases (C-terminal to N-terminal). On the other hand, aza-peptide epoxides bind in the caspases-1 active site in the same direction as the normal peptide substrate (N-terminal to C-terminal). Interestingly, we synthesized di- and tetrapeptide epoxides in the reverse peptide direction (similar to E-64) with both L- and D-amino residues and they showed no inhibition of caspases-3, -6, and -8 and little to no inhibition (k_{obs}/[I] < 10 M^{-1} s^{-1}) with cathepsin B (unpublished results). One such compound was HOOC-EP-Asp-D-Val-OMe.

Conclusion

Aza-peptide epoxides with a P1 Asp are specific and potent inhibitors for caspases-1, -3, -6, and -8. The aza-peptide epoxide inhibitors have been tested with a variety of other proteases including the aspartyl proteases porcine pepsin, human cathepsin D, plasmepsin 2 from *P. falciparum*, HIV-1 protease, and the secreted aspartic proteinase 2 (SAP-2) from *Candida albicans*; the serine proteases granzyme B and α-chymotrypsin; the cysteine proteases cathepsin B, papain, and caspases-1, -3, -6 and -8. They also show little to no cross reactivity with legumain, another clan CD enzyme specific for Asn. The inhibitors have second-order rate constants of up to 10^6 M^{-1} s^{-1} with the most potent epoxides having the *S,S* stereochemistry. Clearly, this aza-peptide epoxide design is specific for clan CD, as they show little to no inhibition of clan CA cysteine proteases, the proteasome, serine proteases, or aspartyl proteases. Currently, we are trying to refine the P′ portion of the inhibitors to obtain greater specificity. We are also extending this class of inhibitors to other caspases, legumain, gingipain and clostripain, other clan CD enzymes.

Aza-peptide epoxides are irreversible peptidomimetic inhibitors specific for caspases and are clearly useful as probes of purified caspases and of their cellular function. In order for these compounds to be useful in animal models of disease, it will be necessary to improve their pharmacokinetics and cell membrane penetration. With other caspase inhibitors such as peptide aldehydes, the low membrane permeability is due to the presence of the P1 Asp and the peptide chain, which mimics the natural peptide substrate. Replacement of the carboxyl group with isosteres and the peptide backbone with nonpeptide moieties has resulted in considerable improvement in bioavailability in other classes of inhibitors.[61] There is no intrinsic reason an aza-peptide[62] or an epoxide moiety[24,26,32,33] could not appear in a drug candidate. In the development of clinical candidates for treatment of rhinovirus infection (common cold), peptide inhibitors containing the vinyl sulfone warhead have been elaborated into structures with little residual peptide characteristics.[11,63,64] We propose that removal of the elements of the peptide backbone in our aza-peptide epoxides could also lead to suitable drug candidates. A few of our most potent aza-peptide epoxides have already been tested in various apoptotic assays. The preliminary results look promising, and we are continuing to synthesize new derivatives with better pharmacokinetic properties.

Experimental Section

Mono- and dipeptidyl methyl esters were purchased from Bachem Bioscience Inc., King of Prussia, PA. Tripeptides were synthesized using standard coupling procedures such as the mixed anhydride method. The ^{1}H NMR spectra were obtained using a Varian Mercury 300 MHz spectrometer. Electrospray ionization (ESI), fast-atom-bombardment (FAB) and high-resolution mass spectrometry were performed using Micromass Quattro LC and VG Analytical 70-SE instruments. Elemental analysis was performed by Atlantic Microlab Inc., Norcross, GA.

Peptidyl Hydrazides. Anhydrous hydrazine (10 equiv) was added to a solution of the peptidyl methyl ester (1 equiv) in MeOH at room temperature, and the resulting mixture was then stirred for 16 h. With most hydrazides, excess hydrazine and solvent were removed by evaporation. The resulting residue was washed with ethanol and ether to give the desired peptidyl hydrazide as a white solid. MS and ^{1}H NMR ($CDCl_3$ or DMSO-d_6) were consistent with the proposed structures. PhPr-Val-Ala-$NHNH_2$, white solid, yield 75%; Cbz-Val-$NHNH_2$, white solid, yield 92%; Cbz-Asp(O-*t*Bu)-Glu(O-*t*Bu)-Val-$NHNH_2$ was purified by chromatography on a silica gel column using 1:9 MeOH:CH_2Cl_2 as the eluent; white solid, yield 56%; Cbz-Glu(O-*t*Bu)-Val-$NHNH_2$ was purified by chromatography on a silica gel column using 1:9 MeOH:CH_2Cl_2 as the eluent; white solid, yield 47−53%; Cbz-Leu-Glu(O-*t*Bu)-Thr-$NHNH_2$, white solid, yield 97%; Cbz-Ile-Glu(O-*t*Bu)-Thr-$NHNH_2$, white solid, yield 91%.

Peptidyl-$(AA)_n$-$NHNHCH_2COO$-tBu. General Procedure. Neat *tert*-butyl bromoacetate (1 equiv) was added to a stirred solution of the peptide hydrazide and NMM (1 equiv) in DMF precooled at −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 20 h. The DMF was removed by evaporation, and the resulting residue was washed with water, filtered, and dried in vacuo. Purification on a silica gel column using the appropriate solvent gave the substituted peptidyl hydrazide (yields = 48−65%). MS and ^{1}H NMR (DMSO-d_6 or $CDCl_3$) were consistent with the proposed structure.

PhPr-Val-Ala-$NHNHCH_2COO$-*t*Bu was purified by column chromatography on silica gel using 1:9 MeOH:CH_2Cl_2 as the eluent; white solid, yield 55%.

Cbz-Val-$NHNHCH_2COO$-*t*Bu was purified by column chromatography on silica gel using 1:20:4.2 MeOH:CH_2Cl_2:EtOAc as the eluent; white solid, yield 64%. ^{1}H NMR (DMSO-d_6): 0.90 (t, 6H, Val), 1.40 (s, 9H, *t*Bu), 1.86 (m, 1H, Val), 3.37 (d, 2H, $NHCH_2COOH$), 3.72 (t, 1H, α-H), 4.99 (s, 2H, Cbz), 5.13 (d, 1H, NH), 7.30 (s, 5H, Ph), 9.38 (d, 1H, NH).

Cbz-Asp(O-*t*Bu)-Glu(O-*t*Bu)-Val-$NHNHCH_2COO$-*t*Bu was purified by column chromatography on silica gel using 2:18:5 MeOH:CH_2Cl_2:EtOAc as the eluent; white solid, yield 65%. MS (ESI) *m/z* 736.6 [$(M + 1)^+$]. ^{1}H NMR (DMSO-d_6): 0.90 (d, 6H, Val), 1.49 (s, 27H, *t*Bu), 1.85−2.20 (m, 3H, Val and Glu), 2.21 (m, 2H, Glu), 2.40−2.70 (m, 2H, Asp CH_2), 3.30 and 3.38 (m, 3H, $NHCH_2$ and $NHCH_2$), 4.05−4.30 (m, 3H, α-H), 5.05 (m, 2H, Cbz), 7.20−7.40 (m, 5H, Ph), 7.60−7.95 (m, 3H, NH), 9.2 (m, 1H, NH).

Cbz-Glu(O-*t*Bu)-Val-$NHNHCH_2COO$-*t*Bu was purified by column chromatography on silica gel using 2:18:5 MeOH:CH_2Cl_2:EtOAc as the eluent; white solid, yield 78%. MS (ESI) *m/z* 565.3 [$(M + 1)^+$]. ^{1}H NMR ($CDCl_3$): 0.95 (t, 6H, Val), 1.49 (s, 18H, *t*Bu), 1.85−2.20 (m, 3H, Val and Glu), 2.21 (m, 2H, Glu), 3.45−3.70 (m, 3H, $NHCH_2$ and $NHCH_2$), 4.25−4.30 (m, 2H, α-H), 5.05 (m, 2H, Cbz), 5.85 (d, 1H, NH), 7.05 (d, 1H, NH), 7.20−7.40 (m, 5H, Ph), 8.00 (m, 1H, NH).

Cbz-Leu-Glu(O-*t*Bu)-Thr-$NHNHCH_2COO$-*t*Bu was purified by column chromatography on silica gel using 1:9 MeOH:CH_2Cl_2 as the eluent; white solid, yield 34%. MS (ESI) *m/z* 680 [$(M + 1)^+$]. ^{1}H NMR (DMSO-d_6): 0.7−0.9 (t, 6H, Leu CH_3), 1.0 (d, 3H, Thr CH_3), 1−1.3 (m, 2H, Leu CH_2), 1.3−1.5 (m, 18H, *t*Bu), 1.5−1.8 (m, 2H, Leu CH and Glu CH_2), 1.8−1.95 (m, 1H, Glu CH_2), 2.1−2.3 (m, 2H, Glu CH_2), 3.4 (d, 2H, NCH_2), 3.9 (m, 1H, α-H), 4.1 (m, 1H, α-H and Thr C*H*-OH), 4.3 (m, 1H,

α-H), 4.9 (d, 1H, NH), 5.03 (m, 2H, Cbz), 7.3−7.4 (m, 5H, phenyl), 7.5 (d, 1H, NH), 7.6 (d, 1H, NH), 8.05 (d, 1H, NH), 9.2 (d, 1H, NH).

Cbz-Ile-Glu(O-*t*Bu)-Thr-NHNHCH$_2$COO-*t*Bu was purified by column chromatography on silica gel using 1:9 MeOH:CH$_2$Cl$_2$ as the eluent; white solid, yield 26%. MS (ESI) *m*/*z* 680 [(M + 1)$^+$]. ^{1}H NMR (DMSO-d_6): 0.7−0.9 (t, 6H, Ile CH$_3$), 0.9−1.0 (d, 3H, Thr CH$_3$), 1−1.2 (m, 2H, Ile CH$_2$), 1.3−1.5 (s, 18H, *t*Bu), 1.6−1.8 (m, 2H, Ile CH and Glu CH$_2$), 1.8−1.9 (m, 1H, Glu CH$_2$), 2.1−2.3 (m, 2H, Glu CH$_2$), 3.4 (d, 2H, NCH$_2$), 3.9 (m, 2H, α-H), 4.1 (m, 1H, α-H), 4.35 (m, 1H, Thr C*H*-OH), 4.8 (d, 1H, NH), 5.03 (s, 2H, Cbz), 5.05 (d, 1H, NH), 7.3−7.4 (m, 5H, phenyl), 7.7 (d, 1H, NH), 8.05 (d, 1H, NH), 9.2 (s, 1H, NH).

(2*S*,3*S*) and (2*R*,3*R*)-Oxirane-2,3-dicarboxylic Acid Monoethyl Esters (Monoethyl Epoxysuccinates, HOOC-EP-COOEt). Enantiomerically pure diethyl epoxysuccinate esters (2*S*,3*S*) and (2*R*,3*R*) were synthesized from diethyl D-(−) and L-(+)-tartrate, respectively, following the general method developed by Mori and Iwasawa.[40,41] This procedure involved three steps including bromination, elimination, and epoxidation. The selective hydrolysis of one ester to yield monoethyl epoxysuccinates was performed by a method similar to that described previously by Rich and Schaschke.[42,43]

***trans*-Oxirane-2,3-dicarboxylic Acid Diethyl Ester (or Diethyl Epoxysuccinate, EtOOC-EP-COOEt).** The *trans*-oxirane-2,3-dicarboxylic acid diethyl ester was synthesized using a general procedure for the stereocontrolled epoxidation of α,*β*-unsaturated carbonyl compounds, which was similar to the method previously described by Meth-Cohn.[38] An anhydrous solution of *tert*-butyl hydroperoxide in toluene (3.3 M solution, 46 mL, 1.5 equiv)[39] was added to freshly distilled THF (240 mL) at −78 °C under argon. This was followed by the addition of a solution of *tert*-butyllithium in pentane (1.7 M solution, 65 mL, 1.1 equiv). The mixture was stirred at −78 °C for 5 min, and then a solution of diethyl fumarate (17.2 g, 0.1 mol, 1 equiv) in THF (50 mL) was added. The reaction mixture was stirred at room temperature for 2 h (monitored by TLC). Sodium sulfite (10 g) was added and the mixture was stirred for 20 min. The mixture was concentrated to ca. 100 mL, diluted with ether (100 mL), filtered through diatamaceous earth (Celite), and evaporated. To the residue was added 1 M HCl (100 mL). The product *trans*-oxirane-2,3-dicarboxylic acid diethyl ester was extracted with EtOAc (3 × 100 mL), and the organic layer was washed with saturated NaCl (3 × 50 mL), dried over MgSO$_4$, and the solvent evaporated. Chromatography on a silica gel column with 2:3 EtOAc:hexane as the eluting solvent system afforded the product *trans*-oxirane-2,3-dicarboxylic acid diethyl ester (yield = 52%). ^{1}H NMR (CDCl$_3$): 1.2−1.4 (t, 6H, OCH$_2$*CH*$_3$), 3.70 (s, 2H, epoxy), 4.2−4.4 (m, 4H, O*CH*$_2$CH$_3$).

***trans*-3-(4-Chlorophenyl)oxirane-2-carboxylic Acid (HOOC-EP-Ph-4-Cl).** The starting material, 4-chloro-*trans*-cinnamic acid (1 equiv), was cooled to −20 °C in dry methanol. Thionyl chloride (3 equiv) was added dropwise to the cooled solution over 1 h. The mixture was stirred at −15 °C for an additional 30 min and subsequently stirred at room temperature for 24 h. Evaporation of the volatiles yielded the methyl ester MeOOC−CH=CH−Ph-4-Cl as a white solid (yield = 89%). Without further purification the acrylate was epoxidized using the procedure described above in the synthesis of *trans*-oxirane-2,3-dicarboxylic acid diethyl ester. The crude product was purified by column chromatography on silica gel using 1:3 EtOAc:hexane as the eluent. ^{1}H NMR (CDCl$_3$): 3.47 (d, 1H, epoxy), 3.83 (s, 3H, CH$_3$), 4.08 (d, 1H, epoxy), 7.21 (d, 2H, Ph), 7.33 (d, 2H, Ph). Standard hydrolysis of the methyl ester with sodium hydroxide (1 N) followed by acidic workup (1 N HCl) yielded the product, 3-(4-chlorophenyl) glycidic acid. ^{1}H NMR (DMSO-d_6): 3.65 (s, 1H, epoxy), 4.14 (s, 1H, epoxy), 7.37 (d, 2H, Ph), 7.43 (d, 2H, Ph).

***trans*-Oxirane-2,3-dicarboxylic Acid (HOOC-EP-COOH).** A solution of 1 M NaOH (98 mL, 1.9 equiv) was added to *trans*-oxirane-2,3-dicarboxylic acid diethyl ester (9.8 g, 52 mmol) in MeOH (30 mL) at 0 °C. The resulting solution was stirred for 1 h at 0 °C, then for 30 min at room temperature, after which the solution was acidified to pH 3. Water and MeOH were evaporated. The residue was treated with EtOH (150 mL) and filtered, and the solvent was evaporated to give *trans*-oxirane-2,3-dicarboxylic acid as a colorless semisolid (yield = 97%).

***trans*-Oxirane-2,3-dicarboxylic Acid Monobenzyl Ester (HOOC-EP-COOCH$_2$Ph).** The reagent EDC (2.32 g, 11 mmol) was added to a stirred solution of *trans*-oxirane-2,3-dicarboxylic acid (1.32 g, 10 mmol), benzyl alcohol (1.08 g, 10 mmol), and DMAP (122 mg, 1 mmol) in DMF (100 mL), which was cooled to 0 °C. The resulting solution was stirred for 15 h at room temperature. After removal of DMF, the residue was purified by column chromatography using 1:9 MeOH:CH$_2$Cl$_2$ as an eluent and was then rechromatographed using 1:4 MeOH:CH$_2$Cl$_2$ as the eluent to give *trans*-oxirane-2,3-dicarboxylic acid monobenzyl ester as a dark yellow oil (yield = 66%). ^{1}H NMR: 3.60 (d, 1H, epoxy), 3.75 (d, 1H, epoxy), 5.18 (s, 2H, PhCH$_2$O), 7.38 (d, 5H, Ph).

Enantiomerically pure monobenzyl esters (2*S*,3*S*) and (2*R*,3*R*) were synthesized using the above procedure for *trans*-oxirane-2,3-dicarboxylic acid monobenzyl ester by using (2*S*,3*S*)-oxirane-2,3-dicarboxylic acid and (2*R*,3*R*)-oxirane-2,3-dicarboxylic acid as the starting materials, respectively.

(2*S*,3*S*)-Oxirane-2,3-dicarboxylic Acid Monobenzyl Ester (HOOC-EP-COOCH$_2$Ph). ^{1}H NMR (CDCl$_3$): 3.70 (d, 2H, epoxy), 5.22 (d, 2H, *CH*$_2$Ph), 7.35 (m, 5H, Ph).

(2*R*,3*R*)-Oxirane-2,3-dicarboxylic Acid Monobenzyl Ester (HOOC-EP-COOCH$_2$Ph). ^{1}H NMR (CDCl$_3$): 3.65 (d, 2H, epoxy), 5.17 (d, 2H, *CH*$_2$Ph), 7.32 (m, 5H, Ph).

(2*S*,3*S*)-Oxirane-2,3-dicarboxylic Acid Monophenethyl Ester (HOOC-EP-COOCH$_2$CH$_2$Ph). This epoxide was synthesized using the above procedure for *trans*-oxirane-2,3-dicarboxylic acid monobenzyl ester by using (2*S*,3*S*)-oxirane-2,3-dicarboxylic acid and phenethyl alcohol as the starting materials. ^{1}H NMR (CDCl$_3$): 2.98 (t, 2H, CH$_2$), 3.65 (d, 2H, epoxy), 4.41 (m, 2H, OCH$_2$), 7.25 (m, 5H, Ph).

***trans*-3-Phenethyloxirane-2-carboxylic Acid (HOOC-EP-CH$_2$CH$_2$Ph).** A stirred solution of 3-phenylpropionaldehyde (6.7 g, 50 mmol) and malonic acid (5.2 g, 50 mmol) in pyridine (4 mL) was heated at 100−105 °C for 6 h.[65] The reaction mixture was poured onto ice/HCl. The solid precipitate was washed with water sufficiently to give 5-phenylpent-2-enoic acid (6.2 g, yield = 70%).

The reagents DCC (33 mmol) and DMAP (30 mmol) were added to a solution of 5-phenylpent-2-enoic acid (5.3 g, 30 mmol) in EtOH (50 mL) and THF (20 mL), which was cooled to 0 °C. The resulting mixture was stirred for 30 min at 0 °C and then allowed to react for 15 h at room temperature. The suspension was filtered, and the filtrate was concentrated. The residue was dissolved in EtOAc and washed with 1 M HCl (3 × 30 mL), H$_2$O (10 mL), saturated NaHCO$_3$ (3 × 30 mL), H$_2$O (10 mL), and saturated NaCl (20 mL), dried over MgSO$_4$, and concentrated. Chromatography on a silica gel column using 1:2 EtOAc:hexane as the eluent afforded 5-phenyl-pent-2-enoic acid ethyl ester (3.75 g, yield = 61%).

This compound was epoxidized using the procedure described above in the synthesis of *trans*-oxirane-2,3-dicarboxylic acid diethyl ester and purified on a silica gel column with 1:1 EtOAc:hexane as the eluent to afford *trans*-3-phenethyloxirane-2-carboxylic acid ethyl ester as a light yellow oil (yield = 46%). ^{1}H NMR: 1.25 (t, 3H, OEt); 1.90 (t, 2H, PhCH$_2$*CH*$_2$), 2.80 (t, 2H, PhCH$_2$), 3.2 (s, 2H, epoxy), 4.2 (q, 2H, OEt), 7.3 (m, 5H, Ph).

