A Thesis
Presented to
The Academic Faculty

By

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In Partial Fulfillment
Of the Requirements for the Degree
Doctor of Philosophy in Chemistry

Georgian Institute of Technology
December 2003

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DEDICATION

To my wife Karrie E. A. Rukamp, my parents Leroy J. Rukamp and Diane M. Rukamp, my brothers David A. Rukamp and Christopher L. Rukamp, my sister Stephanie J. Rukamp, and to the memory of my grandparents Clement A. Rukamp and Georgina E. Rukamp, and David R. Cavil and Dorothy J. Cavil.
I would like to give a sincere thanks to the faculty, staff, and students of the School of Chemistry and Biochemistry for creating the friendly and open atmosphere that encouraged me to ask questions and brainstorm with other disciplines, allowing me to learn so much. I would like to thank my family and friends for encouraging me when I felt I could not go on, for standing by me when I was less than pleasant with them when I was frustrated by my work, and for celebrating with me when I conquered the objectives set before me to reach my ultimate goal.

A special thank you to Dr. James C. Powers for taking me under his wing and guiding me through these years of study, learning, and growth. Thank you Dr. Powers for putting up with me and giving me the opportunity to work for you. I would like to thank Dr. Mark J. Smyth and Dr. Janice M. Kelly of the Peter MacCallum Cancer Institute in Victoria, Australia for their contributions to the granzyme M project. I would also like to thank Dr. Jan Potempa from the University of Georgia for providing the enzymes for the gingipain project.

I would like to thank a number of people personally at the Georgia Tech for their assistance. Thank you to Dr. Les Gelbwax for his help with working and understanding the NMR machines. I would like to thank David Bostwick and Sarah Shealy for their analysis of my compounds with mass spectrometry and assistance in understanding what the results meant. I would also like to thank the undergraduates I had the privilege and pleasure to guide, teach, and learn from. Thank you to Sudah Natarajan and Brad W. Bolton for their work on the granzyme M project.
I am especially grateful to my labmates and co-workers, who helped to guide me through the difficult work and provided a fun and enjoyable environment to work in. Thank you to Karrie Rukamp, Dr. Joel Krauser, Tinh Tran, Özlem Doğan Ekiç, Marion Götz, Sylvia Shadinger, Amy Campbell, Dr. Karen Ellis James, Dr. Juliana Asgian, and Temam Juhar. I would like to give a special thank you to the research scientists who have worked in our group over the years, Zhao Zhao Li and Dr. Chih-Min Kam, whose wisdom and guidance were worth more than they could ever know.

Lastly, I would like to thank my committee members for taking the time to meet with me during my initial and final research reviews, my original research proposal, my oral exam, and read my thesis. Your advice and encouragement was greatly treasured.
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LIST OF ABBREVIATIONS

AA  amino acid residue
AAaa aza-l-lys or aza-Orn
Ac  acetyl
Ala alanine
ALys aza-lysine
AOrn aza-ornithine
AMC 7-amino-4-methylcoumarin
Arg arginine
Asp aspartic acid
Asn asparagine
Azi aziridinyl
Boc t-butyloxycarbonyl
Brij 35 polyoxyethylene (23) lauryl ether
BSA bovine serum albumin
tBu t-butyl
Bz, Bzl benzyl
CEK chloroethyl ketone
CDCl₃ deuterated chloroform
Chaps 3-[(3-cholamidopropyl)dimethylamino]-1-propanesulfonate
CMK chloromethyl ketone
CTL cytotoxic T-lymphocytes
Cys  cysteine

DCC  1,3-dicyclohexylcarbodiimide

DCU  dicyclohexylurea

DMF  N,N-dimethyl formamide

DTT  threo-1,4-dimercapto-2,3-butane diol

DMSO  dimethyl sulfoxide

DMSO-d$_6$  dimethyl sulfoxide-d$_6$ deuterated

DTNB  5,5'-dithiobis(2-nitrobenzoic acid)

