

DESIGN, SYNTHESIS, AND EVALUATION OF NOVEL IRREVERSIBLE
INHIBITORS FOR CASPASES

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Design, Synthesis, and Evaluation of Novel Irreversible Inhibitors for Caspases

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DEDICATION

To my mother Kezban Doğan,

To my father Özcan Doğan,

To my brother Gökhan Doğan,

and

To my husband Eylem Ekici

Cok Sevgili Anneciğim Kezban Doğan ve Babaciğim Özcan Doğan'a

Birtanecik Kardeşim Gökhan Doğan'a

ve

Canım Eşim Eylem Ekici'ye

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LIST OF ABBREVIATIONS

AA	amino acid residue
AAsn	aza-asparagine
AAsp	aza-asparagine
A β	amyloid beta peptide
Ac	acetyl
AcOH	acetic acid
AFC	7-amino-4-trifluoromethylcoumarin
Agm	agmantine (1-amino-4-guanidino-butane)
Ala	alanine
ALS	amyotropic lateral sclerosis
AMC	7-amino-4-methylcoumarin
Apaf-1	apoptosis activating factor-1
Arg	arginine
Asn	asparagine
Asp	aspartic acid
Boc	<i>t</i> -butoxycarbonyl
Brij	polyoxyethylenelaurylether
Bzl	benzyl, CH ₂ Ph
CA-074	<i>N</i> -(L-3- <i>trans</i> -propylcarbamoyloxiran-2-carbonyl)-L-isoleucyl-L-proline
CaSF	calcium-activated sarcoplasmic factor
Cbz	benzyloxycarbonyl

CDCl ₃	deuterated chloroform
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CMK	chloromethyl ketone
Cys	cysteine
ced-3	<i>Caenorhabditise elegans</i> cell death gene
DED	death effector domains
DIC	1,3-diisopropylcarbodiimide
DMAP	4-dimethylaminopyridine
DMF	<i>N,N</i> -dimethylformamide
DMSO	dimethylsulfoxide
DMSO-d ₆	dimethylsulfoxide-d ₆ deuterated
DTT	dithiothreitol
E-64	<i>N</i> -(L-3- <i>trans</i> -carboxyoxiran-2-carbonyl)-L-leucyl-4-guanidinobutanamide
E-64c	<i>N</i> -(L-3- <i>trans</i> -carboxyoxiran-2-carbonyl)-L-leucyl-3-methylbutanamide
EDC	1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride
EDTA	ethylenediaminetetraacetic acid
EP	epoxide, C ₂ H ₂ O
Eps	epoxysuccinyl
ESI-MS	electrospray ionization mass spectrometry
Et ₃ N	triethylamine
EtOAc	ethyl acetate
EtOH	ethanol
FAB	fast atomic bombardment

FMK	fluoromethyl ketone
Fmoc	9-fluorenylmethoxycarbonyl
Gln	glutamine
Glu	glutamic acid
Gly	glycine
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
His	histidine
HOBt	<i>N</i> -hydroxybenzotriazole
HRMS	high resolution mass spectrometry
¹ H NMR	proton nuclear magnetic resonance
<i>i</i> BCF	isobutyl chloroformate
<i>i</i> Bu	isobutyl
ICE	interleukin converting enzyme, caspase-1
Ile	isoleucine
k	rate constant
K _i	inhibition constant
K _M	Michaelis constant
Leu	leucine
Lys	lysine
<i>m/z</i>	mass to charge ratio
M	molarity (moles/liter)
MeOH	methanol
Met	methionine

MHC	major histocompatibility complex
min	minutes
MPP ⁺	1-methyl-4-phenylpyridinium
MS	mass spectrometry
NaOEt	sodium ethoxide
ND	not determined
NI	no inhibition
NMM	<i>N</i> -methyldmorpholine
NMR	nuclear magnetic resonance
OBzl	benzyloxy
6-OHDA	6-hydroxydopamine
Orn	ornothine
PhPr	PhCH ₂ CH ₂ CO-
<i>p</i> NA	<i>p</i> -nitroanilide
Pro	proline
PS-CSL	positional-scanning combinatorial substrate library
RFU	relative fluorescent unit
RP-HPLC	reverse phase-high pressure liquid chromatography
s	second
Ser	serine
SMBA	spinal bulbar muscular atrophy
SOD	superoxide dismutase
Suc	succinyl

<i>t</i> Bu	<i>t</i> -butyl
<i>t</i> BuOOH	<i>t</i> -butylhydroperoxide
<i>t</i> BuLi	<i>t</i> -butyllithium
TFA	trifluoroacetic acid
THF	tetrahydrofuran
Thr	threonine
TIS	triisopropylsilane
TLC	thin layer chromatography
TNF	tumor necrosis factor
Trp	tryptophan
Tyr	tyrosine
Val	valine

SUMMARY

The goal of this research was the design, synthesis, and kinetic evaluation of new classes of inhibitors for cysteine proteases, particularly caspases. We have designed irreversible inhibitors, which resulted in new classes of inhibitors that are highly potent and specific for caspases.

The first project involved the design and synthesis of epoxysuccinyl peptides with caspase specific sequence. This design was based on the ideal substrate sequences for caspases, where the P1 Asp residue would provide selectivity for caspases and the epoxysuccinyl moiety would cause irreversible inhibition of the enzyme. However, this particular design turned out to be clan CA specific and our inhibitors did not show activity against caspases.

In an effort to synthesize potent and specific inhibitors for caspases, we focused our attention to another inhibitor design involving the epoxysuccinyl warhead, which we termed aza-peptide epoxides. Aza-peptide epoxides were designed based on an extended substrate structure of the target enzyme with the placement of the carbonyl group of the epoxide moiety in a location identical to that of the carbonyl of the scissile peptide bond. Replacement of the α -carbon of the amino acid residue at P1 with a nitrogen resulted in the formation of an aza-peptide. Previous studies in our laboratory focusing on the tetrapeptidyl aza-peptide epoxides resulted in potent and selective inhibition of caspases. Therefore, we have synthesized dipeptidyl analogs of aza-peptide epoxides with a P1 aza-Asp residue in the hopes for obtaining broad-spectrum caspase inhibitors. However, we observed that these inhibitors were not as potent as we expected. Yet, the aza-peptide

epoxide design constituted the basis of another new class of inhibitors, which we termed the aza-peptide Michael acceptors.

We have successfully designed, synthesized, and evaluated aza-peptide Michael acceptors targeting caspases. We hypothesized that aza-peptide Michael acceptors would be more potent and selective inhibitors for caspases than other available caspase inhibitors such as aldehydes and halomethyl ketones. Aza-peptide Michael acceptor design consists of the best substrate sequence for the target caspase and the Michael acceptor chemical warhead, where the active site nucleophilic attack is expected to take place. As in the case with aza-peptide epoxides, replacement of the α -carbon of the amino acid residue at P1 with a nitrogen resulted in the formation of an aza-peptide, which allowed easy derivatization on the P' site of the inhibitors.

Aza-peptide Michael acceptors are a new class of irreversible protease inhibitors, which are the most potent and selective inhibitors for caspases yet reported. Second order rate constants are as high as $10^6 \text{ M}^{-1}\text{s}^{-1}$, and yet the selectivity among the caspase family is also conserved. The inhibitors designed specifically for caspase-3 are potent inhibitors of this enzyme, while other caspases such as caspase-6 and -8 are only weakly inhibited. Aza-peptide Michael acceptors are very specific for clan CD cysteine proteases. They do not show any cross reactivity with cysteine proteases from other clans such as clan CA enzymes papain, cathepsins, and calpains. Aza-peptide Michael acceptors are also very specific among the clan CD enzymes. Caspase specific aza-peptide Michael acceptors do not inhibit other clan CD enzymes such as legumain, gingipain K, and clostripain.

Aza-peptide Michael acceptors could lead to the development of potential drugs, where caspases are recognized as novel therapeutic targets for neurodegenerative diseases such as stroke, ALS, Huntington's, Alzheimer's, Parkinson's and MS. Aza-peptide Michael acceptors would also be invaluable in elucidating the roles of individual caspases in biochemical processes such as apoptosis.

CHAPTER 1

INTRODUCTION

Cysteine Proteases

Proteases (proteolytic enzymes, peptidases) form one of the largest and more important group of enzymes with estimated 550-650 members. Proteases selectively catalyze the hydrolysis of peptide bonds. Hydrolysis of a peptide bond is an energetically favorable reaction, but extremely slow.¹ Most proteases are either exopeptidases cleaving one or a few amino acids from the N- or C-terminus, or endopeptidases that act internally in polypeptide chains. The endopeptidases can be grouped into five major classes (or groups): aspartic, serine, cysteine, threonine, and metalloproteases. Proteases are involved in numerous important physiological processes including protein turnover, digestion, blood coagulation and wound healing, fertilization, cell differentiation and growth, cell signaling, the immune response, and apoptosis. Uncontrolled, unregulated or undesired proteolysis can lead to many diseases such as emphysema, stroke, viral infections, cancer, Alzheimer's disease, inflammation, and arthritis. Proteases have been grouped into evolutionary families and clans by Rawlings and Barrett.^{2,3} This effort led to the development of the MEROPS database (<http://www.merops.co.uk>), which now includes a frequently updated listing of all protease sequences.

Cysteine proteases, also known as thiol proteases have been identified in the biological systems of bacteria, protozoa, fungi, plant viruses, and mammals. A family of proteases is a group in which every member shows a statistically significant relationship in amino acid sequence to at least one other member of the family. Related families are

grouped into clans. Clan is the term used to describe a group of families the members of which have evolved from a single ancestral protein, but have diverged so far that we can no longer prove their relationship by comparison of the primary structures.² Most cysteine proteases are members of clan CA, also referred to as papain clan, which contains papain, cathepsins, and calpains (Figure 1).

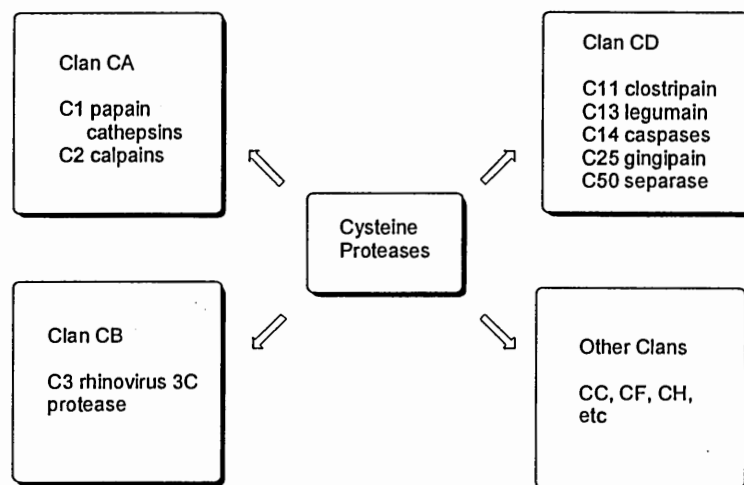


Figure 1. Representative Clans of Cysteine Proteases

Crystal structures of papain and several clan CA proteases have been determined. The catalytic residues have been identified as Cys, His, and Asn, occurring in that order in the sequence. Several important cysteine proteases such as caspases, legumain, gingipain, clostripain, and separase belong to a new but smaller clan of cysteine proteases, which is termed as clan CD. The tertiary structure shows a unique α/β fold unlike that of any other protein, with the catalytic residues in the order His, Cys in the sequence. Structural differences between clans and families of proteases of the same class should be useful for the design of specific inhibitor structures.

Subsite Nomenclature. The subsite nomenclature of Schechter & Berger is used to designate the individual amino acid residues and the corresponding enzyme subsites.⁴ Amino acid residues to left of the scissile bond are numbered P₁, P₂, etc., with numbering increasing in the direction of the N-terminal residue of the substrate or inhibitor (Figure 2). Residues to the right of the scissile bond are numbered P₁', P₂', etc. The corresponding complementary regions of the enzyme's active site are numbered S₁, S₂, S₁', etc.

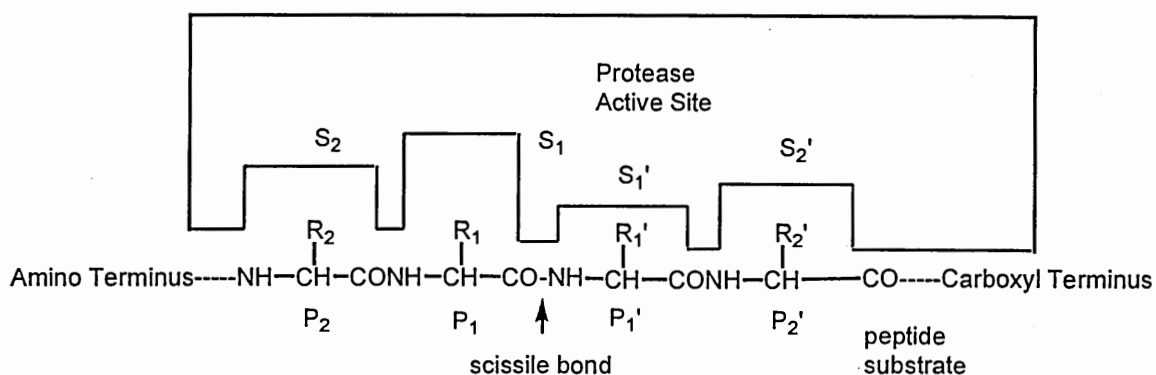


Figure 2. Subsite Nomenclature Used with Proteolytic Enzymes

Specificity. One of the most noticeable differences between clan CA and clan CD cysteine proteases is their substrate specificity. The primary specificities of papain, cathepsin B, and calpain are determined by the shape and electrostatic character of S₂, while the specificity of clan CD proteases is determined by S₁ (Figure 3). With caspases, the P₁ Asp residue interacts with the Arg 179 in S₁. Legumain is specific for a P₁ Asn residue, where gingipains R and K are specific for P₁ Arg or Lys residues, respectively. The primary specificity of most serine proteases is also determined by S₁. However, many proteases have extended substrate binding sites and require longer peptides or

inhibitors for effective binding. Caspases would be one example, where tetrapeptides are required for effective substrate hydrolysis or for inhibitor potency.

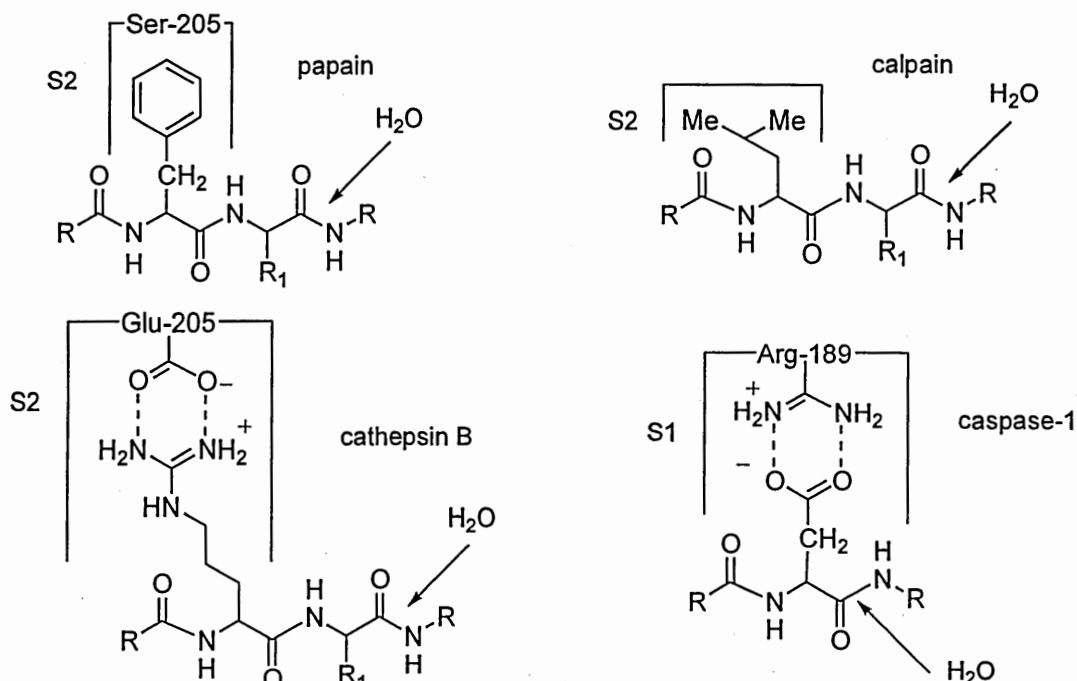


Figure 3. Cysteine Protease Binding Sites

Catalytic Mechanism. The catalytic residues of serine, cysteine, and threonine proteases' consists of an active site nucleophile and a basic residue, which can also function as a general acid in the catalytic mechanism. With cysteine proteases, the thiol group of the active site cysteine and the imidazole group of histidine are believed to exist as a thiolate/imidazolium ion pair.⁵ The thiolate ion is therefore highly nucleophilic and readily attacks the scissile amide bond (Figure 4a). The tetrahedral intermediate produced is stabilized by the oxyanion hole (Figure 4b). Via acid-assisted catalysis, the collapse of this intermediate yields the acyl enzyme and the N-terminal substrate fragment is released (Figure 4c). Finally, hydrolysis by water gives the C-terminal substrate fragment and the enzymes' active site is regenerated (Figure 4d).

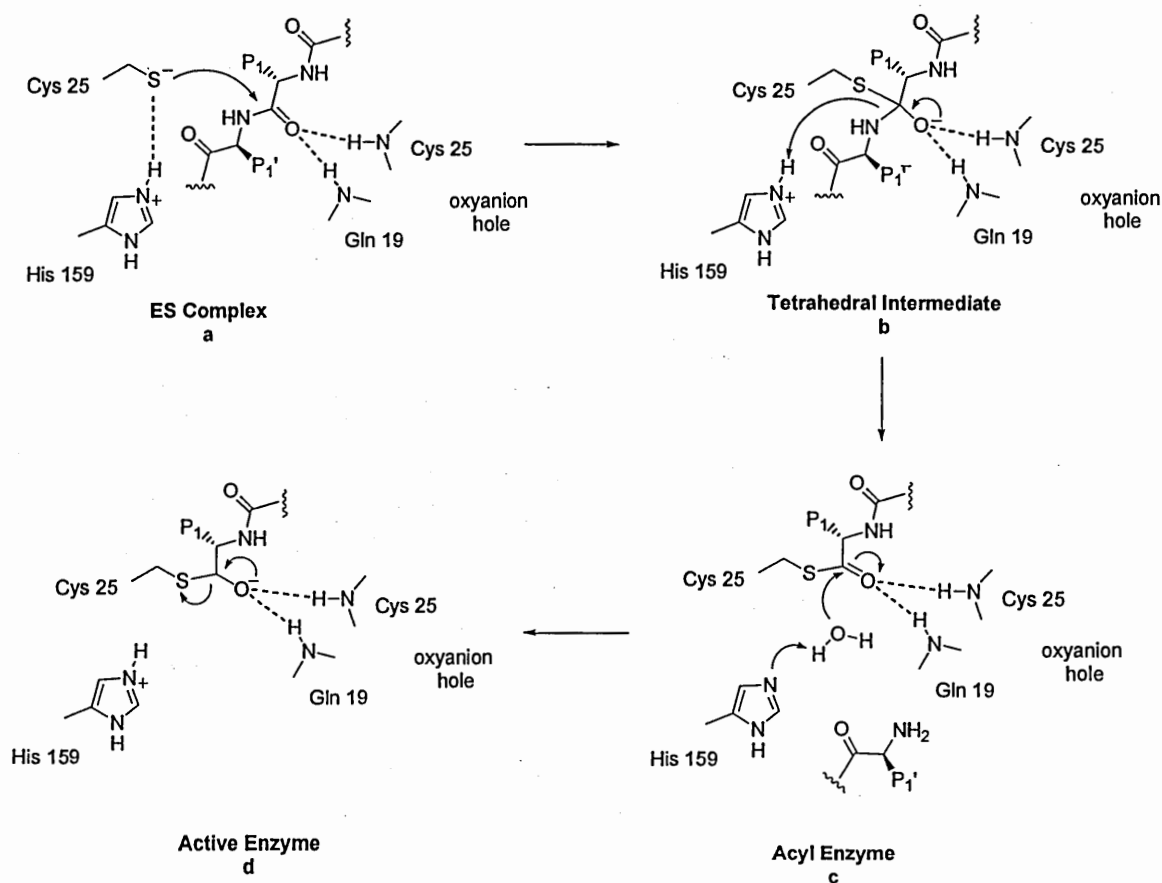


Figure 4. Catalytic Mechanism for Substrate Hydrolysis by Cysteine Proteases

The transition states of serine, cysteine, and threonine proteases all involve a tetrahedral intermediate, and an oxyanion hole. Oxyanion hole is a common term used to describe the stabilization of the oxyanion of the tetrahedral intermediate through hydrogen bonding interactions in the active site. The oxyanion hole of serine proteases is usually quite rigid and involves backbone peptide bond NH groups as hydrogen bond donors. For effective substrate hydrolysis the interaction with the oxyanion hole is essential. With cysteine proteases, the oxyanion hole seems to be more flexible. In papain clan (clan CA), in addition to the catalytic triad Cys-His-Asn, a fourth residue, a Gln preceding the catalytic Cys, participates in the catalytic activity and is believed to

help form the 'oxyanion hole'. In the clan CD enzyme caspase-1, the oxyanion hole is formed from the backbone residues and is very rigid. It resembles the oxyanion hole in serine proteases. These active site differences within the different families and clans have a considerable influence on the design of more selective inhibitor structures. One such example would be the case with the epoxysuccinate inhibitors. Papain clan enzymes are inhibited very potently by epoxysuccinate inhibitors, whereas clan CD enzymes such as caspases are inert to epoxysuccinates such as E-64.

Kinetics and Rate Constants

The rate of reaction of an irreversible inhibitor with a protease is typically measured using the incubation method. The enzyme is mixed with the inhibitor and allowed to incubate at a set of prescribed conditions. At various time intervals, an aliquot of this incubating inhibition mixture is removed, diluted into an assay solution containing a substrate for the protease, and assayed for residual enzymatic activity. The conditions in the assay mixture and the inhibition incubation mixture can be quite similar or quite different. The conditions in the inhibition incubation mixture control the rate of enzyme inhibition, while the conditions in the assay mixture determine the sensitivity of the assay for detection of residual enzyme activity.

Typically, inhibition kinetics are carried out using pseudo first order kinetics where the inhibitor concentration ⁶ is greater than 10-fold higher than the enzyme concentration [E]. A plot of $\ln a$ (activity) against time gives the observed rate of inactivation k_{obs} using the equation $\ln a = -k_{\text{obs}} t$. With very potent irreversible inhibitors, rates are often too fast to measure using pseudo first order kinetics and the inhibitor

concentration is decreased until the concentrations of enzyme and inhibitor are of the same magnitude and then a second order rate constant k_{2nd} can be determined using a second rate equation:

$$k_{2nd}t = (1/(i-e))\ln(e(i-x)/i(e-x)) \text{ where } e-x \text{ is the residual enzyme concentration.}$$

If the inhibitor is very potent and binds in the active site, it is also possible to slow the reaction by incubating the inhibitor with the enzyme in the presence of a substrate or a competitive inhibitor. Inhibition rates can then be determined using various kinetic equations.

A more convenient method for measurement of the inhibition rate constant in the presence of substrates was introduced by Tian and Tsou.⁷ This is referred to as the progress curve method and is suitable for measuring irreversible inhibition rates with fast inhibitors. The progress curve method also has the advantage that relatively few separate kinetic measurements, which consume valuable enzyme and substrate, are needed to measure inhibition rate constants. In this method, the inhibitor, substrate and enzyme are incubated together, and the rate of substrate hydrolysis is measured continuously. Both the inhibitor and the substrate are competing for the same active site, and the observed inhibition rate is decreased. The active enzyme concentration is dropping as it reacts with the inhibitor and a plot of substrate hydrolysis product vs time is hyperbolic as the amount of active enzyme is reduced. From this curve, the observed inhibition rate constant (k_{obs}) at a particular substrate concentration can be calculated. Measurement of k_{obs} at different substrate concentrations and extrapolation to zero substrate concentration, gives the apparent rate constant (k_{app}). This should be similar to k_{obs} determined by the incubation method if the inhibition rates are measured under pseudo first order

conditions. If there is not a large excess of inhibitor relative to the enzyme concentration, then a second rate inhibition constant (k_{2nd}) could be determined.

The inactivation of a protease by an active-site directed irreversible inhibitor usually proceeds by the rapid formation of a reversible enzyme-inhibitor complex ($E \cdot I$, Figure 5). In a slower chemical step, a covalent bond is formed with the enzyme to generate the enzyme-inhibitor adduct ($E-I$).

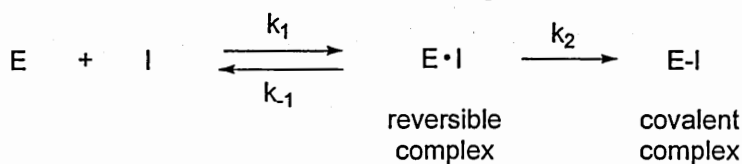


Figure 5. Irreversible Inhibition Kinetics

When $[I] > [E]$, the kinetics are described by the equation of Kitz and Wilson.⁸

$$k_{obs} = k_2 / (1 + K_i/[I])$$

This equation can also be written in a linearized form.

$$1/k_{obs} = K_i/k_2 [I] + 1/k_2$$

$$K_i = [E][I]/[E \cdot I] = k_1/k_{-1}$$

The K_i is the dissociation constant of the enzyme inhibitor complex and k_2 is the maximum (or limiting) inhibition rate if the enzyme is saturated with inhibitor. This is a first order rate constant. The ratio k_2/K_i is a second order inhibition rate, similar to k_{cat}/K_M , and is the most common used parameter to report inhibition data.

If a series of experiments are carried out at different inhibitor concentrations, it is possible to measure k_2 and K_i from a plot of $1/k_{obs}$ vs $1/[I]$. This is only possible if the

inhibitor is forming an E•I complex and the inhibitor concentration used are below the point at which E is saturated and data points are obtained with I concentration in the range of K_i . If $K_i > [I]$ or the enzyme is not forming an E•I complex, then this plot will go through the origin and $k_{obs}/[I] = k_2/K_i$ for an enzyme that forms E•I. However, if $K_i \approx [I]$, then this plot will yield k_2 , K_i , and k_2/K_i . Although the ratio k_2/K_i is a fairly common method to report inhibition data, in some cases the initial assumptions of irreversible inhibition kinetics may not be sufficient to interpret the kinetic data. The enzyme inactivation could occur at several stages during the reaction as summarized by Tsou.⁹ Progress curves for the reaction can then be obtained by determining the formation of enzymic product over time in the presence of various concentrations of substrate and inhibitor.

It is fairly common to report IC_{50} values for inhibitors of all types in medicinal chemistry journals. This represents the inhibitor concentration necessary to effect 50% inhibition of the enzyme under the conditions of the enzyme assay. With an irreversible inhibitor, the IC_{50} value clearly depends on the time during which the enzyme is incubated with the irreversible inhibitor. The longer the incubation time, the lower is the IC_{50} value. It is possible to make a rough estimate of the irreversible inhibition rate constant. If you assume that IC_{50} (in M) is approximately equal to the inhibitor concentration necessary to reduce the enzyme activity by 50% during the time of the incubation, then the assay time (t_{assay}) should be equal to the half-life $t_{1/2}$ for the pseudo first order inactivation rate.

$$k_{obs} = 0.0693/t_{1/2} = 0.693/t_{assay} ; k_{obs}/[I] = 0.693/[t_{assay} \times IC_{50} \text{ (in M)}]$$

Clan CA Enzymes

Papain. Papain is the most widely studied member of the cysteine proteases family of enzymes. It is first isolated from the tropical papaya fruit (*Carica papaya*) in 1879 by Wurtz & Bouchut.¹⁰ Early work on papain is responsible for major advances not only in the field of cysteine proteases but also in enzymology generally. Papain's three-dimensional structure was the first determined among the cysteine proteases (PDB code: 9PAP).¹¹ This enzyme is considered to be the archetype of cysteine proteases and accordingly, family C1 is referred to as the papain family of cysteine proteases.

Specificity. Overall, papain can be considered to have a fairly broad specificity. The S2 subsite of this enzyme is the primary determinant of specificity, having a preference for bulky nonpolar side chains. The S1 subsite is not as selective as the S2 subsite, but there is some preference for Arg and Lys at this position. Very little is known about the S' subsite specificity, which seems to be relatively broad.¹²

Biological Significance. Very little is known about the biology of papain. It is speculated that it plays a protective role, guarding the plant against attack by pests such as insects and fungi.

Cathepsin B. Cathepsin B is the first described member of what has become recognized as the large family of lysosomal cysteine proteases.¹³ Cathepsin B has been isolated from fungi and various mammalian tissues. The term "cathepsin" refers to intracellular proteases, mostly located in the lysosomes, which are active at acidic pH. Cathepsin B possesses both endopeptidase and dipeptidyl carboxypeptidase activities, the primary role being the dipeptidyl carboxypeptidase activity. The first complete cathepsin B protein sequence was published for the rat liver enzyme in 1983¹⁴ and the first three-

dimensional structure of cathepsin B was determined in 1991 from human liver cathepsin B (PDB code: 1HUC).¹⁵ As expected from sequence similarity, the peptide backbone of cathepsin B was essentially superimposable on to that of papain and other papain family members. The active site of cathepsin B greatly resembled papain's active site. However, the cathepsin B prime subsite region differed from that of papain dramatically. In cathepsin B, two His residues which are a part of a peptide segment with extra 20 residues, interact with the carboxy end of the substrates beyond S2' subsite. This segment is termed as the "occluding loop" and equips the enzyme to act as a dipeptidyl peptidase. Deletion of the loop by site-directed mutagenesis abolishes this activity.¹⁶

Specificity. As with the other members of the papain family, the primary determinant of specificity is the S2 subsite. Cathepsin B has a preference for large hydrophobic residues, although it will also tolerate an Arg side chain at this position due to the location of a Glu residue (Glu 245) in the S2 subsite.^{17,18}

Biological Significance. The lysosomal localization of cathepsin B suggests that it participates in the intracellular protein degradation system. It is implicated in disease states such as arthritis, cancer and acute pancreatitis.¹⁹ Findings from many groups have shown alternate locations of cathepsin B, suggesting various physiological and pathological roles. Extensive studies have demonstrated the relationship between increased cathepsin B levels and redistribution of the enzyme to human and animal tumors. A role for the enzyme in invasion and metastasis has been proposed.^{20,21} Cathepsin B is also involved in muscular dystrophy, gingivitis, bone resorption, and apoptosis.²²

Calpain. Calpain is an intracellular, Ca^{+2} -dependent cysteine protease that is ubiquitously distributed, and shows regulated activity at neutral pH. The occurrence of Ca^{+2} -dependent neutral protease activity in rat brain was first described by Guroff.²³ Calpain was then reidentified as a calcium-activated sarcoplasmic factor (CaSF) hydrolyzing Z-lines by the Goll group.²⁴ The name “calpain” originated from **calcium** ion dependent **papain**-like cysteine protease. To date, 14 members of calpain family have been identified.²⁵ In mammals, there are two major types of calpain, μ - and m-calpains. The old names, calpain I (corresponding to μ -calpain), and calpain II (corresponding to m-calpain) are no longer recommended.²⁶ As the names suggest, μ -calpain requires micromolar concentrations of Ca^{+2} , and m-calpain millimolar concentration of Ca^{+2} for activation *in vitro*. Each of these calpains consist of two subunits, the different, large (ca. 80 kDa) and the common, small (ca. 30 kDa) subunits. Calpain activity is regulated by the natural inhibitor calpastatin and Ca^{+2} concentrations. The three-dimensional structures of unactivated m-calpain (PDB code: 1 DF0)²⁷ and Ca^{+2} activated μ -calpain (PDB code: 1KXR)²⁸ reveal that calcium binding induces the changes in the active site necessary for catalytic activity. μ -Calpain requires cooperative binding of two Ca^{+2} atoms. It is also revealed that calpain has an unusual cysteine protease fold, which is associated with calcium binding domains through heterodimerization.

Specificity. Calpain has a preference for hydrophobic residues at P2 position such as Leu or Val and likes various amino acids such as Tyr, Arg, and Gly at P1.²⁹ The P2 residue seems to be more important than P1 residue for the determination of specificity.

Biological Significance. Various pathological conditions that may involve calpain and/or calpastatin have been reported, including cataract formation, Alzheimer's disease, cerebral and cardiac ischemia, inflammation and muscular dystrophy.³⁰ These conditions, characterized by altered Ca^{+2} -homeostasis resulting from some sort of cellular stress, appear to result in disrupted regulation and excessive activation of calpain. Consequently, calpain is a potentially important therapeutic target, since the development of specific inhibitors could be invaluable for the treatment of these diseases.

Clan CD Enzymes

Five protease families constitute the CD clan of cysteine proteases. These families include caspase (C14), legumain (C13), clostripain (C11), gingipain (C25), and separase (C50). These families have been grouped together based on amino acid sequence and catalytic dyad motif. The protein fold for members of clan CD is predicted to be similar to the fold determined for the caspases in family C14. Compared with other cysteine proteases of broader specificity such as clan CA, members of clan CD have a strict requirement for a specific side chain of the P1 amino acid residue.

Clostripain. Clostripain is a well known cysteine protease for its selective hydrolysis of arginyl bonds, although lysyl bonds are cleaved at a lower rate. It has been known for many years that culture filtrates of the anaerobic bacterium *Clostridium histolyticum* contain a cysteine-activated protease. Labouesse and Gros chromatographically purified the enzyme, which they designated clostripain, and observed that the protease hydrolyzes arginine-containing synthetic substrates much more rapidly than analogous compounds containing lysine.³¹ Clostripain depends on thiol and

calcium ions for activity and can also act as a transpeptidase, showing maximal activity in the pH range 7.6-9.0.³²

Gingipain R and Gingipain K. Gingipains are cysteine proteases which are produced by the well-established oral pathogen *Porphyromonas gingivalis*. The term gingipain was suggested by Chen and coworkers since the purified enzyme shared some properties with clostripain including a similar molecular mass, requirements of calcium for stability and narrow specificity at the P1 position. Neither gingipain R nor gingipain K has primary sequence homology with any other cysteine protease.^{33,34} However, the crystal structure of gingipain R revealed that there's some topological homology with those of caspase-1 and caspase-3.

Specificity. Gingipain R has a strict specificity for a P1 Arg residue.³⁵ In contrast, gingipain K cleaves exclusively after Lys residues in various peptides and *p*-nitroanilide substrates. The pH optimum for gingipain K is at pH 8.0 for hydrolysis of small synthetic substrates, whereas with protein substrates, such as azocasein, it is near pH 8.5. Gingipain R has a slightly lower pH optimum of about 7.5, which is the same for degradation of synthetic and protein substrates.

Biological Significance. Gingipains R and K are recognized as important virulent factors of the bacteria in the periodontal cavities associated with the periodontal disease.^{36,37} In addition, the enzymes hydrolyze a variety of proteins, including collagen,³⁸ fibronectin,³⁹ and immunoglobulins.⁴⁰

Legumain. The 'legumains' or asparaginyl endopeptidases are a novel family of cysteine proteases. They represent one of five protease families in clan CD. Legumains (EC.3.4.22.34) were originally identified in leguminous plants and the parasitic blood fluke *Schistosoma mansoni*.^{41,42} Legumains are recently discovered in mammals.⁴³

Specificity. Legumain is the only protease that is selective for Asn residue at P1. The specificities for P2 and P3 are similar, the human enzyme having a preference for Pro residue at P3. Differentiation of the parasite enzyme from that of the host at P3 supports the possibility of selective inhibition.

Biological Significance. The recently discovered mammalian legumain is localized in the lysosome⁴³ and facilitates the processing of bacterial antigens⁴⁴ and the potential autoantigen, myelin basic protein, in the major histocompatibility (MHC) class II system.⁴⁵ Mammalian legumain is also believed to be involved in the inhibition of osteoclast formation and bone resorption⁴⁶ and in the processing of chromatin remodeling-associated prothymosin α , to the immunoregulatory peptides T α_1 and T α_{11} in the thymus.⁴⁷ In *S. mansoni*, legumain may process gut-associated clan CA zymogens to their active forms, thus facilitating the digestion of ingested host serum proteins to absorbable nutrients.⁴¹

Caspases

Caspases were first characterized as cysteinyl aspartyl proteases via the homology of the *Caenorhabditis elegans* cell death gene *ced-3* and the interleukin-1 β converting enzyme (ICE, now designated caspase-1).⁴⁸ Caspases have near absolute specificity for aspartic acid residue at P1.⁴⁹ As a result, the name caspase originates from cysteinyl

aspartate specific protease. The only known eukaryotic proteases with this specificity are the caspases themselves and the cytotoxic T lymphocyte serine protease granzyme B.