A solution of 1 M NaOH (1.1 eq, 5.5 mL) was added to a stirred solution of *trans*-3-phenethyloxirane-2-carboxylic acid ethyl ester (1.1 g, 5 mmol) in MeOH (10 mL) at room temperature. After stirring for 1 h at room temperature, the solution was acidified to pH 3, and extracted with EtOAc (2 × 30 mL). The organic layer was washed with water (2 × 30 mL) and saturated NaCl (20 mL), dried over MgSO$_4$, and concentrated to give *trans*-3-phenethyloxirane-2-carboxylic acid as a light yellow oil (yield = 72%). ^{1}H NMR: 1.90 (t, 2H, PhCH$_2$*CH*$_2$), 2.80 (t, 2H, PhCH$_2$), 3.30 (s, 2H, epoxy), 7.30 (m, 5H, Ph). HRMS (FAB) Calcd for C$_{12}$H$_{12}$O$_5$: 237. Observed *m*/*z* 236.9.

(2*S*,3*S*)-3-(2-Phenethylcarbamoyl)oxirane-2-carboxylic Acid (HOOC-(2*S*,3*S*)-EP-CONHCH$_2$CH$_2$Ph). General Procedure for Coupling Oxirane Dicarboxylic Acid Monoethyl Esters to Amines. The procedure used to synthesize amide derivatives of epoxysuccinate monoethyl esters was similar to that of Therrien et al.[44] To a solution of epoxysuccinate monoethyl ester (1 g, 6.25 mmol), phenethylamine (or another amine/amino acid (1.2 equiv)), and HOBt (1 equiv) in $CHCl_3$ (30 mL) at 0 °C was added EDC (1.1 equiv) slowly in five portions. The reaction was stirred for 1 h at 0 °C and then subsequently at room temperature for 16 h. The solvent was evaporated, and the residue was partitioned between EtOAc (50 mL) and dH_2O (5 mL). The organic layer was washed with 0.5 M HCl (2 × 10 mL) and sat. $NaHCO_3$ (2 × 50 mL), dried over $MgSO_4$, and concentrated. In general, the oxirane-2,3-dicarboxylic acid monoamides were obtained in 43−74% yield. The crude product was purified by chromatography on a silica gel column using 1:1 EtOAc:hexanes as the eluent to yield a white solid. Hydrolysis of the ester by KOH (1.2 equiv) gave the desired amides (yields = 65−95%). ^{1}H NMR ($CDCl_3$): 2.82 (t, 2H, *CH*$_2$Ph), 3.35 and 3.69 (d, 2H, epoxy), 3.54 (m, 2H, NCH_2), 6.20 (b, 1H, NH), 7.15−7.33 (m, 5H, Ph).

(2*R*,3*R*)-3-(2-Phenethylcarbamoyl)oxirane-2-carboxylic Acid (HOOC-(2*R*,3*R*)-EP-CONHCH$_2$CH$_2$Ph). ^{1}H NMR ($CDCl_3$): 2.82 (t, 2H, *CH*$_2$Ph), 3.35 and 3.69 (d, 2H, epoxy), 3.54 (m, 2H, NCH_2), 6.20 (b, 1H, NH), 7.15−7.33 (m, 5H, Ph).

(2*S*,3*S*)-3-(3-Benzylcarbamoyl)oxirane-2-carboxylic Acid (HOOC-(2*S*,3*S*)-EP-CONHCH$_2$Ph). This compound was synthesized using $PhCH_2NH_2$ as the starting material. ^{1}H NMR ($CDCl_3$): 3.53 and 3.78 (d, 2H, epoxy), 4.45 (d, 2H, *CH*$_2$Ph), 6.40 (b, 1H, NH), 7.25−7.33 (m, 5H, Ph).

(2*R*,3*R*)-3-(3-Benzylcarbamoyl)oxirane-2-carboxylic Acid (HOOC-(2*R*,3*R*)-EP-CONHCH$_2$Ph). This compound was synthesized using $PhCH_2NH_2$ as the starting material. ^{1}H NMR (DMSO-d_6): 3.50 and 3.60 (d, 2H, epoxy), 4.29 (d, 2H, *CH*$_2$-Ph), 7.23−7.33 (m, 5H, Ph), 8.89 (b, 1H, NH).

(2*S*,3*S*)-3-(3-Ethylcarbamoyl)oxirane-2-carboxylic Acid (HOOC-(2*S*,3*S*)-EP-CONHCH$_2$CH$_3$). This compound was synthesized using $CH_3CH_2NH_2$ as the starting material.^{1}H NMR ($CDCl_3$): 1.16 (t, 3H, CH_3), 3.29 (q, 2H, CH_2), 3.46 and 3.65 (d, 2H, epoxy), 6.10 (b, 1H, NH).

(2*S*,3*S*)-3-(2-Hydroxy-2-phenylethylcarbamoyl)oxirane-2-carboxylic Acid (PhCH(OH)CH$_2$NHCO-(2*S*,3*S*)-EP-COOH). This compound was synthesized using PhCH(OH)CH_2NH_2 as the starting material.^{1}H NMR ($CDCl_3$): 3.35 (m, 1H, NCH_2), 3.54 (m, 2H, epoxy and NCH_2), 3.63 (s, 1H, epoxy), 4.80 (m, 1H, OCH_2), 7.20 (t, 1H, NH), 7.33−7.41 (m, 5H, Ph and NH).

(2*R*,3*R*)-3-(2-Hydroxy-2-phenylethylcarbamoyl)oxirane-2-carboxylic Acid (PhCH(OH)CH$_2$NHCO-(2*R*,3*R*)-EP-COOH). This compound was synthesized using PhCH(OH)-CH_2NH_2 as the starting material.^{1}H NMR ($CDCl_3$): 3.37 (m, 1H, NCH_2), 3.54 (m, 2H, epoxy and NCH_2), 3.63 (s, 1H, epoxy), 4.80 (m, 1H, NCH_2), 7.20 (t, 1H, NH), 7.33−7.41 (m, 5H, Ph and NH).

(2*S*,3*S*)-3-(1-Benzylcarbamoylethylcarbamoyl)oxirane-2-carboxylic Acid (HOOC-(2*S*,3*S*)-EP-CO-Ala-NHBzl). This compound was synthesized using HCl·$NH_2CH(CH_3)$CONHBzl as the starting material.^{1}H NMR (DMSO-d_6): 1.26 (d, 3H, Ala), 3.45 and 3.65 (d, 2H, epoxy), 4.26 (d, 2H, *CH*$_2$Ph), 4.33 (m, 1H, α-H), 7.19 and 7.29 (m, 5H, Ph), 8.51 (t, 1H, NH), 8.67 (d, 1H, NH).

(2*R*,3*R*)-3-(1-Benzylcarbamoylethylcarbamoyl)oxirane-2-carboxylic Acid (HOOC-(2*R*,3*R*)-EP-CO-Ala-NHBzl). This compound was synthesized using HCl·$NH_2CH(CH_3)$CONHBzl as the starting material.^{1}H NMR (DMSO-d_6): 1.25 (d, 3H, Ala), 3.49 and 3.65 (d, 2H, epoxy), 4.26 (d, 2H, *CH*$_2$Ph), 4.33 (m, 1H, α-H), 7.19 and 7.29 (m, 5H, Ph), 8.51 (t, 1H, NH), 8.67 (d, 1H, NH).

(2*S*,3*S*)-3-(1-Carbamoyl-3-methylbutylcarbamoyl)oxirane-2-carboxylic Acid (HOOC-(2*S*,3*S*)-EP-CO-Leu-NH$_2$). This compound was synthesized using HCl·$NH_2CH(CH_2CH(CH_3)_2)CONH_2$ as the starting material.^{1}H NMR (DMSO-d_6): 0.84 (d, 6H, CH_3), 1.46 (m, 2H, CH_2), 1.55 (m, 1H, CH), 3.48 and 3.64 (d, 2H, epoxy), 4.20 (m, 1H, α-H), 7.00 (s, 1H, NH), 7.45 (s, 1H, NH), 8.50 (d, 1H, NH).

(2*R*,3*R*)-3-(1-Carbamoyl-3-methylbutylcarbamoyl)oxirane-2-carboxylic Acid (HOOC-(2*R*,3*R*)-EP-CO-Leu-NH$_2$). This compound was synthesized using HCl·$NH_2CH(CH_2CH(CH_3)_2)CONH_2$ as the starting material.^{1}H NMR (DMSO-d_6): 0.85 (d, 6H, CH_3), 1.46 (m, 2H, CH_2), 1.56 (m, 1H, CH), 3.48 and 3.64 (d, 2H, epoxy), 4.20 (m, 1H, α-H), 7.00 (s, 1H, NH), 7.45 (s, 1H, NH), 8.50 (d, 1H, NH).

(2*S*,3*S*)-3-(1-Carbamoyl-2-phenylethylcarbamoyl)oxirane-2-carboxylic Acid (HOOC-(2*S*,3*S*)-EP-CO-Phe-NH$_2$). This compound was synthesized using $NH_2CH(CH_2Ph)CONH_2$ as the starting material.^{1}H NMR (DMSO-d_6): 2.78 (m, 1H, *CH*$_2$Ph), 3.02 (m, 1H, *CH*$_2$Ph), 3.25 and 3.56 (d, 2H, epoxy), 4.45 (m, 1H, α-H), 7.13−7.25 (m, 6H, Ph and NH), 7.57 (s, 1H, NH), 8.52 (d, 1H, NH).

(2*R*,3*R*)-3-(1-Carbamoyl-2-phenylethylcarbamoyl)oxirane-2-carboxylic Acid (HOOC-(2*R*,3*R*)-EP-CO-Phe-NH$_2$). This compound was synthesized using $NH_2CH(CH_2Ph)CONH_2$ as the starting material.^{1}H NMR (DMSO-d_6): 2.78 (m, 1H, *CH*$_2$Ph), 3.02 (m, 1H, *CH*$_2$Ph), 3.32 and 3.59 (d, 2H, epoxy), 4.42 (m, 1H, α-H), 7.13−7.25 (m, 6H, Ph and NH), 7.57 (s, 1H, NH), 8.60 (d, 1H, NH).

(2*S*,3*S*)-3-(1-Carbamoyl-2(4-hydroxyphenyl)ethylcarbamoyl)oxirane-2-carboxylic Acid (HOOC-(2*S*,3*S*)-EP-CO-Tyr-NH$_2$). This compound was synthesized using NH_2-$CH(CH_2Ph$-4-$OH)CONH_2$ as the starting material.^{1}H NMR (CD_3COCD_3): 2.85 (m, 1H, *CH*$_2$Ph), 3.10 (m, 1H, *CH*$_2$Ph), 3.40 and 3.59 (d, 2H, epoxy), 4.60 (m, 1H, α-H), 6.50 (b, 1H, OH), 6.74 and 7.07(d of d, 4H, Ph), 7.15 (s, 1H, NH), 7.35 (d, 1H, NH).

***trans*-3-(*N*^{2}-(*N*-3-Phenylpropanoylvalylalanyl)-N^1-*t*-butoxycarbonylmethylhydrazinocarbonyl)oxirane-2-carboxylic Acid Benzyl Ester (PhPr-Val-Ala-AAsp(O-*t*Bu)-*trans*-EP-COOCH$_2$Ph). Aza-peptide Epoxide Synthesis Using the EDC/HOBt Coupling Method.** To a stirred solution of trans-, (2*S*,3*S*)- or (2*R*,3*R*)-oxirane-2,3-dicarboxylic acid monoester (0.44 mmol), or (2*S*,3*S*)/(2*R*,3*R*)-oxirane-2,3-dicarboxylic acid monoamide (0.66 mmol), HOBt (0.44 mmol for esters and 0.66 mmol for amides/amino acids), and PhPr-Val-Ala-NHNHCH$_2$COO-*t*Bu (0.22 mmol) in DMF (20 mL) was added EDC (0.44 mmol for esters and 0.66 mmol for amides). The mixture was allowed to react for 16 h at room temperature. The DMF was evaporated, and the residue was treated with EtOAc (30 mL for esters and 50 mL for amides). The organic layer was washed with 2% citric acid (2 × 20 mL), saturated $NaHCO_3$ (2 × 20 mL), H_2O (10 mL), and saturated NaCl (10 mL), dried over $MgSO_4$, and concentrated. Chromatography on a silica gel column using 1:19 MeOH:CH_2Cl_2 as the eluent (for esters, yields = 59−69%), or 50:35:10:5 CH_2Cl_2:CH_3COOEt:THF:MeOH (for amides, yields = 41−69%) afforded PhPr-Val-Ala-AAsp(O-*t*Bu)-*trans*-EP-COOCH$_2$Ph (or the respective products) as white solids (yield = 36%). MS and ^{1}H NMR ($CDCl_3$) were consistent with the proposed structures.

***trans*-3-(*N*^{2}-(*N*-3-Phenylpropanoylvalylalanyl)-*N*^{1}-*tert*-butoxycarbonylmethylhydrazinocarbonyl)-2-(2-phenethyl)oxirane (PhPr-Val-Ala-AAsp(O-*t*Bu)-*trans*-EP-CH$_2$-CH$_2$Ph).** This compound was obtained using EDC/HOBt coupling of PhPr-Val-Ala-NHNHCH$_2$COO-*t*Bu with *trans*-3-phenethyloxirane-2-carboxylic acid and purified by column chromatography using 1:19 MeOH:CH_2Cl_2 as the eluent; white solid, yield 38%. MS and ^{1}H NMR ($CDCl_3$) were consistent with the proposed structure.

PhPr-Val-Ala-AAsp(O-*t*Bu)-(2*S*,3*S*)-EP-COOEt was obtained using the EDC/HOBt coupling method and purified by column chromatography on silica gel with 19:1 CH_2Cl_2:MeOH as the eluent, and then rechromatographed using 50:35:10:5 CH_2Cl_2:EtOAc:THF:MeOH as the eluent; white solid, yield 31%. ^{1}H NMR ($CDCl_3$): 0.84 (m, 6H, Val CH_3), 1.34 (m, 6H, Ala CH_3 and OEt), 1.44 (s, 9H, *t*Bu), 2.00 (m, 1H, Val CH), 2.58 (m, 2H, Ph*CH*$_2$), 2.97 (t, 2H, PhCH$_2$*CH*$_2$CO), 3.65 (s, 2H, AAsp CH_2), 3.62 and 3.91 (d, 2H, epoxy), 4.23 (m, 4H, α-H and

OEt), 6.00 (d, 1H, NH), 6.60 (d, 1H, NH), 7.16−7.30 (m, 5H, Ph), 9.05 (s, 1H, NH).

PhPr-Val-Ala-AAsp(O-*t*Bu)-(2*R*,3*R*)-EP-COOEt was obtained using the EDC/HOBt coupling method and purified by column chromatography on silica gel with 19:1 CH_2Cl_2:MeOH as the eluent, and then rechromatographed using 50:35:10:5 CH_2Cl_2:EtOAc:THF:MeOH as the eluent; white solid, yield 69%. ^{1}H NMR ($CDCl_3$): 0.82 (m, 6H, Val CH_3), 1.30 (t, 3H, OEt), 1.37 (d, 2H, Ala CH_3), 1.46 (s, 9H, *t*Bu), 2.00 (m, 1H, Val CH), 2.58 (m, 2H, PhCH_2), 2.97 (t, 2H, PhCH$_2$$CH_2$CO), 3.22 (s, 2H, AAsp CH_2), 3.62 and 3.95 (d, 2H, epoxy), 4.23 (m, 4H, α-H and OEt), 6.20 (d, 1H, NH), 6.70 (d, 1H, NH), 7.16−7.27 (m, 5H, Ph), 9.10 (s, 1H, NH).

PhPr-Val-Ala-AAsp(O-*t*Bu)-(2*S*,3*S*)-EP-COOCH$_2$Ph was obtained using the EDC/HOBt coupling method, purified by column chromatography on silica gel with 19:1 CH_2Cl_2:MeOH as the eluent, and then rechromatographed using 50:35:10:5 CH_2Cl_2:EtOAc:THF:MeOH as the eluent; white solid, yield 56%. ^{1}H NMR ($CDCl_3$): 0.82 (m, 6H, Val CH_3), 1.26 (m, 3H, Ala CH_3), 1.42 (s, 9H, *t*Bu), 2.00 (m, 1H, Val CH), 2.56 (m, 2H, PhCH_2), 2.93 (m, 2H, PhCH$_2$$CH_2$CO), 3.67 (s, 2H, AAsp CH_2), 3.75 and 3.94 (d, 2H, epoxy), 4.20 and 4.35 (m, 2H, α-H), 5.19 (m, 2H, benzyl), 6.20 (d, 1H, NH), 6.75 (d, 1H, NH), 7.15−7.37 (m, 10H, Ph), 9.25 (s, 1H, NH).

PhPr-Val-Ala-AAsp(O-*t*Bu)-(2*R*,3*R*)-EP-COOCH$_2$Ph was obtained using the EDC/HOBt coupling method, purified by column chromatography on silica gel with 19:1 CH_2Cl_2:MeOH as the eluent, and then rechromatographed using 50:35:10:5 CH_2Cl_2:EtOAc:THF:MeOH as the eluent; white solid, yield 48%. ^{1}H NMR ($CDCl_3$): 0.82 (m, 6H, Val CH_3), 1.26 (m, 3H, Ala CH_3), 1.42 (s, 9H, *t*Bu), 2.00 (m, 1H, Val CH), 2.56 (m, 2H, PhCH_2), 2.95 (m, 2H, PhCH$_2$$CH_2$CO), 3.62 (s, 2H, AAsp CH_2), 3.70 and 3.94 (d, 2H, epoxy), 4.20 and 4.35 (m, 2H, α-H), 5.20 (m, 2H, benzyl), 6.25 (d, 1H, NH), 6.75 (d, 1H, NH), 7.15−7.37 (m, 10H, Ph), 9.30 (s, 1H, NH).

PhPr-Val-Ala-AAsp(O-*t*Bu)-(2*S*,3*S*)-EP-COOCH$_2$-CH$_2$Ph was obtained using the EDC/HOBt coupling method, purified by column chromatography on silica gel with 19:1 CH_2-Cl_2:MeOH as the eluent, and then rechromatographed using 50:35:10:5 CH_2Cl_2:EtOAc:THF:MeOH as the eluent; white solid, yield 61%. ^{1}H NMR ($CDCl_3$): 0.83 (m, 6H, Val CH_3), 1.34 (d, 3H, Ala CH_3), 1.45 (s, 9H, *t*Bu), 2.00 (m, 1H, Val CH), 2.58 (m, 2H, PhCH_2), 2.96 (m, 4H, PhCH$_2$$CH_2$CO and Ph$CH_2$), 3.63 (s, 2H, AAsp CH_2), 3.75 and 3.90 (d, 2H, epoxy), 4.35 (m, 4H, α-H and OCH_2), 6.20 (m, 1H, NH), 7.85 (d, 1H, NH), 7.12−7.30 (m, 10H, Ph), 9.20 (s, 1H, NH).

PhPr-Val-Ala-AAsp(O-*t*Bu)-(2*S*,3*S*)-EP-CONHCH$_2$CH$_3$ was obtained using the EDC/HOBt coupling method, purified by column chromatography on silica gel with 19:1 CH_2Cl_2:MeOH as the eluent, and then rechromatographed using 50:35:10:5 CH_2Cl_2:EtOAc:THF:MeOH as the eluent; white solid, yield 62%. ^{1}H NMR (acetone-d_6): 0.88 (m, 6H, Val CH_3), 1.10 (t, 3H, CH_3), 1.35 (d, 3H, Ala CH_3), 1.46 (s, 9H, *t*Bu), 2.00 (m, 1H, Val CH), 2.61 (m, 2H, PhCH_2), 2.82 (s, 2H, AAsp CH_2), 2.95 (t, 2H, PhCH$_2$$CH_2$CO), 3.25 (m, 2H, CH_2N), 3.41 (s, 2H, epoxy), 4.20 and 4.40 (m, 2H, α-H), 7.15 (b, 1H, NH), 7.25 (m, 6H, Ph and NH), 7.40 (b, 1H, NH), 7.60 (d, 1H, NH).