[E]  enzyme concentration

[E]$_0$  initial enzyme concentration

[E]$_t$  enzyme concentration at time t

[E*]+[I]$_0$  initial reversible enzyme-inhibitor complex concentration

[E-I]$_0$  initial covalently bound enzyme-inhibitor complex concentration

[E-I]$_t$  covalently bound enzyme-inhibitor complex concentration at time t

$\Delta$ε  change in extinction coefficient

E-64  L-trans-epoxysuccinyl-leucylamido(4-guanidino)butane

EDC  1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride

EDTA  ethylenediaminetetraacetic acid

Et  ethyl

EtO  ethoxy

eq  equivalents

FAB  fast atom bombardment

Glu  glutamic acid

xi
Gly  |  glycine  
Hepes  |  4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid  
His  |  histidine  
HOBI  |  N-hydroxybenzotriazole  
$^1$H NMR  |  proton nuclear magnetic resonance  
Hph  |  homophenylalanine  
HRMS  |  high resolution mass spectrometry  
[I]  |  inhibitor concentration  
$[I]_0$  |  initial inhibitor concentration  
iBA  |  iodobenzoic acid  
IBCF  |  iso-butyldihaloformate  
IBX  |  iodoxybenzoic acid  
Ile  |  isoleucine  
k  |  rate constant  
k_{2nd}  |  second order inactivation rate constant  
k_{obs}  |  pseudo-first order inactivation rate constant  
k_{cat}  |  catalytic rate constant  
k_{d}  |  pseudo-first order rate constant of degradation  
k_{i}  |  second order rate constant of inactivation after degradation  
$K_i$  |  first order inactivation constant for reversible inhibitors  
$K_d$  |  dissociation constant  
$K_M$  |  Michaelis constant  
$K_a$  |  equilibrium constant of dissociation
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
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<tr>
<td>Leu</td>
<td>leucine</td>
</tr>
<tr>
<td>Lys</td>
<td>lysine</td>
</tr>
<tr>
<td>m/z</td>
<td>mass to charge ratio</td>
</tr>
<tr>
<td>Me</td>
<td>methyl</td>
</tr>
<tr>
<td>MeO</td>
<td>methoxy</td>
</tr>
<tr>
<td>Met</td>
<td>methionine</td>
</tr>
<tr>
<td>ND</td>
<td>not determined</td>
</tr>
<tr>
<td>NH</td>
<td>no hydrolysis</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer</td>
</tr>
<tr>
<td>NMM</td>
<td>N-methylmorpholine</td>
</tr>
<tr>
<td>Nle</td>
<td>norleucine</td>
</tr>
<tr>
<td>Orn</td>
<td>ornithine</td>
</tr>
<tr>
<td>pFP</td>
<td>pentafluorophenol</td>
</tr>
<tr>
<td>Ph</td>
<td>phenyl</td>
</tr>
<tr>
<td>Phe</td>
<td>phenylalanine</td>
</tr>
<tr>
<td>PhPr</td>
<td>3-phenylpropanoyl</td>
</tr>
<tr>
<td>Pipes</td>
<td>piperazine-1,4-bis(2-ethanesulfonic acid)</td>
</tr>
<tr>
<td>pNA</td>
<td>p-nitroanilide</td>
</tr>
<tr>
<td>Pro</td>
<td>proline</td>
</tr>
<tr>
<td>Rf</td>
<td>retention factor</td>
</tr>
<tr>
<td>RFU</td>
<td>relative fluorescence units</td>
</tr>
<tr>
<td>RNK</td>
<td>rat natural killer</td>
</tr>
<tr>
<td>rt</td>
<td>room temperature</td>
</tr>
<tr>
<td>Symbol</td>
<td>Definition</td>
</tr>
<tr>
<td>--------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>[S]</td>
<td>substrate concentration</td>
</tr>
<tr>
<td>Ser</td>
<td>serine</td>
</tr>
<tr>
<td>Suc</td>
<td>succinyl</td>
</tr>
<tr>
<td>t</td>
<td>time</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
</tr>
<tr>
<td>Thr</td>
<td>threonine</td>
</tr>
<tr>
<td>TLMK</td>
<td>α-N-tosyl-L-lysine chloromethyl ketone</td>
</tr>
<tr>
<td>TLC</td>
<td>thin-layer chromatography</td>
</tr>
<tr>
<td>Tos-OH</td>
<td>p-toluene sulfonylic acid</td>
</tr>
<tr>
<td>Tris</td>
<td>2-amino-2-(hydroxymethyl)-1,3-propane diol</td>
</tr>
<tr>
<td>Trp</td>
<td>tryptophan</td>
</tr>
<tr>
<td>Tyr</td>
<td>tyrosine</td>
</tr>
<tr>
<td>ν</td>
<td>rate constant</td>
</tr>
<tr>
<td>ν₀</td>
<td>initial rate constant</td>
</tr>
<tr>
<td>Vₜ</td>
<td>maximum velocity obtained at high substrate concentrations</td>
</tr>
<tr>
<td>Val</td>
<td>valine</td>
</tr>
<tr>
<td>Z, Cbz</td>
<td>benzylxoycarbonyl</td>
</tr>
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</table>
SUMMARY