⁵⁰ Caspases reside in the cell as latent proenzymes (zymogens) that are organized into three structural regions: an N-terminal prodomain and the large and small subunits. Most caspases are activated by cleavage at two distinct sites, resulting in the loss of an N-terminal prodomain and the generation of the large and small subunits that comprise the mature enzyme. ⁵¹

Caspases are found in worms to flies to humans. Fourteen distinct family members have been identified to date, with 11 members in humans. ⁵² The caspases can be divided into three groups. The first group are important mediators of inflammation and are called the “cytokine processing” caspases. The other two groups are the “initiator” and “executioner” caspases, where they participate in signaling and effector pathways in apoptosis (programmed cell death). The initiator caspases (caspases-2, -8, -9, and -10) generally have large prodomains and are responsible for initiating the execution phase of apoptosis by triggering activation of distinct caspase cascades. ⁵³ The initiator caspases are activated when they bind specific adapter molecules via defined motifs present in their N-terminal prodomain. The nature of the adapter molecules defines two distinct apoptotic pathways, the mitochondrial pathway and the death receptor pathway. Caspases-8 and -10 contain tandem “death effector domains” (DED) required for binding to adapter proteins during the death receptor ligation. ⁵⁴ The executioner caspases (caspases-3, -6, -7) generally have short prodomains and are activated by other caspases (caspases-8 and -9). Caspase-3 is a key executioner, which is responsible for the cleavage of key structural and functional proteins such as cytoskeletal

proteins, DNA repair enzymes, cell cycle proteins, signal transduction enzymes, apoptosis proteins, and disease-associated proteins.^{55,56} Caspase-12 is located within the endoplasmic reticulum (ER) and activated by either calpain or amyloid-beta (A β) during ER stress-induced apoptosis.⁵⁷ Caspase-14 is the odd one among this family. It may be involved in keratinocyte differentiation.⁵² The cytokine processing caspases (caspases-1, -4, -5, -11, and -13) are generally thought not to be involved in the apoptotic process. Caspases-1, -5, and -11 process and activate proinflammatory mediators such as interleukin-1 β ,⁵⁸ and interleukin-18.⁵⁹

Caspase Specificity. Caspases have a specificity for at least the four amino acids to the left of the cleavage site (P site). The S4 subsite is the single most important determinant of specificity among caspases after the P1 Asp.^{49,54,60,61} The difference in selectivity for the P4 residue divides caspases into three groups (Table 1): Group I caspases (-1, -4, and -5) prefer Trp at P4 with the optimal sequence Trp-Glu-His-Asp. Group II caspases (-2, -3, and -7) are extremely selective for Asp at P4 with the optimal sequence Asp-Glu-X-Asp. Group III caspases (-6, -8, -9) show a wide tolerance of amino acids at P4, with preference for branched, aliphatic side chains such as Val or Leu. The optimal sequence for Group III caspases is (Val,Leu-Glu-X-Asp). The analysis of the available crystal structures and sequence alignments suggest that additional selectivity can be obtained in certain caspases by designing inhibitors which exploit the P' region. Only few caspase inhibitors reported in the literature have utilized the opportunities presented in the P' pocket.

Table 1. Relationship and Specificity in the Caspase Family

Groups	Caspases	Subsite Specificity
Group I Cytokine Activators	Caspase-1	Trp-Glu-His-Asp
	Caspase-4	Trp-Glu-His-Asp
	Caspase-5	Trp-Glu-His-Asp
Group III Initiator Caspases	Caspase-2	Asp-Glu-X-Asp
	Caspase-9	Val, Leu-Glu-X-Asp
	Caspase-8	Val, Leu-Glu-X-Asp
	Caspase-10	
Group II Executioner Caspases	Caspase-3	Asp-Glu-X-Asp
	Caspase-6	Val, Leu-Glu-X-Asp
	Caspase-7	Asp-Glu-X-Asp

X = any amino acid

Crystal Structure and Mechanism. The first caspase crystal structure determined was that of caspase-1 in 1994.^{62,63} Since then, crystal structures of caspases-3,⁶⁴⁻⁶⁶ -7,⁶⁷⁻⁶⁹ -8,^{70,71} and -9,⁷² alone or complexed with various types of inhibitors have been solved and revealed a closely related common fold. In the crystal structure complexes of caspases-1, -3, -7, and -8 with the inhibitor Ac-DEVD-CHO, the inhibitor has adopted an extended antiparallel β -sheet conformation in all four complexes.⁶⁸ The active site involves a conserved dyad composed of Cys 285 (caspase-1 numbering) acting as the nucleophile and His 237 acting as the general base to abstract the proton from the catalytic Cys. During catalysis, the oxyanion of the tetrahedral intermediate is stabilized in the oxyanion hole, which is composed of the backbone amide protons of Cys 285 and Gly 238.⁶⁸ At the P1 site, all four complexes show the binding the P1 Asp in a deep

pocket. There are charge-charge interactions between the P1 Asp side chain and two Arg residues, as well as a hydrogen bond with a conserved Glu. For the P2 Val of Ac-DEVD-CHO, the overall nature of the hydrophobic interaction is conserved. The P3 Glu of the tetrapeptide inhibitor makes at least one charge-charge interaction with a conserved Arg in all four caspase complexes. The major differences for the binding of Ac-DEVD-CHO to caspases-1, -3, -7, and -8 occur at the P4 Asp and acetylated amino terminus of the tetrapeptide due to the differences in the size of the S4 loop among the four caspases. The width of the S4 subsite varies with caspase-8 having the widest, followed by caspase-1. Caspases-3 and -7 have a narrower S4 site. This reduced width can be directly linked to the greater extent of the hydrogen bond network between the P4 portion of the tetrapeptide and the S4 subsite which serves to pull the walls of the S4 pocket towards the P4 group. These observations are in perfect correlation with the previous subsite specificity studies.

Apoptosis

Until about ten years ago, cell suicide or programmed cell death was considered an important process during embryonic development, but not in disease. Programmed cell death (apoptosis) now has such a prominent place in biomedical research that the scientists who uncovered its essentials in the 1980s by studying the development of a microscopic worm, were honored with the 2002 Nobel Prize in Physiology or Medicine.

Apoptosis is an extremely well organized and essential process to eliminate unwanted cells during the development and homeostasis of multicellular organisms.^{73,74} In the early 1990s, scientists discovered a link between apoptosis and cancer. Some

cancer cells became resistant to normal apoptosis process, which allowed them to grow uncontrollably and form tumors. More recent studies have shown that in some neurodegenerative diseases such as stroke, brain trauma, epilepsy, amyotrophic lateral sclerosis (ALS), Huntington's disease, Alzheimer's disease, Parkinson's disease, and multiple sclerosis the affected cells do just the opposite. Hence, through uncontrolled, excessive apoptosis they cause their own destruction.

Many caspases play important roles in the biochemical events that take place in apoptosis. Apoptosis is initiated through two major pathways, the extrinsic pathway and the intrinsic pathway (Figure 6).

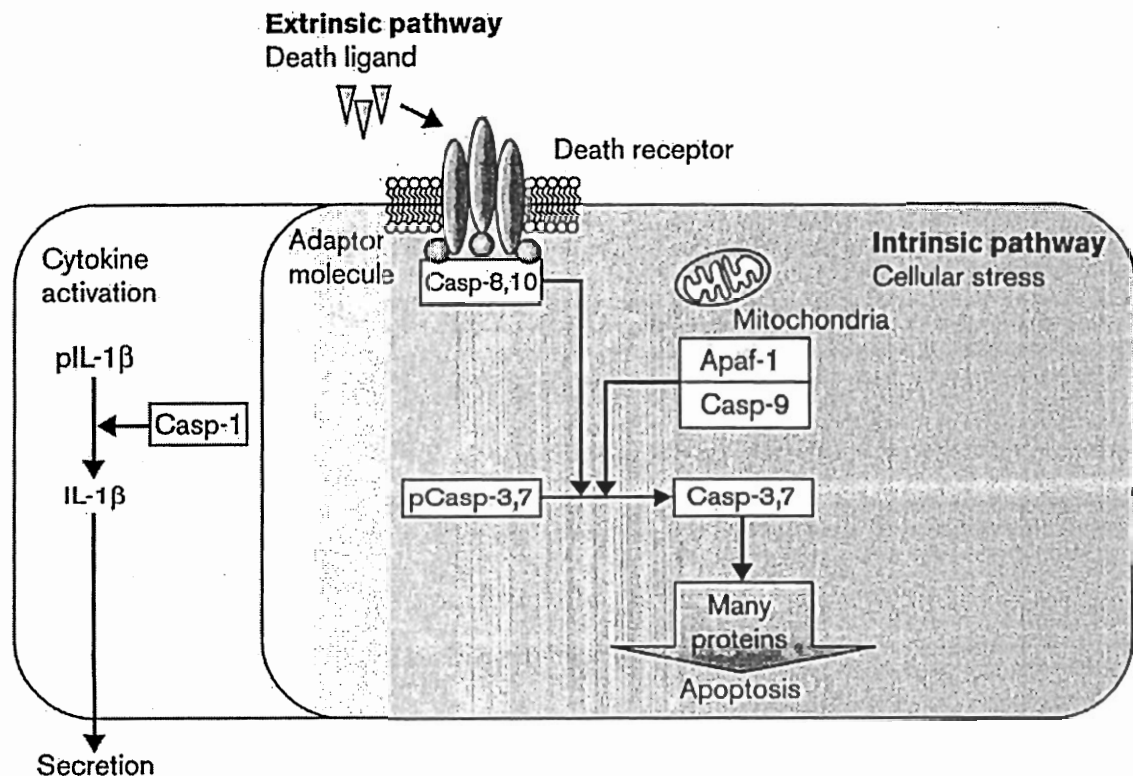


Figure 6. Apoptosis Pathways

In the extrinsic pathway, the apoptotic signal is initiated by Fas ligand (Fas-L) and TNF interaction with their respective receptors (Fas/CD95/APO-1, and TNFR-1) at the

cell surface. This leads to the recruitment of adapter proteins inside the cell and to the activation of initiator caspases-8 and -10, and subsequently to the activation of executioner caspases-3, -7, and possibly -6. In the intrinsic pathway, cellular stress caused by mutagens, drugs, or DNA-damaging agents such as etoposide or γ -irradiation, leads to the release of cytochrome c from the mitochondria through a sequence of events involving Bcl-2 and Bcl-xL proteins. This allows the formation of the apoptosome together with Apaf-1 and the activation of the initiator caspase-9, and thereby caspase-3. Alternatively, calcium aided activation of caspase-12, which is located in the ER, can also lead to the activation of executioner caspases-3, -7, and possibly -6, hence the proteolysis of cellular proteins.

Caspases and Diseases. Caspases are recognized as novel therapeutic targets for central nervous diseases in which cell death occurs mainly by the mechanism of apoptosis. Inappropriate triggering of apoptotic pathways can have severe consequences that may contribute either directly or indirectly to a number of pathologies.

Amyotrophic lateral sclerosis (ALS). Cell and animal models of ALS, along with the finding of a greater than 80% increase in caspase-1 activity in spinal cord samples from humans with ALS indicate that caspases may play an essential role in the pathology of this disease. It has been shown that mutant superoxide dismutase (SOD) promoted apoptotic cell death in differentiated mouse neuroblastoma cells.⁷⁵

Intracerebroventricular administration of the caspase inhibitor Z-VAD-FMK delayed both disease onset and mortality in symptomatic SOD transgenic mouse model.⁷⁶

Huntington's Disease. A role for caspases in the pathogenesis of Huntington's disease was suggested by the finding that the protein huntingtin is a substrate for cleavage

by caspases. Brains of patients with Huntington's disease show increased levels of DNA strand breaks, a process typical of apoptotic cells.⁷⁷ Tetrapeptide aldehyde inhibitors Ac-DEVD-CHO and Ac-YVAD-CHO were used to demonstrate that caspases cleave other polyglutamine-containing protein products representative of neurodegenerative diseases such as spinal bulbar muscular atrophy (SBMA).

Alzheimer's Disease. Amyloid β peptide ($A\beta$), which builds up in the brains of people with Alzheimer's disease (AD), can induce apoptosis in cultured neurons.⁷⁸ Caspases -3, -6, and -8 have been shown to cleave the cytoplasmic tail of APP (VEVD↓A), the precursor protein for $A\beta$ peptides in intact cells.⁷⁹ This data suggests that caspase activation in AD brains may generate more $A\beta$, which in turn leads to additional caspase activation. Thus, a vicious cycle of caspase activation and $A\beta$ production leads to cell death. Inhibition of caspase activity may serve to inhibit apoptosis and decrease the production of $A\beta$. The caspase-6 inhibitor, Z-IETD-FMK, prevents neuronal apoptosis and serum deprivation-mediated increased $A\beta$.⁸⁰ It has been also shown that initiator caspases cleave Alzheimer's gene products, presenilins 1 and 2 proteins.⁸¹

Parkinson's Disease. Several experimental models of Parkinson's disease have supported a role for caspases in apoptotic dopaminergic cell death. Peptide caspase inhibitors have been shown to protect against 1-methyl-4-phenylpyridinium (MPP⁺)-induced apoptosis of cultured cerebellar granular neurons and increase the rate of survival.⁸² Additionally, stereotaxic administration of Z-VAD-FMK prevented the loss of nigral dopaminergic neurons following intrastriatal administration of the neurotoxin 6-

hydroxydopamine (6-OHDA).⁸³ This prevention of dopaminergic neuron loss by Z-VAD-FMK suggests a role for caspases in 6-OHDA-mediated cell death.

Epilepsy. Some, but not all, human epileptic brain samples contain signs of apoptosis, including DNA fragmentation, caspase-1 and caspase-3 proteolysis, and increased expression of Bcl-2 and Bcl-xL, the family of proteins responsible for the release of cytochrome c from mitochondria upon cellular stress.⁸⁴ The inhibitor Z-DEVD-FMK is shown to substantially reduce the apoptotic cell death in brain regions where the seizure propagates, suggesting that caspase-3 plays a key role in status epilepticus induced-apoptosis.⁸⁵

Stroke and Ischemic Brain Injury. Neuronal apoptosis is seen after acute injuries such as stroke⁸⁶, trauma⁸⁷, and ischemia⁸⁸. The effect of irreversible inhibitors (Z-VAD-FMK, Z-DEVD-FMK, and YVAD-CMK) on ischemia-induced tissue shows that caspase-1 and caspase-3 are involved in the mechanism of cell death in ischemic and excitotoxic brain injury.⁸⁸

Hypothesis

Caspases are currently the focus of both commercial and academic research. The exact roles and functions of individual caspase members in the inflammatory and apoptotic signaling pathways have not been fully understood. Although apoptosis is a complicated process, as the biochemical and molecular complexities are elucidated, new therapeutic strategies will arise and synthetic peptides will be the key molecules to validate such strategies.

Several substrate based caspase inhibitors have been extensively used to address the roles of caspases in cellular systems over the past decade.^{48,57,89-92} These include reversible inhibitors such as peptide aldehydes (Ac-YVAD-CHO, Ac-DEVD-CHO, Ac-WEHD-CHO) and irreversible inhibitors such as fluoro- or chloromethyl ketones (Z-VAD-FMK, Z-YVAD-FMK/CMK, Z-DEVD-FMK/CMK). A few of these inhibitors have even entered preclinical studies with animal models of human diseases.⁴⁸

However, the major disadvantage of these inhibitors is their lack of selectivity. Several of these caspase inhibitors have been recently shown to inactivate efficiently other cysteine proteases, particularly cathepsin B (Table 2).^{93-94,98} Cathepsin B inhibition by Ac-YVAD-CMK rescued neuronal cells against glucose/oxygen deprivation-induced apoptosis, suggesting that other proteases may be involved in the processes previously designated only to caspases. Cathepsin B is a lysosomal protease, which belongs to the papain clan of cysteine proteases. Papain clan enzymes have no specific requirements for S1. Furthermore, several lysosomal cysteine proteases were shown to be involved in various apoptosis models, although the mechanisms are not yet clear.^{99,100} Thus, there is a considerable need for potent and selective inhibitors for caspases.

We hypothesize that our new class of inhibitors consisting of the caspase specific recognition sequence and a chemical warhead such as an epoxide or a Michael acceptor moiety, would be potent and selective inhibitors for caspases. We hypothesize that our inhibitors would not inhibit other cysteine proteases from other clans such as cathepsin B or within the same clan, such as the clan CD enzyme legumain. We also propose that our

new inhibitors would be selective among the caspase family members, by utilizing the P' site of our inhibitor design.

Caspases are by far the most popular targets for the development of drugs that potentially modulate the apoptotic process. Currently, we are targeting the key executioner caspase-3, and the initiator caspases-6, and -8. The full control of apoptotic pathways will probably be achieved via a multi-drug therapy due to the different regulatory points that control the process in the cell. An apoptosis-activation based therapy would represent a new class of drugs against cancer, consisting of inhibitors which target Bcl-2 proteins or the release of cytochrome c. In contrast, an apoptosis-inhibition based therapy against neurodegenerative disorders would consist of molecules that efficiently inhibit the formation of apoptosome or direct inhibitors of caspases.

Potent and selective inhibitors of caspases would be invaluable in elucidating the roles of individual caspases in apoptosis and as potential therapeutics. Our peptide based inhibitors comprise a specific design in their structures and could potentially evolve toward new designs of inhibitors, where they might have increased bioavailability and might be able to penetrate the blood-brain barrier to exert their effect on the neurodegenerative disorders and maybe reach the market one day.

Table 2. Inactivation of Papain-like Cysteine Proteases and Legumain by Caspase Inhibitors ⁹³

Enzyme	k_2/K_1 ($M^{-1}s^{-1}$)				
	Inhibitor				
	Z-DEVD-CMK	Ac-YVAD-CMK	Z-DEVD-FMK	Z-YVAD-FMK	Z-VAD-FMK
Papain	1,750	8,100	15,100	10,250	18
Cathepsin B	5,200	10,200	3,800	810	56
Cathepsin L	36,000	1,900	ND	ND	<50
Cathepsin V	355,000	15,000	22,000	180	28
Cathepsin K	290	26	ND	ND	<50
Cathepsin F	22,100	11,900	ND	ND	170
Cathepsin S	28,100	6,900	ND	ND	160
Cathepsin X	850	1,000	ND	600	34
Cathepsin H	<10	13	57	43	1.5
Cathepsin C	<10	<10	ND	ND	<10
Legumain	660	1,300	ND	ND	1,200
Caspase-3	1,000,000	<100	2,000	ND	6,500

ND = not determined.

CHAPTER 2

EPOXYSUCCINYL PEPTIDE INHIBITORS FOR CASPASES

Introduction

The epoxide moiety has been incorporated in a variety of inhibitor structures for cysteine proteases.¹⁰¹ Epoxysuccinyl peptides are derivatives of the natural product E-64, which was initially isolated from *Aspergillus japonicus* in 1978 by Hanada et al (Figure 7).¹⁰² Once the E-64 structure was elucidated, its inhibitory power has been explored towards a number of cysteine proteases such as papain, cathepins B, H, F, K, L, O, S, V, and X, calpains, cruzain, and other cysteine proteases (Table 3).

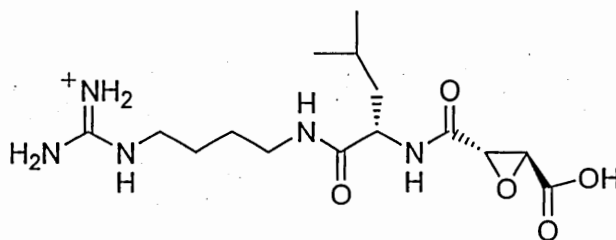


Figure 7. Structure of the Natural Product E-64

Unlike many other microbial inhibitors, E-64 is a potent and irreversible inhibitor of cysteine proteases. E-64 does not inhibit serine proteases, aspartic proteases, or metalloproteases.¹⁰³ Therefore it has been used extensively as a diagnostic reagent for identification of cysteine proteases. E-64 derivatives labeled with biotin, radiolabeled iodine, and fluorescent dyes, such as rhodamine B, have been developed as affinity labels for cysteine proteases.¹⁰⁴⁻¹⁰⁶

Epoxysuccinyl peptide derivatives have the major advantage of being stable toward simple thiols under physiological conditions, which makes them very useful for *in*

vitro and *in vivo* studies. Due to their potent inhibitory activity toward many cysteine proteases and not other proteases, stability, and permeability into cells and tissues, epoxysuccinyl peptide derivatives have been proposed as possible therapeutic agents. They have been shown to have biological activity in a variety of animal disease models, such as muscular dystrophy, ischemic neuronal death, *Leishmania major* infection, glomerulonephritis, osteoclastic bone resorption, and cancer.¹⁰⁷⁻¹¹³ One E-64 derivative, E-64d, prevents both calpain up-regulation and apoptosis in the lesion and penumbra following spinal cord injury in rats.^{114,115}

Although a large number of cysteine proteases are inactivated by E-64 derivatives, fairly recently discovered clan CD enzymes of cysteine proteases are not inactivated by E-64 derivatives (Table 3). Caspases, legumain, and clostripain are examples of clan CD enzymes that are inert to E-64 derivatives. The reason for this behavior of clan CD enzymes was not clear. One reason could be the strict requirement of clan CD enzymes for the side chain of the P1 amino acid residue that provides the carbonyl group in the scissile peptide bond. Clearly, none of the E-64 derivatives tested so far possessed the required P1 amino acid side chains for activity. The other reason could be an unusual binding mode of the E-64 derivatives at the active site of clan CD enzymes. E-64 derivatives are known to have different binding modes among the clan CA enzymes, such as papain and cathepsin B (See Section on Crystal Structures and Binding Modes).

Table 3. Inhibition of Cysteine Proteases by E-64

enzymes inactivated	rate ($M^{-1} s^{-1}$)	ref	enzymes not inactivated	ref
ficin	0.084 (ID ₅₀)	116	trypsin	116
fruit bromelain	0.110 (ID ₅₀)	116	α -chymotrypsin	116
stem bromelain	0.025 (ID ₅₀)	116	kallikrein	116
papain	0.104 (ID ₅₀)	116	pepsin	116
cathepsin B	89,400	117	plasmin	116
cathepsin H	4,000	117	elastase	116
cathepsin L	96,250	117	mold acid protease	116
cathepsin K	1.8 nM (K_i)	118	LDH	116
cathepsin S	99,000	119	thermolysin	117
cathepsin X	775	120	collagenase	117
cathepsin O	>100 μ M (IC ₅₀)	121	clostripain	117
cathepsin F		122	caspase 1 (ICE)	123
cathepsin V	>0.1 μ M (IC ₅₀)	124	legumain	125
DPPI	100	117,126		
streptococcal proteinase	624	117		
papaya proteinase IV	58,000	127		
calpain II	7,500	128		
bleomycin hydrolase	>160 μ M (IC ₅₀)	129		
cruzain	70,600	130		
vignain	32,500	125		

In order to design potent and specific inhibitors for caspases, we have developed a new series of epoxysuccinyl peptides for caspases. The design of epoxysuccinyl peptides was inspired by the structure of E-64, with the placement of the Asp residue adjacent to the epoxysuccinyl moiety (Figure 8). By incorporating the caspase specific substrate sequence, we hoped to obtain selectivity for caspases. We hypothesized that the Asp side chain would meet the strict P1 side chain requirement and result in potent inhibition of caspases. We also have developed both D- and L-amino acid derivatives of this series of compounds in order to explore the nature of the binding mode in the caspase active site. In particular, we were interested in designing peptidyl epoxysuccinyl inhibitors for caspases-3, -6, and -8.

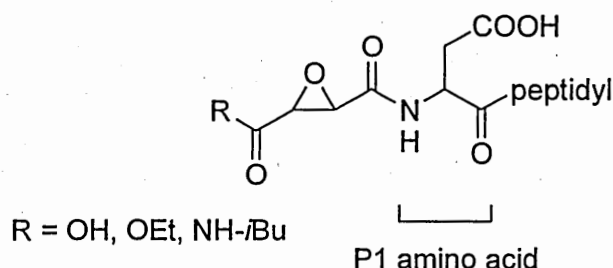
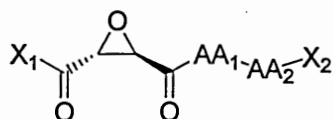


Figure 8. Epoxysuccinyl Peptide Design

Epoxysuccinyl Peptides for Clan CA Enzymes

Once the inhibitory potency of E-64 toward cysteine proteases was recognized, many investigators systematically synthesized a large number of derivatives targeting specific cysteine proteases such as cathepsin B and L (Table 4). Early generation epoxysuccinyl inhibitors are named CA-030 and CA-074, where “CA” represents cathepsin.^{131,132}

Table 4. Common Epoxysuccinate Derivatives

derivative	X ₁	AA ₁	AA ₂ ^a	X ₂
E-64	HO	Leu		Agm ^b
E-64a	HO	Leu		NH(CH ₂) ₄ NH ₂
E-64c (Ep-475)	HO	Leu		NH(CH ₂) ₂ CH(CH ₃) ₂
E-64d (EST, loxistatin)	EtO	Leu		NH(CH ₂) ₂ CH(CH ₃) ₂
circinamide	HO	Leu		N((CH ₂) ₄ NH ₂) ₂
cathestatin C	HO	Tyr		NH(CH ₂) ₅ NH ₂
CA028	HO	Ile	Pro	OH
CA030	EtO	Ile	Pro	OH
CA074	<i>n</i> Pr-NH	Ile	Pro	OH
CLIK148	2-(2-pyridyl)ethyl-NH	Phe		N(CH ₃) ₂

^aAA₂ is the amino acid residue proline or no AA residue. ^bAgm = agmatine (1-amino-4-guanidino-butane).

Nomenclature and Stereochemistry. The stereochemistry of the epoxysuccinyl moiety of E-64 was determined to have 2*S*,3*S*-configuration, whereas the amino acid residues of the peptidyl part of the inhibitor have the L-configuration.^{131,132} On the epoxide ring, C-2 is assigned to the carbon next to the carboxylic acid of the epoxide moiety, whereas C-3 is the carbon close to carbonyl which acylates the peptide (Figure 9). If the carboxylic acid is esterified or converted into an amide derivative, the numbering remains the same. If the epoxysuccinate inhibitor contains two peptides attached to both carboxyls, we number the epoxide such that the C-2 of the epoxide is located at the P side of the active site, whereas C-3 is on the P' site. This is the nomenclature that we will use for our epoxysuccinyl peptides.

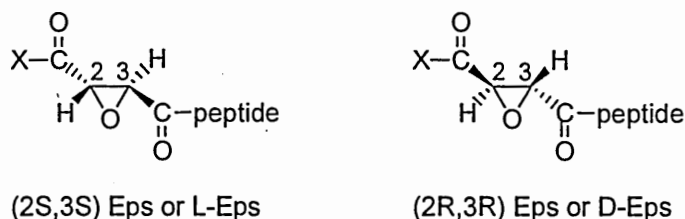


Figure 9. Stereochemistry of *trans*-epoxysuccinates

Mechanism of Inhibition. E-64 and epoxysuccinyl peptides inhibit cysteine proteases by S-alkylation of the active site cysteine, which results in the opening of the epoxide ring.¹³³⁻¹³⁵ Peptidyl epoxysuccinates can inhibit the enzyme by forming a thioether bond via a nucleophilic attack at C-2 or C-3 of the epoxide ring by the active site cysteine residue. The site of the attack depends on the orientation of the epoxysuccinate in the active site. For example, the attack occurs at C-2 when papain is inhibited by E-64c or at C-3 when cathepsin B is inhibited by CA-074 (Figure 10).

Knowledge of how the epoxysuccinate derivative binds is important for determining whether it is the C-2 or C-3 carbon of the oxirane ring that is attacked. The only technique used thus far to determine the structure and stereochemistry of the adducts has been X-ray crystallography and ^{13}C NMR.¹³³

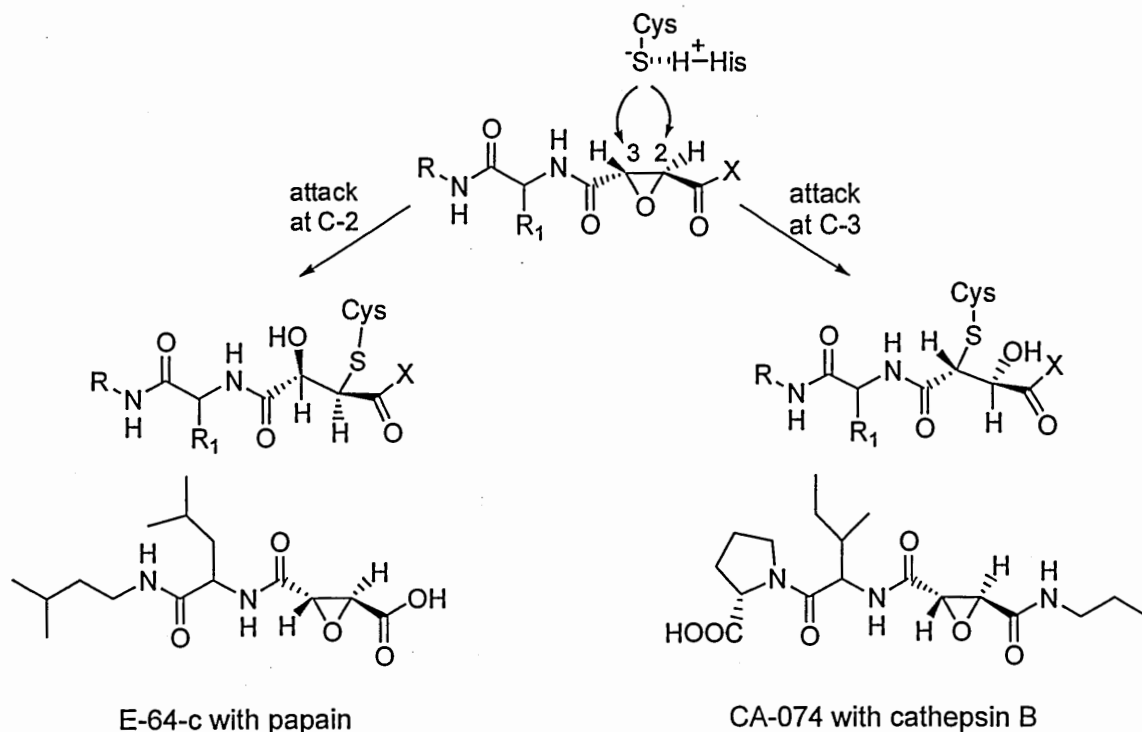


Figure 10. Mechanism of Inhibition of Cysteine Proteases by Epoxysuccinates

The stereochemistry of the enzyme inhibitor adduct has undergone an inversion in configuration at the reaction site due to a nucleophilic attack by the active site thiolate in an $\text{S}_{\text{N}}2$ reaction. For example, E-64, which has the $2\text{S},3\text{S}$ -configuration before the nucleophilic attack, will become $2\text{R},3\text{R}$ after the covalent bond between the cysteine residue and C-2 of the oxirane ring is formed. The 2S to 2R conversion is the result of nucleophilic attack by the Cys thiolate, whereas 3S to 3R conversion is due to a change in the ranking order of the substituents. In only a few cases has X-ray crystallographic

analysis been able to unambiguously assign the stereochemistry in the enzyme inhibitor adducts.

Initially, it was postulated that when E-64 inhibits papain, the oxirane ring would be protonated by His 159, which is analogous to the mechanism of inactivation of papain by peptidyl aldehydes.¹³⁶ However, the crystal structure of papain inhibited by E-64 suggest that the epoxide ring might be protonated by water (Figure 11) due to the distance (5.5 Å) between the His 159 and the resulting hydroxyl group.¹³⁴ Further mechanistic studies confirmed that His 159 is not necessary for protonation of the epoxide and that water is the predominant source of protons.^{137,138}

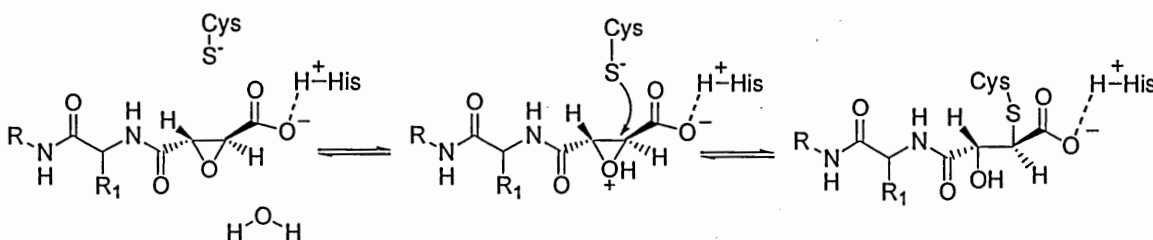


Figure 11. Proposed Mechanism of Inhibition of Cysteine Proteases by Epoxysuccinates

Crystal Structures and Binding Modes. Over a dozen X-ray structures of epoxysuccinate derivatives bound to cysteine proteases have been reported. Initially, it was proposed that E-64 derivatives bound in the S' subsites of the cysteine proteases papain and cathepsin B. However, X-ray structures with papain (PDB codes: 1PEG, 1PPP, and 1CVZ), cathepsin B (1ITO), and cathepsin K (1ATK) revealed that binding instead involved primarily the S subsites. In papain, the the carboxylate group at C-2 position in E-64 orients the epoxide for alkylation by Cys 25 by means of a hydrogen

bonding interaction with His 159 and the oxyanion hole (Gln 19, Figure 13).¹³⁸ As a result, E-64 and several analogous inhibitors bind in S subsites. However, in contrast to the substrate binding mode (N-terminal to C-terminal, Figure 12a), the peptide chain of the inhibitor is oriented in the reverse direction (C-terminal to N-terminal, Figure 12b). In the second binding mode, the inhibitor is located in the S' subsites (Figure 12c). This binding mode has been observed with cathepsin B and several CA inhibitors. Both of the epoxysuccinate analogs CA-074 and CA-030 occupy the S' subsite of cathepsin B in the X-ray structures (Figure 14). Attack of the cysteine thiolate occurs at the C-3 atom of the oxirane ring. This is in contrast to the S subsite binding mode, where the thiolate attack occurs at C-2. Refinement of the crystal structure of the complex of bovine cathepsin B with CA-074 shows the chirality change from 2S,3S to 2R,3R. This chirality change occurs both with S and S' subsite bindings. Newly developed inhibitors, which extend in both directions, are proposed to bind with both the S and S' subsites (Figure 12d). One crystal structure is available so far for a P and P' extended inhibitor (PDB code: 1CVZ).