PhPr-Val-Ala-AAsp(O-*t*Bu)-(2*S*,3*S*)-EP-CONHCH$_2$Ph was obtained using the EDC/HOBt coupling method, purified by column chromatography on silica gel with 19:1 CH_2Cl_2:MeOH as the eluent, and then rechromatographed using 50:35:10:5 CH_2Cl_2:EtOAc:THF:MeOH as the eluent; white solid, yield 49%. ^{1}H NMR ($CDCl_3$): 0.82 (m, 6H, Val CH_3), 1.32 (m, 3H, Ala CH_3), 1.45 (s, 9H, *t*Bu), 2.00 (m, 1H, Val CH), 2.53 (m, 2H, PhCH_2), 2.93 (m, 2H, PhCH$_2$$CH_2$CO), 3.65 (d, 2H, AAsp CH_2), 3.62 and 3.79 (d, 2H, epoxy), 4.15 (m, 2H, α-H), 4.43 (m, 2H, PhCH_2N), 6.35 (b, 1H, NH), 6.90 (b, 1H, NH), 7.00 (b, 1H, NH), 7.13−7.31 (m, 10H, Ph), 9.40 (s, 1H,NH).

PhPr-Val-Ala-AAsp(O-*t*Bu)-(2*R*,3*R*)-EP-CONHCH$_2$Ph was obtained using the EDC/HOBt coupling method, purified by column chromatography on silica gel with 19:1 CH_2Cl_2:MeOH as the eluent, and then rechromatographed using 50:35:10:5 CH_2Cl_2:EtOAc:THF:MeOH as the eluent; white solid, yield 48%. ^{1}H NMR ($CDCl_3$): 0.82 (m, 6H, Val CH_3), 1.32 (m, 3H, Ala CH_3), 1.46 (s, 9H, *t*Bu), 2.00 (m, 1H, Val CH), 2.57 (m, 2H, PhCH_2), 2.95 (m, 2H, PhCH$_2$$CH_2$CO), 3.68 (d, 2H, AAsp CH_2), 3.62 and 3.80 (d, 2H, epoxy), 4.20 (m, 2H, α-H), 4.40 (m, 2H, PhCH_2N), 6.20 (b, 1H, NH), 6.70 (b, 1H, NH), 7.00 (b, 1H, NH), 7.13−7.31 (m, 10H, Ph), 9.20 (s, 1H,NH).

PhPr-Val-Ala-AAsp(O-*t*Bu)-(2*S*,3*S*)-EP-CONHCH$_2$-CH$_2$Ph was obtained using the EDC/HOBt coupling method, purified by column chromatography on silica gel with 19:1 CH_2-Cl_2:MeOH as the eluent, and then rechromatographed using 50:35:10:5 CH_2Cl_2:EtOAc:THF:MeOH as the eluent; white solid, yield 47%. ^{1}H NMR ($CDCl_3$): 0.83 (m, 6H, Val CH_3), 1.35 (m, 3H, Ala CH_3), 1.43 (s, 9H, *t*Bu), 2.05 (m, 1H, Val CH), 2.56 (t, 2H, PhCH_2), 2.78 (t, 2H, PhCH_2CH$_2$N), 2.95 (t, 2H, PhCH$_2$$CH_2$CO), 3.40 and 3.53 (d of m, 2H, AAsp CH_2), 3.53 and 3.69 (d, 2H, epoxy), 4.20 (m, 2H, α-H), 4.45 (m, 2H, NCH_2-CH_2Ph), 6.30 (d, 1H, NH), 6.40 (t, 1H, NH), 7.00 (d, 1H, NH), 7.13−7.31 (m, 10H, Ph), 9.30 (s, 1H, NH).

PhPr-Val-Ala-AAsp(O-*t*Bu)-(2*R*,3*R*)-EP-CONHCH$_2$-CH$_2$Ph was obtained using the EDC/HOBt coupling method, purified by column chromatography on silica gel with 19:1 CH_2-Cl_2:MeOH as the eluent, and then rechromatographed using 50:35:10:5 CH_2Cl_2:EtOAc:THF:MeOH as the eluent; white solid, yield 41%. ^{1}H NMR ($CDCl_3$): 0.83 (m, 6H, Val CH_3), 1.35 (m, 3H, Ala CH_3), 1.43 (s, 9H, *t*Bu), 2.05 (m, 1H, Val CH), 2.56 (t, 2H, PhCH_2), 2.78 (t, 2H, PhCH_2CH$_2$N), 2.95 (t, 2H, PhCH$_2$$CH_2$CO), 3.48 and 3.70 (d of m, 2H, AAsp CH_2), 3.53 and 3.65 (d, 2H, epoxy), 4.17 (m, 2H, α-H), 4.43 (m, 2H, NCH_2-CH_2Ph), 6.30 (d, 1H, NH), 6.40 (t, 1H, NH), 6.80 (d, 1H, NH), 7.15−7.35 (m, 10H, Ph), 9.20 (s, 1H, NH).

PhPr-Val-Ala-AAsp(O-*t*Bu)-(2*S*,3*S*)-EP-CONHCH$_2$CH-(OH)Ph was obtained using the EDC/HOBt coupling method, purified by column chromatography on silica gel with 19:1 CH_2-Cl_2:MeOH as the eluent, and then rechromatographed using 50:35:10:5 CH_2Cl_2:EtOAc:THF:MeOH as the eluent; white solid, yield 33%. ^{1}H NMR (acetone-d_6): 0.86 (m, 6H, Val CH_3), 1.38 (m, 3H, Ala CH_3), 1.46 (s, 9H, *t*Bu), 2.05 (m, 1H, Val CH), 2.49 (m, 2H, PhCH_2), 2.90 (m, 4H, PhCH$_2$$CH_2$ and AAsp CH_2), 3.55 (d, 2H, epoxy), 3.45 and 3.60 (d of m, 2H, NCH_2), 4.20 and 4.37 (m, 2H, α-H), 4.85 (t 1H, CH), 7.15−7.40 (m, 12H, Ph and NH), 7.80 (b, 1H, NH), 7.90 (m, 1H, NH).

PhPr-Val-Ala-AAsp(O-*t*Bu)-(2*R*,3*R*)-EP-CONHCH$_2$CH-(OH)Ph was obtained using the EDC/HOBt coupling method, purified by column chromatography on silica gel with 19:1 CH_2-Cl_2:MeOH as the eluent, and then rechromatographed using 50:35:10:5 CH_2Cl_2:EtOAc:THF:MeOH as the eluent; white solid, yield 37%. ^{1}H NMR (acetone-d_6): 0.86 (m, 6H, Val CH_3), 1.40 (m, 3H, Ala CH_3), 1.46 (s, 9H, *t*Bu), 2.05 (m, 1H, Val CH), 2.45 (m, 2H, PhCH_2), 2.90 (m, 4H, PhCH$_2$$CH_2$ and AAsp CH_2), 3.40 and 3.60 (d of m, 2H, NCH_2), 3.60 and 3.76 (d, 2H, epoxy), 4.20 and 4.35 (m, 2H, α-H), 4.80 (b, 1H, CH), 7.00−7.40 (m, 12H, Ph and NH), 7.80 (b, 2H, NH).

PhPr-Val-Ala-AAsp(O-*t*Bu)-(2*S*,3*S*)-EP-CO-Ala-NHBzl was obtained using the EDC/HOBt coupling method, purified by column chromatography on silica gel with 19:1 CH_2-Cl_2:MeOH as the eluent, and then rechromatographed using 50:35:10:5 CH_2Cl_2:EtOAc:THF:MeOH as the eluent; white solid, yield 41%. ^{1}H NMR (acetone-d_6): 0.87 (m, 6H, Val CH_3), 1.37 (m, 6H, Ala CH_3), 1.46 (s, 9H, *t*Bu), 2.05 (m, 1H, Val CH), 2.63 (m, 2H, PhCH_2), 2.87 (s, 2H, AAsp CH_2), 2.93 (t, 2H, PhCH$_2$$CH_2$CO), 3.55 (s, 2H, epoxy), 4.15 and 4.45 (m, 3H, α-H), 4.43 (d, 2H, PhCH_2N), 7.15 (d, 1H, NH), 7.20−7.30 (m, 10H, Ph), 7.40 (d, 1H, NH), 7.75 (s, 1H, NH), 8.00 (b, 2H, NH).

PhPr-Val-Ala-AAsp(O-*t*Bu)-(2*R*,3*R*)-EP-CO-Ala-NHBzl was obtained using the EDC/HOBt coupling method, purified by column chromatography on silica gel with 19:1 CH_2-Cl_2:MeOH as the eluent, and then rechromatographed using 50:35:10:5 CH_2Cl_2:EtOAc:THF:MeOH as the eluent; white solid, yield 50%. ^{1}H NMR (acetone-d_6): 0.87 (m, 6H, Val CH_3), 1.37 (m, 6H, Ala CH_3), 1.46 (s, 9H, *t*Bu), 2.05 (m, 1H, Val CH), 2.60 (m, 2H, PhCH_2), 2.87 (s, 2H, AAsp CH_2), 2.90 (t, 2H, PhCH$_2$$CH_2$CO), 3.65 (s, 2H, epoxy), 4.25 (m, 3H, α-H), 4.40 (d, 2H, PhCH_2N), 7.15 (d, 1H, NH), 7.20−7.30 (m, 10H, Ph), 7.40 (d, 1H, NH), 7.80−8.00 (m, 3H, NH).

PhPr-Val-Ala-AAsp(O-*t*Bu)-(2*S*,3*S*)-EP-CO-Leu-NH$_2$ was obtained using the EDC/HOBt coupling method, purified by column chromatography on silica gel with 19:1 CH_2Cl_2:MeOH as the eluent, and then rechromatographed using 50:35:10:5 CH_2Cl_2:EtOAc:THF:MeOH as the eluent; white solid, yield 23%. ^{1}H NMR (DMSO-d_6): 0.86−0.96 (m, 12H, Val and Leu CH_3), 1.37 (d, 3H, Ala CH_3), 1.46 (t, 9H, *t*Bu), 1.65 (m, 2H, Leu CH_2), 1.75 (m, 1H, Leu CH), 2.05 (m, 1H, Val CH), 2.63 (m, 2H, PhCH_2), 2.86 (s, 2H, AAsp CH_2), 2.94 (t, 2H, PhCH$_2$$CH_2$-CO), 3.57 (s, 2H, epoxy), 4.19, 4.35 and 4.50 (m, 3H, α-H), 7.15 (d, 1H, NH), 7.25 (s, 5H, Ph), 7.35 (s, 1H, NH), 7.45 (d, 1H, NH), 7.80 (d, 1H, NH), 8.15 (d, 1H, NH), 9.75 (s, 1H, NH).

PhPr-Val-Ala-AAsp(O-*t*Bu)-(2*R*,3*R*)-EP-CO-Leu-NH$_2$ was obtained using the EDC/HOBt coupling method, purified by column chromatography on silica gel with 19:1 CH_2Cl_2:MeOH as the eluent, and then rechromatographed using 50:35:10:5 CH_2Cl_2:EtOAc:THF:MeOH as the eluent; white solid, yield 64%. ^{1}H NMR (DMSO-d_6): 0.86−0.96 (m, 12H, Val and Leu CH_3), 1.37 (d, 3H, Ala CH_3), 1.45 (t, 9H, *t*Bu), 1.65 (m, 2H, Leu CH_2), 1.75 (m, 1H, Leu CH), 2.05 (m, 1H, Val CH), 2.60 (m, 2H, PhCH_2), 2.88 (s, 2H, AAsp CH_2), 2.90 (t, 2H, PhCH$_2$$CH_2$-CO), 3.50 (d, 2H, epoxy), 4.20, 4.40 and 4.45 (m, 3H, α-H), 7.15 (d, 1H, NH), 7.25 (s, 5H, Ph), 7.45 (b, 2H, NH), 7.80 (b, 2H, NH), 9.90 (s, 1H, NH).

PhPr-Val-Ala-AAsp(O-*t*Bu)-(2*S*,3*S*)-EP-CO-Phe-NH$_2$ was obtained using the EDC/HOBt coupling method, purified by column chromatography on silica gel with 19:1 CH_2Cl_2:MeOH as the eluent, and then rechromatographed using 50:35:10:5 CH_2Cl_2:EtOAc:THF:MeOH as the eluent; white solid, yield 39%. ^{1}H NMR (acetone-d_6): 0.88 (m, 6H, Val CH_3), 1.39 (d, 3H, Ala CH_3), 1.46 (s, 9H, *t*Bu), 2.05 (m, 1H, Val CH), 2.60 (m, 2H, PhCH_2), 2.83 (s, 2H, AAsp CH_2), 2.95 (m, 3H, Phe PhCH_2 and PhCH$_2$$CH_2$CO), 3.20 (m, 1H, Phe Ph$CH_2$), 3.50 (d, 2H, epoxy), 4.20 and 4.35 (m, 2H, α-H), 4.65 (m, 1H, Phe α-H), 7.15−7.35 (m, 11H, Ph and NH), 7.50 (s, 1H, NH), 7.80 (d, 1H, NH), 7.95 (d, 1H, NH), 8.15 (b, 1H, NH), 9.80 (s, 1H, NH).

PhPr-Val-Ala-AAsp(O-*t*Bu)-(2*R*,3*R*)-EP-CO-Phe-NH$_2$ was obtained using the EDC/HOBt coupling method, purified by column chromatography on silica gel with 19:1 CH_2Cl_2:MeOH as the eluent, and then rechromatographed using 50:35:10:5 CH_2Cl_2:EtOAc:THF:MeOH as the eluent; white solid, yield 56%. ^{1}H NMR (acetone-d_6): 0.88 (m, 6H, Val CH_3), 1.37 (d, 3H, Ala CH_3), 1.46 (s, 9H, *t*Bu), 2.05 (m, 1H, Val CH), 2.58 (m, 2H, PhCH_2), 2.83 (s, 2H, AAsp CH_2), 2.93 (m, 2H, PhCH$_2$$CH_2$CO), 3.00 and 3.40 (d of m, 2H, Phe PhCH_2), 3.45 (d, 2H, epoxy), 4.25 and 4.35 (m, 2H, α-H), 4.65 (m, 1H, Phe α-H), 7.15−7.35 (m, 11H, Ph and NH), 7.40 (d, 1H, NH), 7.50 (b, 1H, NH), 8.00 (b, 1H, NH), 8.60 (b, 1H, NH), 9.80 (s, 1H, NH).

PhPr-Val-Ala-AAsp(O-*t*Bu)-(2*S*,3*S*)-EP-CO-Tyr-NH$_2$ was obtained using the EDC/HOBt coupling method, purified by column chromatography on silica gel with 19:1 CH_2Cl_2:MeOH as the eluent, and then rechromatographed using 50:35:10:5 CH_2Cl_2:EtOAc:THF:MeOH as the eluent; white solid, yield 50%. ^{1}H NMR (acetone-d_6): 0.87 (m, 6H, Val CH_3), 1.35 (d, 3H, Ala CH_3), 1.45 (s, 9H, *t*Bu), 2.05 (m, 1H, Val CH), 2.60 (m, 2H, PhCH_2), 2.88 (s, 2H, AAsp CH_2), 2.94 (m, 3H, Tyr PhCH_2 and PhCH$_2$$CH_2$CO), 3.10 (m, 1H, Tyr Ph$CH_2$), 3.72 (d, 2H, epoxy), 4.30 and 4.55 (m, 3H, α-H), 6.50 (s, 1H, NH), 6.75 (d, 2H, Ph), 7.15 (m, 5H, Ph), 7.20 (d, 2H, Ph), 7.30 (d, 1H, NH), 7.50 (d, 1H, NH), 7.80 (d, 1H, NH), 8.20 (d, 2H, NH).

Cbz-Val-AAsp(O-*t*Bu)-(2*S*,3*S*)-EP-COOEt was obtained using the EDC/HOBt coupling method, and purified by column chromatography on silica gel with 1:20:4.2 MeOH:CH_2Cl_2:EtOAc as the eluent; white solid, yield 51%. MS (ESI) *m*/*z* 522.2 [(M + 1)$^+$]. ^{1}H NMR ($CDCl_3$): 0.95−1.02 (m, 6H, Val), 1.28 (t, 3H, OCH$_2$$CH_3$), 1.63 (s, 9H, *t*Bu), 2.05 (m, 1H, Val), 3.62 (s, 1H, epoxy), 3.97 (d, 2H, NHCH_2COOH), 4.15 (t, 1H, α-H), 4.25 (q, 2H, OCH_2CH$_3$), 5.12 (m, 2H, Cbz), 7.35 (s, 5H, Ph), 8.61 (s, 1H, NH).

Cbz-Val-AAsp(O-*t*Bu)-*trans*-EP-CH$_2$CH$_2$Ph was obtained using the EDC/HOBt coupling method, and purified by column chromatography on silica gel with 1:50:51 MeOH:CH_2Cl_2:EtOAc as the eluent; white solid, yield 48%. HRMS (FAB) Calcd for $C_{30}H_{39}N_3O_7$: 553.27878. Observed *m*/*z* 553.28663. ^{1}H NMR ($CDCl_3$): 0.90−1.04 (d, 6H, Val), 1.46 (s, 9H, *t*Bu), 1.78−1.96 (d, 2H, CH$_2$CH_2Ph), 2.00−2.14 (m, 1H, Val), 2.66−2.87 (m, 2H, CH_2CH$_2$Ph), 3.16 (t, 1H, epoxy), 3.53 (s, 1H, NHCH_2-COOH), 3.85 (t, 1H, α-H), 4.81 (d, 1H, NH), 4.99−5.20 (m, 2H, Cbz), 7.13−7.37 (m, 10H, Ph), 8.25 (s, 1H, NH).

Cbz-Val-AAsp(O-*t*Bu)-*trans*-EP-Ph-4-Cl was obtained using the EDC/HOBt coupling method, and purified by column chromatography on silica gel with 1:100:100 MeOH:CH_2Cl_2:EtOAc as the eluent; white solid, yield 51%. MS (ESI) *m*/*z* 560.4 [(M + 1)$^+$]. ^{1}H NMR (DMSO-d_6): 0.64−0.92 (d, 6H, Val), 1.20 (s, 9H, *t*Bu), 1.84 (m, 1H, Val), 3.68 (m, 1H, NHCH_2-COOH), 3.75 (s, 1H, epoxy), 4.05 (d, 1H, α-H), 4.80−5.09 (s, 2H, Cbz), 7.18−7.46 (m, 9H, Ph), 11.02 (m, 1H, NH).

Cbz-Glu(O-*t*Bu)-Val-AAsp(O-*t*Bu)-(2*S*,3*S*)-EP-CONHCH$_2$-CH$_2$Ph was obtained using the EDC/HOBt coupling method, purified by column chromatography on silica gel with 7:13:1:1 hexane:EtOAc:MeOH:CH_2Cl_2 as the eluent, and then rechromatographed using 3:1 EtOAc:hexane as the eluent; white solid, yield 35%. MS (ESI) *m*/*z* 782.4 [(M + 1)$^+$]. ^{1}H NMR ($CDCl_3$): 0.95 (d, 6H, Val), 1.45 (s, 18H, *t*Bu), 1.90−2.30 (m, 3H, Val and Glu), 2.45 (m, 2H, Glu), 2.72 (t, 2H, NHCH$_2$$CH_2$-Ph), 3.40 and 3.60 (m, 2 H, NHCH_2CH$_2$Ph), 3.50 and 3.8 (d, 2H, epoxy), 4.10−4.30 (m, 4H, NCH_2 and α-H), 5.10 (m, 2H, Cbz), 6.10 and 6.25 (m, 2H, NH), 7.20−7.40 (m, 5H, Ph), 9.00 (m, 1H, NH).

Aza-peptide Epoxide Synthesis Using the Mixed Anhydride Coupling Method. Coupling of bulky peptides, such as Cbz-Asp(O-*t*Bu)-Glu(O-*t*Bu)-Val-NHNHCH$_2$COO-*t*Bu and Cbz-Leu-Glu(O-*t*Bu)-Thr-NHNHCH$_2$COO-*t*Bu, with the monoethyl epoxysuccinates, was accomplished using the mixed anhydride coupling method. To a solution of the epoxide (5 equiv) in DMF at 0 °C was added *N*-methylmorpholine (NMM, 5 equiv) followed by isobutyl chloroformate (IBCF, 5 equiv). After the reaction mixture was allowed to stir for 30 min, the substituted hydrazide (1 equiv), dissolved in DMF, was added to the mixture. After 10 min the ice bath was removed and the reaction was stirred for 16 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 $CDCl_3$) were consistent with the proposed structures.

Cbz-Asp(O-*t*Bu)-Glu(O-*t*Bu)-Val-AAsp(O-*t*Bu)-(2*S*,3*S*)-EP-COOEt was obtained using the mixed anhydride coupling method, and purified using column chromatography on silica gel using 2:18:5 MeOH:CH_2Cl_2:EtOAc as the eluent; white solid, yield 58%. MS (ESI) *m*/*z* 878.2 [(M + 1)$^+$].^{1}H NMR (DMSO-d_6): 0.92 (m, 6H, Val), 1.20 (t, 3H, OCH$_2$$CH_3$), 1.40 (m, 27H, *t*Bu), 1.7−2.1 (m, 3H, Val and Glu), 2.21 (m, 2H, Glu), 2.40−2.65 (m, 2H, Asp), 3.58 and 4.10 (d, 2H, epoxy), 4.05−4.22 (m, 4H, NCH_2 and OCH_2CH$_3$), 4.50−4.60 (m, 3H, α-H), 5.05 (m, 2H, Cbz), 7.20−7.40 (m, 5H, Ph), 7.60 (1H, NH), 7.85 (m, 2H, NH).

Cbz-Asp(O-*t*Bu)-Glu(O-*t*Bu)-Val-AAsp(O-*t*Bu)-(2*R*,3*R*)-EP-COOEt was obtained using the mixed anhydride coupling method, and purified using column chromatography on silica gel using 2:18:5 MeOH:CH_2Cl_2:EtOAc as the eluent; white solid, yield 57%. MS (ESI) *m*/*z* 878.1 [(M + 1)$^+$].^{1}H NMR (DMSO-d_6): 0.92 (m, 6H, Val), 1.21 (t, 3H, OCH$_2$$CH_3$), 1.40 (m, 27H, *t*Bu), 1.7−2.1 (m, 3H, Val and Glu), 2.21 (m, 2H, Glu), 2.40−2.65 (m, 2H, Asp), 3.58 and 4.10 (d, 2H, epoxy), 4.05−4.22 (m, 4H, NCH_2 and OCH_2CH$_3$), 4.50−4.60 (m, 3H, α-H), 5.05 (m, 2H, Cbz), 7.20−7.40 (m, 5H, Ph), 7.60 (1H, NH), 7.85 (m, 2H, NH).

Cbz-Asp(O-*t*Bu)-Glu(O-*t*Bu)-Val-AAsp(O-*t*Bu)-(2*S*,3*S*)-EP-COOCH$_2$Ph was obtained using the mixed anhydride coupling method, and purified using column chromatography on silica gel using 5:1:19 EtOAc:MeOH:CH_2Cl_2 as the eluent; white solid, yield 23%. MS (ESI) *m*/*z* 940.5 [(M + 1)$^+$].^{1}H NMR ($CDCl_3$): 0.85 (m, 6H, Val), 1.35 (m, 27H, *t*Bu), 1.80−2.24 (m, 3H, Val and Glu), 2.41 (m, 2H, Glu), 2.65−2.90 (m, 2H, Asp), 3.60 and 4.00 (d, 2H, epoxy), 4.05−4.12 (m, 2H, NCH_2), 4.10−4.40 (m, 3H, α-H), 5.05 (m, 4H, Cbz), 6.05 (m, 1H, NH), 7.20−7.40 (m, 10H, Ph), 7.85 (m, 2H, NH), 9.05 (m, 1H, NH).

Cbz-Asp(O-*t*Bu)-Glu(O-*t*Bu)-Val-AAsp(O-*t*Bu)-(2*S*,3*S*)-EP-CONHCH$_2$Ph was obtained using the mixed anhydride coupling method and was purified using column chromatography on silica gel using 1:2 EtOAc:hexane as the eluent; white solid, yield 31%. MS (ESI) *m*/*z* 939.6 [(M + 1)$^+$]. ^{1}H NMR ($CDCl_3$): 0.97 (m, 6H, Val), 1.40 (m, 27H, *t*Bu), 1.9−2.3 (m, 3H, Val and Glu), 2.41 (m, 2H, Glu), 2.80−2.95 (m, 2H, Asp), 3.63 and 3.97 (d, 2H, epoxy), 4.05−4.22 (m, 4H, N*CH*$_2$), 4.20−4.55 (m, 3H, α-H), 4.45 (m, 2H, NH*CH*$_2$Ph), 5.05 (m, 2H, Cbz), 7.20−7.40 (m, 10H, Ph), 6.15 (m, 1H, NH), 6.60 (1H, NH), 7.85 (m, 2H, NH).

Cbz-Asp(O-*t*Bu)-Glu(O-*t*Bu)-Val-AAsp(O-*t*Bu)-(2*S*,3*S*)-EP-CO-Phe-NH$_2$ was obtained using the mixed anhydride method, purified using column chromatography on silica gel using 10:1:9 EtOAc:MeOH:CH_2Cl_2 as the eluent, and then was rechromatographed using 10:7:2:1 CH_2Cl_2:EtOAc:THF:MeOH as the eluent; white solid, yield 28%. ESI (M+1) Calcd for $C_{49}H_{70}N_7O_{15}$: 996.4 Observed *m*/*z* 996.4. ^{1}H NMR ($CDCl_3$): 0.94 (m, 6H, Val), 1.43 (m, 27H, COO-*tBu*), 1.60−2.00 (m, 3H, Val, Glu), 2.21 (m, 2H, Glu), 2.78−3.2 (m, 2H Asp, 2H, Phe), 3.50 and 3.98 (d, 2H, epoxy), 3.90−4.20 (m, 2H, N*CH*$_2$COOH), 4.00−4.40 (m, 3H, α-H), 5.10 (m, 2H, Cbz), 7.0 (s, 2H, NH$_2$), 7.20−7.40 (m, 10H, Ph), 8.00 (m, 3H, NH).

Cbz-Glu(O-*t*Bu)-Val-AAsp(O-*t*Bu)-(2*S*,3*S*)-EP-COOEt was obtained using the mixed anhydride coupling method and was purified using column chromatography on silica gel with 1:2 EtOAc:hexane as the eluent, and recrystallized from EtOAc/hexane; white solid, yield 44%. MS (ESI) *m*/*z* 706.1 [(M + 1)$^+$]. ^{1}H NMR (DMSO-d_6): 0.90 (m, 6H, Val), 1.20 (t, 3H, OCH$_2$*CH*$_3$), 1.40 (m, 18H, *t*Bu), 1.60−2.00 (m, 3H, Val and Glu), 2.21 (m, 2H, Glu), 3.50 and 3.98 (d, 2H, epoxy), 4.00−4.20 (m, 4H, N*CH*$_2$ and O*CH*$_2$CH$_3$), 4.20−4.40 (m, 2H, α-H), 5.05 (m, 2H, Cbz), 7.20−7.40 (m, 5H, Ph), 8.00 (m, 2H, NH).

Cbz-Glu(O-*t*Bu)-Val-AAsp(O-*t*Bu)-(2*R*,3*R*)-EP-COOEt was obtained using the mixed anhydride coupling method and was purified using column chromatography on silica gel with 7:13:1:19 hexane:EtOAc:MeOH:CH_2Cl_2 as the eluent, and then rechromatographed using 3:1 EtOAc:hexane as the eluent; white solid, yield 35%. MS (ESI) *m*/*z* 706.1 [(M + 1)$^+$]. ^{1}H NMR ($CDCl_3$): 0.94 (m, 6H, Val), 1.21 (t, 3H, OCH$_2$*CH*$_3$), 1.43 (m, 18H, *t*Bu), 1.60−2.00 (m, 3H, Val and Glu), 2.21 (m, 2H, Glu), 3.60 and 3.98 (d, 2H, epoxy), 4.00−4.20 (m, 4H, N*CH*$_2$ and O*CH*$_2$CH$_3$), 4.20−4.40 (m, 2H, α-H), 5.10 (m, 2H, Cbz), 7.20−7.40 (m, 5H, Ph), 8.00 (m, 2H, NH).

Cbz-Glu(O-*t*Bu)-Val-AAsp(O-*t*Bu)-(2*R*,3*R*)-EP-CO-Phe-NH$_2$ was obtained using the mixed anhydride coupling method and was purified by column chromatography on silica gel using 10:1:9 EtOAc:MeOH:CH_2Cl_2 as the eluent, and then rechromatographed using 10:7:3:1 CH_2Cl_2:EtOAc:THF:MeOH as the eluent; white solid, yield 32%. MS (ESI) *m*/*z* 825.4 [(M + 1)$^+$]. ^{1}H NMR ($CDCl_3$): 0.94 (m, 6H, Val), 1.43 (m, 18H, *t*Bu), 1.60−2.00 (m, 3H, Val and Glu), 2.21 (m, 2H, Glu), 2.80 and 2.95 (m, 2H, Phe), 3.50 and 3.98 (d, 2H, epoxy), 3.90−4.20 (m, 2H, N*CH*$_2$), 4.00−4.40 (m, 3H, α-H), 5.10 (m, 2H, Cbz), 7.0 (s, 2H, NH$_2$), 7.20−7.40 (m, 10H, Ph), 8.00 (m, 2H, NH).

Cbz-Leu-Glu(O-*t*Bu)-Thr-AAsp(O-*t*Bu)-(2*S*,3*S*)-EP-COOEt was synthesized using the mixed anhydride coupling method, purified by column chromatography using 1:13 MeOH:CH_2Cl_2 as an eluent, and then rechromatographed using 2:1 EtOAc:hexane as an eluent; white solid, yield 44%. MS (ESI) *m*/*z* 822 [(M + 1)$^+$]. ^{1}H NMR (DMSO-d_6): 0.85 (t, 6H, Leu CH$_3$), 1.05 (d, 3H, Thr CH$_3$), 1.2 (t, 3H, OCH$_2$*CH*$_3$), 1.4 (d, 20H, *t*Bu and Leu CH$_2$), 1.6 (m, 1H, CH Leu), 1.75 (m, 1H, Glu CH$_2$), 1.9 (m, 1H, Glu CH$_2$), 2.25 (m, 2H, Glu CH$_2$), 3.5 (s, 1H, epoxy), 3.9−4.1 (m, 4H, N*CH*$_2$COOH, epoxy, α-H), 4.1−4.3 (m, 3H, O*CH*$_2$CH$_3$ and *CH*-OH), 4.3−4.4 (m, 2H, α-H), 5.02 (m, 2H, Cbz), 7.30−7.40 (m, 5H, Ph), 7.50 (d, 1H, NH), 7.75 (d, 1H, NH), 8.1 (d, 1H, NH).

Cbz-Leu-Glu(O-*t*Bu)-Thr-AAsp(O-*t*Bu)-(2*S*,3*S*)-EP-COOCH$_2$Ph was synthesized using the mixed anhydride coupling method, purified by column chromatography using 4:1 (10% MeOH:CH_2Cl_2):EtOAc as an eluent, and then rechromatographed using 4:1 (5% MeOH:CH_2Cl_2):EtOAc as the eluent; white solid, yield 44%. MS (ESI) *m*/*z* 884.4 [(M + 1)$^+$].

Cbz-Leu-Glu(O-*t*Bu)-Thr-AAsp(O-*t*Bu)-(2*S*,3*S*)-EP-CONHCH$_2$CH$_2$Ph was obtained using the EDC/HOBt coupling method, purified by column chromatography on silica gel with 19:1 CH_2Cl_2:MeOH as the eluent, and then rechromatographed using 50:35:10:5 CH_2Cl_2:EtOAc:THF:MeOH as the eluent; white solid, yield 52%. ^{1}H NMR ($CDCl_3$): 0.90 (t, 6H, Leu CH$_3$), 1.14 (m, 3H, Thr CH$_3$), 1.26 (m, 2H, Leu CH$_2$), 1.45 (s, 18H, *t*Bu), 1.65 (m, 1H, Leu CH), 2.02 (m, 2H, Glu CH$_2$), 2.40 (m, 2H, Glu CH$_2$), 2.77 (t, 2H, *CH*$_2$Ph), 3.40−3.70 (m, 6H, epoxy, AAsp CH$_2$ and NCH$_2$), 4.20 (m, 3H, α-H), 4.40 (m, 1H, Thr CH), 5.07 (s, 2H, Z), 6.40 (b, 1H, NH), 7.15−7.35 (m, 12H, Ph and NH), 8.00 (s, 1H, NH), 9.45 (s, 1H, NH).

Cbz-Leu-Glu(O-*t*Bu)-Thr-AAsp(O-*t*Bu)-(2*S*,3*S*)-EP-CO-Ala-NHBzl was obtained using the EDC/HOBt coupling method, purified by column chromatography on silica gel with 19:1 CH_2Cl_2:MeOH as the eluent, and then rechromatographed using 50:35:10:5 CH_2Cl_2:EtOAc:THF:MeOH as the eluent; white solid, yield 42%. ^{1}H NMR (acetone-d_6): 0.90 (t, 6H, Leu CH$_3$), 1.15 (d, 3H, Thr CH$_3$), 1.25 (m, 2H, Leu CH$_2$), 1.30 (d, 3H, Ala CH$_3$), 1.45 (t, 18 H, *t*Bu), 1.60 (m, 1H, Leu CH), 1.95 and 2.10 (d of m, 2H, Glu CH$_2$), 2.40 (m, 2H, Glu CH$_2$), 2.90 (s, 2H, AAsp CH$_2$), 3.56 and 3.64 (d, 2H, epoxy), 4.25 (m, 3H, α-H), 4.35 (m, 1H, α-H), 4.40−4.50(m, 3H, Thr CH and *CH*$_2$-Ph), 5.05 (q, 2H, Z), 6.80 (d, 1H, NH), 7.10−7.35 (m, 10H, Ph), 7.40 (d, 1H, NH), 7.75 (b, 1H, NH), 7.90 (d, 2H, NH), 9.80 (s, 1H, NH).

Cbz-Ile-Glu(O-*t*Bu)-Thr-AAsp(O-*t*Bu)-(2*S*,3*S*)-EP-COOEt was synthesized using the mixed anhydride coupling method, purified by column chromatography using 1:13 MeOH:CH_2Cl_2 as an eluent, and then rechromatographed using 2:1 EtOAc:hexane as the eluent. The crude product was then rechromatographed using 1:13 MeOH:CH_2Cl_2 as an eluent and placed on a preparatory TLC plate using 1:13 MeOH:CH_2Cl_2 as the eluent; white solid, yield 37%. HRMS (FAB) Calcd for $C_{39}H_{60}N_5O_{14}$: 822.41368. Observed *m*/*z* 822.41366. ^{1}H NMR ($CDCl_3$): 0.8−1.0 (m, 6H, Ile CH$_3$), 1.1−1.4 (m, 8H, Thr CH$_3$, Ile CH$_2$, and OCH$_2$*CH*$_3$), 1.4−1.5 (s, 18H, *t*Bu), 1.9−2.2 (m, 3H, CH Ile and Glu CH$_2$), 2.3−2.45 and 2.5−2.6 (m, 2H, Glu CH$_2$), 3.0−3.2 (m, 2H, N*CH*$_2$COOH), 3.6 (d, 1H, epoxy), 4.0−4.3 (m, 5H, epoxy, α-H, O*CH*$_2$CH$_3$, and *CH*-OH), 4.3−4.5 (m, 2H, α-H), 5.1 (m, 2H, Cbz), 5.35 (m, 1H, NH), 7.20−7.40 (m, 5H, Ph), 8.25 (m, 1H, NH), 9.5 (m, 1H, NH).

Cbz-Ile-Glu(O-*t*Bu)-Thr-AAsp(O-*t*Bu)-(2*S*,3*S*)-EP-COOCH$_2$Ph was synthesized using the mixed anhydride coupling method and purified by column chromatography using 4:1 (5% MeOH:CH_2Cl_2):EtOAc as the eluent; white solid, yield 38%. MS (FAB) *m*/*z* 884 [(M + 1)$^+$].

Cbz-Ile-Glu(O-*t*Bu)-Thr-AAsp(O-*t*Bu)-(2*R*,3*R*)-EP-COOCH$_2$Ph was synthesized using the mixed anhydride coupling method and purified by column chromatography using 4:1 (10% MeOH:CH_2Cl_2):EtOAc as the eluent, and then rechromatographed using 4:1 (5% MeOH:CH_2Cl_2):EtOAc as the eluent; white solid, yield 37%. MS (FAB) *m*/*z* 884.4 [(M + 1)$^+$].

Cbz-Ile-Glu(O-*t*Bu)-Thr-AAsp(O-*t*Bu)-(2*R*,3*R*)-EP-CONHCH$_2$Ph was synthesized using the mixed anhydride coupling method, purified by column chromatography using 4:1 (10% MeOH:CH_2Cl_2):EtOAc as the eluent, and then rechromatographed using 4:1 (5% MeOH:CH_2Cl_2):EtOAc as the eluent; white solid, yield 47%. MS (ESI) *m*/*z* 883.6 [(M + 1)$^+$].^{1}H NMR (DMSO-d_6): 0.7−0.9 (m, 6H, Ile CH$_3$), 0.9−1.2 (m, 4H, Thr CH$_3$ and Ile CH$_2$), 1.2−1.5 (m, 19H, *t*Bu and Ile CH$_2$), 1.6−1.8 (m, 2H, CH Ile and Glu CH$_2$), 1.8−1.9 (m, 1H, Glu CH$_2$), 2.1−2.3 (m, 2H, Glu CH$_2$), 3.5 (d, 1H, epoxy), 3.8−4.0 (m, 3H, N*CH*$_2$COOH and *CH*-OH), 4.03 (d, 1H, epoxy), 4.1−4.2 (m, 1H, α-H), 4.2−4.5 (m, 4H, NH*CH*$_2$Ph and α-H), 4.9−5.1 (m, 2H, Cbz), 7.15−7.40 (m, 10H, Ph), 7.85 (m, 1H, NH), 8.05 (m, 1H, NH).

Cbz-Ile-Glu(O-*t*Bu)-Thr-AAsp(O-*t*Bu)-(2*R*,3*R*)-EP-COOEt was synthesized using the mixed anhydride coupling method, purified by column chromatography using 1:13 MeOH:CH_2Cl_2 as an eluent, and then rechromatographed using 2:1 EtOAc:hexane as an eluent; white solid, yield 43%. HRMS (FAB) Calcd for $C_{39}H_{60}N_5O_{14}$: 822.41368. Observed *m*/*z* 822.41287. ^{1}H NMR ($CDCl_3$): 0.8−1.0 (m, 6H, Ile CH$_3$), 1.1−

1.35 (m, 8H, Thr CH_3, Ile CH_2, and OCH_2CH_3), 1.4−1.5 (d, 18H, *t*Bu), 1.9−2.15 (m, 3H, CH Ile and Glu CH_2), 2.3−2.45 and 2.5−2.6 (m, 2H, Glu CH_2), 3.0−3.2 (m, 2H, NCH_2COOH), 3.6 (d, 1H, epoxy), 4.0−4.5 (m, 7H, epoxy, OCH_2CH_3, *CH*-OH, and α-H), 5.1 (m, 2H, Cbz), 5.4 (m, 1H, NH), 7.20−7.40 (m, 5H, Ph), 8.25 (m, 1H, NH), 9.4 (m, 1H, NH).