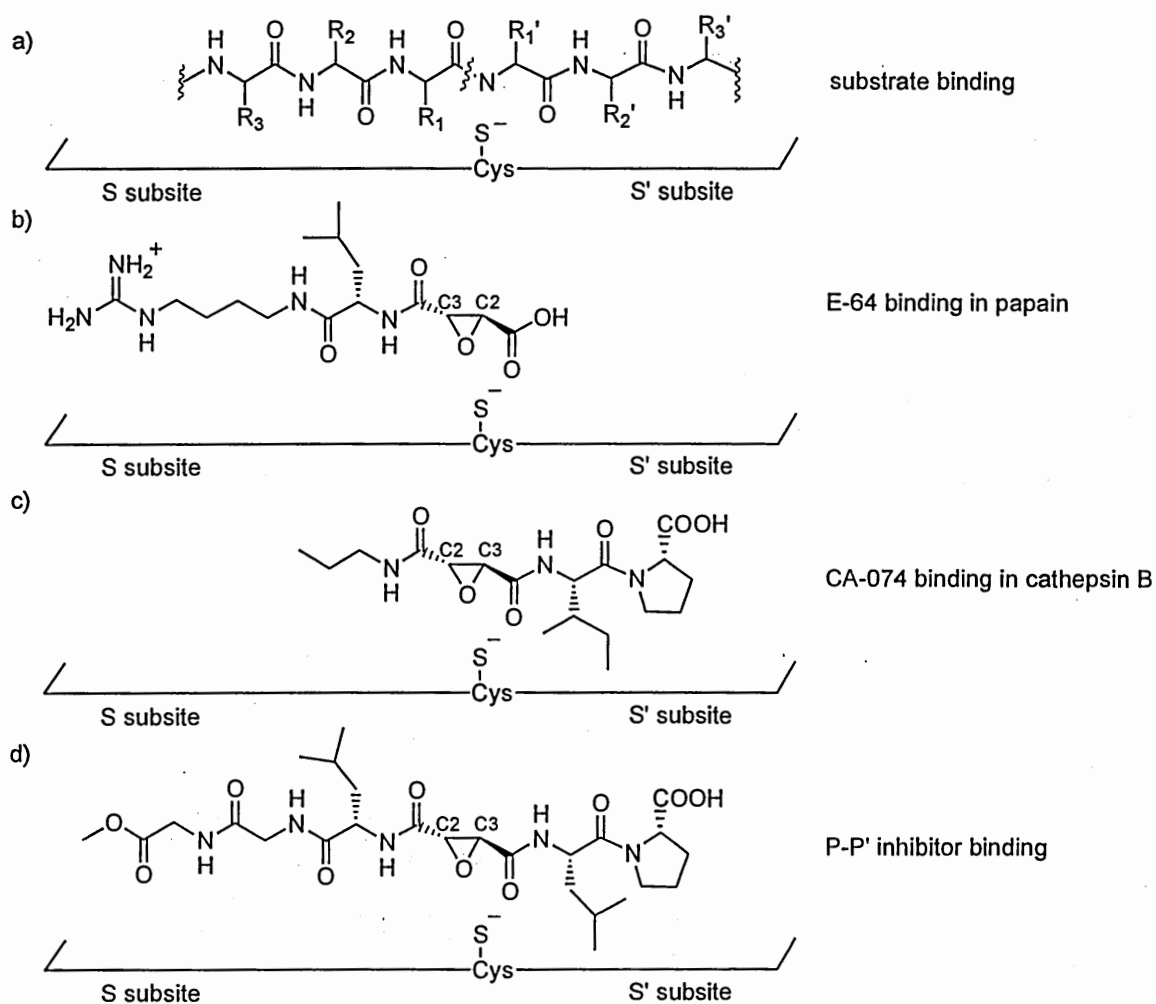


Figure 12. Schematic Representation of the Binding Modes of Peptidyl Epoxysuccinates

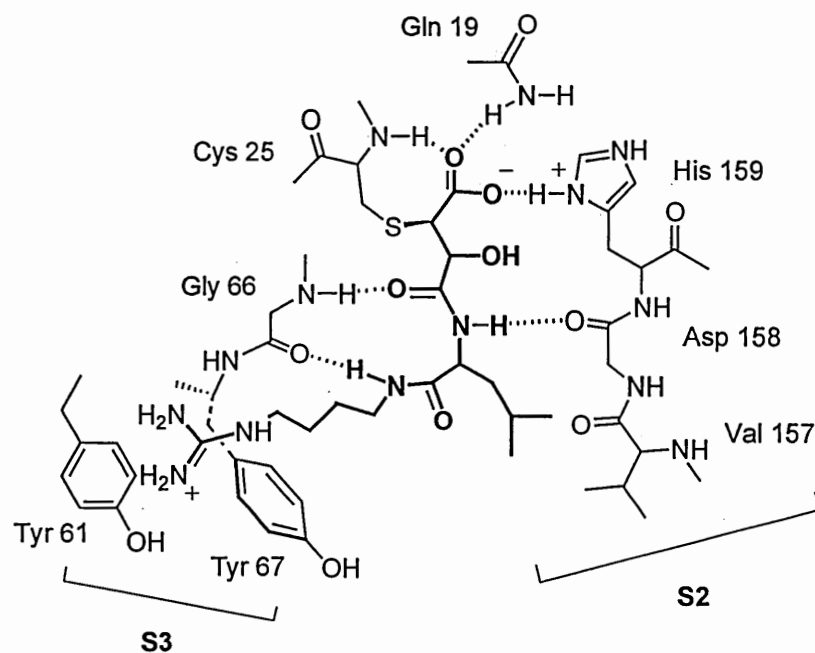


Figure 13. S sub-site binding mode of E-64 in papain-E-64 complex

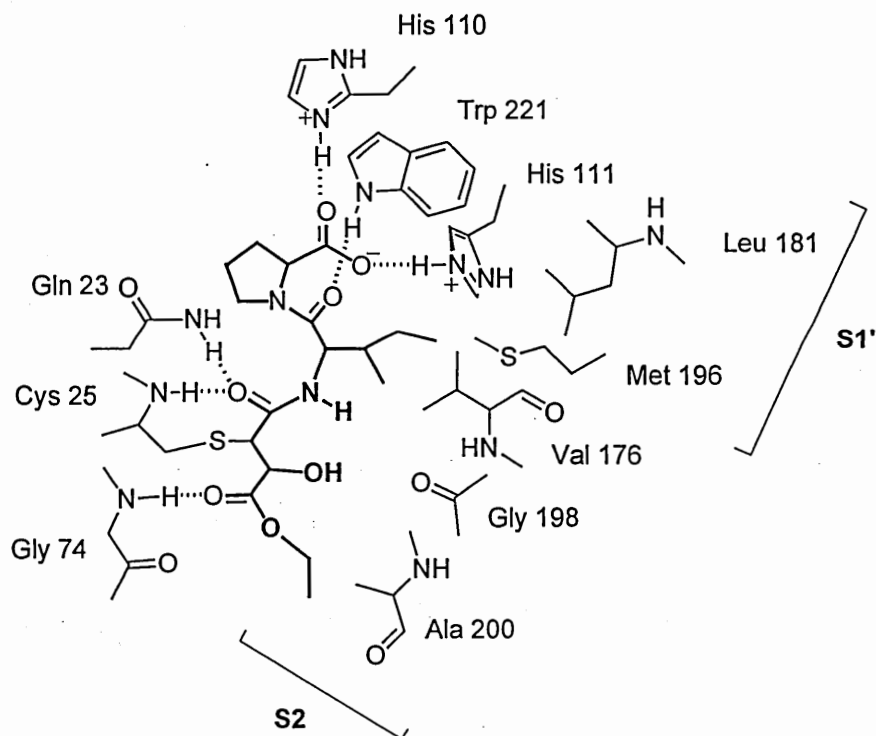


Figure 14. S' sub-site binding of CA-030 in cathepsin B

Structure-Activity Relationships. Many peptidyl epoxysuccinate derivatives were designed and synthesized following the isolation and characterization of E-64. Using structure-activity relationships, researchers have developed derivatives that are selective toward cysteine proteases such as cathepsins B, L, and S, and cruzain. In general, SAR studies revealed the importance of the epoxide moiety, the 2*S*,3*S* stereochemistry of the epoxide ring, and the L-configurations of the amino acid at P1 (except for cruzain). Modifying the substituents on the carboxylic group of the epoxysuccinate and the P1 and P2 residues generated many inhibitors (Table 5).

Studies have shown that all active inhibitors have the *trans*-configuration at the epoxide ring, and the 2*S*,3*S* configuration is 10-1000 times more potent than the corresponding 2*R*,3*R* configuration. The derivatives that contain L-amino acids are 10-100 fold more reactive than the corresponding D-amino acid derivatives. However, recent research has revealed that some inhibitors with 2*R*,3*R* configuration also exhibit high activity¹³⁹ and that certain D-amino acids can be used to obtain selective inhibition of cruzain.¹³⁰

Analogues of E-64c in which the free carboxylate has been replaced by a hydroxamic acid, amide, methyl ketone, hydroxyl, or an ester show that the rates of inhibition of papain and cathepsin B follow the order $\text{CO}_2\text{H} > \text{CONHOH} > \text{CONH}_2 > \text{COR} > \text{CO}_2\text{R} > \text{CH}_2\text{OH} > \text{H}$. The results show that the electrostatic attraction between the carboxylate and the protonated His 159 of papain make the docking of the inhibitor in the active site possible.

Table 5. Inhibition of Cysteine Proteases by Epoxysuccinate Derivatives

inhibitor	rate ($M^{-1}s^{-1}$)					ref
	papain	cathepsin B	cathepsin H	cathepsin L	cathepsin S	
HO-(2 <i>S</i> ,3 <i>S</i>)-Eps-Leu-Agm (E-64)	638,000	89,400	4,000	96,300	99,000 ^c	117 ^a
HO-(2 <i>R</i> ,3 <i>R</i>)-Eps-Leu-Agm	60,900	1,900	65	2,700		117
HO-(2 <i>S</i> ,3 <i>S</i>)-Eps-Leu-NH-(CH ₂) ₇ -NH ₂	874,000	339,000	2070	142,000		117
HO-(2 <i>S</i> ,3 <i>S</i>)-Eps-Leu-NH-(CH ₂) ₂ -CH(CH ₃) ₂	357,000	298,000	2018	206,000		117
HO-(2 <i>S</i> ,3 <i>S</i>)-Eps-D-Leu-NH-(CH ₂) ₂ -CH(CH ₃) ₂	32,500	3,790	28			117
<i>n</i> PrNH-(2 <i>S</i> ,3 <i>S</i>)-Eps-Ile-Pro-OH	112,000	<10	20	<10		140
<i>n</i> PrNH-(2 <i>S</i> ,3 <i>S</i>)-Eps-Ile-Pro-OMe	70	210	180	85		140
HO-(2 <i>S</i> ,3 <i>S</i>)-Eps-Leu-OBzl	52,500	24,700		791,000	171,000	119,141b,c
HO-(2 <i>S</i> ,3 <i>S</i>)-Eps-Leu-NHBzl	90,000	37,600		4,200,000	542,000	119,141
HO-(2 <i>S</i> ,3 <i>S</i>)-Eps-Phe-NHBzl	43,000	9,000		27,800,000	501,000	119,141
HO-(2 <i>S</i> ,3 <i>S</i>)-Eps-D-Phe-NHBzl	3,700	670		117,000	118,000	119,141
HO-(2 <i>S</i> ,3 <i>S</i>)-Eps-Leu-Tyr(I ₂)-NH- <i>i</i> C ₅ H ₁₁	29,000	105,000				142d
HO-(2 <i>R</i> ,3 <i>R</i>)-Eps-Leu-Tyr(I ₂)-NH- <i>i</i> C ₅ H ₁₁	3,500	750				142
EtO-(2 <i>S</i> ,3 <i>S</i>)-Eps-Leu-Pro-OBzl	176,000	8,700				143
<i>i</i> BuNH-(2 <i>S</i> ,3 <i>S</i>)-Eps-Leu-Pro-OBzl	2,070	206				143
<i>i</i> BuNH-(2 <i>S</i> ,3 <i>S</i>)-Eps-Leu-Pro-OH	558	52,000				143

EtO-(2S,3S)-Eps-Leu-Pro-OH	6,130	44,400	170	139 ^e
EtO-(2R,3R)-Eps-Leu-Pro-OH	56	567,000	26	139
HO-(2R,3R)-Eps-Leu-Pro-OH	3270	270	74	139
<i>n</i> PrNH-(2R,3R)-Eps-Leu-Pro-OH	5	29,400	46	139
<i>n</i> PrNH-(2S,3S)-Eps-Leu-Pro-OH	103	153,000	22	139

^aThe apparent second-order rates were measured at 40 °C, pH 6.8 for papain, 40 °C, pH 6.0 for human cathepsin B, 40 °C, pH 5.5 for cathepsin L. ^b k_{inact}/K_i were obtained at 25 °C, pH 6.5 for papain and pH 6.0 for cathepsin B. ^cThe second-order rate constants were measured at 22 °C, pH 5.5 for cathepsin L, and pH 6.5 for cathepsin S. ^dPseudo-first-order conditions at 25 °C and pH 6.8 for both papain and bovine spleen cathepsin B. ^eThe k_i/K_i values were determined at 30 °C and pH 5.5 for papain, human cathepsin B, and cathepsin L.

Epoxy succinyl Peptides for Caspases

The goal of this project was to design a new series of inhibitors for caspases, which we referred to as “epoxy succinyl peptides”. The epoxy succinyl moiety from E-64 has been utilized as the chemical warhead group, where the nucleophilic attack by the active site cysteine thiolate was expected to occur for the inactivation of the enzyme. The epoxide moiety was attached to the caspase specific sequences, which are complementary to the S subsites of caspases-3, -6, and -8 to obtain selectivity. We hypothesized that by introducing the Asp residue at P1, the strict requirement of P1 side chain for caspases would be fulfilled and caspases would be potently inhibited. We also hypothesized that the inhibitors could bind in a reverse fashion relative to the substrate binding mode, as in the case with papain. We have utilized both D- and L-configuration of the amino acids in our design to cover all the possible configurations for a possible binding.

It has been already shown with clan CA enzymes that all the potent inhibitors have the *trans*-configuration at the epoxide moiety, with the 2*S*,3*S* stereochemically pure configuration being most active. We have synthesized epoxy succinyl peptide derivatives including epoxide moieties with *trans*, *cis* and stereochemically pure *S,S* configurations.

We have synthesized several analogs with a free carboxylate group on the epoxide ring, where previous studies have shown that those inhibitors were the most potent ones. We also made ester and amide analogs.

Synthesis

Epoxy succinyl peptides are synthesized by coupling the peptide portion of the inhibitor to the epoxide moiety, which are synthesized separately. For example, the *trans*

epoxide moiety was synthesized from fumaric acid diethyl ester using a general procedure for the stereocontrolled epoxidation of α,β -unsaturated compounds with lithium *t*-butyl hydroperoxide, which was similar to the method developed by Meth-Cohn.¹⁴⁴ This reaction yielded two products. The major product was the diethyl epoxysuccinate and the second product was 2-ethyl-3-*t*-butyl epoxysuccinate. The selective hydrolysis of one of the ethyl esters to yield the monoethyl epoxysuccinate was accomplished by using 1 eq of KOH in ethanol. The selective hydrolysis of the ethyl ester in the presence of the *t*-butyl ester was accomplished by using 1 eq of KOH in ethanol and dry THF (Figure 15a).

The amide derivative of the epoxysuccinate was obtained by coupling the corresponding amine to the monoethyl epoxysuccinate and subsequent hydrolysis of the ethyl ester using 1.2 eq of NaOH in EtOH (Figure 15b).

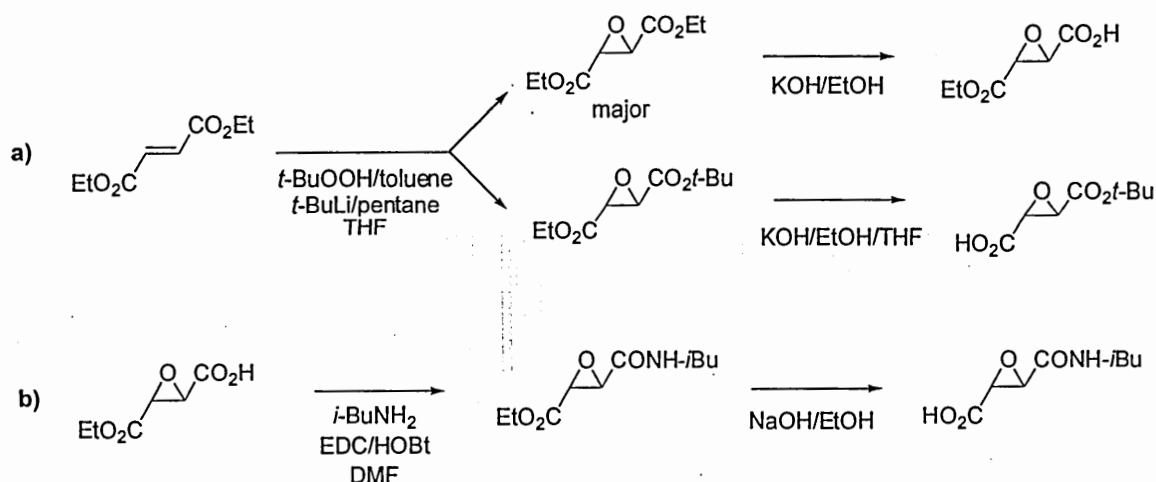


Figure 15. Synthesis of the Epoxide Moiety

The first examples of epoxysuccinyl peptides were synthesized using standard EDC/HOBt coupling method, followed by deprotection of the *t*-butyl protecting groups with TFA (Figure 16).

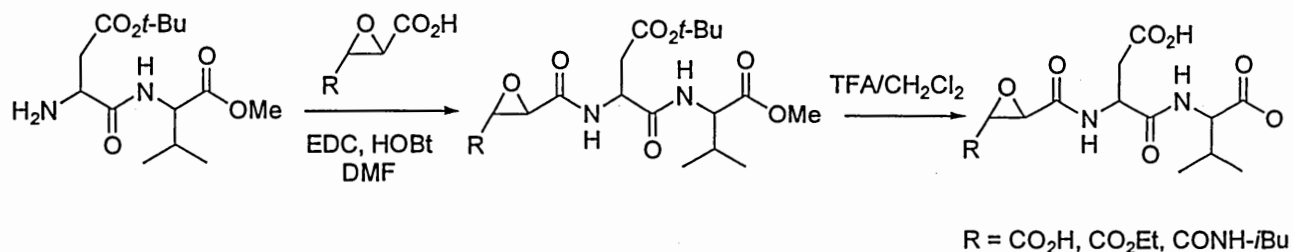


Figure 16. Solution Phase Synthesis of Epoxysuccinyl Peptides

Most of the epoxysuccinyl peptides were synthesized using the polystyrene based Wang resin as the solid support. By utilizing solid phase synthesis, one has the opportunity of making a variety of compounds in a considerably short amount of time. The epoxide moiety was synthesized separately and then attached to the solid support. The peptide portion was synthesized on the solid support using Fmoc protected amino acids. The epoxide moiety was then coupled to the peptide portion on solid support as the last step and the cleavage with TFA from the resin along with the deprotection of the *t*-Bu groups gave the final compounds as shown in the figure below (Figure 17).

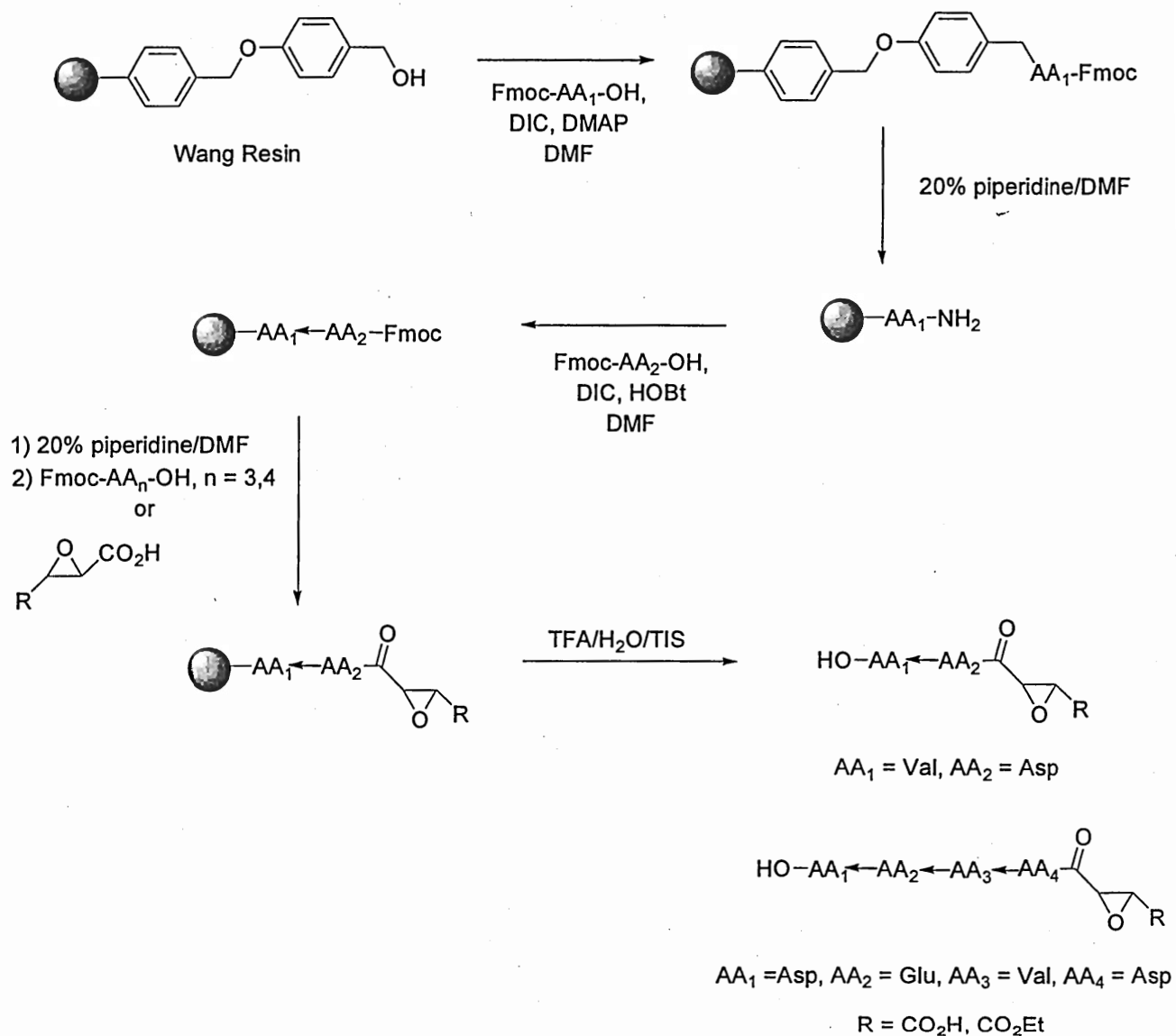


Figure 17. Solid Phase Synthesis of Epoxysuccinyl Peptides

Results and Discussion

We have designed and synthesized a new series of epoxysuccinyl peptides for caspases. We hypothesized that by incorporating an Asp residue adjacent to the epoxide moiety one could obtain selectivity toward caspases. We also hypothesized that the

active site cysteine thiolate would attack the epoxide ring at either C-2 or C-3 position resulting in irreversible alkylation of the enzyme (Figure 18).

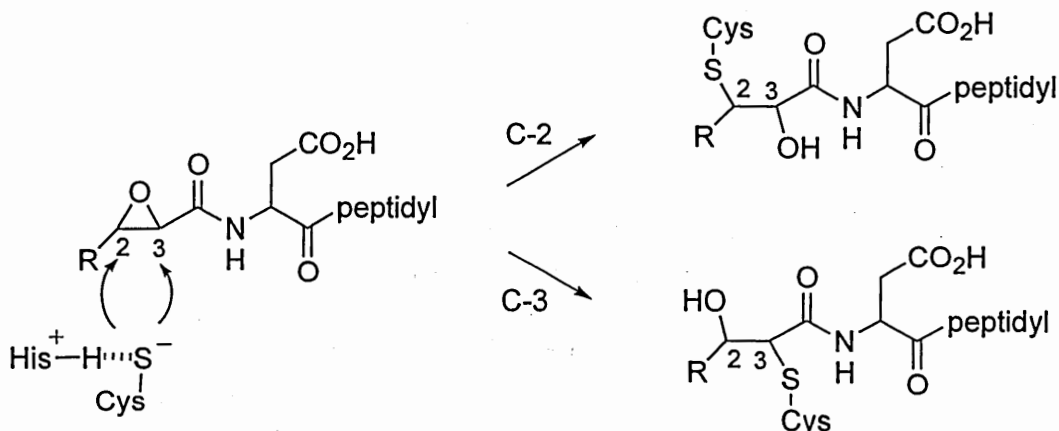


Figure 18. Proposed Mechanism of Inhibition of Caspases by Epoxysuccinyl Peptides

Unfortunately, caspases -3, -6 and -8 were not inactivated by epoxysuccinyl peptides. The epoxysuccinyl dipeptides with Asp-Val sequence, which were designed as broad-spectrum caspase inhibitors, did not show any activity against caspases -3, -6, and -8 (Figure 19). Although both D- and L-configurations of the amino acids were used in the design of inhibitors like HOOC-*trans*-EP-CO-Asp-Val-OMe and HOOC-*trans*-EP-CO-Asp-D-Val-OH, to allow interactions with different subsites of the enzyme active site, neither of the epoxysuccinyl dipeptides produced effective binding at the caspase active site. The inhibitors with the extended general tetrapeptide sequences HOOC-*trans*-EP-CO-Asp-Ala-Ala-Ala-OH and HOOC-*trans*-EP-CO-Asp-D-Ala-D-Ala-D-Ala-OH, and caspase-3 specific sequence EtOOC-2*S*,3*S*-EP-CO-Asp-Val-Glu-Asp-OH did not exhibit improvement in the activity.

However, epoxysuccinyl peptides designed for caspases are weak inhibitors of clan CA enzymes. The epoxysuccinyl dipeptide HOOC-EP-CO-Asp-D-Val-OMe has

second order rate constants of $< 10 \text{ M}^{-1}\text{s}^{-1}$ against papain and cathepsin B. Clearly, the charged P1 side chain is not well accommodated at the active sites of papain and cathepsin B. But even a weak inhibition suggests that the inhibitors are probably positioned such that a nucleophilic attack of the active site cysteine thiolate can take place. One can conclude that the binding of epoxysuccinyl peptides is not in a reverse direction at the caspase active site as is the case with papain. On the contrary, the binding is in the same direction as the substrate binding, which is from N-terminal to C-terminal.

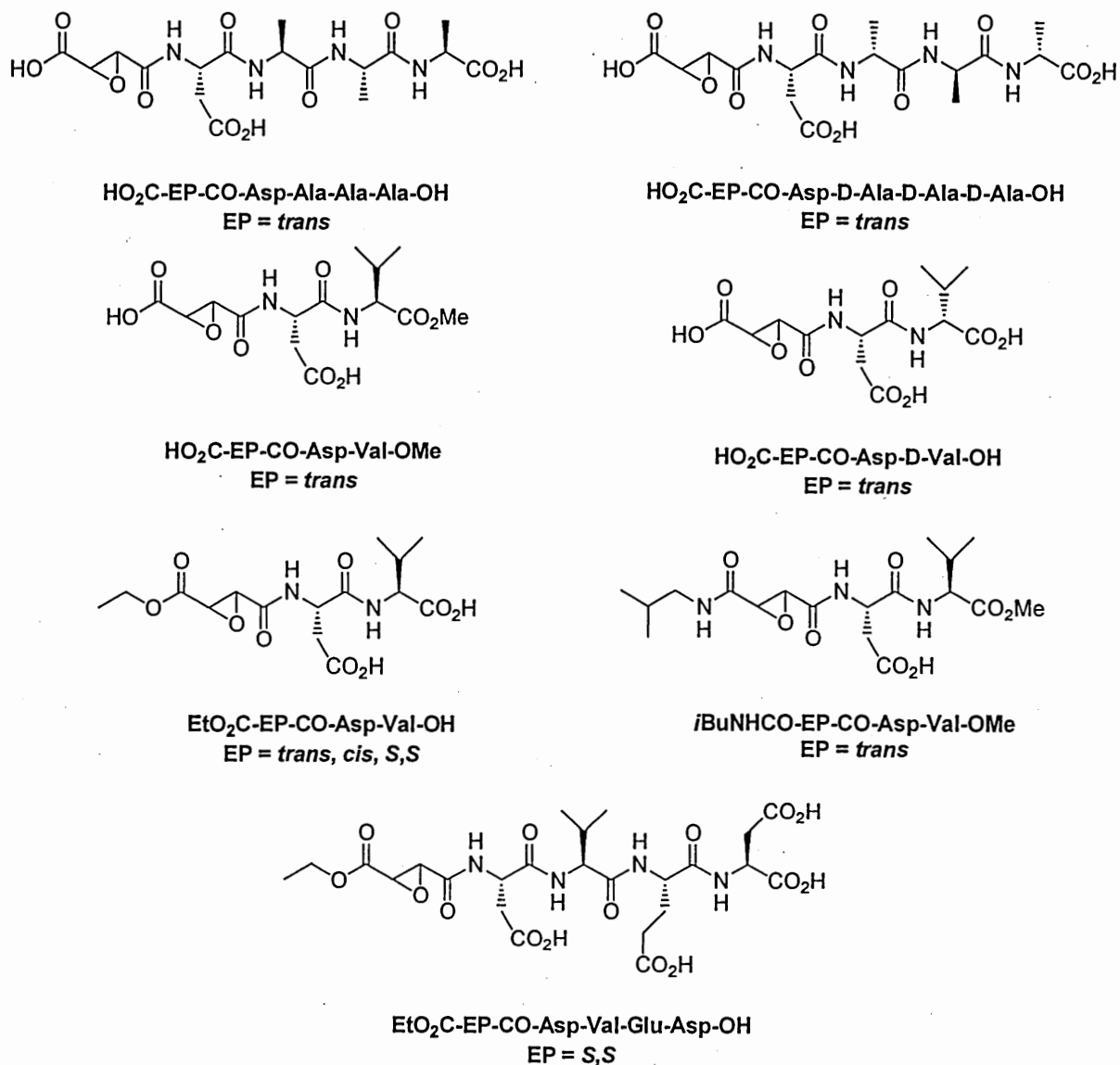


Figure 19. Epoxysuccinyl Peptides Designed for Caspases

Experimental Procedures

Material and Methods. Mono and dipeptidyl methyl esters were purchased from Bachem Bioscience Inc., King of Prussia, PA. The polystyrene based Wang resin and the Fmoc-protected amino acids were purchased from Calbiochem-Novabiochem Corp. San Diego, CA. The Bio-spin chromatography columns were purchased from BioRad,

Hercules, CA. Dipeptides and tetrapeptides were synthesized using standard coupling procedures such as the DIC/HOBt coupling method using solid phase or EDC/HOBt coupling using solution phase chemistry. The ^1H NMR spectra were obtained using a Varian Mercury Vx 400 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. Caspases-3, -6 and -8 were expressed in *E. coli* and purified according to methods previously described by Stennicke and Salvesen.¹⁴⁵ Enzymes and substrates were obtained from Guy Salvesen. The fluorogenic substrate Ac-DEVD-AMC was purchased from Bachem Bioscience Inc., King of Prussia, PA.

***trans*-Oxirane-2,3-dicarboxylic Acid Diethyl Ester (EtOOC-EP-COOEt) and *trans*-3-Ethoxycarbonyloxirane-2-carboxylic Acid *t*-Butyl Ester (EtOOC-EP-COO*t*Bu).** 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.¹⁴⁴ An anhydrous solution of *t*-butyl hydroperoxide in toluene (3.3 M solution, 46 mL, 1.5 eq), was added to freshly distilled THF (240 mL) at -78 °C under argon. This was followed by the addition of a solution of butyl lithium in pentane (1.7 M solution, 65 mL, 1.1 eq). The mixture was stirred at -78 °C for 5 min, then a solution of diethyl fumarate (17.2 g, 0.1 M, 1 eq) in THF (50 mL) was added. The reaction mixture was stirred at room temperature for 2 hours (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 diatomaceous earth (celite), and evaporated. To the residue was added 1 M HCl (100 mL). The products *trans*-oxirane-2,3-dicarboxylic acid diethyl ester and *trans*-3-ethoxycarbonyloxirane-2-carboxylic acid *t*-butyl ester were extracted with EtOAc (3 x 100 mL), and the organic layer was washed with saturated NaCl (3 x 50 mL), dried over MgSO₄, and the solvent evaporated. Chromatography on a silica gel column with 2:3 EtOAc:hexane afforded the major product *trans*-oxirane-2,3-dicarboxylic acid diethyl ester (yield = 52%) and the minor product *trans*-3-ethoxycarbonyloxirane-2-carboxylic acid *t*-butyl ester (yield = 23 %). ¹H NMR (CDCl₃): 1.30 (t, 6H, OCH₂CH₃), 3.65 (s, 2H, ep), 4.27 (q, 4H, OCH₂CH₃). ¹H NMR (CDCl₃): 1.30 (t, 3H, OCH₂CH₃), 1.50 (s, 9H, *t*Bu), 3.58 (d, 2H, ep), 4.27 (q, 2H, OCH₂CH₃).

***trans*-Oxirane-2,3-dicarboxylic Acid Monoethyl Ester (HOOC-EP-COOEt).**

The selective hydrolysis of one ester to yield monoethyl epoxysuccinates was achieved by a method similar to that described previously by Rich and Schaschke.¹³⁹ *trans*-Oxirane-2,3-dicarboxylic acid diethyl ester (1 eq) was dissolved in minimum amount of EtOH and cooled to 4 °C. To that solution 1.08 N KOH (1 eq) in EtOH was added dropwise over 3 hours. During this time a white precipitate formed and the mixture was stored overnight in a freezer. The volatiles were removed after warming to room temperature. The residue was taken up in H₂O and washed with 2 x 25 mL EtOAc. The pH was adjusted to 2 with 3 N HCl, and the solution was extracted with 6 x 25 mL EtOAc. The organics were combined, dried over Na₂SO₄, and concentrated to give a white solid with yield 55 %. ¹H NMR (CDCl₃): 1.30 (t, 3H, OCH₂CH₃), 3.70 (s, 2H, ep), 4.27 (q, 2H, OCH₂CH₃).

***trans*-Oxirane-2,3-dicarboxylic Acid Mono-*t*-butyl Ester (HOOC-EP-COO*t*Bu).** The selective hydrolysis of the ethyl ester of *trans*-3-ethoxycarbonyloxirane-2-carboxylic acid *t*-butyl ester was accomplished with a slightly modified method as described previously by Rich and Schaschke. *trans*-3-Ethoxycarbonyloxirane-2-carboxylic acid *t*-butyl ester was dissolved in minimum amount of dry THF and cooled to 0 °C. To that solution 1.08 N KOH (0.9 eq) in 1:1 EtOH/dry THF was added dropwise over 3 hours. During this time a white precipitate formed and the mixture was stored overnight in a freezer. The volatiles were removed after warming to room temperature. The residue was taken up in H₂O and washed with 2 x 25 mL EtOAc. The pH was adjusted to 2 with 3 N HCl, and the solution was extracted with 6 x 25 mL EtOAc. The organics were combined, dried over Na₂SO₄, and concentrated to give a white solid with yield 61 %. ¹H NMR (DMSO-*d*₆): 1.42 (s, 9H, *t*Bu), 3.53 (s, 2H, ep).

(2*S*,3*S*)-Oxirane-2,3-dicarboxylic Acid Monoethyl Ester (HOOC-EP-COOEt). This compound was a gift from J. Asgian in our laboratory.

***cis*-Oxirane-2,3-dicarboxylic Acid Mono-*t*-butyl Ester (HOOC-EP-COO*t*Bu).** *cis*-(3-Ethoxycarbonyl)oxirane-2-carboxylic acid *t*-butyl ester was a gift from S. Shadinger in our laboratory. The selective hydrolysis of the ethyl ester (1 eq) with KOH (1 eq) in 1:1 EtOH/dry THF gave the desired epoxysuccinate HOOC-EP-COO*t*Bu in 64 % yield. ¹H NMR (CDCl₃): 1.50 (s, 9H, *t*Bu), 3.65 (d, 1H, ep), 3.75 (d, 1H, ep).

***trans*-(3-Isobutylcarbamoyl)oxirane-2-carboxylic Acid (HOOC-EP-CONHCH₂CH(CH₃)₂).** The procedure used to synthesize amide derivatives of epoxysuccinate monoethyl esters was similar to that of Therrien *et al.*¹²⁰ To a solution of epoxysuccinate monoethyl ester (1 g, 6.25 mM), isobutyl amine (1.2 eq) and HOBt (1 eq)

in CHCl_3 (30 mL) at 0 °C was added EDC (1.1 eq) slowly in 5 portions. The reaction was stirred for one hour at 0 °C and then subsequently at room temperature for 16 hours. The solvent was evaporated and the residue was partitioned between EtOAc (50 mL) and H_2O (5 mL). The organic layer was washed with 0.5 M HCl (2 x 10 mL), sat NaHCO_3 (2 x 50 mL), dried over MgSO_4 and concentrated to yield a white solid with 86 % yield. Hydrolysis of the ester by NaOH (1.2 eq) gave the desired amide (yield = 72 %). ^1H NMR (DMSO-d_6): 0.92 (d, 6H, $\text{NHCH}_2\text{CH}(\text{CH}_3)_2$), 1.70 (m, 1H, $\text{NHCH}_2\text{CH}(\text{CH}_3)_2$), 2.92 (t, 2H, $\text{NHCH}_2\text{CH}(\text{CH}_3)_2$), 3.44 (s, 1H, ep), 3.55 (s, 1H, ep), 8.34 (t, 1H, NH).

3-*trans*-Ethoxycarbonyloxiran-2-carbonyl-L-*t*-butoxyaspartic Acid Methyl Ester (EtOOC-*trans*-EP-CO-Asp(O-*t*Bu)-OMe). Epoxysuccinyl Peptide Synthesis Using the Mixed Anhydride Coupling Method. To a solution of the *trans*-oxirane-2,3-dicarboxylic acid monoethyl ester (5 eq) in DMF at 0 °C was added N-methylmorpholine (NMM, 5 eq) followed by isobutyl chloroformate (IBCF, 5 eq). After the reaction mixture was allowed to stir for 30 min, $\text{HCl}\cdot\text{H-Asp}(\text{O-}i\text{Bu})\text{-OMe}$ (1 eq) dissolved in DMF, was added to the mixture. After 10 min the ice bath was removed and the reaction was stirred for 16 hours at room temperature. The DMF was evaporated and the residue was washed and purified using the same procedure as described for the EDC/HOBt coupling method. MS and ^1H NMR (DMSO-d_6 or CDCl_3) were consistent with the proposed structures. Purification by column chromatography on silica gel with 1:19 MeOH: CH_2Cl_2 as the eluent gave the product as yellow oil with 64 % yield. MS (FAB) m/z 346 [$(\text{M} + 1)^+$, 25 %], 290 ($\text{M-}i\text{Bu}$) + 3, 100 %]. ^1H NMR (CDCl_3): 1.31 (t, 3H, OCH_2CH_3), 1.44 (s, 9H, *t*Bu), 2.75-2.90 (dt, 2H, asp), 3.45 (s, $\frac{1}{2}$ H, ep), 3.62 (s, $\frac{1}{2}$ H,

ep), 3.70 (d, 1H, ep), 3.76 (s, 3H, OCH₃), 4.28 (q, 2H, OCH₂CH₃), 4.80 (m, 1H, α -H), 7.00 (d, 1H, NH).