Cbz-Ile-Glu(O-*t*Bu)-Thr-AAsp(O-*t*Bu)-(2*S*,3*S*)-EP-CO-Ala-NHBzl was obtained using the EDC/HOBt coupling method, purified by column chromatography on silica gel with 19:1 CH_2Cl_2:MeOH as the eluent, and then rechromatographed using 50:35:10:5 CH_2Cl_2:EtOAc:THF:MeOH as the eluent; white solid, yield 36%. ^{1}H NMR (acetone-d_6): 0.90 (t, 3H, Ile CH_3), 0.98 (d, 3H, Ile CH_3), 1.14 (d, 3H, Thr CH_3), 1.22 (m, 2H, Ile CH_2), 1.37 (d, 3H, Ala CH_3), 1.42 (m, 19H, Ile CH and *t*Bu), 1.90 and 2.10 (d of m, 2H, Glu CH_2), 2.40 (m, 2H, Glu CH_2), 2.93 (s, 2H, AAsp CH_2), 3.72 (d, 2H, epoxy), 4.20 (m, 3H, α-H), 4.30 (m, 1H, α-H), 4.40−4.50 (m, 3H, Thr CH and CH_2Ph), 5.07 (q, 2H, Z), 6.70 (d, 1H, NH), 7.10−7.35 (m, 10H, Ph), 7.50 (d, 1H, NH), 7.80 (b, 1H, NH), 7.90 (b, 2H, NH), 9.80 (s, 1H, NH).

(2*R*,3*R*)-3-(*N*^{2}-(*N*-Benzyloxycarbonylleucyl-*tert*-butoxyglutamylthreonyl)-*N*^{1}-*tert*-butoxycarbonylmethylhydrazinocarbonyl)oxirane-2-carboxylic Acid Ethyl Ester (Cbz-Leu-Glu(O-*t*Bu)-Thr-AAsp(O-*t*Bu)-(2*R*,3*R*)-EP-COO-Et). Aza-peptide Epoxide Synthesis Using the Pentafluorophenol Coupling Method. Coupling of Cbz-Leu-Glu(O-*t*Bu)-Thr-$NHNHCH_2COO$-*t*Bu with the monoethyl epoxysuccinate was accomplished using the pentafluorophenol coupling method.[41] The epoxide (1 equiv) in DMF was reacted with pentafluorophenol (1 equiv) and DCC (1 equiv) in DMF at 0 °C. The reaction was allowed to reach room temperature and was then stirred for 24 h. Dicyclohexylurea was removed by filtration, and the filtrate was evaporated to dryness under reduced pressure. The peptide Cbz-Leu-Glu(O-*t*Bu)-Thr-$NHNHCH_2COO$-*t*Bu (1 equiv) in DMF was added to the residue, and the reaction was allowed to stir at room temperature for 24 h. The solvent was evaporated and the product was purified by column chromatography using 1:9 MeOH:CH_2Cl_2 as an eluent and was then rechromatographed using 2:1 EtOAc:hexane as the eluent; white solid, yield 16%. MS (ESI) *m*/*z* 822 [$(M + 1)^+$]. ^{1}H NMR (DMSO-d_6): 0.85 (t, 6H, Leu CH_3), 1.0 (d, 3H, Thr CH_3), 1.2 (t, 3H, OCH_2CH_3), 1.4 (d, 20H, *t*Bu and Leu CH_2), 1.6 (m, 1H, CH Leu), 1.7 (m, 1H, Glu CH_2), 1.9 (m, 1H, Glu CH_2), 2.2 (m, 2H, Glu CH_2), 3.5 (s, 1H, epoxy), 3.9−4.0 (m, 2H, NCH_2COOH), 4.0−4.1 (m, 2H, epoxy and α-H), 4.1−4.2 (m, 3H, OCH_2CH_3 and *CH*-OH), 4.3−4.4 (m, 2H, α-H), 5.02 (s, 2H, Cbz), 7.30−7.40 (m, 5H, Ph), 7.50 (d, 1H, NH), 7.75 (d, 1H, NH), 8.1 (d, 1H, NH).

Aza-peptide Epoxides. Deblocking of the *tert*-Butyl Protecting Groups. Epoxysuccinyl peptides were reacted with TFA at 0 °C for 1−1.5 h. The TFA was removed under vacuum, and the final products were recrystallized from methanol/ether and ether/hexane to give the final epoxysuccinyl peptides as white solids (yields 59−85%).

(2*S*,3*S*)-3-(*N*^{2}-(*N*-Benzyloxycarbonylvalyl)-*N*^{1}-carboxymethylhydrazinocarbonyl)oxirane-2-carboxylic Acid Ethyl Ester (Cbz-Val-AAsp-(2*S*,3*S*)-EP-COOEt, 22c). ^{1}H NMR ($CDCl_3$): 0.98 (d, 6H, Val), 1.30 (t, 3H, OCH_2CH_3), 2.15 (m, 1H, Val), 3.65 (s, 1H, epoxy), 3.90 (s, 1H, $NHCH_2COOH$), 4.10 (m, 1H, α-H), 4.22 (q, 2H, OCH_2CH_3), 4.51 (m, 1H, NH), 4.95−5.17 (d, 2H, Cbz), 5.40 (m, 1H, NH), 7.30−7.40 (m, 5H, Ph). HRMS (FAB) Calcd for $C_{21}H_{27}N_3O_9$: 465.1747. Observed *m*/*z* 465.1825. Anal. Calcd for $C_{21}H_{27}N_3O_9 \cdot 1.5H_2O$: C, 51.21; H, 5.52; N, 8.53. Found: C, 51.26; H, 5.69; N, 8.52.

(2*S*,3*S*)-3-(*N*^{2}-(*N*-Benzyloxycarbonylvalyl)-*N*^{1}-carboxymethylhydrazinocarbonyl)oxirane-2-carboxylic Acid (Cbz-Val-AAsp-(2*S*,3*S*)-EP-COOH, 22n). The ethyl ester of Cbz-Val-AAsp-(2*S*,3*S*)-EP-COOEt was hydrolyzed using NaOH in MeOH to yield Cbz-Val-AAsp-(2*S*,3*S*)-EP-COOH.^{1}H NMR (DMSO-d_6): 0.86 (d, 6H, Val), 1.94 (m, 1H, Val), 3.62 (s, 2H, epoxy), 3.84 (s, 1H, α-H), 5.02 (s, 2H, Cbz), 7.33 (s, 5H, Ph), 7.54 (t, 1H, NH), 11.06 (s, 1H, NH). HRMS (ESI) Calcd for $C_{19}H_{23}N_3O_9$: 437.1513. Observed *m*/*z* 437.1493. Anal. Calcd for $C_{19}H_{23}N_3O_9 \cdot 0.21H_2O \cdot 0.19$TFA: C, 50.29; H, 5.14; N, 9.08. Found: C, 50.32; H, 5.39; N, 8.78.

***trans*-3-(*N*^{2}-(*N*-Benzyloxycarbonylvalyl)-*N*^{1}-carboxymethylhydrazinocarbonyl)-2-(2-phenethyl)oxirane (Cbz-Val-AAsp-*trans*-EP-CH_2CH_2Ph, 22a).** ^{1}H NMR ($CDCl_3$): 0.90−0.95 (d, 6H, Val), 1.90 (m, 2H, CH_2CH_2Ph), 2.05 (m, 1H, Val), 2.60−2.90 (m, 2H, CH_2CH_2Ph), 3.14 (m, 1H, epoxy), 3.86 (m, 1H, epoxy), 4.01−4.08 (m, 3H, α-H and $NHCH_2COOH$), 4.53 (m, 1H, NH), 5.01−5.13 (d, 2H, Cbz), 7.08−7.20 (m, 10H, Ph). HRMS (FAB) Calcd for $C_{26}H_{31}N_3O_7$: 497.2162. Observed *m*/*z* 497.2240. Anal. Calcd for $C_{26}H_{31}N_3O_7 \cdot H_2O$: C, 60.57; H, 6.06; N, 8.15. Found: C, 60.70; H, 6.11; N, 7.93.

***trans*-2-(*N*^{2}-(*N*-Benzyloxycarbonylvalyl)-*N*^{1}-carboxymethylhydrazinocarbonyl)-3-(4-chlorophenyl)oxirane (Cbz-Val-AAsp-*trans*-EP-Ph-4-Cl, 22b).** ^{1}H NMR (DMSO-d_6): 0.75−0.88 (d, 6H, Val), 1.80 (m, 1H, Val), 3.58 (s, 1H, epoxy), 4.11 (s, 1H, $NHCH_2COOH$), 4.35 (s, 1H, α-H), 5.02 (s, 2H, Cbz), 6.18 (d, 1H, NH), 7.15−7.53(m, 9H, Ph), 7.92 (d, 1H, NH). HRMS (ESI) Calcd for $C_{24}H_{26}N_3O_7Cl$: 503.1543. Observed *m*/*z* 503.1537. Anal. Calcd for $C_{24}H_{26}N_3O_7Cl \cdot 1.5H_2O$: C, 54.29; H, 4.93; N, 7.91. Found: C, 54.12; H, 5.00; N, 7.68.

***trans*-3-(*N*^{2}-(*N*-3-Phenylpropanoylvalylalanyl)-*N*^{1}-carboxylmethylhydrazinocarbonyl)oxirane-2-carboxylic Acid Benzyl Ester (PhPr-Val-Ala-AAsp-*trans*-EP-$COOCH_2$Ph, 23d).** ^{1}H NMR ($CDCl_3$): 0.80 (m, 6H, Val), 1.21 (m, 3H, Ala), 1.90 (m, 1H, Val), 2.54 (m, 3H, PhCH_2 and Asp), 2.90 (m, 3H, $PhCH_2CH_2CO$ and Asp), 3.64 and 3.71 (d, 2H, epoxy), 4.15 (m, 2H, NCH_2COOH), 4.50 (m, 2H, α-H), 5.20 (m, 2H, benzyl), 6.51 (m, 1H, NH), 6.95 (M, 1H, NH), 7.16−7.37 (m, 10H, Ph), 9.60 (M, 1H, NH). HRMS (FAB) Calcd for $C_{30}H_{36}N_4O_9$: 597.2561. Observed *m*/*z* 597.2647. Anal. Calcd for $C_{30}H_{36}N_4O_9 \cdot 0.5H_2O$: C, 59.48; H, 6.33; N, 9.25. Found: C, 59.47; H, 6.13; N, 9.31.

***trans*-3-(*N*^{2}-(*N*-3-Phenylpropanoylvalylalanyl)-*N*^{1}-carboxylmethylhydrazinocarbonyl)-2-(2-phenethyl)oxirane (PhPr-Val-Ala-AAsp-*trans*-EP-CH_2CH_2Ph, 23a).** ^{1}H NMR ($CDCl_3$): 0.78 (m, 6H, Val), 1.20 (t, 3H, Ala), 1.90 (m, 2H, $PhCH_2$), 2.52 (m, 2H, PhCH_2CH_2CO), 2.67 (m, 2H, $PhCH_2CH_2$-EP), 2.88 (m, 2H, $PhCH_2CH_2CO$), 4.18 (M, 2H, NCH_2COOH), 4.52 (M, 2H, α-H), 6.55 (m, 1H, NH), 6.95 (m, 1H, NH), 7.16−7,26 (M, 10H, Ph), 9.60 (m, 1H, NH). HRMS (FAB) Calcd for $C_{30}H_{38}N_4O_7$: 597.2819. Observed *m*/*z* 567.2868. Anal. Calcd for $C_{30}H_{36}N_4O_9 \cdot H_2O$: C, 61.62; H, 6.88; N, 9.58. Found: C, 61.52; H, 6.58; N, 9.56.

(2*S*,3*S*)-3-(*N*^{2}-(3-Phenylpropanoylvalylalanyl)-*N*^{1}-carboxymethylhydrazinocarbonyl)oxirane-2-carboxylic Acid Ethyl Ester (PhPr-Val-Ala-AAsp-(2*S*,3*S*)-EP-COOEt, 23c). ^{1}H NMR (DMSO-d_6): 0.75 (m, 6H, Val CH_3), 1.17 (m, 6H, Ala CH_3 and OEt), 1.85 (m, 1H, Val CH), 2.42 (m, 2H, PhCH_2), 2.76 (t, 2H, $PhCH_2CH_2CO$), 3.32 (s, 2H, AAsp CH_2COOH), 3.59 (d, 2H, epoxy), 4.15 (m, 4H, α-H and OEt), 7.16−7.24 (m, 6H, Ph and NH), 7.80 (m, 1H, NH), 8.15 (d, 1H, NH), 10.85 (s, 1H, COOH). HRMS (FAB) Calcd for $C_{25}H_{35}N_4O_9$: 535.24040. Observed *m*/*z* 535.24220. Anal. Calcd for $C_{25}H_{34}N_4O_9$: C, 56.17; H, 6.41; N, 10.48. Found: C, 55.90; H, 6.12; N, 10.16.

(2*R*,3*R*)-3-(*N*^{2}-(3-Phenylpropanoylvalylalanyl)-*N*^{1}-carboxymethylhydrazinocarbonyl)oxirane-2-carboxylic Acid Ethyl Ester (PhPr-Val-Ala-AAsp-(2*R*,3*R*)-EP-COOEt, 23c). ^{1}H NMR (DMSO-d_6): 0.77 (m, 6H, Val CH_3), 1.19 (m, 6H, Ala CH_3 and OEt), 1.87 (m, 1H, Val CH), 2.42 (m, 2H, PhCH_2), 2.76 (m, 2H, $PhCH_2CH_2CO$), 3.32 (s, 2H, AAsp CH_2COOH), 3.45 (s, 2H, epoxy), 4.15 (m, 4H, α-H and OEt), 7.11−7.24 (m, 6H, Ph and NH), 7.84 (m, 1H, NH), 8.26 (d, 1H, NH), 10.85 (s, 1H, COOH). HRMS (FAB) Calcd for $C_{25}H_{35}N_4O_9$: 535.24040. Observed *m*/*z* 535.24153. Anal. Calcd for $C_{25}H_{34}N_4O_9 \cdot 0.5H_2O$: C, 55.25; H, 6.44; N, 10.31. Found: C, 55.04; H, 6.36; N, 10.26.

(2*S*,3*S*)-3-(*N*^{2}-(3-Phenylpropanoylvalylalanyl)-*N*^{1}-carboxymethylhydrazinocarbonyl)oxirane-2-carboxylic Acid Benzyl Ester (PhPr-Val-Ala-AAsp-(2*S*,3*S*)-EP-$COOCH_2$Ph, 23d). ^{1}H NMR (DMSO-d_6): 0.77 (m, 6H, Val CH_3), 1.13 (m, 3H, Ala CH_3), 1.87 (m, 1H, Val CH), 2.42 (m, 2H, PhCH_2), 2.77 (m, 2H, $PhCH_2CH_2CO$), 3.32 (s, 2H, AAsp CH_2COOH), 3.61 (d, 2H, epoxy), 4.15 (m, 2H, α-H), 5.17 (m, 2H, benzyl), 7.15−7.37 (m, 11H, Ph and NH), 7.80 (m, 1H, NH), 8.15 (m, 1H, NH), 10.90 (s, 1H, COOH). HRMS (FAB) Calcd for $C_{30}H_{37}$-

N_4O_9: 597.25605. Observed *m*/*z* 597.26012. Anal. Calcd for $C_{30}H_{36}N_4O_9 \cdot 0.5H_2O$: C, 59.49; H, 6.17; N, 9.25. Found: C, 58.68; H, 6.03; N, 9.54.

(2*R*,3*R*)-3-(*N*²-(3-Phenylpropanoylvalylalanyl)-*N*¹-carboxymethylhydrazinocarbonyl)oxirane-2-carboxylic Acid Benzyl Ester (PhPr-Val-Ala-AAsp-(2*R*,3*R*)-EP-COOCH₂Ph, 23d). ^{1}H NMR (DMSO-d_6): 0.77 (m, 6H, Val CH_3), 1.13 (m, 3H, Ala CH_3), 1.87 (m, 1H, Val CH), 2.42 (m, 2H, PhCH_2), 2.77 (t, 2H, PhCH$_2$$CH_2$CO), 3.32 (s, 2H, AAsp CH_2COOH), 3.61 (d, 2H, epoxy), 4.15 (m, 2H, α-H), 5.17 (m, 2H, benzyl), 7.15−7.37 (m, 11H, Ph and NH), 7.84 (m, 1H, NH), 8.24 (m, 1H, NH), 10.90 (b, 1H, COOH). HRMS (FAB) Calcd for $C_{30}H_{37}N_4O_9$: 597.25605. Observed *m*/*z* 597.25875. Anal. Calcd for $C_{30}H_{36}N_4O_9 \cdot 0.5H_2O$: C, 59.49; H, 6.17; N, 9.25. Found: C, 58.75; H, 6.00; N, 9.47.

(2*S*,3*S*)-3-(*N*²-(3-Phenylpropanoylvalylalanyl)-*N*¹-carboxymethylhydrazinocarbonyl)oxirane-2-carboxylic Acid Phenethyl Ester (PhPr-Val-Ala-AAsp-(2*S*,3*S*)-EP-COOCH₂CH₂Ph, 23e). ^{1}H NMR (DMSO-d_6): 0.77 (m, 6H, Val CH_3), 1.18 (m, 3H, Ala CH_3), 1.86 (m, 1H, Val CH), 2.41 (m, 2H, PhCH_2), 2.76 (t, 2H, PhCH$_2$$CH_2$CO), 2.90 (t, 2H, Ph$CH_2$), 3.32 (s, 2H, AAsp CH_2COOH), 3.53 and 3.69 (d, 2H, epoxy), 4.16 (m, 2H, α-H), 4.27 (m, 2H, OCH_2), 7.12−7.30 (m, 11H, Ph and NH), 7.80 (m, 1H, NH), 8.24 (d, 1H, NH), 10.85 (s, 1H, COOH). HRMS (FAB) Calcd for $C_{31}H_{39}N_4O_9$: 611.27170. Observed *m*/*z* 611.27640. Anal. Calcd for $C_{31}H_{38}N_4O_9 \cdot H_2O$: C, 59.23; H, 6.41; N, 8.91. Found: C, 59.19; H, 6.25; N, 9.26.

(2*S*,3*S*)-2-(2-Ethylcarbamoyl)-3-(*N*²-(3-phenylpropanoylvalylalanyl)-*N*¹-carboxymethylhydrazinocarbonyl)oxirane (PhPr-Val-Ala-AAsp-(2*S*,3*S*)-EP-CONHCH₂CH₃, 23f). ^{1}H NMR (DMSO-d_6): 0.76 (m, 6H, Val CH_3), 1.02 (t, 3H, CH_3), 1.19 (d, 3H, Ala CH_3), 1.90 (m, 1H, Val CH), 2.45 (m, 2H, PhCH_2), 2.77 (t, 2H, PhCH$_2$$CH_2$CO), 3.09 (m, 2H, CH_2N), 3.37 (s, 2H, AAsp CH_2COOH), 3.41 (s, 2H, epoxy), 4.17 (m, 2H, α-H), 7.11−7.25 (m, 6H, Ph and NH), 7.82 (d, 1H, NH), 8.23 (s, 1H, NH), 8.36 (t, 1H, NH), 10.85 (s, 1H, COOH). HRMS (FAB) Calcd for $C_{25}H_{36}N_5O_8$: 534.2564. Observed *m*/*z* 534.2535. Anal. Calcd for $C_{25}H_{35}N_5O_8 \cdot H_2O$: C, 54.44; H, 6.76; N, 12.70. Found: C, 54.53; H, 6.65; N, 12.90.

(2*S*,3*S*)-2-(2-Benzylcarbamoyl)-3-(*N*²-(3-phenylpropanoylvalylalanyl)-*N*¹-carboxymethylhydrazinocarbonyl)oxirane (PhPr-Val-Ala-AAsp-(2*S*,3*S*)-EP-CONHCH₂Ph, 23g). ^{1}H NMR (DMSO-d_6): 0.75 (m, 6H, Val CH_3), 1.13 (m, 3H, Ala CH_3), 1.87 (m, 1H, Val CH), 2.43 (m, 2H, PhCH_2), 2.77 (m, 2H, PhCH$_2$$CH_2$CO), 3.37 (m, 2H, AAsp CH_2COOH), 3.50 (s, 2H, epoxy), 4.15 (m, 2H, α-H), 4.31 (t, 2H, PhCH_2N), 7.13−7.31 (m, 11H, Ph and NH), 7.83 (m, 1H, NH), 8.10 (m, 1H, NH), 8.91 (t, 1H, NH), 10.85 (s, 1H, COOH). HRMS (FAB) Calcd for $C_{30}H_{38}N_5O_8$: 596.27204. Observed *m*/*z* 596.27178. Anal. Calcd for $C_{30}H_{37}N_5O_8 \cdot 0.7H_2O$: C, 59.22; H, 6.37; N, 11.52. Found: C, 59.20; H, 6.18; N, 11.52.