3-*trans*-*t*-Butoxycarbonyloxiran-2-carbonyl-L-*t*-butoxyaspartyl-L-valine Methyl Ester (*t*BuOOC-*trans*-EP-CO-Asp(O-*t*Bu)-Val-OMe). Epoxysuccinyl

Peptide Synthesis Using the EDC/HOBt Coupling Method. To a stirred solution of HCl·H-Asp(O-*t*Bu)-Val-OMe (1 eq), HOBt (0.37 mM, 1.1 eq), and *trans*-oxirane-2,3-dicarboxylic acid monot-butyl ester (1.1 eq) in DMF was added EDC (1.1 eq) and the mixture was allowed to react for 16 h at room temperature. The DMF was removed under vacuum and the residue was treated with 10 mL EtOAc. The organic layer was then washed with 2 x 10 mL 2 % citric acid, 1 x 15 mL saturated NaHCO₃, and 2 x 10 mL brine. The organic layer was dried over Na₂SO₄, filtered and concentrated. Column chromatography on silica gel with 1:199 MeOH: CH₂Cl₂ as the eluent gave the product as a yellow oil with 71 % yield. MS and ¹H NMR were consistent with the proposed structure. HRMS (FAB) Calcd. for C₂₂H₃₆N₂O₉: 472.24991. Observed *m/z* 472.25198. ¹H NMR (CDCl₃): 0.91 (dd, 6H, Val), 1.41 (dd, 18H, *t*Bu), 2.17 (m, 6H, Val), 2.60 (dd, 1H, Asp), 2.86 (dt, 1H, Asp), 3.40 (ds, 1H, ep), 3.62 (s, 1H, ep), 3.70 (s, 3H, OCH₃), 4.49 (m, 1H, α -H), 4.77 (m, 1H, α -H), 7.05 (d, 1H, NH).

3-*trans*-*t*-Butoxycarbonyloxiran-2-carbonyl-L-*t*-butoxyaspartyl-D-valine Methyl Ester (*t*BuOOC-*trans*-EP-CO-Asp(O-*t*Bu)-D-Val-OMe) was synthesized using the EDC/HOBt coupling method, purified by column chromatography on silica gel with 1:199 MeOH: CH₂Cl₂ as the eluent; yellow oil, yield 66 %. ¹H NMR (CDCl₃): 0.91 (m, 6H, Val), 1.46 (dd, 18H, *t*Bu), 2.17 (m, 6H, Val), 2.63 (dd, 1H, Asp), 2.81 (td, 1H, Asp),

3.52 (s, 1H, ep), 3.62 (s, 1H, ep), 3.74 (s, 3H, OCH₃), 4.49 (m, 1H, α -H), 4.77 (m, 1H, α -H), 7.05 (d, 1H, NH). MS (ESI) m/z 473.2 [(M + 1)⁺].

Deblocking of the *t*-Butyl Protecting Groups. The epoxysuccinyl peptides were treated with 1:1 TFA: CH₂Cl₂ at 0 °C for 1 h and at room temperature for 2 h. TFA and CH₂Cl₂ were removed under vacuum, and the final products were recrystallized from diethyl ether/hexanes mostly as white solids.

3-*trans*-Carboxyoxiran-2-carbonyl-L-aspartyl-D-valine Methyl Ester (HOOC-*trans*-EP-CO-Asp-D-Val-OMe). ¹H NMR (DMSO-d₆): 0.92 (d, 6H, Val), 2.01 (m, 6H, Val), 2.60 (m, 1H, Asp), 3.52 (s, 1H, ep), 3.42 (s, 1H, ep), 3.62 (s, 3H, OCH₃), 4.16 (m, 1H, α -H), 4.70 (m, 1H, α -H), 8.29 (t, 1H, NH), 8.63 (dd, 1H, NH). MS (ESI) m/z 361.1 [(M + 1)⁺].

3-*trans*-Isobutylcarbamoxyloxiran-2-carbonyl-L-aspartyl-L-valine Methyl Ester (*i*Bu-NHOC-*trans*-EP-CO-Asp-Val-OMe) was synthesized using EDC/HOBt coupling method; white solid, yield 83 %. ¹H NMR (DMSO-d₆): 0.91 (d, 12H, Val and *i*Bu), 1.69 (m, 1H, *i*Bu), 2.01 (m, 1H, Val), 2.62 (m, 2H, Asp), 2.90 (m, 2H, *i*Bu), 3.48 (d, 1H, ep), 3.53 (s, 1H, ep), 3.62 (s, 3H, OCH₃), 4.10 (m, 1H, α -H), 4.68 (m, 1H, α -H), 8.20 (t, 1H, NH), 8.36 (t, 1H, NH), 8.70 (t, 1H, NH). Anal. Calcd. for C₁₈H₂₉N₃O₈·0.52H₂O·0.15TFA: C, 49.74; H, 6.89; N, 9.51 Found: C, 49.75; H, 6.93; N, 9.38.

General Methods for Solid Phase Peptide Synthesis.

Syntheses of epoxysuccinyl peptides were carried out manually on Wang resin (100-200 mesh) in a 12 mL Bio-spin chromatography column. In general, Fmoc-

protected amino acids (Fmoc-AA) such as Ala, Val and Fmoc-protected amino acids with *t*-butyl side chain protecting groups such as Asp and Glu were used. Monoethyl and mono-*t*-butyl epoxysuccinates were synthesized separately as described above.

Attachment of the First Amino Acid Residue to the Resin. The first amino acid (10 eq to resin loading) was attached to the resin (loading: 0.5 -1.2 mmole/g) by reacting it with DIC (5 eq to resin loading)/DMAP(0.1 eq to resin loading). The resin is placed in a Bio-spin chromatography column and sufficient DMF is added to cover the resin, which is swelled for 30 min. To a solution of the Fmoc-AA in CH₂Cl₂, DIC is added and the mixture is stirred for 20 min at 0 °C keeping it free from moisture with CaCl₂ drying tube. CH₂Cl₂ is removed under pressure. The white precipitate is dissolved in minimum amount of DMF and added to the resin. DMAP is dissolved in DMF and also added to the aa/resin solution. The mixture is stirred for 2 hr. The resin is then washed with DMF (6 x 5 mL), CH₂Cl₂ (6 x 5 mL), and DMF again (1 x 5 mL).

Removal of the Fmoc Protecting Group. The Fmoc protecting group is removed by treating the resin with 20 % piperidine/DMF solution for 20 min. The resin is then washed with DMF (6 x 5 mL), CH₂Cl₂ (6 x 5 mL), and DMF again (1 x 5 mL). The reaction completion was assessed using the Kaiser test.

Kaiser Test. The resin is washed several times with EtOH. A few resin beads are transferred to a small test tube and two drops of ninhydrin solution (5 g ninhydrin in 100 mL EtOH), liquified phenol solution (80 g liquified phenol in 20 mL EtOH), and 0.001 M KCN solution in pyridine are added. The mixture is heated to 120 °C for 5 min. A positive test is indicated by blue resin beads.

DIC/HOBt Coupling Method. The Fmoc protected amino acids and monoethyl or mono-*t*-butyl epoxysuccinates (5 eq to resin loading) and HOBt (5 eq to resin loading) were dissolved in DMF and added to the resin. DIC (5 eq to resin loading) also dissolved in DMF is added to the reaction mixture. The reaction time varied between 2-16 hr depending on the Fmoc-aa or the epoxysuccinate coupled. Each coupling step was followed by washing with DMF (6 x 5 mL), CH₂Cl₂ (6 x 5 mL), and DMF (1 x 5 mL), removal of the Fmoc group by treatment with 20 % piperidine in DMF and then another washing step with DMF (6 x 5mL).

Removal of the *t*-Butyl Protecting Groups and Cleavage from the Resin. The deprotection of the side chain protecting groups and the cleavage from the resin were accomplished by treatment with a mixture of 38:1:1 TFA:H₂O:TIS. The reaction time varied between 4-16 hr depending on the sequence. The resin was removed by filtration and washed twice with TFA. The filtrates were combined and 8-10 fold volume of cold ether was added dropwise. The epoxysuccinyl peptides were obtained mostly as white solids. MS and ¹H NMR were consistent with the proposed structures.

3-*trans*-Carboxyoxiran-2-carbonyl-L-aspartyl-L-alanyl-L-alanyl-L-alanine (HOOC-*trans*-EP-CO-Asp-Ala-Ala-Ala-OH) was synthesized using solid phase synthesis; white solid, yield 85 %. ¹H NMR (D₂O): 1.25-1.41 (m, 9H, Ala), 2.65 - 2.85 (m, 2H, Asp), 3.48 (dd, 1H, ep), 3.55 (d, 1H, ep), 4.15 (m, 4H, α-H). HRMS (FAB) Calcd. for C₁₇H₂₄N₄O₁₁: 460.15198. Observed *m/z* 460.15061. Product assayed using reverse phase HPLC at 215 nm on a C8 column using a mobile phase of 97 % water / 3% acetonitrile / 0.1 % TFA at a flow rate of 1mL/minute.

3-*trans*-Carboxyoxiran-2-carbonyl-L-aspartyl-D-alanyl-D-alanyl-D-alanine (HOOC-*trans*-EP-CO-Asp-D-Ala-D-Ala-D-Ala-OH) was synthesized using solid phase synthesis; white solid, yield 91 %. Product assayed using reverse phase HPLC at 215 nm on a C8 column using a mobile phase of 97 % water / 3% acetonitrile / 0.1 % TFA at a flow rate of 1mL/minute. HRMS (FAB) Calcd. for $C_{17}H_{24}N_4O_{11}$: 460.15198. Observed m/z 460.15084.

3-*cis*-Carboxyoxiran-2-carbonyl-L-aspartyl-L-valine (HOOC-*cis*-EP-CO-Asp-Val-OH) was synthesized using solid phase synthesis; white solid, yield 87 %. 1H NMR (DMSO- d_6): 0.91 (d, 6H, Val), 2.03 (m, 1H, Val), 2.60 (m, 2H, Asp), 3.72 (m, 2H, ep), 4.03 (m, 1H, α -H), 4.63 (m, 1H, α -H), 7.65 (d, $\frac{1}{2}$ H, NH), 7.84 (d, $\frac{1}{2}$ H, NH), 8.10 (d, $\frac{1}{2}$ H, NH), 8.33 (d, $\frac{1}{2}$ H, NH). HRMS (ESI) Calcd. for $C_{13}H_{18}N_2O_9$: 346.10906 Observed m/z : 346.10876. Anal. Calcd. for $C_{13}H_{18}N_2O_9 \cdot 0.76 H_2O \cdot 0.49(CH_3CH_2)_2O$: C, 45.34; H, 6.21; N, 7.07 Found: C, 45.35; H, 6.05; N, 6.98.

3-*cis*-Carboxyoxiran-2-carbonyl-D-aspartyl-D-valine (HOOC-*cis*-EP-CO-D-Asp-D-Val-OH) was synthesized using solid phase synthesis; white solid, yield 87 %. 1H NMR (DMSO- d_6): 0.91 (d, 6H, Val), 2.03 (m, 1H, Val), 2.60 (m, 2H, Asp), 3.72 (m, 2H, ep), 4.03 (m, 1H, α -H), 4.63 (m, 1H, α -H), 7.84 (d, 1 H, NH), 8.10 (d, $\frac{1}{2}$ H, NH), 8.33 (d, $\frac{1}{2}$ H, NH). HRMS (ESI) Calcd. for $C_{13}H_{18}N_2O_9$: 346.10906 Observed m/z : 346.10901. Anal. Calcd. for $C_{13}H_{18}N_2O_9 \cdot 1H_2O \cdot 0.43(CH_3CH_2)_2O$: C, 44.63; H, 6.18; N, 7.07 Found: C, 44.62; H, 6.23; N, 7.24.

3-*trans*-Ethoxycarbonyloxiran-2-carbonyl-L-aspartyl-L-valine (EtOOC-*trans*-EP-CO-Asp-Val-OH) was synthesized using solid phase synthesis; white solid, yield 82 %. 1H NMR (DMSO- d_6): 0.91 (d, 6H, Val), 1.20 (t, 3H, OCH_2CH_3), 2.03 (m,

1H, Val), 2.60 (m, 2H, Asp), 3.55 (d, 1H, ep), 3.65 (s, 1H, ep), 4.06 (m, 1H, α -H), 4.13 (q, 2H, OCH₂CH₃), 4.65 (m, 1H, α -H), 7.97 (d, 1H, NH), 8.70 (dd, 1H, NH). HRMS (ESI) Calcd. for C₁₅H₂₂N₂O₉: 374.14036 Observed *m/z*: 374.13850. Anal. Calcd. for C₁₅H₂₂N₂O₉·0.85 H₂O·0.41TFA: C, 43.54; H, 5.57; N, 6.42 Found: C, 43.54; H, 5.62; N, 6.60.

3-*trans*-Carboxyoxiran-2-carbonyl-L-aspartyl-L-valine (HOOC-*trans*-EP-CO-Asp-Val-OH) was synthesized using solid phase synthesis; white solid, yield 79 %. ¹H NMR (DMSO-*d*₆): 0.91 (d, 6H, Val), 2.01 (m, 1H, Val), 2.60 (m, 2H, Asp), 3.43 (d, 1H, ep), 3.60 (s, 1H, ep), 4.08 (m, 1H, α -H), 4.65 (m, 1H, α -H), 7.92 (m, 1H, NH), 8.65 (m, 1H, NH). HRMS (ESI) Calcd. for C₁₃H₁₈N₂O₉: 346.10906 Observed *m/z*: 346.10830. Anal. Calcd. for C₁₃H₁₈N₂O₉·1.39H₂O·0.16EtOAc: C, 42.22; H, 5.80; N, 7.30 Found: C, 42.22; H, 5.57; N, 7.02.

3-(2*S*,3*S*)-Ethoxycarbonyloxiran-2-carbonyl-L-aspartyl-L-valine (EtOOC-(*S,S*)-EP-CO-Asp-Val-OH) was synthesized using solid phase synthesis; white solid, yield 78 %. ¹H NMR (DMSO-*d*₆): 0.91 (d, 6H, Val), 1.20 (t, 3H, OCH₂CH₃), 2.04 (m, 1H, Val), 2.60 (m, 2H, Asp), 3.56 (d, 1H, ep), 3.68 (s, 1H, ep), 4.06 (m, 1H, α -H), 4.14 (q, 2H, OCH₂CH₃), 4.68 (m, 1H, α -H), 7.97 (d, 1H, NH), 8.65 (d, 1H, NH). HRMS (ESI) Calcd. for C₁₅H₂₂N₂O₉: 374.14036 Observed *m/z*: 374.14036. Anal. Calcd. for C₁₅H₂₂N₂O₉·0.80H₂O·0.19(CH₃CH₂)₂O: C, 46.99; H, 6.38; N, 6.95 Found: C, 46.99; H, 6.30; N, 6.96.

3-*cis*-Ethoxycarbonyloxiran-2-carbonyl-L-aspartyl-L-valine (EtOOC-*cis*-EP-CO-Asp-Val-OH) was synthesized using solid phase synthesis; white solid, yield 90 %. ¹H NMR (DMSO-*d*₆): 0.91 (d, 6H, Val), 1.15 (t, 3H, OCH₂CH₃), 2.03 (m, 1H, Val), 2.60

(m, 2H, Asp), 3.75 (dd, 1H, ep), 3.90 (d, 1H, ep), 4.10 (m, 3H, α -H and OCH_2CH_3), 4.64 (m, 1H, α -H), 7.67 (d, $\frac{1}{2}$ H, NH), 7.90 (d, $\frac{1}{2}$ H, NH), 8.25 (d, $\frac{1}{2}$ H, NH), 8.43 (d, $\frac{1}{2}$ H, NH). MS (FAB) m/z 375.1 $[(M + 1)^+]$. Anal. Calcd. for $\text{C}_{15}\text{H}_{22}\text{N}_2\text{O}_9 \cdot 0.27\text{H}_2\text{O}$: C, 47.51; H, 5.99; N, 7.39 Found: C, 47.70; H, 6.29; N, 7.31.

3-(2*S*,3*S*)-Ethoxycarbonyloxiran-2-carbonyl-L-aspartyl-L-valyl-L-glutamyl-L-aspartic Acid (EtOOC-(2*S*,3*S*)-EP-CO-Asp-Val-Glu-Asp-OH) was synthesized using solid phase synthesis; white solid, yield 80 %. ^1H NMR ($\text{DMSO}-d_6$): 0.91 (d, 6H, Val), 1.21 (t, 3H, OCH_2CH_3), 1.72 (m, 1H, Val), 1.90 (dm, 2H, Glu), 2.22 (t, 2H, Glu), 2.60 (m, 2H, Asp), 3.56 (d, 1H, ep), 3.63 (s, 1H, ep), 4.08 (q, 3H, α -H and OCH_2CH_3), 4.24 (m, 1H, α -H), 4.50 (m, 1H, α -H), 4.63 (m, 1H, α -H), 7.80 (t, 1H, NH), 7.98 (t, 1H, NH), 8.10 (d, 1H, NH), 8.70 (dd, 1H, NH). HRMS (FAB) Calcd. for $\text{C}_{24}\text{H}_{33}\text{N}_4\text{O}_{15}\text{Na}$: 640.19184 Observed m/z : 640.19209. Anal. Calcd. for $\text{C}_{24}\text{H}_{34}\text{N}_4\text{O}_{15} \cdot 0.80\text{H}_2\text{O} \cdot 0.68\text{TFA} \cdot 1.05(\text{CH}_3\text{CH}_2)_2\text{O}$: C, 45.04; H, 5.98; N, 7.11 Found: C, 45.04; H, 5.93; N, 6.83.

Enzyme Assays

Caspase-3, -6, and -8. Most of the inhibitors were tested with caspases-3, -6, and -8 by Jowita Mikolajczyk in the laboratory of Guy Salvesen at the Burnham Institute, La Jolla, CA. Caspases-3, -6, and -8 were expressed in *E. coli* and purified according to the methods previously described by Stennicke and Salvesen.¹⁴⁵ Assays using the fluorogenic substrate Cbz-DEVD-AFC ($\lambda_{\text{ex}} = 400$ nm, $\lambda_{\text{em}} = 505$ nm) were carried out on a Molecular DevicesfMax fluorescence microplate reader at 37 °C. Inhibition rates were determined by the progress curve method described by Tian and Tsou.⁷ This method is suitable for measuring irreversible inhibition rates with fast inhibitors, where the

inhibitor, the substrate and the enzyme are incubated together and the rate of substrate hydrolysis is measured continuously. The standard 100 μ L reaction was started by adding enzyme to a mixture of substrate (final concentration of Cbz-DEVD-AFC 100 μ M) and various amounts of inhibitor in 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 caspase were preactivated for 10 minutes at 37 $^{\circ}$ C in the presence of 10 mM DTT.

Cathepsin B. Irreversible kinetic assays were performed by the incubation method with human liver cathepsin B. Enzymatic activities of cathepsin B were measured in 0.1 M KHPO_4 , 1.25 mM EDTA, 0.01% Brij, pH 6.0 buffer and at 23 $^{\circ}$ C using Cbz-Arg-Arg-AMC as the substrate. To a freshly prepared enzyme stock solution of cathepsin B (approximately 6.98×10^{-3} μ g/ μ L) in enzyme buffer containing DTT (0.1 mM) was added an additional 300 μ L of enzyme buffer and 30 μ L of a stock inhibitor solution in DMSO. At various time intervals 50 μ L aliquots were withdrawn from the incubation mixture and added to 200 μ L enzyme buffer containing Cbz-Arg-Arg-AMC (500 μ M). Substrate hydrolysis was monitored using a Tecan Spectra Fluor microplate reader ($\lambda_{\text{ex}} = 360$ nm, $\lambda_{\text{em}} = 465$ nm). Pseudo first-order rate constants (k_{obs}) were obtained from plots of $\ln v_t/v_0$ versus time.

Papain. Irreversible kinetic assays were performed by the incubation method with papaya latex papain. An enzyme stock solution for the papain assays was freshly prepared from 330 μ L of enzyme storage solution (1.19 mg/ml) diluted with 645 μ L papain buffer (50 mM Hepes, and 2.5 mM EDTA at pH 7.5) and 25 μ L of DTT (0.1 M). The incubation mixture was prepared from 300 μ L buffer, 30 μ L of inhibitor stock solution (DMSO), and 30 μ L of enzyme stock solution. At various times aliquots (50 μ L)

were withdrawn and added to 200 μ L of Cbz-Phe-Arg-pNA substrate in buffer (53.7 μ M). The absorbance of released *p*-nitroaniline was monitored at 405 nm on a Molecular Devices Thermomax plate reader.

CHAPTER 3

AZA-PEPTIDE EPOXIDE INHIBITORS FOR CASPASES

Introduction

Only a few irreversible inhibitors have been investigated with caspases and these include peptide halomethyl ketones (peptide-COCH₂X, X = Cl or F), diazomethyl ketones (peptide-CHN₂), and acyloxymethyl ketones (peptide-COCH₂OCOR). Although these classes of inhibitors inhibit caspases very potently, it has been recently shown that they were not as specific for caspases as previously hoped. Peptide fluoromethyl ketones and chloromethyl ketones designed with caspase-specific sequence efficiently inhibit other cysteine proteases such as cathepsins, papain, and legumain.⁹³

In an effort to synthesize potent and selective irreversible inhibitors for caspases, our laboratory was also focused on another new class of epoxide incorporated inhibitor design. We refer to this novel class of inhibitors as aza-peptide epoxides.

Aza-peptide epoxides were designed based on an extended substrate structure with the placement of the carbonyl group of the epoxide moiety in a location identical to that of the carbonyl of the scissile peptide bond (Figure 20). Replacement of the α -carbon of the amino acid residue at P1 with a nitrogen results in the formation of an aza-peptide, which allows the ready synthesis of a variety of derivatives. There is no change in the overall polarity of the molecule and aligning of the side chain groups. This manipulation decreases the electrophilicity of the P1 carbonyl and causes the loss of the chirality at the α -position. As a result, the geometry around the nitrogen atom changes from tetrahedral to trigonal planar. This is a configuration that is between D- and L-

configurations of amino acids. Therefore, some aza-peptides are more resistant to the hydrolysis by proteases compared to their parent peptide analogs.

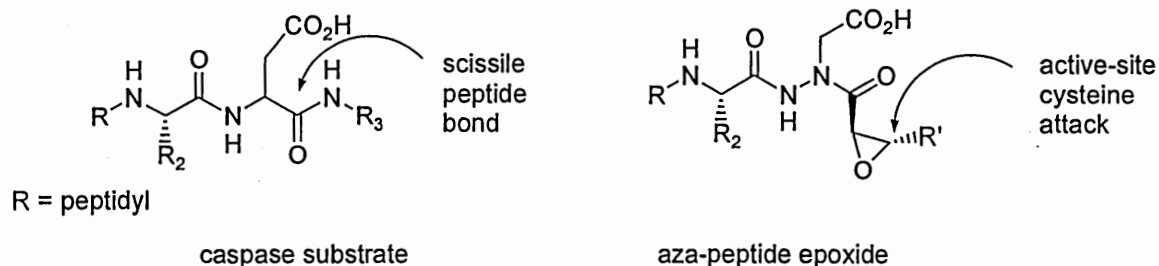


Figure 20. Aza-peptide Epoxide Design

Aza-peptide epoxides and epoxysuccinyl peptides have considerable differences in their designs (Figure 21). The epoxide moiety in the epoxysuccinyl peptide design is attached to the peptide sequence from the N-terminal, in the hopes of making interactions in a reverse fashion at the active site. The epoxide moiety in the aza-peptide epoxide design on the other hand, is attached to the aza-amino acid residue at the C-terminal so that the binding at the active site mimics the substrate binding mode. The major structural difference between the two designs is that the carbonyl group in the aza-peptide epoxide design that is equivalent to the scissile carbonyl in the substrate structure is a part of the aza-amino acid residue, whereas in the epoxysuccinyl peptide design the equivalent carbonyl is a part of the epoxysuccinate moiety.

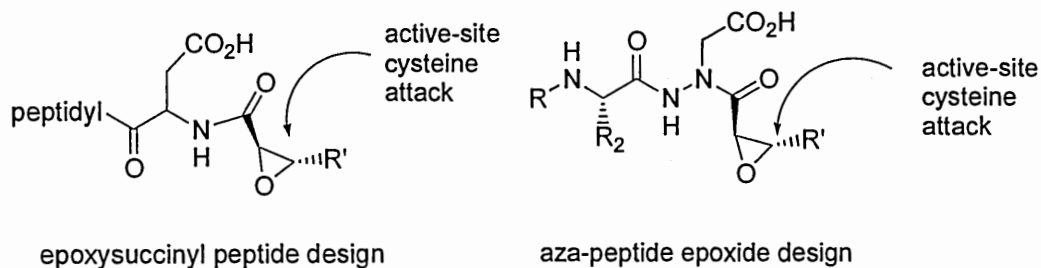


Figure 21. Comparison of the Two Designs of Inhibitors

We hypothesized that the aza-peptide epoxide binding mode would be opposite that of E-64 and E-64 derivatives. As discussed in Chapter 2, E-64 and its derivatives bind in the reverse direction, relative to the substrate binding mode, in the active site of papain.

Nomenclature

The aza-amino acid residue in a peptide sequence will be abbreviated using four letters starting with the letter 'A' followed by the three letter amino acid abbreviation. For example, the aza-aspartic acid residue will be abbreviated as AAsp. The nomenclature and stereochemistry of the epoxide moiety in the aza-peptide epoxides will be the same as that of epoxysuccinyl peptides as discussed in Chapter 2, page 32.

Crystal Structure and Mechanism

Aza-peptide epoxides were first synthesized by Z. Li in our laboratory with support from BASF. These compounds were specifically designed to target caspase-1 by utilizing the PhPr-Val-Ala-Asp truncated sequence, which is a good substrate for caspase-1. The stereochemistry of the epoxide moiety in both structures was *trans*. The two inhibitors **I** and **II** were potent irreversible inhibitors of caspase-1 (Figure 22).

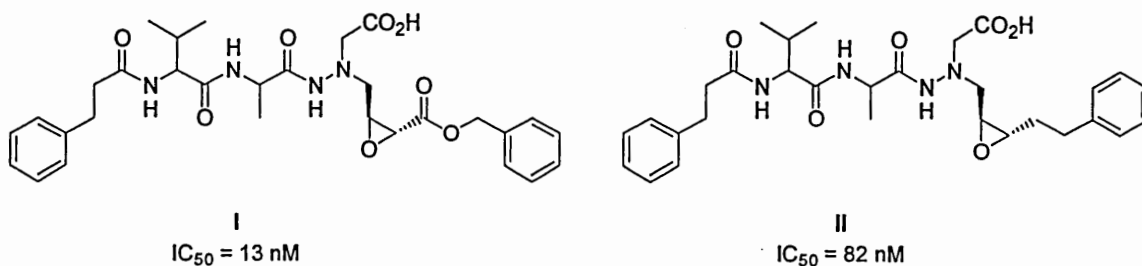


Figure 22. Aza-peptide Epoxides for Caspase-1

X-ray crystal structures of caspase-1 inhibited by **I** and **II** have been determined by Ron Rubin (Parke-Davis, now Pfizer, unpublished results). A schematic representation of the interactions between the inhibitor and the enzyme's active site is illustrated below (Figure 23).

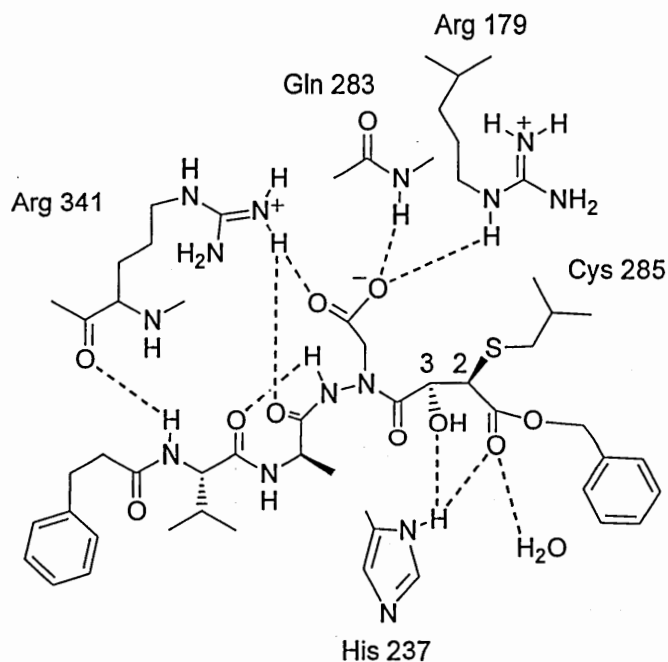


Figure 23. Inhibition of Caspase-1 by PhPr-Val-Ala-AAsp-*trans*-EP-CO₂Bzl

The active site nucleophile Cys 285 thiolate attacks the C-2 carbon of the epoxide ring resulting in a thioester bond between the enzyme and the inhibitor. Our initial

expectation for the site of nucleophilic attack was the C-3 carbon of the epoxide ring. With previously designed transition-state inhibitors for caspases, such as peptide aldehydes, the site of nucleophilic attack is the aldehyde carbonyl, which is equivalent to the scissile peptide carbonyl group in a substrate. However, the position of the attack by the active site cysteine with our inhibitor clearly indicates that the nucleophilic attack at chemical warhead groups can take place quite distant from the P1 aza-amino acid residue. In the light of the crystal structural data, the mechanism of inhibition of caspases by aza-peptide epoxides is proposed as illustrated in the following figure (Figure 24).

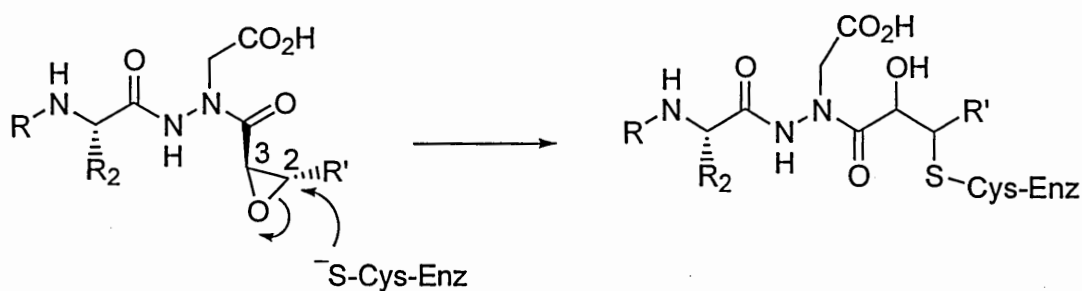


Figure 24. Mechanism of Inhibition of Caspases by Aza-peptide Epoxides

Synthesis

The synthesis of aza-peptide epoxides was achieved by coupling the two components, the substituted peptidyl hydrazide and the epoxide moiety, which are synthesized separately.

Peptidyl hydrazides were obtained from dipeptidyl methyl esters by reacting them with excess hydrazine. The subsequent introduction of the aza-amino acid side chain was accomplished by alkylation. According to the caspase specificity for Asp residue at P1, the Asp side chain was obtained by alkylation with *t*-butyl bromoacetate to form the substituted hydrazide (Figure 25).

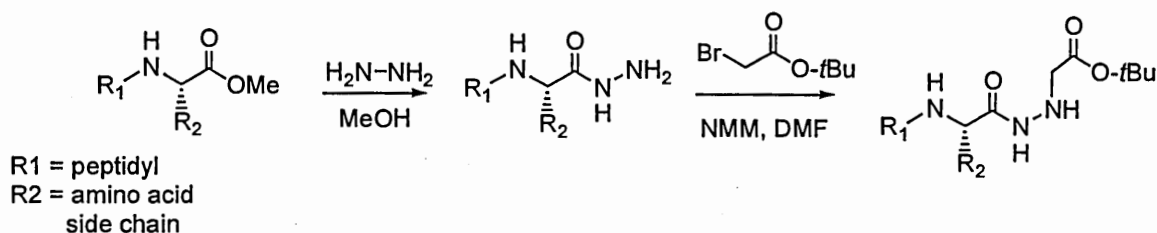


Figure 25. Synthesis of Substituted Hydrazine

Enantiomerically pure diethyl epoxysuccinate ester (2S,3S) was synthesized from diethyl D-(-)-tartrate, following the general method developed by Mori and Iwasawa (Figure 26a).¹⁴⁶ The selective hydrolysis of one ester to yield the monoethyl epoxysuccinate was accomplished using 1 eq of KOH in ethanol. *Trans*-3-phenethyloxirane-2-carboxylic acid was synthesized utilizing the Doebner modification, starting with 3-phenylpropionaldehyde and malonic acid to form the α,β -unsaturated acid, which was then transformed into the ethyl ester. The double bond was epoxidized using *t*-butyl peroxide and *t*-butyl lithium.¹⁴⁴ The ethyl ester was then hydrolyzed using NaOH in ethanol to yield *trans*-3-phenethyloxirane-2-carboxylic acid (Figure 26b). *Trans*-3-(4-chlorophenyl)-oxirane-2-carboxylic acid methyl ester was epoxidized using the same method followed by deblocking of the methyl ester using NaOH in methanol to yield *trans*-3-(4-chlorophenyl)-oxirane-2-carboxylic acid (Figure 26c).

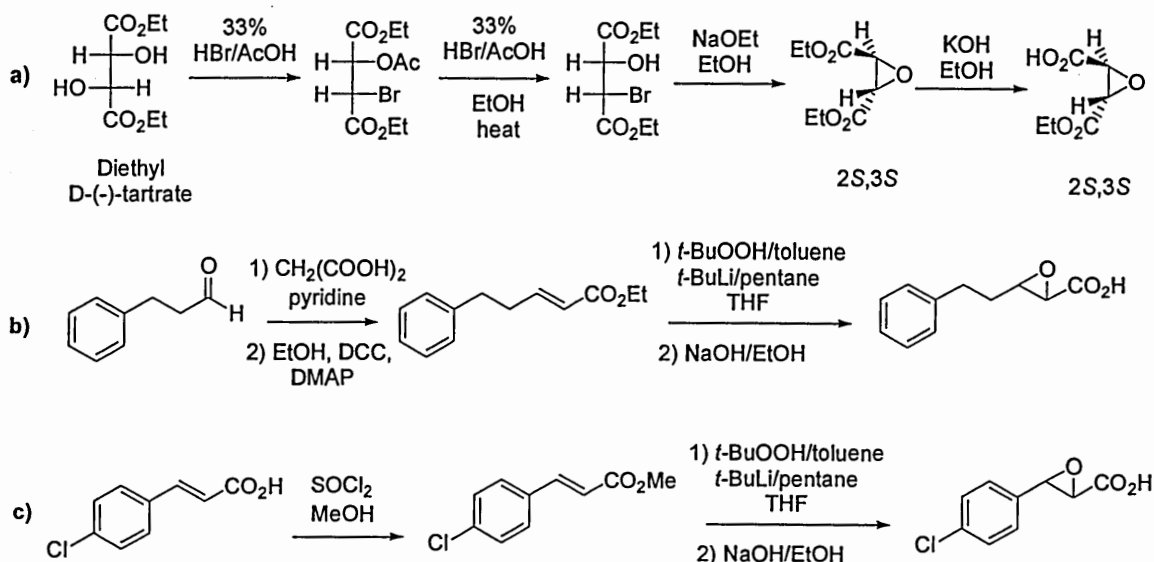


Figure 26. Synthesis of the Epoxide Moiety

The substituted hydrazides were coupled to the corresponding epoxide moieties using the EDC/HOBt coupling method. The final aza-peptide epoxides were then obtained by deblocking the *t*-butyl protective group with TFA in methylene chloride (Figure 27). The aza-peptide epoxide Cbz-Val-AAsp-(2S,3S)-EP-COOH was obtained from Cbz-Val-AAsp-(2S,3S)-EP-COOEt by deblocking the ethyl ester with NaOH in methanol as the last step.

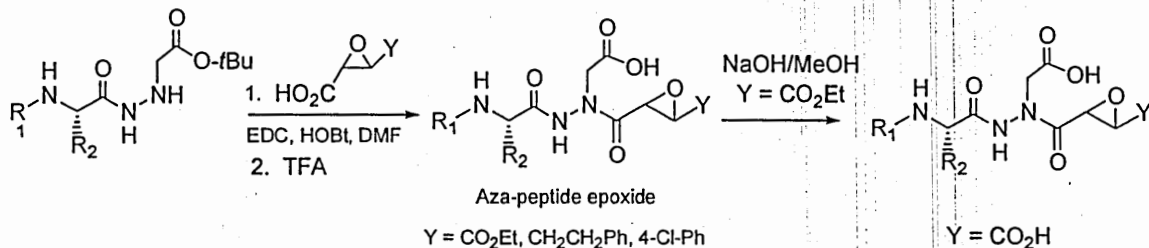


Figure 27. Coupling of the Substituted Hydrazide with the Epoxide Moiety

Stability Studies

Aza-peptide epoxides exhibit considerable stability in buffer solutions. One inhibitor, Cbz-Leu-Glu-Thr-AAsp-(*S,S*)-EP-CO₂Bzl designed for caspase-8 in our laboratory, was incubated in the caspase assay buffer containing DTT (50 mM Hepes, 100 mM NaCl, 0.1% (w/v) CHAPS, 10% (w/v) sucrose, 10 mM DTT, at pH 7.2) for 32 minutes. The substrate (Ac-DEVD-AMC, 100 μ M) and the enzyme (caspase-6, 10 nM) were added and the progress of the reaction was monitored. The inhibitor was equally potent as when not incubated with DTT.

Epoxysuccinyl peptide inhibitors reported in the literature are stable toward simple thiols under physiological conditions. It has been shown that E-64 does not react with 100 mM cysteine nor does it inactivate lactate dehydrogenase, a non-proteolytic thiol-dependent enzyme.

Results and Discussion

Aza-peptide epoxides are potent and specific inhibitors of caspases. Our laboratory has successfully synthesized a variety of aza-peptide epoxides targeting caspases-3, -6, and -8.¹⁴⁷ The goal of this particular project was to design a general caspase inhibitor that would target all caspases equally in potency (Figure 28). Although previous irreversible inhibitors such as fluoromethyl ketones and chloromethyl ketones are potent inhibitors of all the caspases (caspases-1,-3,-6,-7,and -8), their lack of selectivity toward other cysteine proteases makes them unsuitable for the studies of cell and animal models of apoptosis, as well as for future therapeutic utility.

The Cbz-Val-Asp dipeptide sequence is chosen based on the previous caspase substrate studies reported in the literature.^{61,148} Caspases have a specificity for four amino acids to the left of the cleavage site. The S4 subsite is the most important determinant of specificity among caspases after the P1 Asp residue. Group II caspases such as caspase-3, prefer Asp residue at P4 position. Group III caspases such as caspase-6 and -8 prefer branched, aliphatic side chains at P4. We eliminated the P4 amino acid completely in order to obtain a broad spectrum activity. We also eliminated the P3 amino acid Glu, in order to minimize the number of charged residues of the inhibitor so that it would become more suitable for cell and animal studies. It is clear that higher inhibition rates can be obtained with individual caspases when chemical warhead groups would be attached to their optimum tetrapeptide sequences. We hypothesized that caspases would still be inhibited by a dipeptidyl aza-peptide epoxide inhibitor potently, and that inhibitor would be a general inhibitor.

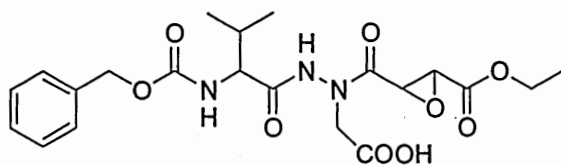
We have synthesized four dipeptidyl aza-epoxide inhibitors. The inhibition rates are listed in Table 6. Truncation of the peptide portion to the dipeptide Cbz-Val-AAsp resulted in a loss of caspase inhibitory potency as expected. In general, the inhibitors with *S,S* stereochemistry at the epoxide ring are moderate inhibitors of caspase-3, and weak inhibitors of caspases-6 and -8. The inhibitor Cbz-Val-AAsp-(*S,S*)-EP-CO₂Et is the most potent dipeptidyl aza-peptide epoxide with a second order rate constant of 3,650 M⁻¹s⁻¹ for caspase-3 and 2,750 M⁻¹s⁻¹ for caspase-6. The free carboxylic acid analog Cbz-Val-AAsp-(*S,S*)-EP-CO₂H is about 3 fold less potent than its ester analog for all caspases. Cbz-Val-AAsp-*trans*-EP-CH₂CH₂Ph is a weak inhibitor, suggesting that the *S,S* stereochemistry is an important factor affecting the inhibition rates. Cbz-Val-AAsp-

trans-EP-4-Ph-Cl inhibits caspase-3 very weakly, and does not inhibit caspases-6 and -8 at all. This result provides additional evidence for the mechanism of inhibition, where the active site cysteine attacks the C-2 carbon of the epoxide ring. The phenyl group adjacent to the epoxide ring probably made the inhibitor structure more rigid and site of nucleophilic attack less accessible.

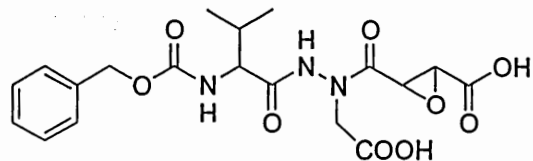
Table 6. Inhibition of Caspases by Aza-peptide Epoxide Inhibitors

Inhibitor	EP ^a	$k_{\text{obs}}/[\text{I}] \text{ (M}^{-1}\text{s}^{-1}\text{)}$		
		Caspase-3	Caspase-6	Caspase-8
Cbz-Val-AAsp-EP-COOH	<i>S,S</i>	1,110	695	270
Cbz-Val-AAsp-EP-COOEt	<i>S,S</i>	3,650	2,750	905
Cbz-Val-AAsp-EP-CH ₂ CH ₂ Ph	<i>trans</i>	310	350	130
Cbz-Val-AAsp-EP-4-Ph-Cl	<i>trans</i>	146	NI	NI

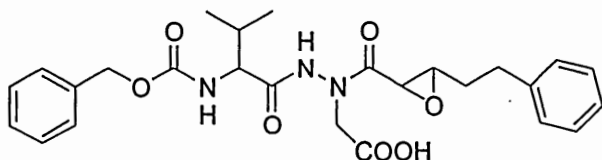
EP = epoxide, *trans* = racemic mixture of *S,S* and *R,R* epoxide, ^a = The stereochemistry at the epoxide, Cbz = Ph-CH₂-CO-, AAsp = aza-Asp, NI = no inhibition



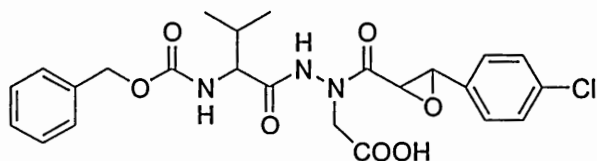
Cbz-Val-AAsp-(S,S)-EP-COOEt



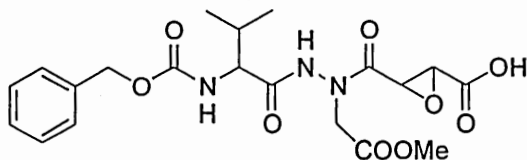
Cbz-Val-AAsp-(S,S)-EP-COOH



Cbz-Val-AAsp-trans-EP-CH₂CH₂Ph



Cbz-Val-AAsp-trans-EP-Ph-4-Cl



Cbz-Val-AAsp(OMe)-(S,S)-EP-COOH

Figure 28. Dipeptidyl Aza-peptide Epoxides for Caspases

Experimental Procedures

Material and Methods. 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 ^1H NMR spectra were obtained using a Varian Mercury Vx 400 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. Caspases-3, -6 and -8 were expressed in *E. coli* and purified according to methods previously described by Stennicke and Salvesen.¹⁴⁵ Enzymes and substrates were obtained from G. Salvesen. The fluorogenic substrate Ac-DEVD-AMC was purchased from Bachem Bioscience Inc., King of Prussia, PA.

Peptidyl Hydrazides. Anhydrous hydrazine (10 eq) was added to a stirred solution of a peptidyl methyl ester (1 eq) in MeOH and the reaction mixture was stirred vigorously for 16 h at room temperature. Excess hydrazine and solvent were removed under vacuum and the residue was transferred to water and extracted from EtOAc, dried over Na_2SO_4 to give the peptidyl hydrazide as a white solid. Cbz-Val-NHNH₂, white solid, yield 92 %. ^1H NMR ($\text{DMSO}-d_6$): 0.90 (t, 6H, Val), 1.86 (m, 1H, Val), 3.77 (t, 1H, α -H), 4.20 (s, 2H, NH₂), 4.99 (s, 2H, Cbz), 7.30 (s, 5H, Ph), 9.08 (d, 1H, NH)

Peptidyl AA₂-NH-NH-CH₂COO*t*Bu. To a stirred solution of the peptidyl hydrazide (1 eq) in DMF, NMM (1.1 eq) and neat *t*-butyl bromoacetate (1.1 eq) were added dropwise at -15 °C and the mixture was allowed to react for 16 h at room temperature. The solvent was removed under vacuum and the residue was dried *in*

vacuo. Cbz-Val-NH-NH-CH₂COO*t*Bu was purified by column chromatography on silica gel using 1:20:4.2 MeOH:CH₂Cl₂:EtOAc as the eluent; white solid, yield 64 %. ¹H NMR (DMSO-d₆): 0.90 (t, 6H, Val), 1.40 (s, 9H, *t*Bu), 1.86 (m, 1H, Val), 3.37 (d, 2H, NHCH₂COOH), 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)

Peptidyl AA₂-NH-NH-CH₂COOMe. To a stirred solution of the peptidyl hydrazide (1 eq) in DMF, NMM (1.1 eq) and neat methyl bromoacetate (1.1 eq) were added dropwise at -15 °C and the mixture was allowed to react for 16 h at room temperature. The solvent was removed under vacuum and the residue was dried *in vacuo*. Cbz-Val-NH-NH-CH₂COOMe was purified by column chromatography on silica gel using 1:19:20 MeOH:CH₂Cl₂:EtOAc as the eluent; white solid, yield 69 %. ¹H NMR (CDCl₃): 0.93 (m, 6H, Val), 2.08 (m, 1H, Val), 3.65 (s, 2H, NHCH₂COOMe), 3.76 (s, 3H, OCH₃), 3.90 (t, 1H, α-H), 5.08 (s, 2H, Cbz), 5.29 (d, 1H, NH), 7.30 (s, 5H, Ph), 7.77 (d, 1H, NH).

Epoxide Synthesis

***trans*-3-Phenethyloxirane-2-carboxylic Acid (HOOC-EP-CH₂CH₂Ph).** This epoxide was synthesized from the precursor 5-phenyl-pent-2-enoic acid ethyl ester, which was a gift from J. Asgian in our laboratory. 5-Phenyl-pent-2-enoic acid ethyl ester was epoxidized using the general procedure for the stereocontrolled epoxidation of α,β-unsaturated compounds with lithium *t*-butyl hydroperoxide, which was similar to the method developed by Meth-Cohn.¹⁴⁴ The purification on a silica gel column with 1:5 EtOAc:hexane as the eluent afforded *trans*-3-phenethyloxirane-2-carboxylic acid ethyl

ester as a yellow oil (yield = 51 %). ^1H NMR (CDCl_3): 1.25 (t, 3H, OCH_2CH_3), 1.90 (t, 2H, $\text{CH}_2\text{CH}_2\text{Ph}$), 2.80 (t, 2H, $\text{CH}_2\text{CH}_2\text{Ph}$), 3.21 (s, 2H, epoxy), 4.20 (q, 2H, OCH_2CH_3), 7.32 (m, 5H, Ph).

A solution of 1 M NaOH (1.1 eq) was added to a stirred solution of *trans*-3-phenethyloxirane-2-carboxylic acid ethyl ester (1 eq) in methanol (12 mL) at 0 °C. After stirring for 45 minutes at 0 °C, and 90 minutes at room temperature, the solution was acidified to pH 3 with 1 N HCl, and extracted with EtOAc (2 x 30 mL). The organic layer was washed with water (1 x 30 mL), saturated NaCl (1 x 30 mL), dried over Na_2SO_4 , and concentrated to give *trans*-3-phenethyloxirane-2-carboxylic acid as a yellow oil (yield = 76 %). ^1H NMR (CDCl_3): 1.90 (t, 2H, $\text{CH}_2\text{CH}_2\text{Ph}$), 2.80 (t, 2H, $\text{CH}_2\text{CH}_2\text{Ph}$), 3.30 (s, 2H, epoxy), 7.32 (m, 5H, Ph).

***trans*-3-(4-Chlorophenyl)oxirane-2-carboxylic Acid (HOOC-EP-Ph-Cl).** This compound was synthesized by M. Götz in our laboratory.

Aza-peptide Epoxides

Cbz-Val-AAsp(*t*Bu)-(2*S*,3*S*)-EP-CO₂Et was obtained using the EDC/HOBt coupling method, and purified using 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)⁺]. ^1H NMR (CDCl_3): 0.95-1.02 (m, 6H, Val), 1.28 (t, 3H, OCH_2CH_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(*t*Bu)-*trans*-EP-CH₂CH₂Ph was obtained using the EDC/HOBt coupling method, and purified using column chromatography on silica gel with 1:50:51

MeOH:CH₂Cl₂:EtOAc as the eluent; white solid, yield 48 %. HRMS (FAB) calcd. for C₃₀H₃₉N₃O₇: 553.27878 Observed *m/z* 553.28663. ¹H NMR (CDCl₃): 0.90-1.04 (d, 6H, Val), 1.46 (s, 9H, *t*Bu), 1.78-1.96 (d, 2H, CH₂CH₂Ph), 2.00-2.14 (m, 1H, Val), 2.66-2.87 (m, 2H, CH₂CH₂Ph), 3.16 (t, 1H, epoxy), 3.53 (s, 1H, NHCH₂COOH), 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(*t*Bu)-*trans*-EP-4-Cl-Ph was obtained using the EDC/HOBt coupling method, and purified using column chromatography on silica gel with 1:100:100 MeOH:CH₂Cl₂:EtOAc as the eluent; white solid, yield 51 %. MS (ESI) *m/z* 560.4 [(M + 1)⁺]. ¹H 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, NHCH₂COOH), 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-Val-AAsp(OMe)-(2*S*,3*S*)-EP-CO₂Et was obtained using the EDC/HOBt coupling method, and purified using column chromatography on silica gel with 1:19:20 MeOH:CH₂Cl₂:EtOAc as the eluent; white solid, yield 56 %. MS (ESI) *m/z* 480.2 [(M + 1)⁺]. ¹H NMR (CDCl₃): 0.84 (m, 6H, Val), 1.16 (t, 3H, OCH₂CH₃), 1.94 (m, 1H, Val), 3.52 (s, 1H, epoxy), 3.65 (s, 3H, OCH₃), 3.82 (t, 1H, α-H), 4.09 (q, 2H, OCH₂CH₃), 5.00 (m, 2H, Cbz), 7.33 (s, 5H, Ph), 7.57 (d, 1H, NH), 11.08 (s, 1H, NH).

Deblocking of the *t*-Butyl Group in the Aza-Asp Peptides

(2*S*,3*S*)-3-(N²-(N-Benzyloxycarbonylvalyl)-N¹-carboxymethylhydrazinocarbonyl)oxirane-2-carboxylic Acid Ethyl Ester (Z-Val-AAsp-(2*S*,3*S*)-EP-CO₂Et). ¹H NMR (CDCl₃): 0.98 (d, 6H, Val), 1.30 (t, 3H, OCH₂CH₃), 2.15 (m, 1H, Val), 3.65 (s, 1H, epoxy), 3.90 (s, 1H, NHCH₂COOH), 4.10 (m,

1H, α -H), 4.22 (q, 2H, OCH₂CH₃), 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 C₂₁H₂₇N₃O₉: 465.1747. Observed *m/z* 465.1825. Anal. Calcd. for C₂₁H₂₇N₃O₉·1.5H₂O: C, 51.21; H, 5.52; N, 8.53. Found: C, 51.26; H, 5.69; N, 8.52.

(2*S*,3*S*)-3-(N²-(N-Benzylloxycarbonylvalyl)-N¹-carboxymethylhydrazinocarbonyl)oxirane-2-carboxylic Acid (Z-Val-AAsp-(2*S*,3*S*)-EP-CO₂H). ¹H 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 C₁₉H₂₃N₃O₉: 437.1513 Observed *m/z* 437.1493. Anal. Calcd. for C₁₉H₂₃N₃O₉·0.21H₂O·0.19TFA: C, 50.29; H, 5.14; N, 9.08. Found: C, 50.32; H, 5.39; N, 8.78.

***trans*-2-(N²-(N-Benzylloxycarbonylvalyl)-N¹-carboxymethylhydrazinocarbonyl)-3-(2-phenethyl)oxirane (Z-Val-AAsp-*trans*-EP-CH₂CH₂Ph).** ¹H NMR (CDCl₃): 0.90-0.95 (d, 6H, Val), 1.90 (m, 2H, CH₂CH₂Ph), 2.05 (m, 1H, Val), 2.60-2.90 (m, 2H, CH₂CH₂Ph), 3.14 (m, 1H, epoxy), 3.86 (m, 1H, epoxy), 4.01-4.08 (m, 3H, α -H and NHCH₂COOH), 4.53 (m, 1H, NH), 5.01-5.13 (d, 2H, Cbz), 7.08-7.20 (m, 10H, Ph). HRMS (FAB) calcd. for C₂₆H₃₁N₃O₇: 497.2162 Observed *m/z* 497.2240. Anal. Calcd. for C₂₆H₃₁N₃O₇·H₂O: C, 60.57; H, 6.06; N, 8.15. Found: C, 60.70; H, 6.11; N, 7.93.

***trans*-2-(N²-(N-Benzylloxycarbonylvalyl)-N¹-carboxymethylhydrazinocarbonyl)-3-(4-chlorophenyl)oxirane (Z-Val-AAsp-*trans*-EP-4-Cl-Ph).** ¹H 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, NHCH₂COOH), 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.

(2*S*,3*S*)-3-(N^2 -(*N*-Benzyloxycarbonylvalyl)- N^1 -methoxycarbonylmethylhydrazinocarbonyl)oxirane-2-carboxylic Acid (Z-Val-AAsp(OMe)-(2*S*,3*S*)-EP-CO₂H). ¹H NMR (DMSO-*d*₆): 0.86 (t, 6H, Val), 1.96 (m, 1H, Val), 3.41 (s, 1H, epoxy), 3.66 (s, 3H, OCH₃), 3.81 (m, 1H, α-H), 4.93-5.06 (dd, 2H, Cbz), 7.33 (s, 5H, Ph), 7.57 (t, 1H, NH), 11.10 (s, 1H, NH). HRMS (ESI) calcd. for $C_{20}H_{25}N_3O_9$: 451.1669 Observed m/z 451.1652.

Enzyme Assays

Caspase-3, -6, and -8. Assays using the fluorogenic substrate Ac-DEVD-AMC ($\lambda_{ex} = 360$ nm, $\lambda_{em} = 465$ nm) were carried out on a Tecan Spectra Fluor microplate reader at 37 °C. The K_M values for Ac-DEVD-AMC with caspase-3 ($K_M = 9.7$ μM), caspase-6 ($K_M = 236.35$ μM), and caspase-8 ($K_M = 6.79$ μM) were determined in the laboratory of Guy Salvesen. The k_2 values are 11.309-fold higher than the apparent rate for caspase-3 because of the 100 mM [S] and $K_M = 9.7$ μM. The k_2 values are 1.4231-fold higher than the apparent rate for caspase-6 because of the 100 mM [S] and $K_M = 236.35$ μM. The k_2 values are 15.7275-fold higher than the apparent rate for caspase-8 because of the 100 mM [S] and $K_M = 6.79$ μM. Inhibition rates were determined by the progress curve method described by Tian and Tsou.⁷ This method is suitable for measuring irreversible inhibition rates with fast inhibitors, where the inhibitor, the substrate and the enzyme are incubated together and the rate of substrate hydrolysis is

measured continuously.

The concentration of the caspase-3 stock solution was 2 nM in the assay buffer. Assay buffer is a 1:1 mixture of 40 mM Pipes, 200 mM NaCl, 0.2% (w/v) CHAPS, and sucrose 20% (w/v) to 20 mM DTT solution in H₂O at pH 7.2. The concentration of the substrate stock solution was 2 mM in DMSO. The enzyme was pre-activated for 10 min at 37 °C in the assay buffer. The standard 100 µL reaction was started by adding 40 µL of assay buffer, 5 µL of various amounts of inhibitor (stock solution concentrations varied from 5×10^{-3} M to 4.84×10^{-7} M in DMSO), and 5 µL of substrate in DMSO (100 µM final concentration) at 37 °C. 50 µL of 2 nM enzyme stock solution (final concentration: 1 nM) was added to the mixture after 1 min and reading started immediately for 20 min at 37 °C. Inhibition experiments were repeated in duplicate and standard deviations determined.

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

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

CHAPTER 4

AZA-PEPTIDE MICHAEL ACCEPTOR INHIBITORS FOR CASPASES

Introduction

Aza-peptide Michael acceptors are a novel class of irreversible inhibitors for cysteine proteases. Inspired by the success of the aza-peptide epoxide inhibitors designed in our laboratory,¹⁴⁷ we have designed this new class of inhibitors for clan CD cysteine proteases. We have termed this new class of inhibitors “aza-peptide Michael acceptors”.

Aza-peptide Michael acceptors with an aza-Asp residue at P1, are very potent and selective, irreversible inhibitors of caspases-3, -6, and -8. The inhibition rate constants are as high as $3,000,000 \text{ M}^{-1}\text{s}^{-1}$, where selectivity up to 588-fold among the family members is achieved. Aza-peptide Michael acceptors are specific for clan CD enzymes. Like aza-peptide epoxides, they do not inhibit clan CA enzymes such as papain, cathepsin B, and calpain. Selective and specific caspase inhibitors would be invaluable for elucidation of the roles of individual caspases in apoptosis and as potential therapeutic, where currently available inhibitors of caspases exhibit nonspecific inhibition of caspases and other cysteine proteases such as cathepsins.

Clearly, the aza-peptide epoxide design is unique to clan CD cysteine proteases. We hypothesized that by changing the chemical warhead group from an epoxide to a double bond, we would obtain a new class of inhibitors that are specific for clan CD enzymes, particularly caspases. So far, there's no report of Michael acceptor inhibitors for clan CD enzymes in the literature.

Aza-peptide Michael Acceptor Design

The aza-peptide Michael acceptor design is based on an optimal substrate sequence, where the carbonyl group of the scissile bond is replaced by the carbonyl group of the double bond moiety (Figure 29). As in the case with the aza-peptide epoxides, the α -carbon of the P1 amino acid is replaced by a nitrogen atom, which forms the basis for the unique clan CD specific design. For the overall design, the effects of this manipulation such as the loss of chirality at the α -position and decreased electrophilicity of the P1 carbonyl, were expected to be similar to aza-peptide epoxide inhibitors. This replacement also allowed a ready synthesis for a variety of derivatives.

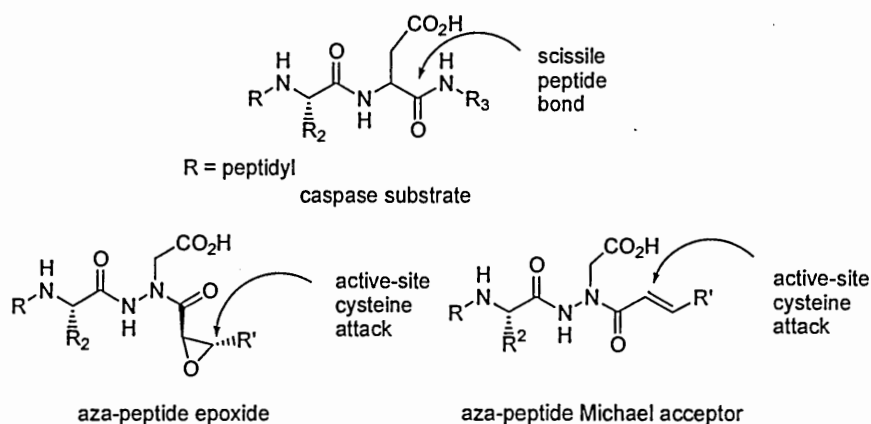


Figure 29. Aza-peptide Michael Acceptor Design

Nomenclature

The aza-amino acid residue in a peptide sequence will be abbreviated using four letters starting with the letter 'A' followed by the three letter amino acid abbreviation. For example, the aza-aspartic acid residue will be abbreviated as AAsp. The stereochemistry of the double bond will be designated as *cis* and *trans*. On the double

bond, C-2 is assigned to the carbon next to the carboxylic acid of the double bond moiety, whereas C-3 is the carbon close to peptide sequence (Figure 30). If the carboxylic acid is esterified or converted into an amide derivative, the numbering remains the same.

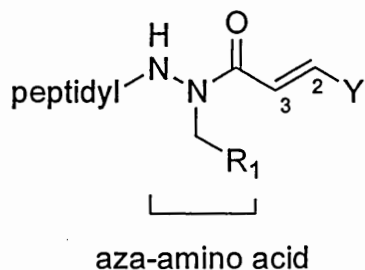
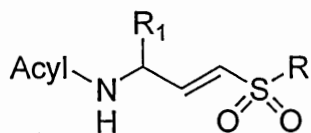


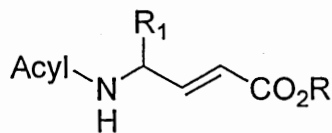
Figure 30. Structure of a General Aza-peptide Michael Acceptor

Other Michael Acceptor Inhibitors

A variety of irreversible inhibitors with a Michael acceptor warhead are reported in the literature for cysteine proteases. This class of inhibitors includes vinyl sulfones and α,β -unsaturated carbonyl derivatives, which are highly potent inhibitors for endopeptidases such as DPPI and many clan CA proteases including papain, cathepsins B, L, S, and K, calpains, and cruzain (Figure 31). They inhibit cysteine proteases by forming covalent bonds with the active site thiol of cysteine proteases. These cysteine protease inhibitors are very successful and have second-order inhibition rates as high as $14,000,000 \text{ M}^{-1}\text{s}^{-1}$.



vinyl sulfone



α,β -unsaturated carbonyl derivative

R_1 = amino acid side chain, R = alkyl or aryl

Figure 31. Other Michael Acceptor Inhibitors

It should be noted that with these class of inhibitors, the $[\text{CH}=\text{CH}]$ moiety replaces the carbonyl group of the scissile peptide bond and is attached to the Michael acceptor electrophiles such as sulfone and ester groups. In the aza-peptide Michael acceptor design however, the carbonyl group that is equivalent to the scissile bond carbonyl is a part of the aza-amino acid residue.

One of the first Michael acceptors described in the literature is the fumarate derivative of E-64c (Figure 32). This inhibitor contains an α,β -unsaturated carboxyl moiety and was found to be an irreversible inhibitor of cathepsin B ($k_{\text{app}} = 625 \text{ M}^{-1}\text{s}^{-1}$), cathepsin H ($k_{\text{app}} = 11 \text{ M}^{-1}\text{s}^{-1}$), and cathepsin L ($k_{\text{app}} = 2272 \text{ M}^{-1}\text{s}^{-1}$).¹¹⁷

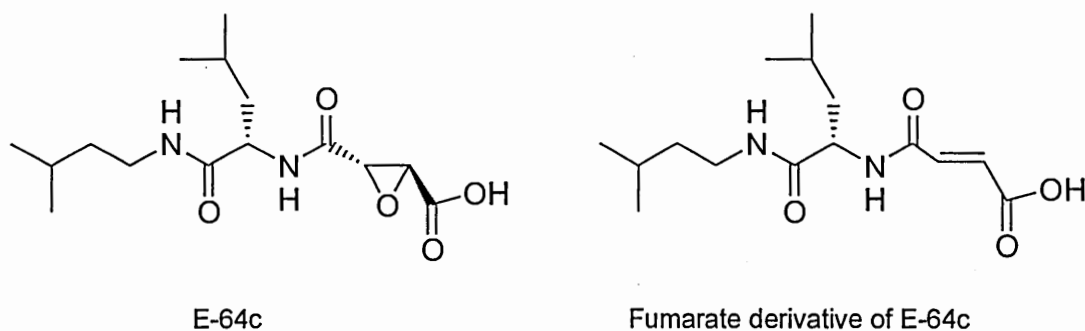


Figure 32. Fumarate Derivative of E-64c

Hanzlik and his coworkers were the first to design a series of amino acid derived Michael acceptor inhibitors for cysteine proteases.^{149,150} They showed that various vinyl sulfone and α,β -unsaturated carbonyl derivatives of phenylalanine are inhibitors of papain and DPPI. These vinyl sulfone cysteine protease inhibitors were later developed into a class of inhibitors for a variety of other cysteine proteases by Palmer and

coworkers.¹⁵¹ They designed potent inhibitors against disease-associated cysteine proteases, such as cathepsin B, L, S, and K (also called cathepsin O2), calpains, and cruzain. This class of irreversible inhibitors, which contain a double bond activated by an electron withdrawing sulfone functional group, was subsequently shown also to inhibit the rhinovirus 3C protease and cathepsin V.^{152,153} Vinyl sulfones will not irreversibly inhibit serine proteases although some derivatives act as weak competitive inhibitors.¹⁵¹ Peptidyl vinyl sulfones and other Michael acceptors are stable, unreactive toward nucleophiles, and need the catalytic machinery of the cysteine proteases for activation. Vinyl sulfones are less reactive toward nucleophiles than vinyl ketones or vinyl esters, which is an advantage for *in vivo* studies. In addition, vinyl sulfones can be manipulated on both the P and P' side of the molecule, allowing for greater selectivity and reactivity toward target enzymes. Thus, most of the Michael acceptors, which have been studied, are vinyl sulfones.

Synthesis

Aza-peptide Michael acceptors were obtained by coupling a substituted hydrazide to an acrylic or carbamoylacrylic acid moiety. The synthesis of the substituted hydrazides with the Asp P1 side chain is as previously described in Chapter 3, page 66.

Carbamoylacrylic acids with *trans* configuration were obtained by coupling monoethyl fumarate with the desired amine by mixed anhydride coupling method using NMM and iBCF, followed by deblocking the ethyl ester with NaOH in ethanol (Figure 33).

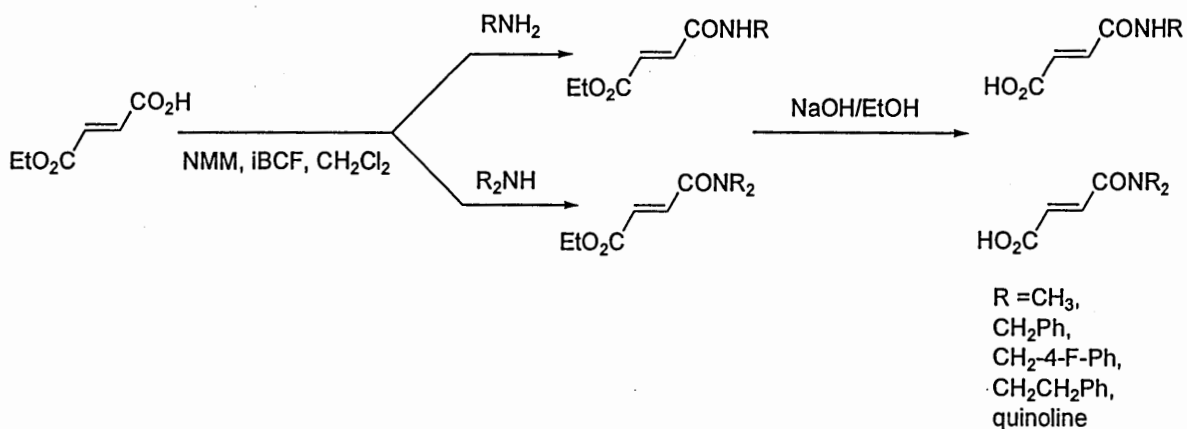


Figure 33. Synthesis of the Carbamoylacrylic Acids

Monoethyl maleate was obtained by alcoholysis of maleic anhydride with ethanol (Figure 34).

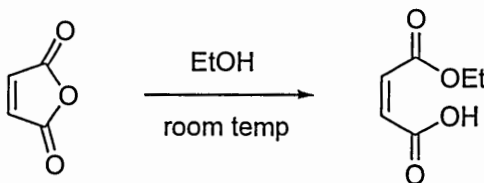


Figure 34. Synthesis of Monoethyl Maleate

The substituted hydrazides were coupled to the desired acrylic or carbamoylacrylic acids using the EDC/HOBt coupling method. The final aza-peptide Michael acceptors were obtained by deblocking the protective *t*-butyl group with TFA (Figure 35).

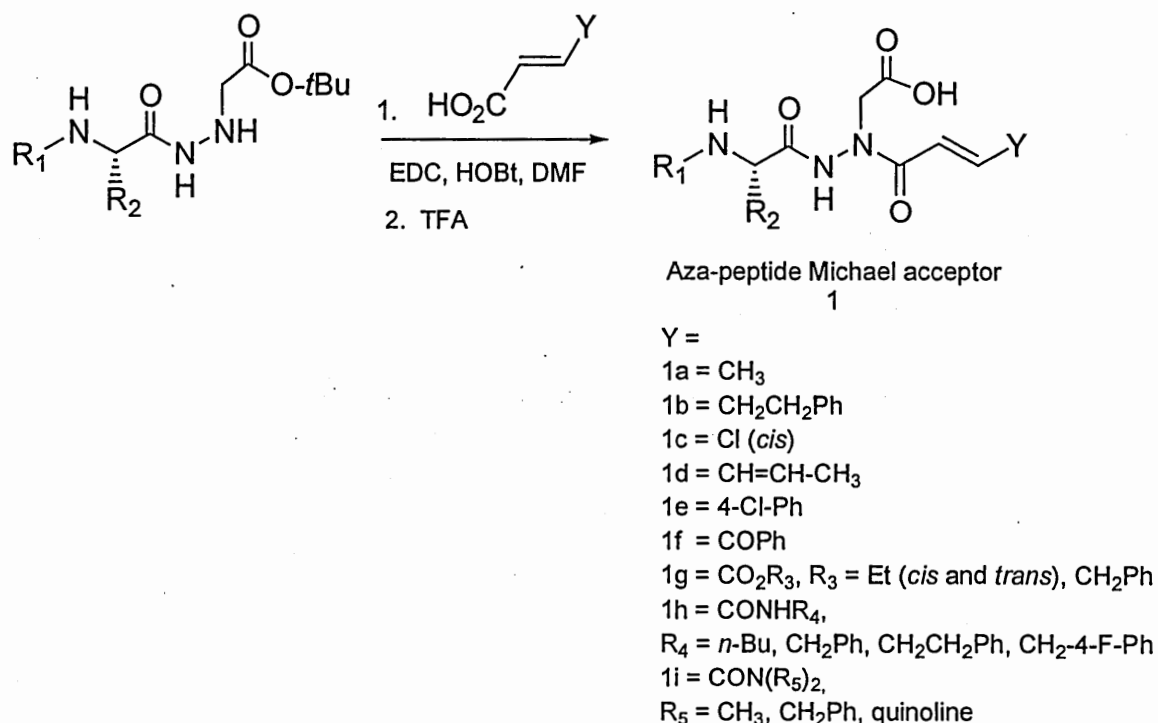


Figure 35. Synthesis of Aza-peptide Michael Acceptors

Mechanism of Inhibition

We hypothesized that aza-peptide Michael acceptors are irreversible inhibitors of cysteine proteases. We also hypothesized that aza-peptide Michael acceptors alkylate the cysteine protease active site either via pathway **a** or **b** (Figure 36). We have observed differences in activities toward the enzyme with different substituents on the C-2 position of the double bond moiety. The inhibitors with an alkyl or aryl group substituent, which possess only one position of a Michael attack (pathway **b**) showed no inhibition of caspases. However, the inhibitors with an adjacent carbonyl group to the double bond moiety, such as ester or amide groups, which possess two Michael addition positions (pathways **a** or **b**), exhibited potent inhibition of caspases. One inhibitor, designed with a

benzoyl group substituent also showed activity against caspases, although not as potent as the ester and amide analogs.

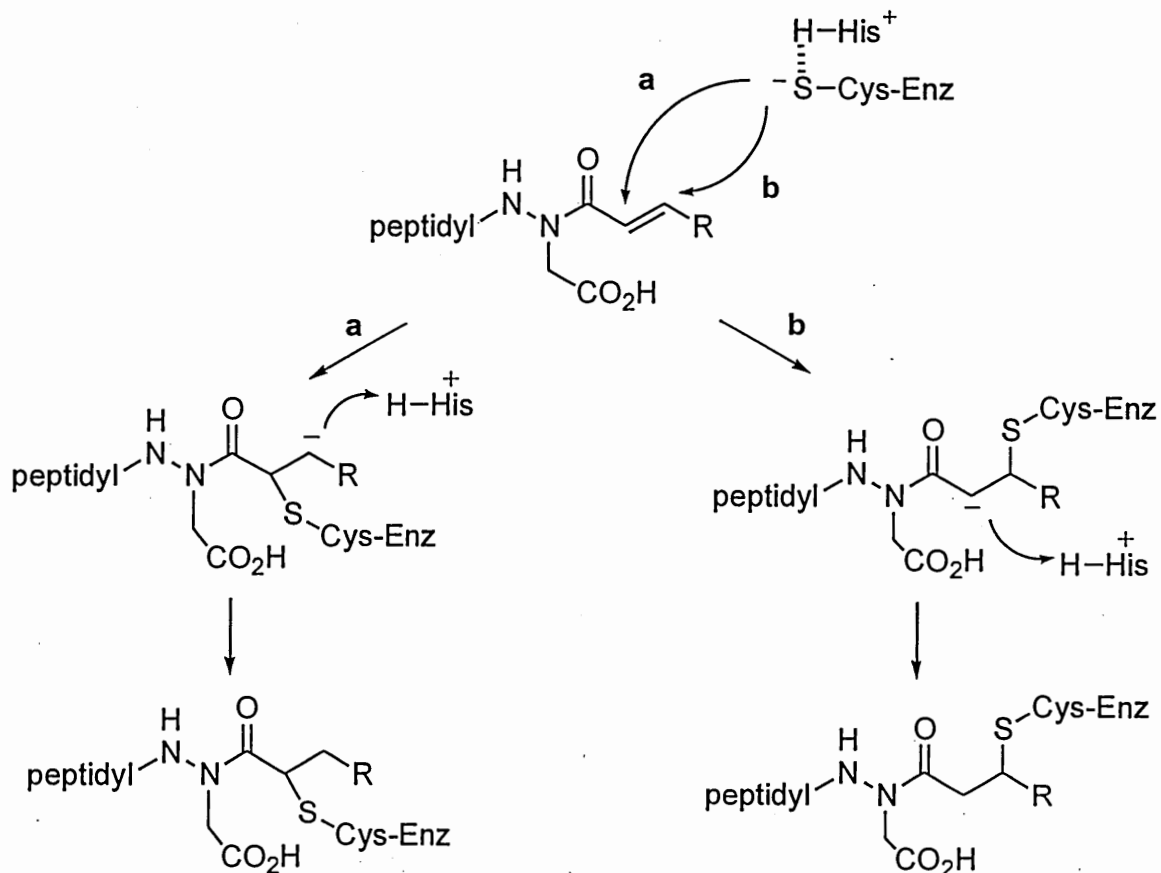


Figure 36. Possible Pathways for the Mechanism of Inhibition of Caspases by Azapeptide Michael Acceptors

We also have conducted stability studies, where we observed the behavior of our inhibitors toward simple thiol reagents such as DTT (See section on Stability Studies). Interestingly, the inhibitors with an alkyl or aryl group substituent, did not show any reactivity against DTT. On the other hand, the inhibitors with ester and amide substituents on the double bond showed reacted with DTT. These observations gave us

insights about the mechanism of inhibition. Mechanistically, we concluded that the point of attack by the DTT nucleophile is the C-3 carbon on the double bond.