(2*R*,3*R*)-2-(2-Benzylcarbamoyl)-3-(*N*²-(3-phenylpropanoylvalylalanyl)-*N*¹-carboxymethylhydrazinocarbonyl)oxirane (PhPr-Val-Ala-AAsp-(2*R*,3*R*)-EP-CONHCH₂Ph, 23g). ^{1}H NMR (DMSO-d_6): 0.75 (m, 6H, Val CH_3), 1.13 (m, 3H, Ala CH_3), 1.87 (m, 1H, Val CH), 2.42 (m, 2H, PhCH_2), 2.77 (m, 2H, PhCH$_2$$CH_2$CO), 3.37 (m, 2H, AAsp CH_2COOH), 3.55 (d, 2H, epoxy), 4.15 (m, 2H, α-H), 4.28 (t, 2H, PhCH_2N), 7.14−7.30 (m, 11H, Ph and NH), 7.85 (m, 1H, NH), 8.24 (m, 1H, NH), 8.90 (t, 1H, NH), 10.94 (s, 1H, COOH). HRMS (FAB) Calcd for $C_{30}H_{38}N_5O_8$: 596.27204. Observed *m*/*z* 596.27030. Anal. Calcd for $C_{30}H_{37}N_5O_8 \cdot H_2O$: C, 58.72; H, 6.41; N, 11.41. Found: C, 58.42; H, 6.05; N, 11.62.

(2*S*,3*S*)-2-(2-Phenethylcarbamoyl)-3-(*N*²-(3-phenylpropanoylvalylalanyl)-*N*¹-carboxymethylhydrazinocarbonyl)oxirane (PhPr-Val-Ala-AAsp-(2*S*,3*S*)-EP-CONHCH₂CH₂Ph, 23h). ^{1}H NMR (DMSO-d_6): 0.75 (m, 6H, Val CH_3), 1.18 (m, 3H, Ala CH_3), 1.88 (m, 1H, Val CH), 2.43 (m, 2H, PhCH_2), 2.47−2.80 (m, 4H, PhCH$_2$$CH_2$CO and Ph$CH_2$$CH_2$N), 3.32 (m, 4H, N$CH_2$-$CH_2$Ph and AAsp CH_2COOH), 3.43 (s, 2H, epoxy), 4.16 (m, 2H, α-H), 7.13−7.31 (m, 11H, Ph and NH), 7.82 (m, 1H, NH), 8.25 (m, 1H, NH), 8.48 (t, 1H, NH), 10.85 (s, 1H, COOH). HRMS (FAB) Calcd for $C_{31}H_{40}N_5O_8$: 610.28769. Observed *m*/*z* 610.28714. Anal. Calcd for $C_{31}H_{39}N_5O_8 \cdot 0.8H_2O$: C, 59.65; H, 6.57; N, 11.22. Found: C, 59.59; H, 6.42; N, 11.26.

(2*R*,3*R*)-2-(2-Phenethylcarbamoyl)-3-(*N*²-(3-phenylpropanoylvalylalanyl)-*N*¹-carboxymethylhydrazinocarbonyl)oxirane (PhPr-Val-Ala-AAsp-(2*R*,3*R*)-EP-CONHCH₂CH₂Ph, 23h). ^{1}H NMR (DMSO-d_6): 0.75 (m, 6H, Val CH_3), 1.12 (m, 3H, Ala CH_3), 1.86 (m, 1H, Val CH), 2.40 (m, 2H, PhCH_2), 2.76 (m, 4H, PhCH$_2$$CH_2$CO and Ph$CH_2$$CH_2$N)), 3.33 (m, 4H, N$CH_2$$CH_2$-Ph and AAsp CH_2COOH), 3.67(s, 2H, epoxy), 4.15 (m, 2H, α-H), 7.13−7.31 (m, 11H, Ph and NH), 7.83 (m, 1H, NH), 8.15 (m, 1H, NH), 8.24 (t, 1H, NH), 10.93 (s, 1H, COOH). HRMS (FAB) Calcd for $C_{31}H_{40}N_5O_8$: 610.28769. Observed *m*/*z* 610.29073. Anal. Calcd for $C_{31}H_{39}N_5O_8$: C, 59.32 H, 6.58; N, 11.16. Found: C, 59.49; H, 6.45; N, 11.59.

(2*S*,3*S*)-2-(2-Hydroxy-2-phenylethylcarbamoyl)-3-(*N*²-(3-phenylpropanoylvalylalanyl)-*N*¹-carboxymethylhydrazinocarbonyl)oxirane (PhPr-Val-Ala-AAsp-(2*S*,3*S*)-EP-CONHCH₂CH(OH)Ph, 23i). ^{1}H NMR (DMSO-d_6): 0.76 (m, 6H, Val CH_3), 1.18 (m, 3H, Ala CH_3), 1.85 (m, 1H, Val CH), 2.43 (m, 2H, PhCH_2), 2.77 (m, 2H, PhCH$_2$$CH_2$), 3.37 (m, 4H, NCH_2 and AAsp CH_2COOH), 3.53 (s, 2H, epoxy), 4.16 (m, 2H, α-H), 4.60 (b, 1H, CH), 7.14−7.39 (m, 11H, Ph and NH), 7.80 (m, 1H, NH), 8.20 (m, 1H, NH), 8.50 (t, 1H, NH), 10.90 (s, 1H, COOH). HRMS (FAB) Calcd for $C_{31}H_{40}N_5O_9$: 626.28260. Observed *m*/*z* 626.28608. Anal. Calcd for $C_{31}H_{39}N_5O_9 \cdot H_2O$: C, 57.84; H, 6.42; N, 10.88. Found: C, 58.03; H, 6.35; N, 10.65.

(2*R*,3*R*)-2-(2-Hydroxy-2-phenylethylcarbamoyl)-3-(*N*²-(3-phenylpropanoylvalylalanyl)-*N*¹-carboxymethylhydrazinocarbonyl)oxirane (PhPr-Val-Ala-AAsp-(2*R*,3*R*)-EP-CONHCH₂CH(OH)Ph, 23i). ^{1}H NMR (DMSO-d_6): 0.77 (m, 6H, Val CH_3), 1.17 (m, 3H, Ala CH_3), 1.85 (m, 1H, Val CH), 2.43 (m, 2H, PhCH_2), 2.77 (m, 2H, PhCH$_2$$CH_2$), 3.32 (m, 4H, NCH_2 and AAsp CH_2COOH), 3.40 (s, 2H, epoxy), 4.16 (m, 2H, α-H), 4.60 (b, 1H, CH), 7.13−7.39 (m, 11H, Ph and NH), 7.80 (m, 1H, NH), 8.21 (m, 1H, NH), 8.50 (t, 1H, NH), 10.90 (s, 1H, COOH). HRMS (FAB) Calcd for $C_{31}H_{40}N_5O_9$: 626.28260. Observed *m*/*z* 626.28250. Anal. Calcd for $C_{31}H_{39}N_5O_9 \cdot H_2O$: C, 57.84; H, 6.42; N, 10.88. Found: C, 57.92; H, 6.40; N, 10.76.

(2*S*,3*S*)-2-(1-Benzylcarbamoylethylcarbamoyl)-3-(*N*²-(3-phenylpropanoylvalylalanyl)-*N*¹-carboxymethylhydrazinocarbonyl)oxirane (PhPr-Val-Ala-AAsp-(2*S*,3*S*)-EP-CO-Ala-NHBzl, 23j). ^{1}H NMR (DMSO-d_6): 0.76 (m, 6H, Val CH_3), 1.16 (d, 3H, Ala CH_3), 1.26 (d, 3H, Ala CH_3), 1.88 (m, 1H, Val CH), 2.43 (m, 2H, PhCH_2), 2.78 (t, 2H, PhCH$_2$$CH_2$-CO), 3.33 (d, 2H, AAsp CH_2COOH), 3.60 (s, 2H, epoxy), 4.15 (m, 3H, α-H), 4.27 (d, 2H, PhCH_2N), 7.13−7.30 (m, 11H, Ph and NH), 7.80 (d, 1H, NH), 8.25 (d, 1H, NH), 8.45 (s, 1H, NH), 8.70 (b, 1H, NH), 10.85 (s, 1H, COOH). HRMS (FAB) Calcd for $C_{33}H_{43}N_6O_9$: 667.30915. Observed *m*/*z* 667.31344. Anal. Calcd for $C_{33}H_{42}N_6O_9 \cdot 0.5H_2O$: C, 58.67; H, 6.37; N, 12.44. Found: C, 58.11; H, 6.31; N, 12.30.

(2*R*,3*R*)-2-(1-Benzylcarbamoylethylcarbamoyl)-3-(*N*²-(3-phenylpropanoylvalylalanyl)-*N*¹-carboxymethylhydrazinocarbonyl)oxirane (PhPr-Val-Ala-AAsp-(2*R*,3*R*)-EP-CO-Ala-NHBzl, 23j). ^{1}H NMR (DMSO-d_6): 0.77 (m, 6H, Val CH_3), 1.13 (m, 3H, Ala CH_3), 1.25 (d, 3H, Ala CH_3), 1.88 (m, 1H, Val CH), 2.43 (m, 2H, PhCH_2), 2.78 (m, 2H, PhCH$_2$$CH_2$-CO), 3.36 (d, 2H, AAsp CH_2COOH), 3.50 (s, 2H, epoxy), 4.15 (m, 3H, α-H),), 4.26 (m, 2H, PhCH_2N), 7.13−7.30 (m, 11H, Ph and NH), 7.85 (d, 1H, NH), 8.25 (d, 1H, NH), 8.45 (s, 1H, NH), 8.70 (b, 1H, NH), 10.93 (s, 1H, COOH). HRMS (FAB) Calcd for $C_{33}H_{43}N_6O_9$: 667.30915. Observed *m*/*z* 667.31059. Anal. Calcd for $C_{33}H_{42}N_6O_9 \cdot H_2O$: C, 57.88; H, 6.48; N, 12.27. Found: C, 57.83; H, 6.37; N, 12.40.

(2*S*,3*S*)-2-(1-Carbamoyl-3-methylbutylcarbamoyl)-3-(*N*²-(3-phenylpropanoylvalylalanyl)-*N*¹-carboxymethylhydrazinocarbonyl)oxirane (PhPr-Val-Ala-AAsp-(2*S*,3*S*)-EP-CO-Leu-NH₂, 23k). ^{1}H NMR (DMSO-d_6): 0.74−0.87 (m, 12H, Val and Leu CH_3), 1.17 (d, 3H, Ala CH_3), 1.45 (m, 2H, Leu CH_2), 1.54 (m, 1H, Leu CH), 1.88 (m, 1H, Val CH), 2.40 (m, 2H, PhCH_2), 2.78 (t, 2H, PhCH$_2$$CH_2$CO), 3.32 (s, 2H, AAsp CH_2COOH), 3.56 (s, 2H, epoxy), 4.16 (m, 2H, α-H), 4.26 (m, 1H, α-H), 7.00 (s, 1H, NH), 7.15−7.23 (m, 6H, Ph and NH), 7.42 (s, 1H, NH), 7.85 (d, 1H, NH), 8.25 (s, 1H, NH), 8.55 (b,

1H, NH), 10.85 (b, 1H, COOH). HRMS (FAB) Calcd for $C_{29}H_{43}N_6O_9$: 619.30915. Observed *m/z* 619.3129. Anal. Calcd for $C_{29}H_{42}N_6O_9 \cdot H_2O$: C, 54.71; H, 6.97; N, 13.20. Found: C, 54.40; H, 6.89; N, 13.18.

(2*R*,3*R*)-2-(1-Carbamoyl-3-methylbutylcarbamoyl)-3-(*N*^{2}-(3-phenylpropanoylvalylalanyl)-*N*^{1}-carboxymethylhydrazinocarbonyl)oxirane (PhPr-Val-Ala-AAsp-(2*R*,3*R*)-EP-CO-Leu-NH$_2$, 23k). ^{1}H NMR (DMSO-d_6): 0.73−0.87 (m, 12H, Val and Leu CH_3), 1.17 (d, 3H, Ala CH_3), 1.43 (m, 2H, Leu CH_2), 1.53 (m, 1H, Leu CH), 1.87 (m, 1H, Val CH), 2.40 (m, 2H, PhCH_2), 2.77 (t, 2H, PhCH$_2$$CH_2$CO), 3.32 (s, 2H, AAsp CH_2COOH), 3.48 (s, 2H, epoxy), 4.20 (m, 3H, α-H), 6.97 (s, 1H, NH), 7.15−7.24 (m, 6H, Ph and NH), 7.42 (s, 1H, NH), 7.85 (d, 1H, NH), 8.25 (s, 1H, NH), 8.55 (d, 1H, NH), 10.85 (b, 1H, COOH). HRMS (FAB) Calcd for $C_{29}H_{43}N_6O_9$: 619.30915. Observed *m/z* 619.31200. Anal. Calcd for $C_{29}H_{42}N_6O_9 \cdot 0.7H_2O$: C, 55.18; H, 6.88, N, 13.32. Found: C, 55.34; H, 6.81; N, 13.31.

(2*S*,3*S*)-2-(1-Carbamoyl-2-phenylethylcarbamoyl)-3-(*N*^{2}-(3-phenylpropanoylvalylalanyl)-*N*^{1}-carboxymethylhydrazinocarbonyl)oxirane (PhPr-Val-Ala-AAsp-(2*S*,3*S*)-EP-CO-Phe-NH$_2$, 23l). ^{1}H NMR (DMSO-d_6): 0.76 (m, 6H, Val CH_3), 1.15 (d, 3H, Ala CH_3), 1.90 (m, 1H, Val CH), 2.42 (m, 2H, PhCH_2), 2.80 (m, 3H, Phe PhCH_2 and PhCH$_2$$CH_2$CO), 3.00 (m, 1H, Phe Ph$CH_2$), 3.32 (s, 2H, AAsp CH_2COOH), 3.56 (s, 2H, epoxy), 4.16 (m, 2H, α-H), 4.50 (m, 1H, Phe α-H), 7.13−7.30 (m, 12H, Ph and NH), 7.54 (s, 1H, NH), 7.84 (d, 1H, NH), 8.25 (s, 1H, NH), 8.70 (d, 1H, NH), 10.85 (b, 1H, COOH). HRMS (FAB) Calcd for $C_{32}H_{41}N_6O_9$: 653.29350. Observed *m/z* 653.29936. Anal. Calcd for $C_{32}H_{40}N_6O_9 \cdot H_2O$: C, 57.30; H, 6.31; N, 12.53. Found: C, 57.53; H, 6.14; N, 12.16.

(2*R*,3*R*)-2-(1-Carbamoyl-2-phenylethylcarbamoyl)-3-(*N*^{2}-(3-phenylpropanoylvalylalanyl)-*N*^{1}-carboxymethylhydrazinocarbonyl)oxirane (PhPr-Val-Ala-AAsp-(2*R*,3*R*)-EP-CO-Phe-NH$_2$, 23l). ^{1}H NMR (DMSO-d_6): 0.77 (m, 6H, Val CH_3), 1.15 (d, 3H, Ala CH_3), 1.88 (m, 1H, Val CH), 2.43 (m, 2H, PhCH_2), 2.80 (m, 3H, Phe PhCH_2 and PhCH$_2$$CH_2$CO), 2.95 (m, 1H, Phe Ph$CH_2$), 3.32 (s, 2H, AAsp CH_2COOH), 3.47 (s, 2H, epoxy), 4.15 (m, 2H, α-H), 4.45 (m, 1H, Phe α-H), 7.13−7.30 (m, 11H, Ph and NH), 7.45 (s, 1H, NH), 7.83 (d, 1H, NH), 8.00 (d, 1H, NH), 8.15 (b, 1H, NH), 8.30 (s, 1H, NH), 10.88 (s, 1H, COOH). HRMS (FAB) Calcd for $C_{32}H_{41}N_6O_9$: 653.29350. Observed *m/z* 653.29525. Anal. Calcd for $C_{32}H_{40}N_6O_9 \cdot 0.5H_2O$: C, 58.09; H, 6.20; N, 12.70. Found: C, 57.83; H, 6.31; N, 12.84.

(2*S*,3*S*)-2-(1-Carbamoyl-2(4-hydroxyphenyl)ethylcarbamoyl)-3-(*N*^{2}-(3-phenylpropanoylvalylalanyl)-*N*^{1}-carboxymethylhydrazinocarbonyl)oxirane (PhPr-Val-Ala-AAsp-(2*S*,3*S*)-EP-CO-Tyr-NH$_2$, 23m). ^{1}H NMR (DMSO-d_6): 0.78 (m, 6H, Val CH_3), 1.08 (d, 3H, Ala CH_3), 1.90 (m, 1H, Val CH), 2.42 (m, 2H, PhCH_2), 2.77 (m, 3H, Tyr PhCH_2 and PhCH$_2$$CH_2$CO), 2.88 (m, 1H, Tyr Ph$CH_2$), 3.32 (s, 2H, AAsp CH_2COOH), 3.65 (d, 2H, epoxy), 4.16 (t, 1H, α-H), 4.32 (m, 2H, α-H), 6.59 (d, 2H, Tyr Ph), 6.95 (d, 2H, Tyr Ph), 7.10−7.22 (m, 6H, Ph and NH), 7.38 (s, 1H, NH), 7.50 (d, 1H, NH), 7.85 (d, 1H, NH), 8.23 (s, 1H, NH), 9.13 (d, 1H, NH), 10.80 (b, 1H, COOH). HRMS (FAB) Calcd for $C_{32}H_{41}N_6O_{10}$: 669.2884. Observed *m/z* 669.2879. Anal. Calcd for $C_{32}H_{40}N_6O_{10} \cdot H_2O$: C, 55.97; H, 6.16; N, 12.24. Found: C, 55.72; H, 6.04; N, 11.98.

(2*S*,3*S*)-3-(*N*^{2}-(*N*-Benzyloxycarbonylaspartylglutamylvalyl)-*N*^{1}-carboxymethylhydrazinocarbonyl)oxirane-2-carboxylic Acid Ethyl Ester (Cbz-Asp-Glu-Val-AAsp-(2*S*,3*S*)-EP-COOEt, 25c). ^{1}H NMR (DMSO-d_6): 0.84 (m, 6H, Val), 1.20, 1.21 (t, 3H, OCH$_2$$CH_3$), 1.7−2.1 (m, 3H, Val and Glu), 2.21 (m, 2H, Glu), 2.40−2.65 (m, 2H, Asp), 3.58 and 4.10 (d, 2H, epoxy), 4.05−4.22 (m, 4H, NCH_2COOH and OCH_2CH$_3$), 4.50−4.60 (m, 3H, α-H), 5.05 (m, 2H, Cbz), 7.20−7.40 (m, 5H, Ph), 7.60 (1H, NH), 7.85 (m, 2H, NH), 11.00 (m, COOH). HRMS (FAB) Calcd for $C_{30}H_{39}N_5O_{15}$: 710.25209. Observed *m/z* 710.2550. Anal. Calcd for $C_{30}H_{38}N_5O_{15} \cdot 1.65\ H_2O$: C, 48.73; H, 5.76; N, 9.47. Found: C, 48.73; H, 5.63; N, 9.45.