We investigated this further by conducting an NMR study of the reaction of our inhibitors with another thiol reagent, benzylmercaptan. The inhibitor Cbz-Asp-Glu-Val-AAsp-*trans*-CH=CH-CO₂Et was reacted with benzylmercaptan, and the reaction was monitored using ¹H NMR over a period of 48 hours. After 24 hours, we have observed the formation of a new signal at 4.05 ppm and the disappearance of the double bond hydrogen signal at 6.63 ppm. The observed spectrum was consistent with a thioether adduct that is formed by the attack of the benzylmercaptan nucleophile at the C-3 carbon (Figure 37). An attack at the C-2 carbon would result in an adduct, where the signal of the C-2 hydrogen would be observed at 3.59 ppm. There was essentially no change in the spectrum after 48 hours, which indicates that probably a stable thioether adduct has formed.

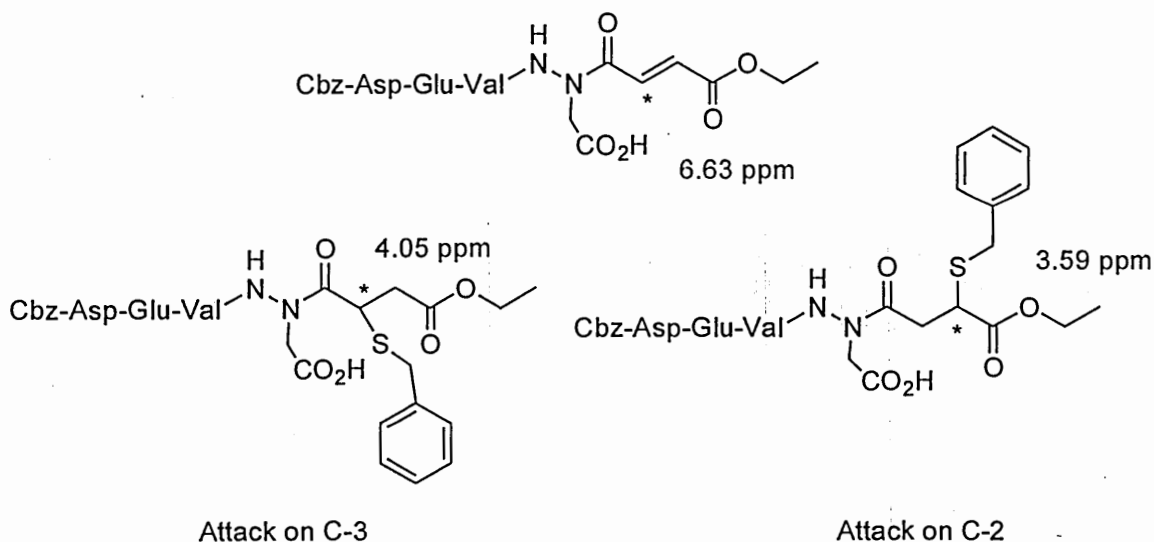


Figure 37. Thioether Adducts with Benzylmercaptan

Another inhibitor, Cbz-Ala-Ala-AAsn-*trans*-CH=CH-CO₂Et, which was designed in our laboratory for legumain, another clan CD enzyme, was also reacted with benzylmercaptan for 48 hours. A new signal at 4.05 ppm and the disappearance of the double bond signal at 6.60 ppm was observed. The alkyl derivative Cbz-Val-AAsp-*trans*-CH=CH-*trans*-CH=CH-CH₃ showed no change in spectrum when reacted with benzylmercaptan for a period of 48 hours.

Hence, based on the observations of the stability and NMR studies, we propose that the mechanism of inhibition of aza-peptide Michael acceptors by cysteine proteases goes through alkylation of the C-3 carbon. The alkylation of the active site is proposed to proceed via a Michael addition to the C-3 carbon on the double bond by the active site cysteine, followed by protonation of the α -carbon to form the thioether derivative (Figure 38).

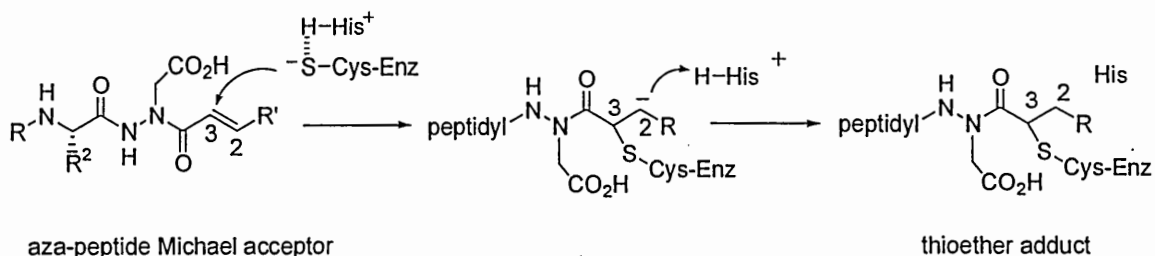


Figure 38. Mechanism of Inhibition of Caspases by Aza-peptide Michael Acceptors

Stability Studies

Aza-peptide Michael acceptors show limited activity against simple thiol reagents such as DTT, benzylmercaptan, mercaptoethanol or cysteine. In order to determine the extent of the reactivity, we have performed several stability tests. We have observed the

reactivity of the double bond moiety in the presence of DTT in the caspase kinetic assay buffer. The stability of a representative group of inhibitors was studied by monitoring the UV spectrum of solutions at 250 nm, at 25 °C over a period of 15 hours. The concentrations of the inhibitors were 2.5-4.0 mM and of DTT was 10 mM. The pH of the buffer solution was 7.2 and it contained about 50 % (v/v) DTT. The half-lives ($t_{1/2}$) were obtained from first-order rate plots of $\ln(A_t - A_f)$ versus time and the correlation coefficients were greater than 0.99.

The disubstituted amide analog Cbz-Asp-Glu-Val-AAsp-*trans*-CH=CH-CON(Me)Bzl is the most stable inhibitor with a half life of 116 minutes (Figure 39). Interestingly, the half life of the monosubstituted analog Cbz-Asp-Glu-Val-AAsp-*trans*-CH=CH-CONHBzl is just half as short as the disubstituted amide analog with 58 minutes. The amide analogs, both mono- and disubstituted analogs are significantly more stable than the ester analog. The ester analog Cbz-Asp-Glu-Val-AAsp-*trans*-CH=CH-CO₂Et is the least stable inhibitor with a half life of about 10 minutes. The alkyl analog Cbz-Val-AAsp-*trans*-CH=CH-*trans*-CH=CH-CH₃ was completely stable over the period of 15 hours.

Aza-peptide Michael acceptors designed for other clan CD enzymes such as legumain, gingipain and clostripain specific sequences were also tested for stability against DTT. The half life of the legumain inhibitor Cbz-Ala-Ala-AAsn-*trans*-CH=CH-COOEt in the legumain assay buffer (pH = 5.8) with DTT was 19 minutes. It should be noted that the pH of the assay buffer has a considerable effect on the stability of the inhibitors against DTT. The lower the pH, the lower is the concentration of the

deprotonated thiol group of DTT. Thus, a nucleophilic attack on the double bond is less favored at lower pH values.

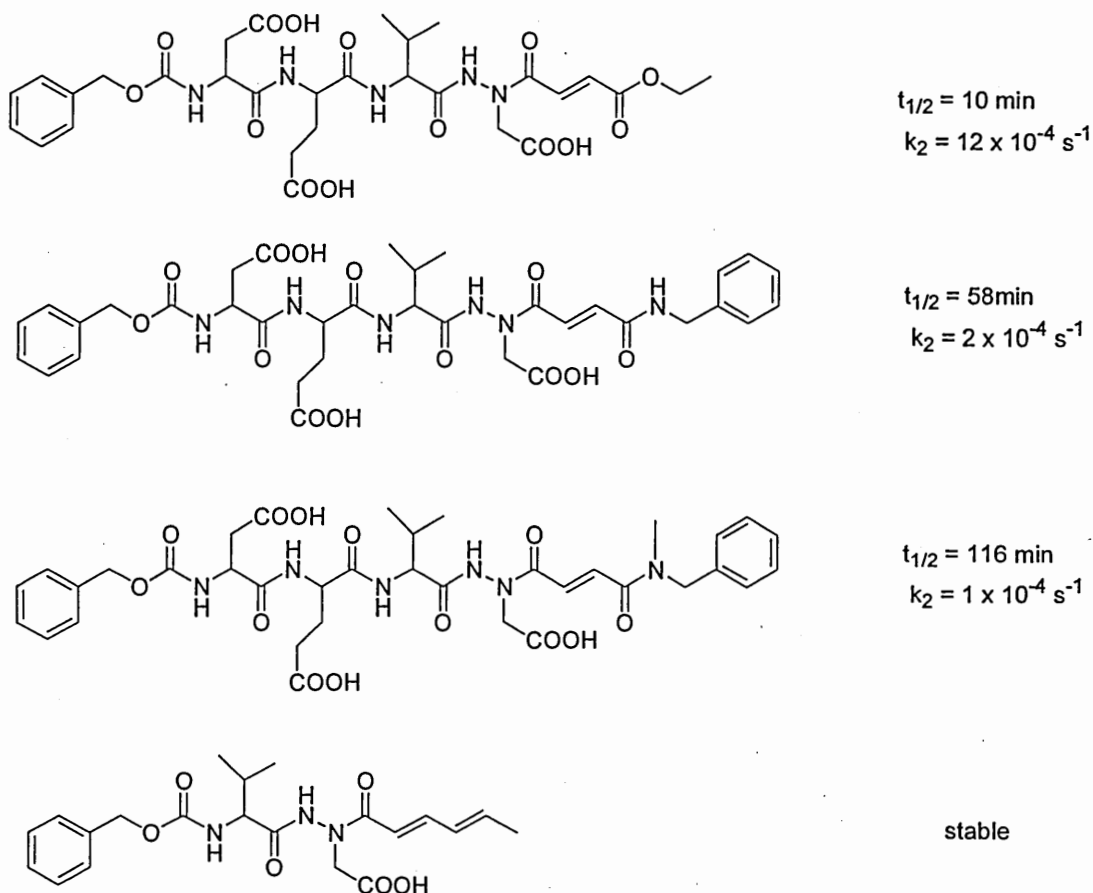


Figure 39. Half Lives of Some Aza-peptide Michael Acceptors in the Assay Buffer in the Presence of DTT

Results and Discussion

In the design of the aza-peptide Michael acceptors, we utilized peptide sequences derived from natural caspase cleavage sites or obtained by peptide mapping of caspases with libraries of AMC substrates.¹⁵⁴ The DEVD and LETD sequences are optimal sequences for caspase-3 and caspase-8, respectively, and were determined using a

positional scanning synthetic combinatorial library.¹⁴⁸ The IETD sequence for caspases-6 and -8 is the cleavage sequence of a natural caspase-8 substrate, the caspase-3 proenzyme.¹⁵⁴ The VEVD sequence is the optimal sequence for caspase-6.¹⁵⁵

Aza-peptide Michael acceptors with an aza-Asp residue at P1 are excellent irreversible inhibitors for caspases-3, -6, and -8 (Table 8). The second order inhibition rates are as high as $3,000,000 \text{ M}^{-1}\text{s}^{-1}$. The inhibitors designed with caspase-3 specific DEVD sequence are the best inhibitors for that enzyme. The inhibitors with LETD and IETD sequences work best with caspase-8, and the ones with VEVD sequence work best with caspase-6. We have made several dipeptidyl aza-peptide Michael acceptor inhibitors, where we utilized the VD sequence. Aza-peptide Michael acceptors are easily extended at their P' site. We have modified the substituents at the P' site using various groups like simple alkyl, alkenyl, and aryl groups, halogens, esters, and amides.

Dipeptidyl aza-peptide Michael acceptor derivatives with simple alkyl, alkenyl, aryl and halogen substituents did not inhibit caspases-3, -6, and -8. The ester and amide derivatives showed weak to moderate inhibition of caspase-3. One of the first aza-peptide Michael acceptor inhibitors designed in our laboratory for caspases is the inhibitor Cbz-Val-AAsp-*trans*-CH=CH-CO₂Et. This compound is found to inhibit caspase-3 with a second order inhibition rate of $8,000 \text{ M}^{-1}\text{s}^{-1}$, which is more than twice as potent as the parent dipeptidyl epoxide inhibitor Cbz-Val-AAsp-2*S*,3*S*-EP-CO₂Et ($k_2 = 3,650 \text{ M}^{-1}\text{s}^{-1}$). This result led us to further investigate the inhibitory potential of this type of compounds toward caspases. As a result, we focused on modifying the substituent on the double bond moiety of a dipeptidyl sequence to find the best compound suitable for a tetrapeptidyl aza-peptide Michael acceptor design. The next choice for a substituent was

the amide group, where we have synthesized one alkyl and one benzyl amide analog. The inhibitors Cbz-Val-AAsp-*trans*-CH=CH-CONH-*n*Bu and Cbz-Val-AAsp-*trans*-CH=CH-CONHBzl were weak inhibitors of caspase-3 ($k_2 = 175 \text{ M}^{-1}\text{s}^{-1}$ and $185 \text{ M}^{-1}\text{s}^{-1}$, respectively) and did not inhibit caspases-6 and -8. We investigated further by introducing simple alkyl and alkenyl groups, such as methyl, 2,4-hexadienoyl, phenethyl, and 4-chlorophenyl, and a halogen (Cl) on the double bond moiety. These compounds did not turn out to be inhibitors of caspases (Figure 40).

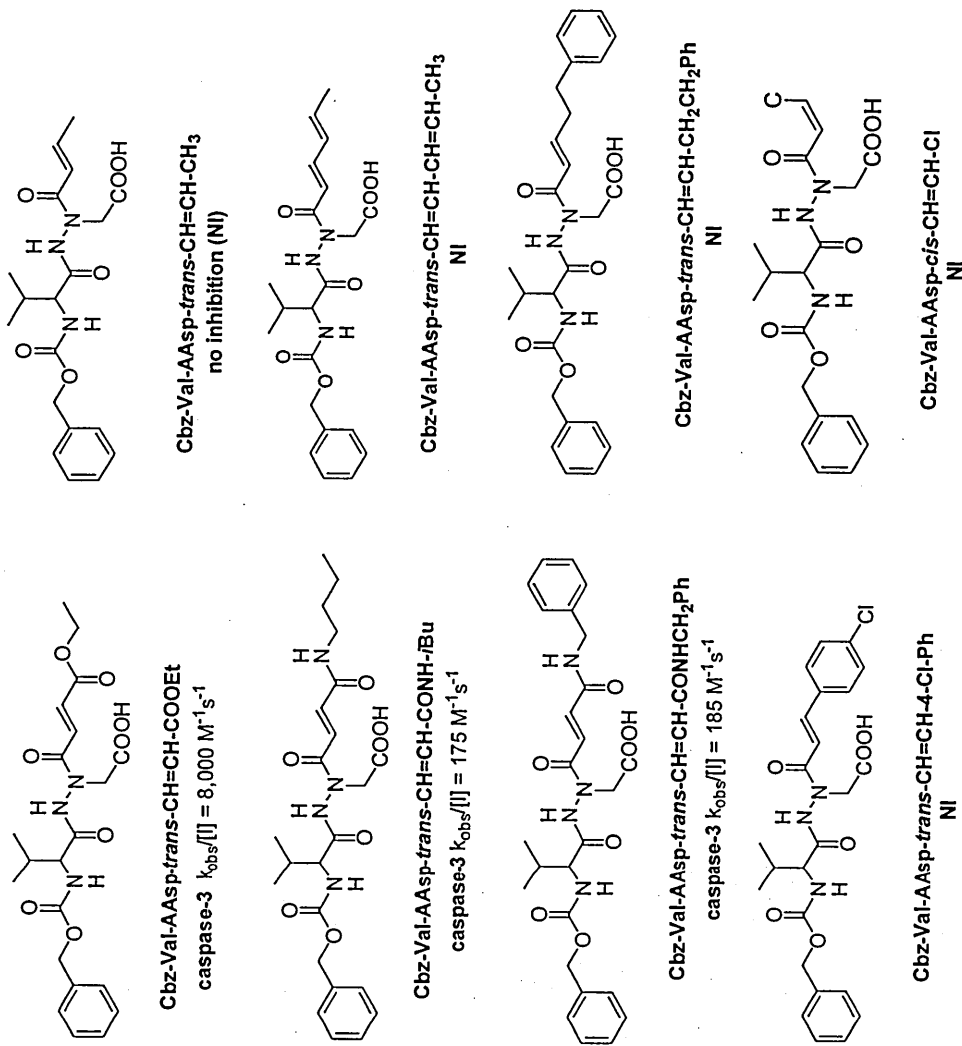
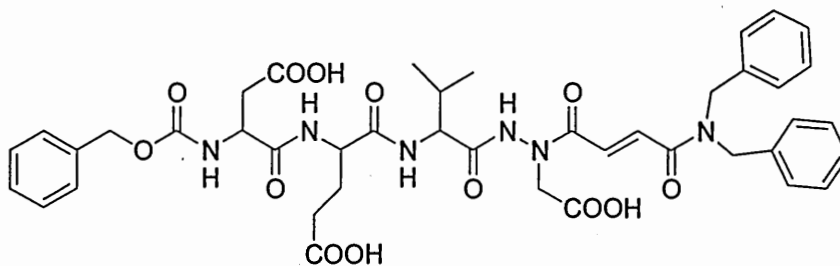


Figure 40. Dipeptidyl Aza-peptide Michael Acceptor Inhibitors

We then focused our attention to designing tetrapeptidyl aza-peptide Michael acceptors with optimal sequences for caspases-3, -6, and -8, hoping to obtain more potent inhibitors. The inhibitors with the caspase-3 specific sequence DEVD showed second order inhibition rates in the range of $10^6 \times \text{M}^{-1}\text{s}^{-1}$. The most reactive caspase-3 inhibitor was the dibenzyl amide Cbz-Asp-Glu-Val-AAsp-*trans*-CH=CH-CON(CH₂Ph)₂ with a second order inhibition rate of $3,000,000 \text{ M}^{-1}\text{s}^{-1}$ (Figure 41). This inhibitor is the only highly potent inhibitor for caspase-3 that is also selective over caspases-6 and -8 reported so far. It is 588 fold more selective for caspase-3 over caspase-6 ($k_2 = 5,100 \text{ M}^{-1}\text{s}^{-1}$) and 348 fold more selective over caspase-8 ($k_2 = 8,600 \text{ M}^{-1}\text{s}^{-1}$). One aza-peptide epoxide inhibitor Cbz-Asp-Glu-Val-AAsp-2*R*,3*R*-EP-CO₂Et designed in our laboratory for caspase-3 was also a very selective inhibitor, but it was not as potent inhibitor for caspase-3 ($k_2 = 464,000 \text{ M}^{-1}\text{s}^{-1}$). This inhibitor was 977-fold and 591-fold more reactive with caspase-3 over caspase-6 and caspase-8, respectively.¹⁴⁷ Among the aza-peptide Michael acceptor inhibitors with DEVD sequence the range of selectivity for caspase-3 over caspases-6 is usually in the range of 500-fold. However, when one obtained selectivity over caspase-6, it was difficult to obtain selectivity also for caspase-8. The range of selectivity for caspase-3 over caspase-8 was only up to 37-fold. It has been previously shown that the prime site of caspase-8 differs from that of the other caspases by the presence of a helix-turn-helix insertion ranging from residues 245-253, which is unique to caspase-8.⁶⁸ Clearly, we have taken advantage of this structural difference by designing an inhibitor with a dibenzyl amide substituent on the double bond moiety, which resulted in optimal selectivity among caspases-3, -6, and -8.



Cbz-Asp-Glu-Val-AAsp-*trans*-CH=CH-CON(CH₂Ph)₂

caspase-3
 $k_{\text{obs}}/[I] = 3,000,000 \text{ M}^{-1}\text{s}^{-1}$

caspase-6
 $k_{\text{obs}}/[I] = 5,100 \text{ M}^{-1}\text{s}^{-1}$

caspase-8
 $k_{\text{obs}}/[I] = 8,600 \text{ M}^{-1}\text{s}^{-1}$

Figure 41. The Most Potent and Selective Caspase-3 Aza-peptide Michael acceptor inhibitor

With caspase-3, the order of reactivity of the inhibitors with various substituents on the double bond moiety was $\text{CON}(\text{CH}_3)\text{CH}_2\text{Ph} > \text{CON-quinoline} > \text{CO}_2\text{Et} (\textit{trans}) > \text{CONHCH}_2\text{-4-F-Ph} > \text{CONHCH}_2\text{CH}_2\text{Ph} > \text{CONHCH}_2\text{Ph} > \text{CO}_2\text{CH}_2\text{Ph} > \text{CON}(\text{CH}_3)\text{CH}_2\text{CH}_2\text{Ph} > \text{CO}_2\text{Et} (\textit{cis}) > \text{COPh}$. We have made one inhibitor with the *cis* configuration of the double bond moiety. This compound **2** was still a very potent inhibitor of caspase-3 ($k_2 = 1,060,000 \text{ M}^{-1}\text{s}^{-1}$), whereas the *trans* ethyl ester **1** however, was twice as potent against caspase-3 ($k_2 = 2,130,000 \text{ M}^{-1}\text{s}^{-1}$).

The caspase-8 specific inhibitor Cbz-Leu-Glu-Thr-AAsp-*trans*-CH=CH-CO₂CH₂Ph was a potent inhibitor of caspase-8 with a second order inhibition rate of $98,000 \text{ M}^{-1}\text{s}^{-1}$. The caspase-6 specific inhibitor Cbz-Val-Glu-Val-AAsp-*trans*-CH=CH-CO₂Et is one of the best inhibitors reported yet for this enzyme with an inhibition rate constant of $83,600 \text{ M}^{-1}\text{s}^{-1}$. Unfortunately, this inhibitor also potently inactivates caspase-8 ($k_2 = 175,800 \text{ M}^{-1}\text{s}^{-1}$).

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 $1,000,000 \text{ M}^{-1}\text{s}^{-1}$.⁹³ More importantly, the aza-peptide Michael acceptors are much more selective (See the Cross-reactivity Section).

Cross Reactivity

The aza-peptide Michael acceptors designed for caspases show little to no inhibition of the clan CA cysteine proteases papain, cathepsin B, and calpain (Table 7). The inhibitors Cbz-Asp-Glu-Val-AAsp-*trans*-CH=CH-CO₂Et, Cbz-Asp-Glu-Val-AAsp-*trans*-CH=CH-CO₂CH₂Ph, Cbz-Asp-Glu-Val-AAsp-*trans*-CH=CH-CONHCH₂Ph, and Cbz-Leu-Glu-Thr-AAsp-*trans*-CH=CH-CO₂CH₂Ph showed essentially no inhibition against papain, cathepsin B, and calpain after an incubation period of one hour.

We also tested the cross reactivity of our inhibitors with other members of clan CD proteases such as legumain, clostripain, and gingipain K. The inhibitors did not show any activity against those enzymes after being incubated for one hour. Likewise, the aza-peptide Michael acceptor inhibitors with legumain, clostripain, and gingipain K specific sequences did not show any cross reactivity with clan CA enzymes or between each other. Clearly, the aza-peptide Michael acceptor design is specific for clan CD enzymes, as was the case with the parent aza-peptide epoxide inhibitors.

Table 7. Cross Reactivity of Caspase Inhibitors with Clan CA and Other Clan CD Enzymes

Inhibitor	k_2 ($M^{-1}s^{-1}$)					
	Cathepsin B	Papain	Calpain	Legumain	Clostripain	Gingipain K
Cbz-Asp-Glu-Val-AAsp- <i>trans</i> -CH=CH-COOEt	NI	NI	NI	NI	NI	NI
Cbz-Asp-Glu-Val-AAsp- <i>trans</i> -CH=CH-COOBzl	NI	ND	ND	ND	NI	NI
Cbz-Asp-Glu-Val-AAsp- <i>trans</i> -CH=CH-CONHBzl	NI	ND	ND	ND	NI	NI
Cbz-Leu-Glu-Thr-AAsp- <i>trans</i> -CH=CH-COOEt	<10	NI	<10	NI	NI	NI

NI = No inhibition, ND = not determined.

Conclusion

We have discovered that aza-peptide Michael acceptors are a new class of inhibitors that are highly specific for clan CD cysteine proteases. We have successfully synthesized aza-peptide Michael acceptors with an aza-Asp residue at P1 position, where potent and selective inhibitors of caspases-3, -6, and -8 were obtained. The second order inhibition constants are in the order of $10^6 \text{ M}^{-1}\text{s}^{-1}$. Aza-peptide Michael acceptors do not show cross reactivity with clan CA cysteine proteases such as papain, cathepsin B, and calpain. Aza-peptide Michael acceptors are also selective among their own clan. Clan CD enzymes such as legumain, clostripain, and gingipain K, which are specific for Asn, and Lys at P1 position respectively, are not inhibited by aza-peptide Michael acceptors designed for caspases.

We have determined the mechanism of inhibition of clan CD cysteine proteases by aza-peptide Michael acceptors by utilizing techniques such as NMR and UV spectroscopy. Our results indicate that the nucleophilic attack of the active site cysteine occurs at the C-3 carbon of the double bond moiety. This is in contrast with our aza-peptide epoxide inhibitors, where the nucleophilic attack occurs at the C-2 carbon.

In general, aza-peptide Michael acceptors were not as stable as the parent aza-peptide epoxides toward simple thiol reagents such as DTT, benzylmercaptan, and cysteine. However, the disubstituted amide analogs showed considerable stability against DTT, which might be suitable for *in vivo* studies.

Future Studies

Aza-peptide Michael acceptor design can certainly be extended to a variety of derivatives by modifying the substituents on the double bond moiety. In an effort to obtain more stable derivatives, placement of an R group at the C-2 position on the double bond moiety could give promising results (Figure 42). A variety of different groups such as halogens, alkyl, and aryl groups can be selected for R group substitution to obtain stability and selectivity on the prime side at the active site. One similar derivative, where an R group is placed on the C-3 position would be a control compound to provide additional information about the inhibition mechanism, where we have shown that this position was not the point of attack by the active site cysteine. This compound should exhibit no or little inhibition of caspases.

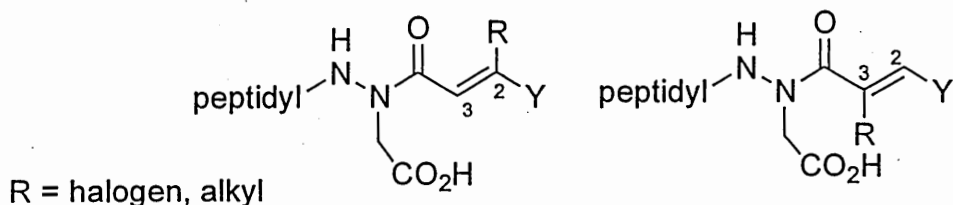


Figure 42. Derivatives of Aza-peptide Michael Acceptors

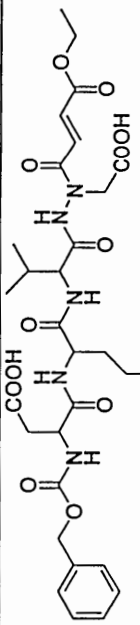
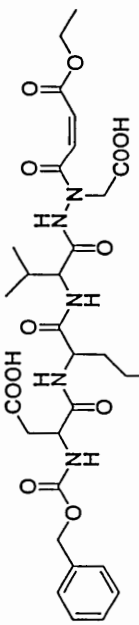
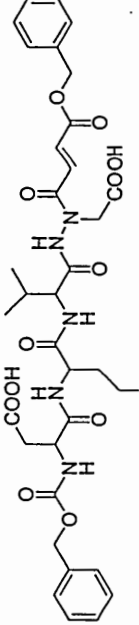
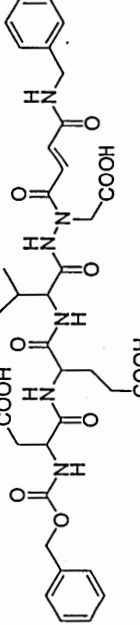
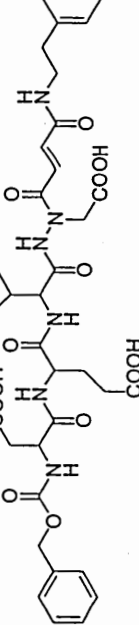
Extending the design of the dibenzyl amide analog (Figure 41) to a variety of structures with different substituents on the phenyl ring would give opportunities for more selectivity among the caspases.

It would be worthwhile to synthesize the α -carbon derivatives of our aza-peptide epoxide and Michael acceptor inhibitors. Although the synthesis of such compounds

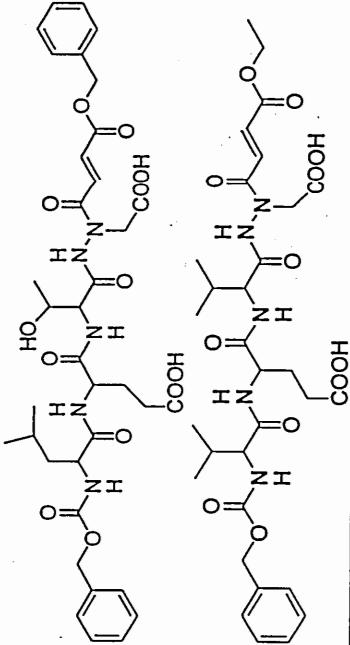
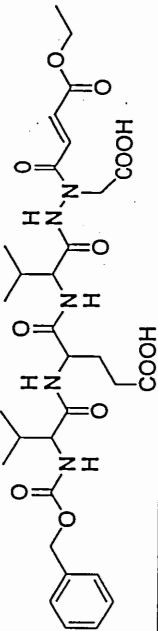
would be more challenging, they would provide complete understanding about how important the aza-amino acid residue actually is in terms of binding at the active site.

Lastly, in order to establish the selectivity of our aza-peptide Michael acceptor inhibitors for cysteine proteases it would be a good idea to test them with the Asp specific serine protease granzyme B. Aza-peptide Michael acceptors should show no to little inhibition of granzyme B.

Table 8. Inhibition of Caspases by Aza-peptide Michael Acceptors

		k_2 ($M^{-1}s^{-1}$)		
Code	Inhibitor	Caspase-3	Caspase-6	Caspase-8
1		2,130,000 $\pm$ 99,100	35,600 $\pm$ 0	273,000 $\pm$ 18,400
2		1,060,000 $\pm$ 92,500	ND	181,000 $\pm$ 13,700
3		1,700,000 $\pm$ 106,000	8,470	121,000 $\pm$ 13,700
4		1,750,000 $\pm$ 18,500	3,210 $\pm$ 105	78,235 $\pm$ 8,000
5		1,950,000 $\pm$ 53,000	3,470 $\pm$ 52	129,000 $\pm$ 27,400

	2,640,000 $\pm$ 397,000	9,500 $\pm$ 210	90,300 $\pm$ 18,200
	1,180,000 $\pm$ 264,000	4,000 $\pm$ 413	31,900 $\pm$ 2,800
	3,000,000 $\pm$ 80,000	5,100 $\pm$ 0	8,600 $\pm$ 1,600
	2,300,000 $\pm$ 26,500	5,700 $\pm$ 0	118,000 $\pm$ 14,800
	2,100,000 $\pm$ 26,400	4,400 $\pm$ 361	85,100 $\pm$ 9,700
	122,400	1,800 $\pm$ 13	9,720 $\pm$ 500

12		4,600 ± 330	47,600 ± 2,500	98,400 ± 9,130
13		41,200 ± 8,600	83,600 ± 8,400	175,800 ± 6,800

The kinetics were performed in our laboratory. ND = not determined, AAsp = aza-Asp

Perspectives

Inhibitor Design. A number of different approaches have been utilized in the design of potent inhibitors for proteases. The most common approach is the attachment of a warhead (for example alkylating agents) to the optimal peptide sequence. Frequently, library screening is used to discover new inhibitors, although this approach is less common with irreversible inhibitors. Once an inhibitor is developed for a protease, extensive modifications are often made to the inhibitor structure to improve the specificity and potency of the inactivator. In most cases, a large number of crystal structures of enzyme inhibitor complexes are determined and molecular modeling is used to guide the design work. Clearly, most of the important warhead functional groups have already been described in the literature. However, there are still new classes of inhibitor structures which remain to be discovered. In addition, systematic modification of current inhibitor structures offers many opportunities for improving inhibitor potency and selectivity and bioavailability.

Protease Biological Function and Functional Proteonomics. Protease inhibitors are useful in elucidating the roles of various proteases in models of biological function. Most of the time, the inhibitors used in biological studies do not have the expected specificity or selectivity, which has led to many incomplete conclusions in the literature. The specificity of many new inhibitors is usually not examined with many other proteases and yet the inhibitor is often claimed to be "specific". For example, the fluoromethyl ketone Asp-Glu-Val-Asp-CH₂F, frequently used as a caspase specific inhibitor in apoptosis models, inhibits many clan CA enzymes very effectively. Therefore, it is necessary to further test the specificity of new inhibitors or widely used

inhibitors with a greater library of potential protease targets before using them in biological studies.

With irreversible inhibitors, once the target enzyme is killed, it can't usually be reactivated and the organism must resynthesize the enzyme. The inhibitor will usually stay covalently bound to the inhibited enzyme until the enzyme is degraded into its constituent pieces. If the protease inhibitor is attached to a fluorophore or biotin molecule, the inhibitor can be used to localize various proteases in cells and tissues. Irreversible inhibitors have a distinct advantage in proteomic profiling in the future.

Therapeutic Potential of Irreversible Inhibitors. Although there is great potential for the use of irreversible protease inhibitors for therapy, no such compounds have reached the market as drugs. Several reversible protease inhibitors have reached the market, most notably angiotensin converting enzyme inhibitors for the treatment of hypertension and HIV protease inhibitors for the treatment of AIDS.

If one wants to develop potential drugs, high enzyme inhibition rates are usually not sufficient. Unfortunately, it is frequently the case that potent inhibitor structures have very poor bioavailability. In general, by minimization of the number of hydrogen bond donors and acceptors, and charges one can obtain good bioavailability.¹⁵⁶ It is important to tune the delicate balance between reaching for distant subsites and trying to keep the size of the inhibitor within a reasonable molecular weight range. It has been shown that orally bioavailable drugs have a roughly Gaussian distribution of molecular weight between 150 to 550, with oral bioavailability quite low in compounds beyond 550 Da.¹⁵⁷ Thus the challenge of the medicinal chemist is always to keep the molecule small, while maintaining potency and specificity.

Experimental Procedures

Material and Methods. 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 ^1H NMR spectra were obtained using a Varian Mercury Vx 400 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. Caspases-3, -6 and -8 were expressed in *E. coli* and purified according to methods previously described by Stennicke and Salvesen.¹⁴⁵ Enzymes and substrates were obtained from G. Salvesen. The fluorogenic substrate Ac-DEVD-AMC was purchased from Bachem Bioscience Inc., King of Prussia, PA.

Peptidyl Hydrazides. Anhydrous hydrazine (10 eq) was added to a stirred solution of a peptidyl methyl ester (1 eq) in MeOH and the reaction mixture was stirred vigorously for 16 h at room temperature. Excess hydrazine and solvent were removed under vacuum and the residue was washed with ether several times to give the peptidyl hydrazide as a white solid. Compounds were purified further on silica gel column using the eluent 2:18:5 MeOH:CH₂Cl₂:EtOAc when needed. MS and ^1H NMR (CDCl₃ or DMSO-d₆) were consistent with the proposed structures.

Cbz-Val-NH-NH₂, white solid, yield 92 %; Cbz-Asp(O-*t*Bu)-Glu(O-*t*Bu)-Val-NH-NH₂, white solid, yield 43 %; Cbz-Val-Glu(O-*t*Bu)-Val-NH-NH₂, white solid, yield 75 %; Cbz-Leu-Glu(O-*t*Bu)-Thr-NH-NH₂, white solid, yield 92 %.

Peptidyl-AA₂-NH-NH-CH₂COO*t*Bu. To a stirred solution of a peptidyl hydrazide (1 eq) in DMF, NMM (1.1 eq) and neat *t*-butyl bromoacetate (1.1 eq) were added dropwise at -15 °C and the mixture was allowed to react for 16 h at room temperature. The solvent was removed under vacuum and the residue was dried *in vacuo*. Purification on a silica gel column with the proper eluent gave the product with yields of 18-68 %. MS and ¹H NMR (CDCl₃ or DMSO-*d*₆) were consistent with the proposed structures.

Cbz-Val-NH-NH-CH₂COO*t*Bu was purified by column chromatography on silica gel using 1:20:4.2 MeOH: CH₂Cl₂: EtOAc as the eluent; white solid, yield 64 %. ¹H NMR (DMSO-*d*₆): 0.90 (t, 6H, Val), 1.40 (s, 9H, *t*Bu), 1.86 (m, 1H, Val), 3.37 (d, 2H, NHCH₂COOH), 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-NH-NH-CH₂COO*t*Bu was purified by column chromatography on silica gel using 2:18:5 MeOH:CH₂Cl₂:EtOAc as the eluent, and then rechromatographed using 1:9:5 MeOH:CH₂Cl₂:EtOAc as the eluent; white solid, yield 54 %. ¹H NMR (DMSO-*d*₆): 0.81 (d, 6H, Val), 1.35-1.41 (t, 27H, *t*Bu), 1.71 (m, 1H, Val), 1.84 (m, 2H, Glu), 2.17 (m, 2H, Glu), 2.44 (dd, 1H, Asp), 2.49 (dd, 1H, Asp), 4.02 (t, 1H, α-H), 4.26-4.35 (dm, 2H, α-H), 5.02 (q, 2H, Cbz), 7.32 (s, 5H, Ph), 7.61 (d, 1H, NH), 7.79 (d, 1H, NH), 7.91 (d, 1H, NH), 9.43 (d, 1H, NH).

Cbz-Val-Glu(O-*t*Bu)-Val-NH-NH-CH₂COO*t*Bu was purified by column chromatography on silica gel using 1:9:5 MeOH:CH₂Cl₂:EtOAc as the eluent, and then rechromatographed using 1:9:5 MeOH:CH₂Cl₂:EtOAc as the eluent; white solid, yield 18 %. ¹H NMR (DMSO-*d*₆): 0.78-0.85 (dd, 12H, Val), 1.40 (d, 18H, *t*Bu), 1.71 (m, 1H,

Val), 1.83 (m, 2H, Glu), 1.91 (m, 1H, Val), 2.17 (m, 2H, Glu), 3.86 (t, 1H, α -H), 4.04 (t, 1H, α -H), 4.32 (m, 1H, α -H), 5.01 (s, 2H, Cbz), 5.12 (m, 1H, NH), 7.33 (s, 5H, Ph), 7.76 (d, 1H, NH), 8.00 (d, 1H, NH), 9.43 (d, 1H, NH).

Cbz-Leu-Glu(O-*t*Bu)-Thr-NH-NH-CH₂COO*t*Bu was purified by column chromatography on silica gel using 2:18:5 MeOH:CH₂Cl₂:EtOAc as the eluent, white solid, yield 68 %. ¹H NMR (DMSO-*d*₆): 0.84 (t, 6H, Leu), 0.98 (d, 3H, Thr), 1.37-1.44 (m, 20H, *t*Bu and Leu), 1.62-1.73 (dm, 2H, Glu), 1.88 (m, 1H, Leu), 2.22 (m, 2H, Glu), 3.94 (m, 1H, α -H), 4.03-4.13 (dm, 2H, α -H), 4.37 (m, 1H, Thr), 4.99 (s, 2H, Cbz), 6.65 7.33 (m, 5H, Ph), 7.42 (d, 1H, NH), 7.63 (d, 1H, NH), 8.03 (d, 1H, NH), 9.26 (s, 1H, NH).

Acrylic and Carbamoylacrylic Acid Synthesis

Monoethyl maleate (HOOC-CH=CH-COOEt). Monoethyl maleate was obtained as a colorless oil from the reaction of maleic anhydride with absolute ethanol at room temperature for 18 hours (yield = 86%). ¹H NMR (DMSO-*d*₆): 1.19 (t, 3H, OCH₂CH₃), 4.11 (q, 2H, OCH₂CH₃), 6.33 (s, 2H, CH=CH).

***trans*-3-Benzylcarbamoylacrylic Acid (HOOC-CH=CH-CONHBzl).** **General Procedure.** To a stirred solution of benzyl amine (1 eq), HOBt (1.1 eq), and monoethyl fumarate (1.1 eq) in DMF was added EDC (1.1 eq) and the mixture was allowed to react for 16 h at room temperature. The DMF was removed under vacuum and the residue was treated with EtOAc. The organic layer was then washed with 2 % citric acid, saturated NaHCO₃, and brine. The organic layer was dried over Na₂SO₄, filtered and concentrated. Column chromatography on silica gel with 1:1 EtOAc: hexanes as the eluent gave the *trans*-3-benzylcarbamoylacrylic acid ethyl ester as a white solid with a 63 % yield.

Hydrolysis of the ethyl ester with NaOH (1.1 eq) followed by acidic workup gave the *trans*-3-benzylcarbamoylacrylic acid as a white solid in 65 % yield. ^1H NMR (DMSO- d_6): 4.37 (d, 2H, CH_2Ph), 6.55 (d, 1H, $\text{CH}=\text{CH}-\text{CONH}$), 6.93 (d, 1H, $\text{CH}=\text{CH}-\text{COOH}$), 7.29 (m, 5H, Ph), 8.96 (t, 1H, NH). In general, carbamoylacrylic acids were obtained in 60-79 % yield.

***trans*-3-Butylcarbamoylacrylic Acid ($\text{HOOC}-\text{CH}=\text{CH}-\text{CONH}-n\text{Bu}$).** This compound was obtained by EDC/HOBt coupling in CHCl_3 followed by standard NaOH hydrolysis (white solid, yield = 60 %). ^1H NMR (DMSO- d_6): 0.86 (t, 3H, $\text{NHCH}_2\text{CH}_2\text{CH}_2\text{CH}_3$), 1.28 (m, 2H, $\text{NHCH}_2\text{CH}_2\text{CH}_2\text{CH}_3$), 1.41 (m, 2H, $\text{NHCH}_2\text{CH}_2\text{CH}_2\text{CH}_3$), 3.12 (m, 2H, $\text{NHCH}_2\text{CH}_2\text{CH}_2\text{CH}_3$), 6.49 (d, 1H, $\text{CH}=\text{CH}-\text{CONH}$), 6.87 (d, 1H, $\text{CH}=\text{CH}-\text{COOH}$), 8.43 (t, 1H, NH).

***trans*-3-Phenethylcarbamoylacrylic Acid ($\text{HOOC}-\text{CH}=\text{CH}-\text{CONHCH}_2\text{CH}_2\text{Ph}$).** Monoethyl fumarate (1 eq) was dissolved in dry THF and cooled to $-15\text{ }^\circ\text{C}$. NMM (1eq) and IBCF were added dropwise and the mixture was allowed to react for 20 min. Phenethylamine was added to the above mixture at $-15\text{ }^\circ\text{C}$. The mixture was stirred for 1 hour at $-15\text{ }^\circ\text{C}$ and was allowed to react for 16 h at room temperature. The solvent was evaporated and the residue was treated with EtOAc and H_2O . The organic layer was washed with 1 M HCl, saturated NaHCO_3 , and brine, dried over Na_2SO_4 , filtered and concentrated to give *trans*-3-phenethylcarbamoylacrylic acid ethyl ester. Hydrolysis of the ethyl ester with NaOH (1.1 eq) followed by acidic workup gave the *trans*-3-phenethylcarbamoylacrylic acid as a yellow solid in 79 % yield. ^1H NMR (DMSO- d_6): 2.75 (t, 2H, $\text{NHCH}_2\text{CH}_2\text{Ph}$), 3.38 (q, 2H, $\text{NHCH}_2\text{CH}_2\text{Ph}$), 6.55 (d, 1H, $\text{CH}=\text{CH}-\text{CONH}$), 6.98 (d, 1H, $\text{CH}=\text{CH}-\text{COOH}$), 7.20 (m, 5H, Ph), 8.59 (t, 1H, NH).

Aza-peptide Michael Acceptors

Cbz-Val-AAsp(O-*t*Bu)-*trans*-CH=CH-COOEt. EDC/HOBt Coupling

Method. To a stirred solution of Cbz-Val-NH-NH-CH₂-COO*t*Bu (0.34 mM, 1 eq), HOBt (0.37 mM, 1.1 eq), and monoethyl fumarate (0.37 mM, 1.1 eq) in DMF was added EDC (0.37 mM, 1.1 eq) and the mixture was allowed to react for 16 h at room temperature (2 eq of EDC/HOBt were used with the tetrapeptides). The DMF was removed under vacuum and the residue was treated with 10 mL EtOAc. The organic layer was then washed with 2 x 10 mL 2 % citric acid, 1 x 15 mL saturated NaHCO₃, and 2 x 10 mL brine. The organic layer was dried over Na₂SO₄, filtered and concentrated. Column chromatography on silica gel with 1:49:50 MeOH:CH₂Cl₂:EtOAc as the eluent gave the product as a white solid with 63 % yield. MS and ¹H NMR (DMSO-*d*₆) were consistent with the proposed structure. MS (ESI) *m/z* 506.3 [(M + 1)⁺]. ¹H NMR (CDCl₃): 0.97-1.02 (dd, 6H, Val), 1.28 (t, 3H, OCH₂CH₃), 1.46 (s, 9H, *t*Bu), 2.18 (m, 1H, Val), 3.98 (t, 1H, α-H), 4.21 (q, 2H, OCH₂CH₃), 5.11 (s, 2H, Cbz), 5.21 (d, 1H, NH), 6.86 (d, 1H, db), 7.33 (s, 6H, Ph and db), 8.49 (s, 1H, NH-N).

Cbz-Val-AAsp(O-*t*Bu)-*trans*-CH=CH-CH₃ was obtained by the EDC/HOBt coupling method, and purified by column chromatography on silica gel with 0.5:49.5:50 MeOH:CH₂Cl₂:EtOAc as the eluent; white solid, yield 53 %. MS (ESI) *m/z* 448.4 [(M + 1)⁺]. ¹H NMR (DMSO-*d*₆): 0.85-0.89 (dd, 6H, Val), 1.39 (s, 9H, *t*Bu), 1.94 (m, 1H, Val), 3.83 (t, 1H, α-H), 5.02 (q, 2H, Cbz), 6.41 (m, 1H, db), 6.72 (m, 1H, db), 7.32 (s, 5H, Ph), 7.58 (d, 1H, NH).

Cbz-Val-AAsp(O-*t*Bu)-*trans*-CH=CH-*trans*-CH=CH-CH₃ was obtained by the EDC/HOBt coupling method, and purified by column chromatography on silica gel with

0.5:49.5:50 MeOH:CH₂Cl₂:EtOAc as the eluent; white solid, yield 8 %. MS (ESI) m/z 474.3 [(M + 1)⁺]. ¹H NMR (DMSO-*d*₆): 0.83-0.89 (dd, 6H, Val), 1.39 (s, 9H, *t*Bu), 1.75 (d, 3H, CH₃), 1.92 (m, 1H, Val), 3.83 (t, 1H, α-H), 5.02 (q, 2H, Cbz), 6.14 (m, 2H, db), 6.40 (m, 1H, db), 7.09 (m, 1H, db), 7.33 (s, 5H, Ph), 7.58 (d, 1H, NH), 10.77 (s, 1H, *NH-N*).

Cbz-Val-AAsp(O-*t*Bu)-*trans*-CH=CH-CH₂CH₂Ph was obtained by the EDC/HOBt coupling method, and purified by column chromatography on silica gel with 0.5:49.5:50 MeOH:CH₂Cl₂:EtOAc as the eluent; white solid, yield 53 %. MS (ESI) m/z 538.3 [(M + 1)⁺].

Cbz-Val-AAsp(O-*t*Bu)-*cis*-CH=CH-Cl was obtained by the EDC/HOBt coupling method, and purified by column chromatography on silica gel with 0.5:49.5:50 MeOH:CH₂Cl₂:EtOAc as the eluent; white solid, yield 25 %. MS (ESI) m/z 468.4 [(M + 1)⁺]. ¹H NMR (DMSO-*d*₆): 0.89 (d, 6H, Val), 1.43 (s, 9H, *t*Bu), 1.95 (m, 1H, Val), 3.84 (t, 1H, α-H), 5.04 (s, 2H, Cbz), 6.59 (s, 1H, db), 6.81 (d, 1H, db), 7.35 (s, 5H, Ph), 7.56 (d, 1H, NH), 10.81 (s, 1H, *NH-N*).

Cbz-Val-AAsp(O-*t*Bu)-*trans*-CH=CH-Ph-4-Cl was obtained by the EDC/HOBt coupling method, and purified by column chromatography on silica gel with 0.5:49.5:50 MeOH:CH₂Cl₂:EtOAc as the eluent; white solid, yield 62 %. MS (ESI) m/z 544.3 [(M + 1)⁺]. ¹H NMR (DMSO-*d*₆): 0.86-0.93 (dd, 6H, Val), 1.41 (s, 9H, *t*Bu), 1.98 (m, 1H, Val), 3.80 (t, 1H, α-H), 5.03 (q, 2H, Cbz), 7.30 (s, 5H, Ph), 7.40 (d, 1H, db), 7.52 (d, 1H, db), 7.76 (m, 4H, Ph-Cl), 10.95 (s, 1H, *NH-N*).

Cbz-Val-AAsp(O-*t*Bu)-*trans*-CH=CH-CONH-*n*Bu was obtained by the EDC/HOBt coupling method, and purified by column chromatography on silica gel with

1:9:10 MeOH:CH₂Cl₂:EtOAc as the eluent; white solid, yield 53 %. MS (ESI) *m/z* 533.2 [(M + 1)⁺]. ¹H NMR (DMSO-*d*₆): 0.83-0.87 (m, 9H, Val and NHCH₂CH₂CH₂CH₃), 1.27 (m, 2H, NHCH₂CH₂CH₂CH₃), 1.41 (s, 11H, *t*Bu and NHCH₂CH₂CH₂CH₃), 1.96 (m, 1H, Val), 3.12 (dd, 2H, NHCH₂CH₂CH₂CH₃), 3.90 (t, 1H, α-H), 5.03 (q, 2H, Cbz), 6.91 (d, 1H, db), 7.08 (d, 1H, db), 7.28-7.34 (m, 5H, Ph), 7.47 (d, 1H, NH), 8.37 (t, 1H, NHCH₂CH₂CH₂CH₃), 10.93 (s, 1H, NH-N).

Cbz-Val-AAsp(O-*t*Bu)-*trans*-CH=CH-CONHBzl was obtained by the EDC/HOBt coupling method, and purified by column chromatography on silica gel with 1:49:50 MeOH:CH₂Cl₂:EtOAc as the eluent; white solid, yield 72 %. MS (ESI) *m/z* 567.4 [(M + 1)⁺]. ¹H NMR (DMSO-*d*₆): 0.85 (d, 6H, Val), 1.40 (s, 9H, *t*Bu), 1.96 (m, 1H, Val), 3.90 (t, 1H, α-H), 4.33 (d, 2H, NHCH₂Ph), 5.00 (s, 2H, Cbz), 6.97 (d, 1H, db), 7.14-7.32 (m, 11H, Ph and db), 7.49 (d, 1H, NH), 8.90 (t, 1H, NHCH₂Ph), 10.92 (s, 1H, NH-N).

Cbz-Asp(O-*t*Bu)-Glu(O-*t*Bu)-Val-AAsp(O-*t*Bu)-*cis*-CH=CH-COOEt was obtained using the EDC/HOBt coupling method, purified by column chromatography on silica gel with 1:19:20 MeOH:CH₂Cl₂:EtOAc as the eluent; white solid, yield 53%. MS (ESI) *m/z* 862.5 [(M + 1)⁺]. ¹H NMR (DMSO-*d*₆): 0.85 (d, 6H, Val), 1.18 (t, 3H, OCH₂CH₃), 1.33-1.39 (m, 27H, *t*Bu), 1.74 (m, 1H, Glu), 1.86 (m, 1H, Glu), 1.95 (m, 1H, Val), 2.18 (m, 2H, Glu), 2.45 (dd, 1H, Asp), 2.61 (dd, 1H, Asp), 4.09 (t, 1H, α-H), 4.16 (q, 2H, OCH₂CH₃), 4.33 (m, 2H, α-H), 5.01 (q, 2H, Cbz), 6.61 (d, 1H, db), 7.25 (d, 1H, db), 7.33 (s, 5H, Ph), 7.62 (d, 1H, NH), 7.93 (t, 2H, NH), 11.05 (s, 1H, NH-N).

Cbz-Asp(O-*t*Bu)-Glu(O-*t*Bu)-Val-AAsp(O-*t*Bu)-*trans*-CH=CH-COOBzl was obtained by the EDC/HOBt coupling method, and purified by column chromatography on

silica gel with 1:499:500 MeOH:CH₂Cl₂:EtOAc as the eluent; white solid, yield 49 %. MS (ESI) m/z 924.5 [(M + 1)⁺]. ¹H NMR (DMSO-d₆): 0.80 (t, 6H, Val), 1.35-1.41 (m, 27H, *t*Bu), 1.75 (m, 1H, Glu), 1.84 (m, 1H, Glu), 1.94 (m, 1H, Val), 2.17 (m, 2H, Glu), 2.45 (dd, 1H, Asp), 2.60 (dd, 1H, Asp), 4.13 (t, 1H, α-H), 4.28-4.35 (dm, 2H, α-H), 5.02 (q, 2H, Cbz), 5.19 (s, 2H, Bzl), 6.70 (d, 1H, db), 7.25-7.37 (m, 11H, Ph and db), 7.57 (d, 1H, NH), 7.92 (d, 1H, NH), 7.98 (d, 1H, NH), 11.02 (s, 1H, *NH*-N).

Cbz-Asp(O-*t*Bu)-Glu(O-*t*Bu)-Val-AAsp(O-*t*Bu)-*trans*-CH=CH-CONHBzl was obtained by the EDC/HOBt coupling method, and purified by column chromatography on silica gel with 1:9:10 MeOH:CH₂Cl₂:EtOAc as the eluent; white solid, yield 49 %. MS (ESI) m/z 923.5 [(M + 1)⁺]. ¹H NMR (DMSO-d₆): 0.85 (t, 6H, Val), 1.35-1.41 (m, 27H, *t*Bu), 1.72 (m, 1H, Glu), 1.84 (m, 1H, Glu), 1.96 (m, 1H, Val), 2.17 (m, 2H, Glu), 2.44 (dd, 1H, Asp), 2.60 (dd, 1H, Asp), 4.17 (t, 1H, α-H), 4.28 (m, 2H, α-H), 4.36 (d, 2H, NHCH₂Ph), 5.02 (q, 2H, Cbz), 6.95 (d, 1H, db), 7.12 (d, 1H, db), 7.22-7.31 (m, 10H, Ph), 7.57 (d, 1H, NH), 7.94 (d, 1H, NH), 8.92 (t, 1H, NHCH₂Ph), 10.96 (s, 1H, *NH*-N).

Cbz-Asp(O-*t*Bu)-Glu(O-*t*Bu)-Val-AAsp(O-*t*Bu)-*trans*-CH=CH-CONHCH₂CH₂Ph was obtained by the EDC/HOBt coupling method, and purified by column chromatography on silica gel with 1:9:5 MeOH:CH₂Cl₂:EtOAc as the eluent, and then rechromatographed using 1:1 EtOAc:CH₂Cl₂ as the eluent; white solid, yield 29 %. MS (ESI) m/z 937.5 [(M + 1)⁺]. ¹H NMR (DMSO-d₆): 0.85 (t, 6H, Val), 1.35-1.41 (m, 27H, *t*Bu), 1.72 (m, 1H, Glu), 1.86 (m, 1H, Glu), 2.00 (m, 1H, Val), 2.17 (m, 2H, Glu), 2.43 (dd, 1H, Asp), 2.61-2.64 (dd, 1H, Asp), 2.73 (t, 2H, NHCH₂CH₂Ph), 3.35 (q, 2H, NHCH₂CH₂Ph), 4.17 (t, 1H, α-H), 4.29-4.35 (m, 2H, α-H), 5.02 (q, 2H, Cbz), 6.89 (d,

1H, db), 7.07 (d, 1H, db), 7.17-7.31 (m, 10H, Ph), 7.59 (d, 1H, NH), 7.94 (d, 1H, NH), 8.51 (t, 1H, *NHCH*₂*CH*₂Ph), 10.95 (s, 1H, *NH-N*).

Cbz-Asp(O-*t*Bu)-Glu(O-*t*Bu)-Val-AAsp(O-*t*Bu)-*trans*-CH=CH-CON(CH₃)Bzl

was obtained by the EDC/HOBt coupling method, and purified by column chromatography on silica gel with 1:9:5 MeOH:CH₂Cl₂:EtOAc as the eluent, and then rechromatographed using 1:1 EtOAc:hexanes as the eluent; white solid, yield 34 %. MS (ESI) *m/z* 937.5 [(M + 1)⁺]. ¹H NMR (DMSO-*d*₆): 0.86 (t, 6H, Val), 1.34-1.41 (m, 27H, *t*Bu), 1.65 (m, 1H, Glu), 1.88 (m, 1H, Glu), 1.97 (m, 1H, Val), 2.17 (m, 2H, Glu), 2.43 (dd, 1H, Asp), 2.59 (dd, 1H, Asp), 2.88-2.98 (d, 3H, N(CH₃)CH₂Ph), 4.16 (t, 1H, α-H), 4.29-4.34 (dm, 2H, α-H), 4.54 (q, 1H, N(CH₃)CH₂Ph), 4.67 (s, 1H, N(CH₃)CH₂Ph), 5.02 (q, 2H, Cbz), 7.14 (d, 1H, db), 7.20 (d, 1H, db), 7.28-7.37 (m, 10H, Ph), 7.56 (d, 1H, NH), 7.94 (t, 1H, NH), 10.97 (s, 1H, *NH-N*).

Cbz-Asp(O-*t*Bu)-Glu(O-*t*Bu)-Val-AAsp(O-*t*Bu)-*trans*-CH=CH-

CON(CH₃)CH₂CH₂Ph was obtained by the EDC/HOBt coupling method, and purified by column chromatography on silica gel with 1:19:20 MeOH:CH₂Cl₂:EtOAc as the eluent, and then rechromatographed using 1:1 EtOAc:CH₂Cl₂ as the eluent; white solid, yield 45 %. MS (ESI) *m/z* 951.5 [(M + 1)⁺]. ¹H NMR (acetone-*d*₆): 0.86 (t, 6H, Val), 1.41-1.48 (m, 27H, *t*Bu), 1.75 (m, 1H, Glu), 1.89 (m, 1H, Glu), 2.01 (m, 1H, Val), 2.21 (m, 2H, Glu), 2.46 (dd, 1H, Asp), 2.62 (dd, 1H, Asp), 2.80 (t, 2H, N(CH₃)CH₂CH₂Ph), 2.85-2.98 (d, 3H, N(CH₃)CH₂CH₂Ph), 3.73 (m, 2H, N(CH₃)CH₂CH₂Ph), 4.18 (t, 1H, α-H), 4.32 (dm, 2H, α-H), 5.00 (q, 2H, Cbz), 6.88 (t, 1H, NH), 7.17-7.34 (m, 12H, Cbz-Ph, N(CH₃)CH₂CH₂Ph, and CH=CH), 7.59 (d, 1H, NH), 7.85 (d, 1H, NH), 7.97 (d, 1H, NH), 9.89 (d, 1H, *NH-N*).

Cbz-Asp(O-*t*Bu)-Glu(O-*t*Bu)-Val-AAsp(O-*t*Bu)-*trans*-CH=CH-CON(Bzl)₂

was obtained by the EDC/HOBt coupling method, and purified by column chromatography on silica gel with 2:1 EtOAc:hexane as the eluent, and then rechromatographed using 1:1 EtOAc:hexanes as the eluent; white solid, yield 62 %. MS (ESI) m/z 1013.4 $[(M + 1)^+]$. ¹H NMR (DMSO-*d*₆): 0.87 (t, 6H, Val), 1.35-1.39 (m, 27H, *t*Bu), 1.73 (m, 1H, Glu), 1.87 (m, 1H, Glu), 2.02 (m, 1H, Val), 2.23 (m, 2H, Glu), 2.48 (dd, 1H, Asp), 2.65 (dd, 1H, Asp), 4.19 (t, 1H, α-H), 4.33 (m, 2H, α-H), 4.55 (d, 2H, N(CH₂Ph)₂), 4.63 (s, 2H, N(CH₂Ph)₂), 5.00 (q, 2H, Cbz), 7.14 (d, 1H, db), 7.21-7.34 (m, 16H, db, Cbz-Ph, and N(CH₂Ph)₂), 7.60 (d, 1H, NH), 7.91-7.98 (dd, 2H, NH), 11.00 (s, 1H, NH-N).

Cbz-Asp(O-*t*Bu)-Glu(O-*t*Bu)-Val-AAsp(O-*t*Bu)-*trans*-CH=CH-CO-

tetrahydroquinoline was obtained by the EDC/HOBt coupling method, and purified by column chromatography on silica gel with 1:2 CH₂Cl₂:EtOAc as the eluent; yellow solid, yield 54 %. MS (ESI) m/z 949.4 $[(M + 1)^+]$. ¹H NMR (DMSO-*d*₆): 0.89 (t, 6H, Val), 1.35-1.42 (m, 27H, *t*Bu), 1.75 (m, 1H, Glu), 1.87 (m, 3H, Glu and NCH₂CH₂CH₂), 2.02 (m, 1H, Val), 2.20 (m, 2H, Glu), 2.48 (dd, 1H, Asp), 2.65 (dd, 1H, Asp), 2.70 (t, 2H, NCH₂CH₂CH₂), 3.73 (t, 2H, NCH₂CH₂CH₂), 4.19 (t, 1H, α-H), 4.33 (m, 2H, α-H), 5.01 (q, 2H, Cbz), 6.95 (m, 1H, db), 7.04 (d, 1H, db), 7.14-7.33 (m, 9H, Cbz-Ph, and quinoline), 7.60 (d, 1H, NH), 7.96 (dd, 2H, NH), 11.02 (s, 1H, NH-N).

Cbz-Asp(O-*t*Bu)-Glu(O-*t*Bu)-Val-AAsp(O-*t*Bu)-*trans*-CH=CH-CONH-Bzl-4-

F was obtained by the EDC/HOBt coupling method, and purified by column chromatography on silica gel with 1:19:20 MeOH:CH₂Cl₂:EtOAc as the eluent; white solid, yield 67 %. MS (ESI) m/z 941.4 $[(M + 1)^+]$. ¹H NMR (DMSO-*d*₆): 0.86 (t, 6H,

Val), 1.34-1.41 (m, 27H, *t*Bu), 1.75 (m, 1H, Glu), 1.88 (m, 1H, Glu), 2.01 (m, 1H, Val), 2.20 (m, 2H, Glu), 2.48 (dd, 1H, Asp), 2.65 (dd, 1H, Asp), 4.18 (t, 1H, α -H), 4.34 (m, 4H, α -H and $\text{NHCH}_2\text{Ph-4-F}$), 5.01 (q, 2H, Cbz), 6.93 (d, 1H, db), 7.12 (d, 2H, $\text{NHCH}_2\text{Ph-4-F}$), 7.25-7.32 (m, 8H, Cbz-Ph, $\text{NHCH}_2\text{Ph-4-F}$, and db), 7.59 (d, 1H, NH), 7.90-7.99 (dd, 2H, NH), 8.94 (t, 1H, $\text{NHCH}_2\text{Ph-4-F}$), 10.98 (s, 1H, *NH-N*).

Cbz-Asp(O-*t*Bu)-Glu(O-*t*Bu)-Val-AAsp(O-*t*Bu)-*trans*-CH=CH-COPh was obtained by the EDC/HOBt coupling method, and purified by column chromatography on silica gel with 1:499:500 MeOH:CH₂Cl₂:EtOAc as the eluent; white solid, yield 40 %. MS (ESI) *m/z* 894.4 [(M + 1)⁺]. ¹H NMR (DMSO-*d*₆): 0.82 (t, 6H, Val), 1.34-1.42 (m, 27H, *t*Bu), 1.70 (m, 1H, Glu), 1.85 (m, 1H, Glu), 1.96 (m, 1H, Val), 2.15 (m, 2H, Glu), 2.44 (dd, 1H, Asp), 2.58-2.60 (dd, 1H, Asp), 4.14 (t, 1H, α -H), 4.26-4.33 (dm, 2H, α -H), 5.02 (q, 2H, Cbz), 7.23 (d, 1H, db), 7.31 (s, 5H, Cbz), 7.51 (m, 3H, Ph), 7.66 (t, 1H, NH), 7.80 (d, 1H, db), 7.99 (d, 2H, Ph), 11.02 (s, 1H, *NH-N*).

Cbz-Leu-Glu(O-*t*Bu)-Thr-AAsp(O-*t*Bu)-*trans*-CH=CH-COOBzl was obtained by the EDC/HOBt coupling method, and purified by column chromatography on silica gel with 1:499:500 MeOH:CH₂Cl₂:EtOAc as the eluent; white solid, yield 52 %. MS (ESI) *m/z* 868.4 [(M + 1)⁺]. ¹H NMR (DMSO-*d*₆): 0.83 (t, 6H, Leu), 1.02 (d, 3H, Thr), 1.36-1.40 (t, 20H, *t*Bu and Leu), 1.60 (m, 1H, Glu), 1.72 (m, 1H, Glu), 1.88 (m, 1H, Leu), 2.21 (m, 2H, Glu), 3.92 (m, 1H, α -H), 4.03 (m, 1H, α -H), 4.17 (m, 1H, α -H), 4.34 (m, 1H, Thr), 4.99 (s, 2H, Cbz), 5.19 (s, 2H, Bzl), 6.67-6.71 (d, 1H, db), 7.32-7.38 (m, 10H, Ph), 7.40 (d, 1H, db), 7.76 (d, 1H, NH), 8.04 (d, 1H, NH), 10.80 (s, 1H, *NH-N*).

Cbz-Val-Glu(O-*t*Bu)-Val-AAsp(O-*t*Bu)-*trans*-CH=CH-COOEt was obtained by the EDC/HOBt coupling method, and purified by column chromatography on silica

gel with 1:9:5 MeOH:CH₂Cl₂:EtOAc as the eluent; white solid, yield 71 %. MS (ESI) m/z 790.3 [(M + 1)⁺]. ¹H NMR (DMSO-d₆): 0.81-0.87 (dd, 12H, Val), 1.22 (t, 3H, OCH₂CH₃), 1.24-1.41 (d, 18H, *t*Bu), 1.72 (m, 1H, Glu), 1.83 (m, 1H, Glu), 1.96 (m, 1H, Val), 2.19 (m, 2H, Glu), 3.86 (t, 1H, α-H), 4.17 (m, 3H, α-H and OCH₂CH₃), 4.32 (m, 1H, α-H), 5.01 (s, 2H, Cbz), 6.64 (d, 1H, db), 7.20 (d, 1H, db), 7.30-7.33 (m, 5H, Ph), 7.97 (t, 2H, NH), 11.02 (s, 1H, NH-N).

Deblocking of the *t*-Butyl Group in the Aza-Asp Peptides. The aza-peptide inhibitors were treated with 1:1 TFA: CH₂Cl₂ at 0 °C for 1 h and at room temperature for 2 h. TFA and CH₂Cl₂ were removed under vacuum and the final products were recrystallized from ether/hexanes mostly as white solids.

N²-(N-Benzyloxycarbonylvalyl)-N¹-carboxymethyl-N¹-*trans*-(3-ethoxycarbonylacryloyl)hydrazine (Cbz-Val-AAsp-*trans*-CH=CH-COOEt). ¹H NMR (DMSO-d₆): 0.87 (d, 6H, Val), 1.15-1.23 (m, 3H, OCH₂CH₃), 1.98 (m, 1H, Val), 3.89 (t, 1H, α-H), 4.13 (q, 2H, OCH₂CH₃), 5.03 (s, 2H, Cbz), 6.65 (d, 1H, db), 7.28-7.34 (m, 6H, db and Ph), 7.54 (d, 1H, NH), 11.00 (s, 1H, NH). HRMS (FAB) Calcd. for C₂₁H₂₇N₃O₈: 449.1798 Observed m/z : 449.1876. Anal. Calcd. for C₂₁H₂₇N₃O₈·0.29H₂O: C, 55.47; H, 6.13; N, 9.26. Found: C, 55.47; H, 6.11; N, 9.24.

N²-(N-Benzyloxycarbonylvalyl)-N¹-*trans*-(3-but-2-enoyl)-N¹-carboxymethylhydrazine (Cbz-Val-AAsp-*trans*-CH=CH-CH₃). HRMS (ESI) Calcd. for C₁₉H₂₅N₃O₆: 391.1822; Observed m/z : 391.1797.

N²-(N-Benzyloxycarbonylvalyl)-N¹-carboxymethyl-N¹-*trans*-(3-hexa-2,4-dienoyl)hydrazine (Cbz-Val-AAsp-*trans*-CH=CH-*trans*-CH=CH-CH₃). ¹H NMR (DMSO-d₆): 0.90 (d, 6H, Val), 1.78 (d, 3H, CH₃), 1.97 (m, 1H, Val), 3.87 (t, 1H, α-H),

5.05 (q, 2H, Cbz), 6.14 (m, 2H, db), 6.38 (m, 1H, db), 7.09 (dd, 2H, db), 7.33 (m, 5H, Ph), 7.59 (d, 1H, NH), 10.81 (s, 1H, *NH-N*). HRMS (ESI) Calcd. for $C_{21}H_{27}N_3O_6$: 417.1978 Observed m/z : 417.1974. Anal. Calcd. for $C_{21}H_{27}N_3O_6 \cdot 1.43H_2O \cdot 0.05TFA$: C, 56.45; H, 6.72; N, 9.36. Found: C, 56.36; H, 6.43; N, 9.66.

N^2 -(*N*-Benzyloxycarbonylvalyl)- N^1 -carboxymethyl- N^1 -*trans*-(3-phenethylacryloyl)hydrazine (Cbz-Val-AAsp-*trans*-CH=CH-CH₂CH₂Ph). HRMS (ESI) Calcd. for $C_{26}H_{31}N_3O_6$: 482.2291 Observed m/z : 482.2301. Anal. Calcd. for $C_{26}H_{31}N_3O_6 \cdot 0.84H_2O$: C, 62.87; H, 6.63; N, 8.46. Found: C, 62.87; H, 6.51; N, 8.28.

N^2 -(*N*-Benzyloxycarbonylvalyl)- N^1 -carboxymethyl- N^1 -*cis*-(3-chloroacryloyl)hydrazine (Cbz-Val-AAsp-*cis*-CH=CH-Cl). ¹H NMR (DMSO-*d*₆): 0.86 (d, 6H, Val), 1.93 (m, 1H, Val), 3.84 (t, 1H, α-H), 5.01 (s, 2H, Cbz), 6.57 (s, 1H, db), 6.76 (d, 1H, db), 7.29-7.34 (m, 5H, Ph), 7.53 (d, 1H, NH), 10.80 (s, 1H, *NH-N*). HRMS (ESI) Calcd. for $C_{18}H_{22}N_3O_6Cl$: 411.1275 Observed m/z : 411.1195. Anal. Calcd. for $C_{18}H_{22}N_3O_6Cl \cdot 0.04H_2O$: C, 52.40; H, 5.39; N, 10.19. Found: C, 52.40; H, 5.46; N, 10.30.

N^2 -(*N*-Benzyloxycarbonylvalyl)- N^1 -carboxymethyl- N^1 -*trans*-(3-(4-chlorophenyl)acryloyl)hydrazine (Cbz-Val-AAsp-*trans*-CH=CH-Ph-4-Cl). ¹H NMR (DMSO-*d*₆): 0.86-0.95 (dd, 6H, Val), 1.94 (m, 1H, Val), 3.84 (t, 1H, α-H), 5.03 (q, 2H, Cbz), 7.27-7.32 (m, 5H, Ph), 7.42 (d, 1H, db), 7.55 (d, 1H, db), 7.77 (m, 4H, Ph-Cl), 10.97 (s, 1H, *NH-N*). HRMS (ESI) Calcd. for $C_{24}H_{26}N_3O_6Cl$: 488.1588 Observed m/z : 488.1563. Anal. Calcd. for $C_{24}H_{26}N_3O_6Cl \cdot 0.29H_2O$: C, 58.45; H, 5.43; N, 8.51. Found: C, 58.46; H, 5.43; N, 8.51.