(2*R*,3*R*)-3-(*N*^{2}-(*N*-Benzyloxycarbonylaspartylglutamylvalyl)-*N*^{1}-carboxymethylhydrazinocarbonyl)oxirane-2-carboxylic Acid Ethyl Ester (Cbz-Asp-Glu-Val-AAsp-(2*R*,3*R*)-EP-COOEt, 25c). ^{1}H NMR (DMSO-d_6): 0.84 (m, 6H, Val), 1.20, 1.21 (t, 3H, OCH$_2$$CH_3$), 1.7−2.1 (m, 3H, Val and Glu), 2.21 (m, 2H, Glu), 2.40−2.65 (m, 2H, Asp), 3.58 and 4.10 (d, 2H, epoxy), 4.05−4.22 (m, 4H, NCH_2COOH and OCH_2CH$_3$), 4.50−4.60 (m, 3H, α-H), 5.05 (m, 2H, Cbz), 7.20−7.40 (m, 5H, Ph), 7.60 (1H, NH), 7.85 (m, 2H, NH), 11.00 (m, COOH). HRMS (FAB) Calcd for $C_{30}H_{39}N_5O_{15}$: 710.25209. Observed *m/z* 710.25195. Anal. Calcd for $C_{30}H_{38}N_5O_{15} \cdot 0.9H_2O$: C, 49.64; H, 5.66; N, 9.65. Found: C, 49.59; H, 5.56; N, 9.66.

(2*S*,3*S*)-3-(*N*^{2}-(*N*-Benzyloxycarbonylaspartylglutamylvalyl)-*N*^{1}-carboxymethylhydrazinocarbonyl)oxirane-2-carboxylic Acid Benzyl Ester (Cbz-Asp-Glu-Val-AAsp-(2*S*,3*S*)-EP-COOCH$_2$Ph, 25d). ^{1}H NMR (DMSO-d_6): 0.85 (m, 6H, Val), 1.80−2.24 (m, 3H, Val, Glu), 2.41 (m, 2H, Glu), 2.65−2.90 (m, 2H, Asp), 3.60 and 4.00 (d, 2H, epoxy), 4.05−4.12 (m, 2H, NCH_2COOH), 4.10−4.40 (m, 3H, α-H), 5.05 (m, 4H, Cbz), 6.05 (m, 1H, NH), 7.20−7.40 (m, 10H, Ph), 7.85 (m, 2H, NH), 9.05 (m, 1H, NH). HRMS (ESI) Calcd for $C_{47}H_{66}N_5O_{15}$: 940.5. Observed *m/z* 940.5. Anal. Calcd for $C_{35}H_{41}N_5O_{15} \cdot 1.5\ H_2O$: C, 52.60; H, 5.55; N, 8.76. Found: C, 52.59; H, 5.37; N, 8.76.

(2*S*,3*S*)-2-(2-Benzylcarbamoyl)-3-(*N*^{2}-(*N*-benzyloxycarbonylaspartylglutamylvalyl)-*N*^{1}-carboxymethylhydrazinocarbonyl)oxirane (Cbz-Asp-Glu-Val-AAsp-(2*S*,3*S*)-EP-CONHCH$_2$Ph, 25g). ^{1}H NMR ($CDCl_3$): 0.97 (m, 6H, Val), 1.9−2.3 (m, 3H, Val and Glu), 2.41 (m, 2H, Glu), 2.80−2.95 (m, 2H, Asp), 3.63 and 3.97 (d, 2H, epoxy), 4.05−4.22 (m, 4H, NCH_2COOH), 4.20−4.55 (m, 3H, α-H), 4.45 (m, 2H, NHCH_2-Ph), 5.05 (m, 2H, Cbz), 7.20−7.40 (m, 10H, Ph), 6.15 (m, 1H, NH), 6.60 (1H, NH), 7.85 (m, 2H, NH). HRMS (ESI) Calcd for $C_{35}H_{43}N_6O_{14}$: 771.3. Observed *m/z* 771.4. Anal. Calcd for $C_{35}H_{42}N_6O_{14} \cdot 2.2\ H_2O$: C, 51.86; H, 5.77; N, 10.36. Found: C, 51.92; H, 5.66; N, 10.39.

(2*S*,3*S*)-2-(1-Carbamoyl-2-phenylethylcarbamoyl)-3-(*N*^{2}-(*N*-benzyloxycarbonylaspartylglutamylvalyl)-*N*^{1}-carboxymethylhydrazinocarbonyl)oxirane (Cbz-Asp-Glu-Val-AAsp-(2*S*,3*S*)-EP-CO-Phe-NH$_2$, 25l). ^{1}H NMR (DMSO-d_6): 0.94 (m, 6H, Val), 1.62−2.00 (m, 3H, Val, Glu), 2.20 (m, 2H, Glu), 2.60 and 3.40 (m, 2H, Phe, 2H, Asp), 3.60 and 4.05 (d, 2H, epoxy), 3.70 (m, 2H, NCH_2COOH), 4.05−4.40 (m, 4H, α-H), 5.10 (m, 2H, Cbz), 7.0 (s, 2H, NH$_2$), 7.10−7.40 (m, 10H, Ph), 7.60 and 8.00 (m, 4H, NH). HRMS Calcd for $C_{37}H_{45}N_7O_{15}$: 827.2974 Observed *m/z* 827.2935 Anal. Calcd for $C_{37}H_{45}N_7O_{15} \cdot 3.0\ H_2O \cdot 0.75$ EtOAc: C, 50.68; H, 6.06; N, 10.34. Found: C, 50.67; H, 5.71; N, 10.08.

(2*S*,3*S*)-3-(*N*^{2}-(*N*-Benzyloxycarbonylglutamylvalyl)-*N*^{1}-carboxymethylhydrazinocarbonyl)oxirane-2-carboxylic Acid Ethyl Ester (Cbz-Glu-Val-AAsp-(2*S*,3*S*)-EP-COOEt, 24c). ^{1}H NMR (DMSO-d_6): 0.90 (d, 6H, Val), 1.20 (t, 3H, OCH$_2$$CH_3$), 1.60−2.10 (m, 3H, Val and Glu), 2.21 (m, 2H, Glu), 3.60 and 4.10 (d, 2H, epoxy), 3.90−4.20 (m, 4H, NCH_2COOH, and OCH_2CH$_3$), 4.20−4.30 (m, 2H, α-H), 5.05 (m, 2H, Cbz), 7.20−7.40 (m, 5H, Ph), 8.00 (m, 1H, NH), 11.00 (m, COOH). HRMS (FAB) Calcd for $C_{26}H_{35}N_4O_{12}$: 595.2251. Observed *m/z* 595.2268. Anal. Calcd for $C_{26}H_{35}N_4O_{12} \cdot 0.5\ H_2O$: C, 51.74; H, 5.84; N, 9.28. Found: C, 51.70; H, 5.80; N, 9.17.

(2*S*,3*S*)-2-(2-Phenethylcarbamoyl)-3-(*N*^{2}-(*N*-benzyloxycarbonylglutamylvalyl)-*N*^{1}-carboxymethylhydrazinocarbonyl)oxirane (Cbz-Glu-Val-AAsp-(2*S*,3*S*)-EP-CONHCH$_2$-CH$_2$Ph, 24h). ^{1}H NMR (DMSO-d_6): 0.95 (d, 6H, Val), 1.20 (t, 3H, OCH$_2$$CH_3$), 1.60−2.10 (m, 3H, Val and Glu), 2.21 (m, 2H, Glu), 3.60 and 4.10 (d, 2H, epoxy), 3.90−4.20 (m, 4H, NCH_2-COOH, and OCH_2CH$_3$), 4.20−4.30 (m, 2H, α-H), 5.05 (m, 2H, Cbz), 7.20−7.40 (m, 5H, Ph), 8.00 (m, 1H, NH), 11.00 (m, COOH). HRMS (FAB) Calcd for $C_{32}H_{40}N_5O_{11}$: 670.2724. Observed *m/z* 670.2822. Anal. Calcd for $C_{32}H_{39}N_5O_{11} \cdot H_2O$: C, 55.88; H, 6.00; N, 10.19. Found: C, 55.91; H, 5.89; N, 10.32.

(2*R*,3*R*)-2-(1-Carbamoyl-2-phenylethylcarbamoyl)-3-(*N*^{2}-(*N*-benzyloxycarbonylaspartylglutamylvalyl)-*N*^{1}-carboxymethylhydrazinocarbonyl)oxirane (Cbz-Glu-Val-AAsp-(2*R*,3*R*)-EP-CO-Phe-NH$_2$, 24l). ^{1}H NMR (DMSO-d_6): 0.94 (m, 6H, Val), 1.62−2.00 (m, 3H, Val and Glu), 2.20 (m, 2H, Glu), 2.80 and 3.00 (m, 2H, Phe), 3.60 and 4.05 (d, 2H, epoxy), 4.05 (m, 2H, NCH_2COOH), 4.05−4.40 (m, 3H, α-H), 5.10 (m, 2H, Cbz), 7.0 (s, 2H, NH$_2$), 7.10−7.40 (m, 10H, Ph), 8.00 (m, 2H, NH). HRMS Calcd for $C_{33}H_{41}N_6O_{12}$: 713.2782

Observed *m*/*z* 713.2811. Anal. Calcd for $C_{33}H_{40}N_6O_{12}\cdot 1.0\ H_2O$: C, 54.21; H, 5.68; N, 11.34. Found: C, 54.24; H, 5.79; N, 10.50.

(2*R*,3*R*)-3-((N^2-(*N*-Benzyloxycarbonylleucylglutamylthreonyl)-N^1-carboxymethylhydrazinocarbonyl)oxirane-2-carboxylic Acid Ethyl Ester (Cbz-Leu-Glu-Thr-AAsp-(2*R*,3*R*)-EP-COOEt, 26c). ^{1}H NMR (DMSO-d_6): 0.82 (t, 6H, Leu CH_3), 1.0 (d, 3H, Thr CH_3), 1.2 (t, 3H, OCH_2CH_3), 1.4 (m, 2H, Leu CH_2), 1.6 (m, 1H, CH Leu), 1.78 (m, 1H, Glu CH_2), 1.9 (m, 1H, Glu CH_2), 2.25 (m, 2H, Glu), 3.5 (s, 1H, epoxy), 3.9−4.0 (m, 1H, α-H), 4.0−4.1 (m, 3H, NCH_2COOH, epoxy), 4.1−4.2 (m, 3H, OCH_2CH_3 and *CH*-OH), 4.32−4.41 (m, 2H, α-H), 5.05 (m, 2H, Cbz), 7.30−7.40 (m, 5H, Ph), 7.45 (d, 1H, NH), 7.80 (m, 1H, NH), 8.05 (m, 1H, NH), 11.00 (m, COOH). HRMS (FAB) Calcd for $C_{31}H_{44}N_5O_{14}$: 710.28848. Observed *m*/*z* 710.29480. Anal. Calcd for $C_{31}H_{43}N_5O_{14}\cdot H_2O$: C, 51.17; H, 6.19; N, 9.63. Found: C, 51.33; H, 6.02; N, 9.66.

(2*S*,3*S*)-3-((N^2-(*N*-Benzyloxycarbonylleucylglutamylthreonyl)-N^1-carboxymethylhydrazinocarbonyl)oxirane-2-carboxylic Acid Ethyl Ester (Cbz-Leu-Glu-Thr-AAsp-(2*S*,3*S*)-EP-COOEt, 26c). ^{1}H NMR (DMSO-d_6): 0.85 (t, 6H, Leu CH_3), 1.05 (d, 3H, Thr CH_3), 1.2 (t, 3H, OCH_2CH_3), 1.4 (m, 2H, Leu CH_2), 1.6 (m, 1H, CH Leu), 1.77 (m, 1H, Glu CH_2), 1.9 (m, 1H, Glu CH_2), 2.3 (m, 2H, Glu CH_2), 3.5 (s, 1H, epoxy), 3.9−4.1 (m, 4H, NCH_2COOH, epoxy, α-H), 4.1−4.3 (m, 3H, OCH_2CH_3 and *CH*-OH), 4.3−4.4 (m, 2H, α-H), 4.95 (m, 1H, NH), 5.02 (m, 2H, Cbz), 7.30−7.40 (m, 5H, Ph), 7.50 (d, 1H, NH), 7.78 (m, 1H, NH), 8.05 (m, 1H, NH), 11.00 (m, COOH). HRMS (FAB) Calcd for $C_{31}H_{44}N_5O_{14}$: 710.28848. Observed *m*/*z* 710.28450. Anal. Calcd for $C_{31}H_{43}N_5O_{14}\cdot 1.25H_2O$: C, 50.85; H, 6.22; N, 9.57. Found: C, 50.74; H, 6.27; N, 9.67.

(2*S*,3*S*)-3-((N^2-(*N*-Benzyloxycarbonylleucylglutamylthreonyl)-N^1-carboxymethylhydrazinocarbonyl)oxirane-2-carboxylic Acid Benzyl Ester (Cbz-Leu-Glu-Thr-AAsp-(2*S*,3*S*)-EP-COOCH$_2$Ph, 26d). ^{1}H NMR (DMSO-d_6): 0.7−0.9 (t, 6H, Leu CH_3), 0.9−1.1 (d, 3H, Thr CH_3), 1.3−1.5 (m, 2H, Leu CH_2), 1.6−1.7 (m, 1H, CH Leu), 1.7−1.8 (m, 1H, Glu CH_2), 1.8−2.0 (m, 1H, Glu CH_2), 2.2−2.35 (m, 2H, Glu CH_2), 3.6 (s, 1H, epoxy), 3.9−4.1 (m, 4H, NCH_2COOH, epoxy, α-H), 4.1−4.3 (m, 1H, *CH*-OH), 4.3−4.4 (m, 2H, α-H), 4.95 (m, 1H, NH), 4.95−5.05 and 5.1−5.3 (m, 4H, Cbz), 7.20−7.50 (m, 10H, Ph), 7.78 (m, 1H, NH), 8.05 (m, 1H, NH), 11.00 (m, COOH). HRMS (FAB) Calcd for $C_{36}H_{46}N_5O_{14}$: 772.30413. Observed *m*/*z* 772.30610. Anal. Calcd for $C_{36}H_{46}N_5O_{14}\cdot H_2O$: C, 54.75; H, 5.96; N, 8.87. Found: C, 54.78; H, 5.94; N, 8.87.

(2*S*,3*S*)-2-(2-Phenethylcarbamoyl)-3-((N^2-(*N*-benzyloxycarbonylleucylglutamylthreonyl)-N^1-carboxymethylhydrazinocarbonyl)oxirane (Cbz-Leu-Glu-Thr-AAsp-(2*S*,3*S*)-EP-CONHCH$_2$CH$_2$Ph, 26h). ^{1}H NMR (DMSO-d_6): 0.83 (t, 6H, Leu CH_3), 1.00 (m, 3H, Thr CH_3), 1.40 (m, 2H, Leu CH_2), 1.60 (m, 1H, Leu CH), 1.75 and 1.90 (d of m, 2H, Glu CH_2), 2.23 (m, 2H, Glu CH_2), 2.71 (t, 2H, CH_2Ph), 3.32 (s, 2H, AAsp CH_2), 3.43 and 3.52 (d, 2H, epoxy), 3.92 (b, 2H, α-H), 4.02 (b, 1H, α-H), 4.19 (m, 1H, Thr CH), 4.34 (m, 2H, NCH_2), 4.99 (s, 2H, Z), 7.15−7.35 (m, 10H, Ph), 7.40 (d, 1H, NH), 7.78 (b, 1H, NH), 8.00 (b, 1H, NH), 8.15 (s, 1H, NH), 8.20 (b, 1H, NH), 10.80 (b, 2H. COOH). HRMS (FAB) Calcd for $C_{37}H_{49}N_6O_{13}$: 785.3358. Observed *m*/*z* 785.3400. Anal. Calcd for $C_{37}H_{48}N_6O_{13}\cdot H_2O$: C, 55.35; H, 6.28; N, 10.47. Found: C, 54.96; H, 6.13; N, 10.21.

(2*S*,3*S*)-2-(1-Benzylcarbamoylethylcarbamoyl)-3-((N^2-(*N*-benzyloxycarbonylleucylglutamylthreonyl)-N^1-carboxymethylhydrazinocarbonyl)oxirane (Cbz-Leu-Glu-Thr-AAsp-(2*S*,3*S*)-EP-CO-Ala-NHBzl, 26j). ^{1}H NMR (DMSO-d_6): 0.83 (t, 6H, Leu CH_3), 0.99 (d, 3H, Thr CH_3), 1.25 (d, 3H, Ala CH_3), 1.39 (m, 2H, Leu CH_2), 1.60 (m, 1H, Leu CH), 1.75 and 1.90 (d of m, 2H, Glu CH_2), 2.24 (m, 2H, Glu CH_2), 3.32 (s, 2H, AAsp CH_2), 3.56 and 3.64 (d, 2H, epoxy), 3.95 (b, 1H, α-H), 4.02 (b, 2H, α-H), 4.19 (m, 1H, α-H), 4.26 (d, 1H, Thr CH), 4.35 (m, 2H, CH_2Ph), 4.99 (s, 2H, Z), 7.15−7.35 (m, 11H, Ph and NH), 7.40 (d, 1H, NH), 7.75 (b, 1H, NH), 8.05 (d, 1H, NH), 8.45 (m, 2H, NH), 10.85 (b, 2H. COOH). HRMS (FAB) Calcd for $C_{39}H_{52}N_7O_{14}$: 842.3572. Observed *m*/*z* 842.3336. Anal. Calcd for $C_{39}H_{51}N_7O_{14}\cdot H_2O$: C, 54.48; H, 6.21; N, 11.40. Found: C, 53.83; H, 5.96; N, 11.10.

(2*S*,3*S*)-3-((N^2-(*N*-Benzyloxycarbonylisoleucylglutamylthreonyl)-N^1-carboxymethylhydrazinocarbonyl)oxirane-2-carboxylic Acid Ethyl Ester (Cbz-Ile-Glu-Thr-AAsp-(2*S*,3*S*)-EP-COOEt, 27c). ^{1}H NMR (DMSO-d_6): 0.8 (m, 6H, Ile CH_3), 1−1.2 (m, 4H, Thr CH_3 and Ile CH_2), 1.2 (t, 3H, OCH_2CH_3), 1.4 (m, 1H, Ile CH_2), 1.6−1.8 (m, 2H, CH Ile and Glu CH_2), 1.8−1.9 (m, 1H, Glu CH_2), 2.1−2.3 (m, 2H, Glu CH_2), 3.5 (d, 1H, epoxy), 3.85−4.05 (m, 3H, NCH_2COOH and *CH*-OH), 4.1 (d, 1H, epoxy), 4.1−4.3 (m, 3H, OCH_2CH_3 and α-H), 4.3−4.45 (m, 2H, α-H), 4.6 (m, 1H, NH), 4.9 (m, 1H, NH), 5.05 (m, 2H, Cbz), 7.30−7.40 (m, 5H, Ph), 7.80 (m, 1H, NH), 8.1 (m, 1H, NH), 11.00 (m, COOH). HRMS (FAB) Calcd for $C_{31}H_{44}N_5O_{14}$: 710.28848. Observed *m*/*z* 710.29205. Anal. Calcd for $C_{31}H_{43}N_5O_{14}\cdot 2H_2O$: C, 49.93; H, 6.31; N, 9.40. Found: C, 49.71; H, 6.12; N, 9.42.

(2*S*,3*S*)-3-((N^2-(*N*-Benzyloxycarbonylisoleucylglutamylthreonyl)-N^1-carboxymethylhydrazinocarbonyl)oxirane-2-carboxylic Acid Benzyl Ester (Cbz-Ile-Glu-Thr-AAsp-(2*S*,3*S*)-EP-COOCH$_2$Ph, 27d). ^{1}H NMR (DMSO-d_6): 0.8 (m, 6H, Ile CH_3), 0.9−1.2 (m, 4H, Thr CH_3 and Ile CH_2), 1.3−1.5 (m, 1H, Ile CH_2), 1.6−1.8 (m, 2H, CH Ile and Glu CH_2), 1.8−2.0 (m, 1H, Glu CH_2), 2.2−2.3 (m, 2H, Glu CH_2), 3.55 (d, 1H, epoxy), 3.9−4.0 (m, 3H, NCH_2COOH and *CH*-OH), 4.05 (d, 1H, epoxy), 4.2 (m, 1H, α-H), 4.4 (m, 2H, α-H), 4.95 (m, 1H, NH), 5−5.05 and 5.1−5.2 (m, 4H, Cbz), 7.20−7.40 (m, 10H, Ph), 7.80 (m, 1H, NH), 8.1 (m, 1H, NH), 11.00 (m, COOH). HRMS (FAB) Calcd for $C_{36}H_{46}N_5O_{14}$: 772.30413. Observed *m*/*z* 772.30898. Anal. Calcd for $C_{36}H_{45}N_5O_{14}\cdot H_2O$: C, 54.75; H, 5.96; N, 8.87. Found: C, 54.97; H, 5.80; N, 8.91.