N²-(N-Benzyloxycarbonylvalyl)-N¹-*trans*-(3-butylcarbamoylacryloyl)-N¹-carboxymethylhydrazine (Cbz-Val-AAsp-*trans*-CH=CH-CONH-*n*Bu). ¹H NMR (DMSO-*d*₆): 0.83-0.87 (m, 9H, Val and NHCH₂CH₂CH₂CH₃), 1.27 (m, 2H, NHCH₂CH₂CH₂CH₃), 1.37-1.41 (m, 2H, NHCH₂CH₂CH₂CH₃), 1.97 (m, 1H, Val), 3.12 (dd, 2H, NHCH₂CH₂CH₂CH₃), 3.93 (t, 1H, α-H), 5.03 (q, 2H, Cbz), 6.91 (d, 1H, db), 7.08 (d, 1H, db), 7.28-7.34 (m, 5H, Ph), 7.47 (d, 1H, NH), 8.37 (t, 1H, NHCH₂CH₂CH₂CH₃), 10.93 (s, 1H, NH-N). HRMS (ESI) Calcd. for C₂₃H₃₂N₄O₇: 477.2349 Observed *m/z*: 477.2357. Anal. Calcd. for C₂₃H₃₂N₄O₇·0.46H₂O: C, 56.98; H, 6.84; N, 11.56. Found: C, 56.99; H, 6.85; N, 11.56.

N²-(N-Benzyloxycarbonylvalyl)-N¹-*trans*-(3-benzylcarbamoylacryloyl)-N¹-carboxymethylhydrazine (Cbz-Val-AAsp-*trans*-CH=CH-CONHBzl). ¹H NMR (DMSO-*d*₆): 0.85 (d, 6H, Val), 1.97 (m, 1H, Val), 3.96 (t, 1H, α-H), 4.35 (d, 2H, NHCH₂Ph), 5.01 (s, 2H, Cbz), 6.97 (d, 1H, db), 7.15 (d, 1H, db), 7.19-7.32 (m, 10H, Ph), 7.47 (d, 1H, NH), 8.91 (t, 1H, NHCH₂Ph), 10.94 (s, 1H, NH-N). HRMS (ESI) Calcd. for C₂₆H₃₀N₄O₇: 510.2193 Observed *m/z*: 510.2208. Anal. Calcd. for C₂₆H₃₀N₄O₇·0.56H₂O: C, 59.98; H, 6.02; N, 10.79. Found: C, 59.99; H, 6.08; N, 10.76.

N²-(N-Benzyloxycarbonylaspartylglutamylvalyl)-N¹-carboxymethyl-N¹-*trans*-(3-ethoxycarbonylacryloyl)hydrazine (Cbz-Asp-Glu-Val-AAsp-*trans*-CH=CH-COOEt). This compound was synthesized by Z. Li in our laboratory.

N²-(N-Benzyloxycarbonylaspartylglutamylvalyl)-N¹-carboxymethyl-N¹-*cis*-(3-ethoxycarbonylacryloyl)hydrazine (Cbz-Asp-Glu-Val-AAsp-*cis*-CH=CH-COOEt). ¹H NMR (DMSO-*d*₆): 0.85 (d, 6H, Val), 1.18 (t, 3H, OCH₂CH₃), 1.74 (m, 1H, Glu), 1.86 (m, 1H, Glu), 1.95 (m, 1H, Val), 2.18 (m, 2H, Glu), 2.45 (dd, 1H, Asp), 2.61

(dd, 1H, Asp), 4.09 (t, 1H, α -H), 4.16 (q, 2H, OCH₂CH₃), 4.33 (m, 2H, α -H), 5.01 (s, 2H, Cbz), 6.61 (d, 1H, db), 7.25 (d, 1H, db), 7.33 (s, 5H, Ph), 7.62 (d, 1H, NH), 7.93 (t, 2H, NH), 11.05 (s, 1H, NH-N). HRMS (ESI) Calcd. for C₃₀H₃₉N₅O₁₄: 693.2566 Observed *m/z*: 693.2516. Anal. Calcd. for C₃₀H₃₉N₅O₁₄·1H₂O: C, 50.63; H, 5.81; N, 9.67. Found: C, 50.61; H, 5.82; N, 9.67.

N²-(N-Benzyloxycarbonylaspartylglutamylvalyl)-N¹-*trans*-(3-benzyloxycarbonylacryloyl)-N¹-carboxymethylhydrazine (Cbz-Asp-Glu-Val-AAsp-*trans*-CH=CH-COOBzl). ¹H NMR (DMSO-*d*₆): 0.81 (d, 6H, Val), 1.74 (m, 1H, Glu), 1.86 (m, 1H, Glu), 1.95 (m, 1H, Val), 2.18 (m, 2H, Glu), 2.45 (dd, 1H, Asp), 2.61 (dd, 1H, Asp), 4.13 (t, 1H, α -H), 4.29 (m, 2H, α -H), 4.99 (s, 2H, Cbz), 5.18 (s, 2H, Bzl), 6.69 (d, 1H, db), 7.25-7.37 (m, 11H, Ph, and db), 7.60 (d, 1H, NH), 7.93 (t, 2H, NH), 11.02 (s, 1H, NH-N). HRMS (ESI) Calcd. for C₃₅H₄₁N₅O₁₄: 755.2728 Observed *m/z*: 755.2631. Anal. Calcd. for C₃₅H₄₁N₅O₁₄·0.59H₂O: C, 54.86; H, 5.55; N, 9.14. Found: C, 54.86; H, 5.67; N, 9.11.

N²-(N-Benzyloxycarbonylaspartylglutamylvalyl)-N¹-*trans*-(3-benzylcarbamoylacryloyl)-N¹-carboxymethylhydrazine (Cbz-Asp-Glu-Val-AAsp-*trans*-CH=CH-CONHBzl). ¹H NMR (DMSO-*d*₆): 0.85 (t, 6H, Val), 1.75 (m, 1H, Glu), 1.88 (m, 1H, Glu), 2.01 (m, 1H, Val), 2.19 (m, 2H, Glu), 2.48 (dd, 1H, Asp), 2.61 (dd, 1H, Asp), 4.17 (t, 1H, α -H), 4.30 (m, 2H, α -H), 4.35 (d, 2H, NHCH₂Ph), 5.00 (s, 2H, Cbz), 6.96 (d, 1H, db), 7.12 (d, 1H, db), 7.21-7.32 (m, 10H, Ph), 7.58 (d, 1H, NH), 7.90 (d, 1H, NH), 7.96 (d, 1H, NH), 8.92 (t, 1H, NHCH₂Ph), 10.97 (s, 1H, NH-N). HRMS (ESI) Calcd. for C₃₅H₄₂N₆O₁₃: 754.2888 Observed *m/z*: 754.2853. Anal. Calcd. for C₃₅H₄₂N₆O₁₃·1.63H₂O: C, 53.61; H, 5.82; N, 10.72. Found: C, 53.82; H, 5.74; N, 10.42.

N²-(N-Benzyloxycarbonylaspartylglutamylvalyl)-N¹-carboxymethyl-N¹-*trans*-(3-phenethylcarbamoylacryloyl)hydrazine (Cbz-Asp-Glu-Val-AAsp-*trans*-CH=CH-CONHCH₂CH₂Ph). ¹H NMR (DMSO-d₆): 0.87 (t, 6H, Val), 1.75 (m, 1H, Glu), 1.89 (m, 1H, Glu), 2.01 (m, 1H, Val), 2.21 (m, 2H, Glu), 2.46 (dd, 1H, Asp), 2.62 (dd, 1H, Asp), 2.73 (t, 2H, NHCH₂CH₂Ph), 3.73 (d, 2H, NHCH₂CH₂Ph), 4.18 (t, 1H, α-H), 4.32 (m, 2H, α-H), 5.01 (s, 2H, Cbz), 6.89 (d, 1H, db), 7.08 (d, 1H, db), 7.16-7.32 (m, 10H, Ph), 7.59 (d, 1H, NH), 7.89 (d, 1H, NH), 7.98 (d, 1H, NH), 8.52 (t, 1H, NHCH₂CH₂Ph), 10.97 (s, 1H, NH-N). HRMS (ESI) Calcd. for C₃₆H₄₄N₆O₁₃: 768.3044 Observed *m/z*: 768.2949. Anal. Calcd. for C₃₆H₄₄N₆O₁₃·1.22H₂O: C, 54.68; H, 5.92; N, 10.63. Found: C, 54.86; H, 5.91; N, 10.33.

N²-(N-Benzyloxycarbonylaspartylglutamylvalyl)-N¹-*trans*-(3-benzylmethylcarbamoylacryloyl)-N¹-carboxymethylhydrazine (Cbz-Asp-Glu-Val-AAsp-*trans*-CH=CH-CON(CH₃)Bzl). ¹H NMR (DMSO-d₆): 0.85 (d, 6H, Val), 1.73 (m, 1H, Glu), 1.87 (m, 1H, Glu), 1.97 (m, 1H, Val), 2.20 (m, 2H, Glu), 2.48 (dd, 1H, Asp), 2.63 (dd, 1H, Asp), 2.88-2.98 (d, 3H, N(CH₃)CH₂Ph), 4.17 (t, 1H, α-H), 4.32 (m, 2H, α-H), 4.47-4.58 (q, 1H, N(CH₃)CH₂Ph), 4.66 (s, 1H, N(CH₃)CH₂Ph), 5.00 (s, 2H, Cbz), 7.14 (d, 1H, db), 7.20 (d, 1H, db), 7.28-7.36 (m, 10H, Ph), 7.57 (d, 1H, NH), 7.91-7.95 (m, 2H, NH), 10.99 (s, 1H, NH-N). HRMS (ESI) Calcd. for C₃₆H₄₄N₆O₁₃: 768.3045 Observed *m/z*: 768.3039. Anal. Calcd. for C₃₆H₄₄N₆O₁₃·EtOAc: C, 55.44; H, 6.20; N, 9.95. Found: C, 55.32; H, 6.20; N, 9.95.

N²-(N-Benzyloxycarbonylaspartylglutamylvalyl)-N¹-carboxymethyl-N¹-*trans*-(3-phenethylmethylcarbamoylacryloyl)hydrazine (Cbz-Asp-Glu-Val-AAsp-*trans*-CH=CH-CON(CH₃)CH₂CH₂Ph). ¹H NMR (DMSO-d₆): 0.86 (t, 6H, Val), 1.75 (m, 1H,

Glu), 1.89 (m, 1H, Glu), 2.01 (m, 1H, Val), 2.21 (m, 2H, Glu), 2.46 (dd, 1H, Asp), 2.62 (dd, 1H, Asp), 2.80 (t, 2H, N(CH₃)CH₂CH₂Ph), 2.85-2.98 (d, 3H, N(CH₃)CH₂CH₂Ph), 3.73 (d, 2H, N(CH₃)CH₂CH₂Ph), 4.18 (t, 1H, α-H), 4.32 (m, 2H, α-H), 5.00 (s, 2H, Cbz), 6.94 (d, 1H, db), 7.05 (dd, 1H, db), 7.15-7.32 (m, 10H, Ph), 7.59 (d, 1H, NH), 7.91 (d, 1H, NH), 7.97 (d, 1H, NH), 10.93 (d, 1H, NH-N). HRMS (ESI) Calcd. for C₃₇H₄₆N₆O₁₃: 783.3196 Observed *m/z*: 783.3182. Anal. Calcd. for C₃₇H₄₆N₆O₁₃·1.09H₂O·0.24TFA: C, 54.32; H, 5.77; N, 10.14. Found: C, 54.32; H, 5.91; N, 9.92.

N²-(N-Benzyloxycarbonylaspartylglutamylvalyl)-N¹-carboxymethyl-N¹-*trans*-(3-dibenzylcarbamoylacryloyl)hydrazine (Cbz-Asp-Glu-Val-AAsp-*trans*-CH=CH-CON(Bzl)₂). ¹H NMR (DMSO-d₆): 0.87 (t, 6H, Val), 1.73 (m, 1H, Glu), 1.87 (m, 1H, Glu), 2.02 (m, 1H, Val), 2.23 (m, 2H, Glu), 2.48 (dd, 1H, Asp), 2.65 (dd, 1H, Asp), 4.19 (t, 1H, α-H), 4.33 (m, 2H, α-H), 4.55 (d, 2H, N(CH₂Ph)₂), 4.63 (s, 2H, N(CH₂Ph)₂), 5.00 (s, 2H, Cbz), 7.14 (d, 1H, db), 7.21-7.34 (m, 16H, db, Cbz-Ph, and N(CH₂Ph)₂), 7.60 (d, 1H, NH), 7.91-7.98 (dd, 2H, NH), 11.00 (s, 1H, NH-N). HRMS (ESI) Calcd. for C₄₂H₄₈N₆O₁₃: 844.3352 Observed *m/z*: 844.3210. Anal. Calcd. for C₄₂H₄₈N₆O₁₃·0.53H₂O·0.40TFA: C, 57.12; H, 5.54; N, 9.34. Found: C, 57.12; H, 5.64; N, 9.11.

N²-(N-Benzyloxycarbonylaspartylglutamylvalyl)-N¹-carboxymethyl-N¹-*trans*-(3-(3,4-dihydro-2H-quinolin-1-yl)oxo)acryloyl)hydrazine (Cbz-Asp-Glu-Val-AAsp-*trans*-CH=CH-CO-tetrahydroquinoline). ¹H NMR (DMSO-d₆): 0.89 (t, 6H, Val), 1.75 (m, 1H, Glu), 1.87 (m, 3H, Glu and NCH₂CH₂CH₂), 2.02 (m, 1H, Val), 2.20 (m, 2H, Glu), 2.48 (dd, 1H, Asp), 2.65 (dd, 1H, Asp), 2.70 (t, 2H, NCH₂CH₂CH₂), 3.73 (t, 2H, NCH₂CH₂CH₂), 4.19 (t, 1H, α-H), 4.33 (m, 2H, α-H), 5.01 (s, 2H, Cbz), 6.95 (m, 1H,

db), 7.04 (d, 1H, db), 7.14-7.33 (m, 9H, Cbz-Ph, and quinoline), 7.60 (d, 1H, NH), 7.96 (dd, 2H, NH), 11.02 (s, 1H, *NH-N*). HRMS (ESI) Calcd. for $C_{37}H_{44}N_6O_{13}$: 780.3039 Observed m/z : 780.2954. Anal. Calcd. for $C_{37}H_{44}N_6O_{13} \cdot 0.48H_2O \cdot 0.47TFA$: C, 54.06; H, 5.43 N, 9.97. Found: C, 54.16; H, 5.72; N, 9.67.

N^2 -(N-Benzyloxycarbonylaspartylglutamylvalyl)- N^1 -carboxymethyl- N^1 -*trans*-(3-(4-fluorobenzyl)carbamoylacryloyl)hydrazine (Cbz-Asp-Glu-Val-AAsp-*trans*-CH=CH-CONH-Bzl-4-F). 1H NMR (DMSO- d_6): 0.86 (t, 6H, Val), 1.75 (m, 1H, Glu), 1.88 (m, 1H, Glu), 2.01 (m, 1H, Val), 2.20 (m, 2H, Glu), 2.48 (dd, 1H, Asp), 2.65 (dd, 1H, Asp), 4.18 (t, 1H, α -H), 4.34 (m, 4H, α -H and $NHCH_2Ph$ -4-F), 5.01 (s, 2H, Cbz), 6.93 (d, 1H, db), 7.12 (d, 2H, $NHCH_2Ph$ -4-F), 7.25-7.32 (m, 8H, Cbz-Ph, $NHCH_2Ph$ -4-F, and db), 7.59 (d, 1H, NH), 7.90-7.99 (dd, 2H, NH), 8.94 (t, 1H, $NHCH_2Ph$ -4-F), 10.98 (s, 1H, *NH-N*). HRMS (ESI) Calcd. for $C_{35}H_{41}N_6O_{13}F$: 772.2788 Observed m/z : 772.2747. Anal. Calcd. for $C_{35}H_{41}N_6O_{13}F \cdot 2.08H_2O$: C, 51.92; H, 5.62; N, 10.38. Found: C, 51.92; H, 5.40; N, 10.21.

N^2 -(N-Benzyloxycarbonylaspartylglutamylvalyl)- N^1 -*trans*-(3-benzoylacryloyl)- N^1 -carboxymethylhydrazine (Cbz-Asp-Glu-Val-AAsp-*trans*-CH=CH-COPh). 1H NMR (DMSO- d_6): 0.82 (d, 6H, Val), 1.73 (m, 1H, Glu), 1.85 (m, 1H, Glu), 1.98 (m, 1H, Val), 2.20 (m, 2H, Glu), 2.48 (dd, 1H, Asp), 2.62 (dd, 1H, Asp), 4.16 (t, 1H, α -H), 4.33 (m, 2H, α -H), 5.01 (s, 2H, Cbz), 7.24 (d, 1H, db), 7.32 (s, 5H, Ph), 7.54 (t, 3H, Ph), 7.67 (t, 1H, NH), 7.79 (d, 1H, NH), 7.93 (t, 1H, NH), 7.97 (d, 2H, Ph), 11.06 (s, 1H, *NH-N*). HRMS (ESI) Calcd. for $C_{34}H_{39}N_5O_{13}$: 725.2623 Observed m/z : 725.2543. Anal. Calcd. for $C_{34}H_{39}N_5O_{13} \cdot H_2O \cdot 0.61TFA$: C, 52.02; H, 5.16; N, 8.61. Found: C, 52.01; H, 5.10; N, 8.54.

N²-(N-Benzylloxycarbonylleucylglutamylthreonyl)-N¹-*trans*-(3-benzylloxycarbonylacryloyl)-N¹-carboxymethylhydrazine (Cbz-Leu-Glu-Thr-AAsp-*trans*-CH=CH-COOBzl). ¹H NMR (DMSO-d₆): 0.84 (t, 6H, Leu), 1.02 (d, 3H, Thr), 1.40 (m, 2H, Leu), 1.60 (m, 1H, Glu), 1.73 (m, 1H, Glu), 1.90 (m, 1H, Leu), 2.22 (m, 2H, Glu), 3.94 (m, 1H, α-H), 4.03 (m, 1H, α-H), 4.20 (m, 1H, α-H), 4.37 (m, 1H, Thr), 4.99 (s, 2H, Cbz), 5.19 (s, 2H, Bzl), 6.65 (d, 1H, db), 7.32-7.36 (m, 10H, Ph), 7.42 (d, 1H, db), 7.76 (d, 1H, NH), 8.03 (d, 1H, NH), 10.84 (s, 1H, NH-N). HRMS (ESI) Calcd. for C₃₆H₄₅N₅O₁₃: 755.3092 Observed *m/z*: 755.3150. Anal. Calcd. for C₃₆H₄₅N₅O₁₃·0.27H₂O·0.28TFA: C, 55.41; H, 5.83; N, 8.84. Found: C, 55.41; H, 5.77; N, 8.77.

N²-(N-Benzylloxycarbonylvalylglutamylvalyl)-N¹-carboxymethyl-N¹-*trans*-(3-ethoxycarbonylacryloyl)hydrazine (Cbz-Val-Glu-Val-AAsp-*trans*-CH=CH-COOEt). ¹H NMR (DMSO-d₆): 0.80-0.87 (dd, 12H, Val), 1.21 (t, 3H, OCH₂CH₃), 1.74 (m, 1H, Glu), 1.89 (m, 1H, Glu), 1.94 (m, 1H, Val), 2.20 (m, 2H, Glu), 3.87 (t, 1H, α-H), 4.16 (m, 3H, α-H and OCH₂CH₃), 4.33 (t, 1H, α-H), 5.01 (s, 2H, Cbz), 6.63 (d, 1H, db), 7.20 (d, 1H, db), 7.33 (m, 5H, Ph), 7.97 (t, 2H, NH), 11.02 (s, 1H, NH-N). HRMS (ESI) Calcd. for C₃₁H₄₃N₅O₁₂: 677.2986 Observed *m/z*: 677.2978. Anal. Calcd. for C₃₁H₄₃N₅O₁₂·1.41H₂O·0.51hex: C, 54.84; H, 7.02; N, 9.39. Found: C, 54.84; H, 6.73; N, 9.16.

Enzyme Assays

Caspase-3, -6, and -8. Caspases-3, -6, and -8 were expressed in *E. coli* and purified in Guy Salvesen's laboratory at the Burnham Institute, La Jolla, CA, according to the methods previously described by Stennicke and Salvesen.¹⁴⁵ Inhibition rates were

determined by the progress curve method described by Tian and Tsou.⁷ This method is suitable for measuring irreversible inhibition rates with fast inhibitors, where the inhibitor, the substrate and the enzyme are incubated together and the rate of substrate hydrolysis is measured continuously. The rate of substrate hydrolysis in the presence of the inhibitor was monitored for 20 minutes. The progress curve of inhibition, where the product formation approaches an asymptote is described in the equation

$$\ln ([P_{\infty}] - [P]) = \ln [P_{\infty}] - A[I]t$$

where $[P]$ and $[P_{\infty}]$ are the product concentrations at t and $t = \infty$ respectively, A is the apparent rate constant in the presence of the substrate. The apparent rate constants were determined from the slopes of plots of $\ln ([P_{\infty}] - [P])$ versus time (t) in seconds as previously described, where $A = \text{slope} / [I]$.

For competitive and irreversible inhibition, the apparent rate constant is converted to the second order rate constant k_2 by taking into consideration the effect of the substrate concentration on the apparent rate constant. The second order rate constant is described as in the equation:

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

Assays using the fluorogenic substrate Ac-DEVD-AMC ($\lambda_{\text{ex}} = 360 \text{ nm}$, $\lambda_{\text{em}} = 465 \text{ nm}$) were carried out on a Tecan Spectra Fluor microplate reader at 37°C . The K_M values for Ac-DEVD-AMC with caspase-3 ($K_M = 9.7 \text{ }\mu\text{M}$), caspase-6 ($K_M = 236.35 \text{ }\mu\text{M}$), and caspase-8 ($K_M = 6.79 \text{ }\mu\text{M}$) were determined in the laboratory of Guy Salvesen. The k_2 values are 11.309-fold higher than the apparent rate for caspase-3 because of the 100 mM $[S]$ and $K_M = 9.7 \text{ }\mu\text{M}$. The k_2 values are 1.4231-fold higher than the apparent rate for caspase-6 because of the 100 mM $[S]$ and $K_M = 236.35 \text{ }\mu\text{M}$. The k_2 values are

15.7275-fold higher than the apparent rate for caspase-8 because of the 100 mM [S] and $K_M = 6.79 \mu\text{M}$.

The concentration of the caspase-3 stock solution was 2 nM in the assay buffer. Assay buffer is a 1:1 mixture of 40 mM Pipes, 200 mM NaCl, 0.2% (w/v) CHAPS, and sucrose 20% (w/v) to 20 mM DTT solution in H₂O at pH 7.2. The concentration of the substrate stock solution was 2 mM in DMSO. The enzyme was pre-activated for 10 min at 37 °C in the assay buffer. The standard 100 μL reaction was started by adding 40 μL of assay buffer, 5 μL of various amounts of inhibitor (stock solution concentrations varied from 5×10^{-3} M to 4.84×10^{-7} M in DMSO), and 5 μL of substrate in DMSO (100 μM final concentration) at 37 °C. 50 μL of 2 nM enzyme stock solution (final concentration: 1 nM) was added to the mixture after 1 min and reading started immediately for 20 min at 37 °C. Inhibition experiments were repeated in duplicate and standard deviations determined.

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

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

***S. mansoni* Legumain.** Conor R. Caffrey in James McKerrow's laboratory performed the *S. mansoni* legumain kinetic assays at the Sandler Center for Basic

Research in Parasitic Diseases, University of California at San Francisco, San Francisco, CA. The zymogen form of schistosome legumain SmAE was expressed in *Pichia*. The lyophilized enzyme (50-100 mg) was reconstituted in 1.5 mL 0.5 M sodium acetate, pH 4.5 containing 4 mM DTT, and left to stand at 37 °C for 3-4 hours to allow for auto-activation of the zymogen. In a black 96-well microtiter plate, 50 µL of activated enzyme was added to an equal volume of 0.1 M citrate-phosphate buffer pH 6.8 containing 4 mM DTT. The inhibitors, added as 1 µL aliquots (serial water dilutions of DMSO stock solutions, 2 to 0.00002 µM [final]), were preincubated with the protease at room temperature for 20 min. After incubation, 100 µL of the same buffer containing 20 µM substrate (Cbz-Ala-Ala-Asn-AMC) was added to the wells and the reaction monitored for 20 minutes. A plot of the RFU/min versus the inhibitor concentration [µM] permitted calculation of an IC₅₀ value.

Pichia cell culture medium containing recombinant *S. mansoni* pro-legumain was incubated overnight at room temperature in 0.3 M sodium acetate, pH 4.5, 2 mM DTT to allow autoactivation of the endogenous zymogen. Inhibitor (1 µl) at 6 concentrations (to yield 0 to 1 µM [final]) was spotted into a 96-well black microtiter plate. To this was added 180 µl 0.1 M citrate-phosphate buffer, pH 6.8, containing 2 mM DTT and 20 µM Cbz-AAN-AMC substrate. Activated legumain (20 µl) was added to the mix and the progress of inhibition followed every 2 seconds for 30 minutes at 25 °C (Molecular Devices Flex Station fluorometer in the injection mode; ex 355/em 460). The k_{inact}/K_i values were determined using non-linear regression analysis (GraphPad Prism 3.X) and corrected for substrate ($1+S/K_m$; $1 + 20/60 = 1.33$).

Clostripain. Brian Rukamp performed the assays with clostripain in our laboratory. Clostripain was purchased from Sigma Chemical Co. (St. Louis, MO) as a solid which was dissolved in an activation solution of 8 mM DTT at a concentration of 5.962 μ M and stored at -20 °C prior to use. The inhibition of clostripain began with the addition of 25 μ l of stock inhibitor solution (concentration varies by inhibitor) in DMSO to a solution of 250 μ l of 20 mM Tris/HCl, 10 mM CaCl₂, 0.005% Brij 35, 2 mM DTT buffer at pH 7.6 (clostripain buffer) and 5 μ l of the stock enzyme solution. Aliquots (25 μ l) of this incubation mixture were taken at various time points and added to a solution containing 100 μ l of the clostripain buffer and 5 μ l of Z-Phe-Arg-AMC substrate solution (0.139 mM) in DMSO. The enzymatic activity was monitored by following the change in fluorescence at 465 nm. The all data obtained was processed by pseudo-first order kinetics.

Gingipain K. Brian Rukamp performed the assays with gingipain K in our laboratory. Gingipain K stock solution was obtained from Jan Potempa's lab (University of Georgia, Athens, GA) in a buffer containing 20 mM Bis-Tris, 150 mM NaCl, 5 mM CaCl₂, 0.02% NaN₃, at pH 8.0 at a concentration of 9 μ M, which was stored at -20 °C prior to use. Before using the enzyme, an aliquot (1 μ l) of the stock enzyme was diluted to a concentration of 4.61 nM in 1.951 ml of a solution of 0.2 M Tris/HCl, 0.1 M NaCl, 5 mM CaCl₂, 2 mM DTT at pH 8.0 (gingipain K buffer) and kept at 0 °C. This solution was used only for one day, as freezing the enzyme at this concentration destroyed all activity. The inhibition of gingipain K began with the addition of 25 μ l of stock inhibitor solution (concentration varies by inhibitor) in DMSO to 244 μ l of the diluted enzyme solution (4.61 nM) in gingipain K buffer warmed to rt. Aliquots (20 μ l) of this were

taken at various time points and added to a solution containing 100 μ l of the gingipain K buffer and 5 μ l of Suc-Ala-Phe-Lys-AMC • TFA as the substrate (0.910 mM stock) in DMSO. The enzymatic activity was monitored by following the change in fluorescence at 465 nm. The data for gingipain K was processed by pseudo-first order kinetics.

Cathepsin B. Cathepsin B kinetic assays were performed by Marion Götz in our laboratory. Irreversible kinetic assays were performed by the incubation method with human liver cathepsin B. Enzymatic activities of cathepsin B were measured in 0.1 M KHPO_4 , 1.25 mM EDTA, 0.01% Brij, pH 6.0 buffer and at 23 °C using Cbz-Arg-Arg-AMC as the substrate. To a freshly prepared enzyme stock solution of cathepsin B (approximately 6.98×10^{-3} $\mu\text{g}/\mu\text{L}$) in enzyme buffer containing DTT (0.1 mM) was added an additional 300 μL of enzyme buffer and 30 μL of a stock inhibitor solution in DMSO. At various time intervals 50 μL aliquots were withdrawn from the incubation mixture and added to 200 μL enzyme buffer containing Cbz-Arg-Arg-AMC (500 μM). Substrate hydrolysis was monitored using a Tecan Spectra Fluor microplate reader ($\lambda_{\text{ex}} = 360$ nm, $\lambda_{\text{em}} = 465$ nm). Pseudo first-order rate constants (k_{obs}) were obtained from plots of $\ln v_t/v_0$ versus time.

Papain. Papain kinetic assays were performed by Marion Götz in our laboratory. Irreversible kinetic assays were performed by the incubation method with papaya latex papain. An enzyme stock solution for the papain assays was freshly prepared from 330 μL of enzyme storage solution (1.19 mg/ml) diluted with 645 μL papain buffer (50 mM Hepes, and 2.5 mM EDTA at pH 7.5) and 25 μL of DTT (0.1 M). The incubation mixture was prepared from 300 μL buffer, 30 μL of inhibitor stock solution (DMSO), and 30 μL of enzyme stock solution. At various times aliquots (50 μL) were withdrawn and

added to 200 μL of Cbz-Phe-Arg-pNA substrate in buffer (53.7 μM). The absorbance of released *p*-nitroaniline was monitored at 405 nm on a Molecular Devices Thermomax plate reader.

Calpain I. Calpain kinetic assays were performed by Marion Götz in our laboratory. Irreversible kinetic assays were performed by the incubation method with calpain I from porcine erythrocytes. Enzymatic activities of calpain I were measured at 23 °C in 50 mM Hepes buffer (pH 7.5) containing 10 mM cysteine and 5mM CaCl_2 , using Suc-Leu-Tyr-AMC as the substrate. To 30 μL of an enzyme stock solution (1 mg/ml) of calpain I was added 300 μL of incubation buffer and 30 μL of a stock inhibitor solution in DMSO. At various time intervals 50 μL aliquots were withdrawn from the incubation mixture and added to 200 μL enzyme buffer containing Suc-Leu-Tyr-AMC (1.6 mM). Substrate hydrolysis was monitored using a Tecan Spectra Fluor microplate reader ($\lambda_{\text{ex}} = 360 \text{ nm}$, $\lambda_{\text{em}} = 465 \text{ nm}$). Pseudo first-order rate constants (k_{obs}) were obtained from plots of $\ln v_t/v_0$ versus time.

Determination of the Stability of the Inhibitors in Buffer and in the Presence of DTT

The stability of a representative group of inhibitors was studied by monitoring the UV spectrum of solutions at 250 nm, at 25 °C. The concentrations of the inhibitors were 2.5-4.0 mM and of DTT was 10 mM. The pH of the buffer solution was 7.2 and it contained about 50 % (v/v) DTT. The half-lives ($t_{1/2}$) were obtained from first-order rate plots of $\ln(A_t - A_f)$ versus time (15 hours) and the correlation coefficients were greater than 0.99.

An aliquot (50 μ L) of 4 mM Z-DEVaD-*trans*-CH=CH-COOEt was added to 450 μ L of assay buffer, and the stability of the inhibitor was monitored spectrophotometrically at 250 nm for every 30 minutes for 4 hours. A half-life of 10 min was obtained from the first order rate plot from absorbance (250 nm) versus time (15 hours).

An aliquot (50 μ L) of 2.5 mM Z-DEVaD-*trans*-CH=CH-CONHCH₂Ph was added to 450 μ L of assay buffer, and the stability of the inhibitor was monitored spectrophotometrically at 250 nm for every 30 minutes for 4 hours. A half-life of 58 min was obtained from the first order rate plot from absorbance (250 nm) versus time (15 hours).

An aliquot (50 μ L) of 2.5 mM Z-DEVaD-*trans*-CH=CH-CON(CH₃)CH₂Ph was added to 450 μ L of assay buffer, and the stability of the inhibitor was monitored spectrophotometrically at 250 nm for every 30 minutes for 4 hours. A half-life of 116 min was obtained from the first order rate plot from absorbance (250 nm) versus time (15 hours).

An aliquot (50 μ L) of 2.5 mM Z-VaD-*trans*-CH=CH-*trans*-CH=CH-CH₃ was added to 450 μ L of assay buffer, and the stability of the inhibitor was monitored spectrophotometrically at 250 nm for every 30 minutes for 4 hours. The inhibitor was essentially stable over the course of the study.

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OBJECTIVE Organic/Medicinal Chemist

EDUCATION

- Aug 1999-Dec 2003* **Georgia Institute of Technology, Atlanta, Georgia**
Ph.D. in Chemistry 2003
Major: Organic Chemistry, Minor: Biochemistry and Bioinorganic Chemistry, GPA: 3.38
Advisor: Dr. James C. Powers
Thesis: Design, Synthesis, and Evaluation of Novel Irreversible Inhibitors for Caspases
- Aug 1998-May 1999* **Case Western Reserve University, Cleveland, Ohio**
Graduate Studies in Organic Chemistry (Transfer)
- Oct 1994-June 1998* **Boğaziçi University, Istanbul, Turkey**
B.S. in Chemistry
GPA: 2.92, Rank: 3/38

WORK EXPERIENCE

- June 2000-Dec 2003* **Graduate Research Assistant**
School of Chemistry and Biochemistry
Georgia Institute of Technology, Atlanta, Georgia
- Structure-based design, multi-step organic synthesis, solid phase peptide synthesis, and kinetic evaluation of novel peptide-based inhibitors for clan CD cysteine proteases, with the emphasis on caspases.
- Worked on caspase specific dipeptidyl aspartic acid derivatives of E-64, a natural inhibitor of clan CA proteases.
 - Worked on dipeptidyl aza-peptide epoxide derivatives designed for caspases.

- Worked on aza-peptide Michael acceptor derivatives as irreversible inhibitors targeting caspases.

Aug 1999-May 2000 **Graduate Teaching Assistant**
 School of Chemistry and Biochemistry
 Georgia Institute of Technology, Atlanta, Georgia
 • Instructed General Chemistry Laboratory.

Aug 1998-May 1999 **Graduate Teaching Assistant**
 Department of Chemistry
 Case Western Reserve University, Cleveland, Ohio
 • Instructed Organic Chemistry Laboratory.

Summer 1996 **Intern**
 Bilim Pharmaceuticals, Inc., Istanbul, Turkey
 Product Development and Quality Assurance Departments
 • Worked on the development of the alimentary tract and metabolism product Neurovit®.
 • Performed qualitative and quantitative analysis of drugs to maintain product quality assurance.

SKILLS

Proficient in:

- Multi-step organic synthesis, solid phase peptide synthesis, structure-based design, molecular modeling and SAR
- Product purification and characterization using thin-layer and column chromatography, NMR and mass spectroscopy
- Enzyme kinetics, fluorescence and UV/VIS spectroscopy
- Molecular modeling using programs MOE and Rasmol

Proficient in PC, Mac, MS Word, MS Excel, MS Power Point, Chem Draw, Adobe Illustrator

PUBLICATIONS/PRESENTATIONS

- Powers, J. C., Asgian, J. L., Ekici, O. D., James, K. E. Irreversible Inhibitors of Serine, Cysteine, and Threonine Proteases. *Chem. Rev.*, **2002**, 102, 4639-4750.
- Ekici, O. D., Asgian, J. L., James, K. E., Li, Z., Powers, J. C. Design and Synthesis of Aza-Peptide Epoxide Inhibitors for Caspases; General Poster Session on September 7-11th, 2003 at the 226th ACS National Meeting in New York, New York
- James, K. E., Asgian, J. L., Li, Z., Ekici, O. D., Mikolajczyk, J., Salvesen, G. S., Powers, J. C. Design, Synthesis, and Evaluation of Aza-Peptide Epoxides as Selective and Potent Inhibitors of Caspases-1, -3, -6, and -8. *Submitted for publication*

- Ekici, O. D., Götz, G. M., James, K. E., Li, Z., Rukamp, B. J., Asgian, J. L., Caffrey, C. R., McKerrow, J. H., Mikolajczyk, J., Salvesen, G. S., Potempa, J., Powers, J. C. Aza-Peptide Michael Acceptors: A New Class of Inhibitors for Clan CD Cysteine Proteases *Manuscript in preparation*
- Ekici, O. D., Li, Z., James, K. E., Asgian, J. L., Mikolajczyk, J., Salvesen, G. S., Powers, J. C. Design, Synthesis, and Evaluation of Aza-Peptide Michael Acceptors as Selective and Potent Inhibitors of Caspases-3, -6, and -8. *Manuscript in preparation*

TECHNICAL ACTIVITIES

- Recruiter of prospective graduate students for the School of Chemistry and Biochemistry, Georgia Tech
- American Chemical Society, student member

LANGUAGES

Fluent in English, German, Turkish.