(2*R*,3*R*)-3-((N^2-(*N*-Benzyloxycarbonylisoleucylglutamylthreonyl)-N^1-carboxymethylhydrazinocarbonyl)oxirane-2-carboxylic Acid Benzyl Ester (Cbz-Ile-Glu-Thr-AAsp-(2*R*,3*R*)-EP-COOCH$_2$Ph, 27d). ^{1}H NMR (DMSO-d_6): 0.7−0.9 (m, 6H, Ile CH_3), 0.9−1.2 (m, 4H, Thr CH_3 and Ile CH_2), 1.4 (m, 1H, Ile CH_2), 1.6−1.8 (m, 2H, CH Ile and Glu CH_2), 1.8−2.0 (m, 1H, Glu CH_2), 2.2−2.35 (m, 2H, Glu CH_2), 3.55−3.6 (d, 1H, epoxy), 3.8−4.0 (m, 3H, NCH_2COOH and *CH*-OH), 4.05 (d, 1H, epoxy), 4.15 (m, 1H, α-H), 4.3−4.5 (m, 2H, α-H), 4.9−5.1 and 5.1−5.3 (m, 4H, Cbz), 7.20−7.50 (m, 10H, Ph), 7.7−7.9 (m, 1H, NH), 8−8.15 (m, 1H, NH), 11.00 (m, COOH). HRMS (FAB) Calcd for $C_{36}H_{46}N_5O_{14}$: 772.30413. Observed *m*/*z* 772.30837. Anal. Calcd for $C_{36}H_{45}N_5O_{14}\cdot H_2O$: C, 54.75; H, 5.96; N, 8.87. Found: C, 54.51; H, 5.82; N, 8.82.

(2*R*,3*R*)-2-(2-Benzylcarbamoyl)-3-((N^2-(*N*-benzyloxycarbonylisoleucylglutamylthreonyl)-N^1-carboxymethylhydrazinocarbonyl)oxirane (Cbz-Ile-Glu-Thr-AAsp-(2*R*,3*R*)-EP-CONHCH$_2$Ph, 27g). ^{1}H NMR (DMSO-d_6): 0.7−0.9 (m, 6H, Ile CH_3), 0.9−1.15 (m, 4H, Thr CH_3 and Ile CH_2), 1.4 (m, 1H, Ile CH_2), 1.65−1.8 (m, 2H, CH Ile and Glu CH_2), 1.8−2.0 (m, 1H, Glu CH_2), 2.2−2.3 (m, 2H, Glu CH_2), 3.5 (d, 1H, epoxy), 3.8−4.0 (m, 3H, NCH_2COOH and *CH*-OH), 4.03 (d, 1H, epoxy), 4.1−4.2 (m, 1H, α-H), 4.3−4.4 (m, 2H, $NHCH_2Ph$), 4.4 (m, 2H, α-H), 4.9−5.1 (m, 2H, Cbz), 7.15−7.40 (m, 10H, Ph), 7.85 (m, 1H, NH), 8.05 (m, 1H, NH), 11.00 (m, COOH). HRMS (FAB) Calcd for $C_{36}H_{47}N_6O_{13}$: 771.32011. Observed *m*/*z* 771.33322. Anal. Calcd for $C_{36}H_{46}N_6O_{13}\cdot 1.25H_2O$: C, 54.51; H, 6.12; N, 10.60. Found: C, 54.30; H, 6.04; N, 10.36.

(2*R*,3*R*)-3-((N^2-(*N*-Benzyloxycarbonylisoleucylglutamylthreonyl)-N^1-carboxymethylhydrazinocarbonyl)oxirane-2-carboxylic Acid Ethyl Ester (Cbz-Ile-Glu-Thr-AAsp-(2*R*,3*R*)-EP-COOEt, 27c). ^{1}H NMR (DMSO-d_6): 0.8 (m, 6H, Ile CH_3), 1.0 (d, 3H, Thr CH_3), 1−1.2 and 1.4 (m, 2H, Ile CH_2), 1.2 (t, 3H, OCH_2CH_3), 1.65−1.8 (m, 2H, CH Ile and Glu CH_2), 1.8−2.0 (m, 1H, Glu CH_2), 2.1−2.3 (m, 2H, Glu CH_2), 3.5 (d, 1H, epoxy), 3.8−4.0 (m, 3H, NCH_2COOH and *CH*-OH), 4.05 (d, 1H, epoxy), 4.1−4.23 (m, 3H, OCH_2CH_3 and α-H), 4.3−4.45 (m, 2H, α-H), 5.05 (m, 2H, Cbz), 7.30−7.40 (m, 5H, Ph), 7.80 (m, 1H, NH), 8.1 (m, 1H, NH), 11.00 (m, COOH). HRMS (FAB) Calcd for $C_{31}H_{44}N_5O_{14}$: 710.28848. Observed *m*/*z* 710.28779. Anal. Calcd for $C_{31}H_{43}N_5O_{14}\cdot 1H_2O$: C, 51.17; H, 6.19; N, 9.63. Found: C, 51.07; H, 6.10; N, 9.55.

(2*S*,3*S*)-2-(1-Benzylcarbamoylethylcarbamoyl)-3-((N^2-(*N*-benzyloxycarbonylisoleucylglutamylthreonyl)-N^1-carboxymethylhydrazinocarbonyl)oxirane (Cbz-Ile-Glu-

Thr-AAsp-(2*S*,3*S*)-EP-CO-Ala-NHBzl, 27j). ^{1}H NMR (DMSO-d_6): 0.79 (m, 6H, Ile CH_3), 1.01 (d, 3H, Thr CH_3), 1.10 (m, 2H, Ile CH_2), 1.25 (d, 3H, Ala CH_3), 1.39 (m, 1H, Ile CH), 1.70 and 1.90 (d of m, 2H, Glu CH_2), 2.24 (m, 2H, Glu CH_2), 3.32 (s, 2H, AAsp CH_2), 3.57 (s, 2H, epoxy), 3.88 (t, 1H, α-H), 3.95 (b, 2H, α-H), 4.20 (m, 1H, α-H), 4.28 (d, 1H, Thr CH), 4.36 (m, 2H, CH_2Ph), 4.99 (s, 2H, Z), 7.15−7.35 (m, 11H, Ph and NH), 7.40 (d, 1H, NH), 7.75 (b, 1H, NH), 8.05 (d, 1H, NH), 8.45 (m, 3H, NH), 10.85 (b, 2H, COOH). HRMS (FAB) Calcd for $C_{39}H_{52}N_7O_{14}$: 842.35722. Observed *m*/*z* 842.36598. Anal. Calcd for $C_{39}H_{51}N_7O_{14}\cdot 2H_2O$: C, 53.42; H, 6.31; N, 11.18. Found: C, 53.06; H, 6.25; N, 10.97.

Enzyme Assays. Progress Curve Method. For the progress curve method, the rate of substrate hydrolysis in the presence of inhibitor was monitored for 20 min. The progressive inhibition curve may be described by the equation

$$[P] = [P]_\infty(1 - e^{-A[I]t})$$

where [P] and $[P]_\infty$ are the product concentrations at t and $t = \infty$, respectively, and A is the apparent rate constant for the formation of the inhibited enzyme. The apparent rate constants (A) were determined from the slopes of plots of ln($[P]_\infty$ - [P]) versus time t in seconds as described by Tian and Tsou[66] by using the equation

$$A = \text{slope}/\{(-0.43)[I]\}$$

Assuming competitive and irreversible inhibition, the apparent rate constant was then converted to the second-order rate constant (k_2) using the equation below:

$$k_2 = A(1 + [S]/K_M)$$

Caspase-1. Caspase-1 and substrate Ac-YVAD-AMC (K_M = 14 μM) were purchased from Biomol Research Laboratories Inc, Plymouth Meeting, PA. Assays using the fluorogenic substrate Ac-YVAD-AMC (λ_{ex} = 360 nm, λ_{em} = 465 nm) were carried out on a Tecan Spectra Fluor microplate reader at 30 °C. Inhibition rates were determined by the progress curve method. The standard 140 μL reaction was started by adding 17 μL of enzyme (173 nM stock, final concentration 21 nM) to a mixture of 5 μL of substrate (666 μM stock, final concentration 23.8 μM) and 5 μL of inhibitor in DMSO (final concentrations ranging from 1.78×10^{-4} M to 2.14×10^{-8} M) in 113 μL buffer (100 mM HEPES, 0.5 mM EDTA, glycerol 20% (v/v), and 5 mM DTT, at pH 7.5). To make the 666 μM substrate stock, 13.3 μL of 10 mM substrate in DMSO was combined with 186.7 μL buffer. Caspase-1 (173 nM stock) was preactivated for 10 min at 30 °C in the presence of 5 mM DTT by combining 5 μL of enzyme stock (4.3 μM) with 120 μL of buffer. Inhibition experiments were repeated in triplicate and standard deviations determined. The k_2 values are 2.699-fold higher than the apparent rate because of the 23.8 μM final substrate concentration and K_M = 14 μM.

Caspases-3, -6, and -8. Caspases-3, -6, and -8 were expressed in *E. coli* and purified according to methods previously described by Stennicke and Salvesen.[67] Assays using the fluorogenic substrate Ac-DEVD-AFC (λ_{ex} = 405 nm, λ_{em} = 535 nm) or Ac-DEVD-AMC (λ_{ex} = 360 nm, λ_{em} = 465 nm) were carried out on a Tecan Spectra Fluor microplate reader at 37 °C. Inhibition rates were determined by the progress curve method. For caspases-3, -6, and -8, the standard 100 μL reaction was started by adding 50 μL of enzyme stock to a mixture of 5 μL of substrate (2 mM stock, 100 μM final concentration) and 5 μL of inhibitor in DMSO (final concentration varied from 2.5×10^{-4} M to 3×10^{-8} M) in 40 μL of buffer (50 mM HEPES, 100 mM NaCl, 0.1% (w/v) CHAPS, sucrose 10% (w/v), and 10 mM DTT, at pH 7.4). The final concentration of caspase-3, caspase-6, and caspase-8 in the wells was 1 nM, 10 nM, and 10 nM, respectively. To make the 2 mM substrate stock for caspases-3, -6, and -8, 50 μL of 10 mM substrate in DMSO was combined with 200 μL of buffer. Caspase-3 (2 nM stock) was preactivated for 10 min at 37 °C in the presence of 10 mM DTT by combining 1 μL of enzyme stock (10 μM) with 4999 μL of buffer. Caspase-6 (20 nM stock) was preactivated for 10 min at 37 °C in the presence of 10 mM DTT by combining 5 μL of enzyme stock (3 μM) with 745 μL of buffer. Caspase-8 (20 nM stock) was preactivated for 10 min at 37 °C in the presence of 10 mM DTT by combining 1 μL of enzyme stock (22 μM) with 1099 μL of buffer. Inhibition experiments were repeated in triplicate and standard deviations determined. The K_M value for Ac-DEVD-AFC or Ac-DEVD-AMC with caspase-3 is K_M = 9.7 μM. The K_M value for Ac-DEVD-AFC with caspase-6 is K_M = 350 μM and with caspase-8 is K_M = 18 μM. The K_M value for Ac-DEVD-AMC with caspase-6 is K_M = 236.35 μM and with caspase-8 is K_M = 6.79 μM.

For caspase-3, the k_2 values are 11.3-fold higher than the apparent rate because of the 100 μM final substrate concentration and K_M = 9.7 μM. For caspase-6 using Ac-DEVD-AMC, the k_2 values are 1.42-fold higher than the apparent rate because of the 100 μM final substrate concentration and K_M = 236.35 μM. For caspase-6 using Ac-DEVD-AFC, the k_2 values are 1.28-fold higher than the apparent rate because of the 100 μM final substrate concentration and K_M = 350 μM. For caspase-8 using Ac-DEVD-AMC, the k_2 values are 15.73-fold higher than the apparent rate because of the 100 μM final substrate concentration and K_M = 6.79 μM. For caspase-8 using Ac-DEVD-AFC, the k_2 values are 6.55-fold higher than the apparent rate because of the 100 μM final substrate concentration and K_M = 18 μM.

Papain and Cathepsin B. The incubation method was used to measure the irreversible inhibition of papain and cathepsin B. With cathepsin B, 30 μL of a stock inhibitor solution was added to 300 μL of 0.1 M potassium phosphate buffer containing 1.25 mM EDTA, 0.01% Brij 35 at pH 6.0, followed by the addition of 30 μL of a freshly prepared cathepsin B solution (approximate concentration 6.98×10^{-3} μg/μL) in the same potassium phosphate buffer containing 1 mM DTT (freshly prepared). Aliquots (50 μL) from the inhibition mixture were withdrawn at various time intervals and added to 200 μL of a 0.1 M potassium phosphate buffer containing 1.25 mM EDTA, 0.01% Brij 35 at pH 6.0, and the substrate Cbz-Arg-Arg-AMC (499 μM). The release of 7-amino-4-methylcoumarin was monitored (λ_{ex} = 360 nm, λ_{em} = 465 nm) using a Tecan Spectra Fluor microplate reader. Pseudo first-order inactivation rate constants were obtained from plots of ln v_t/v_o versus time.

The incubation method was also used for papain. The inhibition incubation buffer for papain was 50 mM HEPES buffer at pH 7.5, containing 2.5 mM DTT and 2.5 mM EDTA. The assay used the substrate Cbz-Phe-Arg-*p*NA (53.7 μM) in the same buffer. The approximate concentration of papain added to the incubation buffer was 0.29 mg/mL. The release of *p*-nitroanilide was monitored at 405 nm with a Molecular Devices Thermomax microplate reader.

Chymotrypsin. The incubation method was also used for the α-chymotrypsin assays. The buffer used was 0.1 M HEPES buffer at pH 7.5, containing 0.5 M NaCl. The assay used the substrate Suc-Ala-Ala-Pro-Phe-*p*NA (2 mM) in DMSO. An enzyme solution (20 μM) was prepared in 1 mM HCl. For the incubation assay, 10 μL of the enzyme solution (20 μM) was added to 0.5 mL buffer containing 50 μL of inhibitor (2.5 mM stock in DMSO). At various times, an aliquot of this incubation mixture (50 μL) was removed and added to a well containing buffer (0.2 mL) and substrate (10 μL, 2 mM). The release of *p*-nitroanilide was monitored at 405 nm with a Molecular Devices Thermomax microplate reader. Measurements were performed in duplicates and multiple inhibitor concentrations were used (2.5 mM, 1.25 mM stock). Pseudo first-order inactivation rate constants were obtained from plots of ln v_t/v_o versus time.

Calpain I. The incubation method was used to measure the rate of irreversible inhibition of calpain I from porcine erythrocytes. The buffer used was 50 mM HEPES containing 10 mM cysteine, 5 mM $CaCl_2$ at pH 7.5. For the incubation, 30 μL of a stock inhibitor solution (2.5 mM in DMSO) was added to 300 μL of the buffer, followed by the addition of 30 μL of

enzyme (1 mg/mL). Aliquots (50 μL) from the incubation mixture were withdrawn at various time intervals and added to a 200 μL buffer and substrate solution ([S] = 1.6 mM). The substrate was Suc-Leu-Tyr-AMC (stock solution was 0.2 M in DMSO). The release of 7-amino-4-methylcoumarin was monitored (λ_{ex} = 360 nm, λ_{em} = 465 nm) using a Tecan Spectra Fluor microplate reader. Measurements were performed in duplicates and multiple inhibitor concentrations were used (2.5 mM, 1.25 mM stock). Pseudo first-order inactivation rate constants were obtained from plots of ln v_t/v_o versus time.

Cathepsins F, K, L, and S. Dieter Brömme at the Mount Sinai School of Medicine, NY, tested Suc-Np2-ALeu-*trans*-EP-COOEt with cathepsins F, K, L, and S. The measurements were done at room temperature in 100 mM acetate buffer, pH 5.5 containing 2.5 mM DTT and 2.5 mM EDTA. The inhibitor was dissolved in DMF. Since the compound was not very potent, a range of inhibition (μM) was obtained.

Granzyme B. Granzyme B assays were performed by Zhenguo Wang and Dr. Froelich at Northwestern. In preliminary assays, inhibitors (10 μL, 5 mM stock solution in DMSO) were incubated with granzyme B (90 μL) for 1 h. After the incubation period, to these wells was added Cbz-IETD-*p*NA (100 μL) and the rate was monitored at 405 nm. A more detailed kinetic study was performed as some compounds showed inhibition. The inhibitor (60 μL, 5 mM) was incubated in PBS buffer (600 μL, phosphate-buffered saline pH 7.3, containing 1.4 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, and 4.3 mM $Na_2HPO_4 \cdot 7H_2O$) with granzyme B (1.2 μM, or DMSO for control) for different amounts of time. At the various times, an aliquot of incubation mixture (50 μL) was removed and added to a well containing substrate (Cbz-IETD-*p*NA, 100 μL, 200 μM) and buffer (50 μL). The release of *p*-nitroanilide was monitored at 405 nm. Pseudo first-order inactivation rate constants were obtained from plots of ln v_t/v_o versus time. This method did not allow incubation times of less than 15 min so a different procedure was attempted. PBS buffer (47.9 μL) and inhibitor (2.1 μL, 5 mM) were added to the wells. At the following times, 0, 8, 12, 14, 15, and 15.5 min, the enzyme granzyme B (50 μL, 0.1 μM) was added to all the wells. The substrate Cbz-IETD-*p*NA (100 μL, 0.2 mM) was then added to the wells, and the release of *p*-nitroanilide was monitored at 405 nm. One well was the control using DMSO instead of inhibitor, and one well was the blank consisting of just PBS buffer and DMSO.

Aspartyl Proteases. Various inhibitors were tested with the following aspartyl proteases: porcine pepsin, human cathepsin D, plasmepsin 2 (from *P. falciparum*), the secreted aspartic proteinase 2 (SAP-2) from *Candida albicans*, and the HIV-1 protease by Bret Beyer, Jose Clemente, and Dr. Dunn at the University of Florida. The inhibitors were preincubated for 30 min with HIV-1 protease and 20 min with the other enzymes. Substrate was then added, and the substrate hydrolysis was monitored. The following buffers were used: 0.05 M sodium acetate, 0.15 M NaCl, and 1 mM DTT for HIV-1 protease; 0.1 M sodium formate at pH 4.5 for plasmepsin-2 and SAP-2; and 0.1 M sodium formate at pH 3.5 for pepsin and cathepsin D.

Legumain. The fluorometric assay for pig kidney legumain performed by Wendy Carter and Dr. Barrett at the Babraham Institute, UK, has been described previously.[68] Legumain, purified from pig kidney tissue, was assayed at 30 °C in buffer (39.5 mM citric acid, 121 mM Na_2HPO_4 at pH 5.8 containing 1 mM EDTA, 1 mM TCEP, tris-(2-carboxyethyl)phosphine, and 0.01% CHAPS) with Cbz-Ala-Ala-Asn-AMC as the substrate (10 μM final concentration; AMC, 7-amino-4-methylcoumarin). The assays were carried out in a Perkin-Elmer LS 3B fluorescence spectrometer (λ_{ex} = 360 nm, λ_{em} = 460 nm) under the control of an IBM-compatible computer running the FLUSYS software. The rate of substrate hydrolysis in the presence of inhibitor (100 μM to 100 nM) was monitored, and rate constants for irreversible inactivation were found by nonlinear regression analysis of the pseudo first-order curves using the FLUSYS software, giving k_{obs}.

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