In the first project, a series of tripeptide thienobenzyl esters substrates with the structure of Z-\text{AA}_1-\text{AA}_2-Met-SBzl (where AA = various amino acids) were designed and synthesized to study the optimal P2 and P3 amino acid preferences of the recombinant serine proteases murine and human granzyme M. Initially, P2 specificity was investigated by varying \text{AA}_2 (\text{Ala}, \text{Phe}, \text{Pro}, \text{Lys}, \text{Ser}, \text{Asp}) with alanine in the P3 position (\text{AA}_3). After finding the most favorable P2 residue, the synthetic substrates were further optimized by varying the P3 amino acid (\text{Phe}, \text{Pro}, \text{Lys}, \text{Ser}, \text{Asp}). The preferred substrate sequences for granzymes M are Z-\text{Ala-Pro-Met-SBzl}, Z-\text{Asp-Pro-Met-SBzl}, and Z-\text{Ser-Pro-Met-SBzl}. We also compared the results of the thienobenzyl ester hydrolysis by granzyme M to those obtained with \text{a-chymotrypsin}, an enzyme more homologous to granzyme M than any other serine protease. A comparison of the substrate preferences of the two enzymes showed that, despite the sequence similarities between the enzymes, their S2 and S3 binding pockets have significant differences, suggesting different cellular functions.

In the second project, aza-peptide Michael acceptors were developed and synthesized based on the combination of aza-peptide active ester and α,β-unsaturated Michael acceptor peptide inhibitors of cysteine proteases, as well as the results obtained by our lab with aza-peptide epoxide inhibitors. The aza-peptide Michael acceptor inhibitors were designed with the clan CD enzymes, clastipain and gingipains K and R, in mind. Aza-peptide Michael acceptors represent a novel type of inhibitor capable of being potent cysteine protease inhibitors. The inhibitors design started with a dipeptide
substrate sequences that all three enzymes could potentially hydrolyze. The α-carbon of
the P1 amino acid residue was then replaced with a nitrogen atom, creating the aza-
peptide, which is resistant to hydrolysis. The scissile peptide bond was replaced with a
reactive α,β-unsaturated moiety to create a set of compounds with the structure PhPr-
Leu-AAaa-CH=CH-CO2Et (AAaa = Lys or Orn). These inhibitors proved to be potent
inactivators of clostripain, but were especially effective against gingipain K. The aza-Lys
derivative was 8-fold more potent with gingipain K than with clostripain, while the aza-
Orn derivative was 1200-fold more potent toward gingipain K. They were also tested
with a variety of other enzymes, including a serine protease, but proved to have little or
no inhibitor activity toward those enzymes.
CHAPTER 1
PEPTIDE THIOBENZYL ESTERS AS SUBSTRATES FOR GRANZYME M

INTRODUCTION

Mature natural killer cells (NK) and cytotoxic T-lymphocytes (CTL) represent the major defense a host has against tumor cells and viral infections (Herberman and Oraltodo, 1981; Young and Cohn, 1986; Tschopp and Jongeneel, 1988; Young, 1989; Smyth et al., 1996a). Lymphocytes protect the body by initiating apoptosis, or cell death, in target cells through granule exocytosis (Young and Cohn, 1986; Peters et al., 1990; Podack and Kupfer, 1991; Doherty, 1993). However, these protective cells have also been associated with autoimmune diseases and organ graft rejection (Herberman and Oraltodo, 1981; Young and Cohn, 1986; Tschopp and Jongeneel, 1988; Young, 1989). One of the active components of these cells is a class of serine proteases from the trypsin/chymotrypsin family known as granzymes (Shi et al., 1992a; Shi et al., 1992b; Shiver et al., 1992; Smyth et al., 1992).

Granzymes (granule-associated enzymes) have a high degree of amino acid sequence similarity (at least 38% positional identities) to chymotrypsin and other well-known serine proteases (Jenne and Tschopp, 1988), suggesting that they are structurally related to the other members of the chymotrypsin superfamily (Murphy et al., 1988; Smyth et al., 1992; Woodard et al., 1994; Smyth et al., 1996a; Smyth et al., 1996b). Granzymes hydrolyze typical serine protease substrates and are inhibited by compounds that selectively inactivate serine proteases (Jenne and Tschopp, 1988; Otake et al., 1991).
One of the granzymes appears essential for the initiation of pore formation in cell membranes (Jenne and Tschopp, 1988; Young, 1989; Ojeiis et al., 1991). This granzyme promotes perforin activation, and then perforin polymerizes in cell membranes to form hydrophobic pores, allowing other granzymes entry into target cells. The enzymes also initiate the process of DNA degradation in target cells by cleaving several internal substrates, such as histones, which are proteins associated with DNA that prevent transcription of genetic code not needed for that particular cell (Munger et al., 1988; Shi et al., 1992b; Hudig et al., 1993). Granzymes alone cannot cause cell lysis, nor can perforin cause DNA degradation (Young et al., 1986; Smyth et al., 1996b). The enzymes are stored fully processed in lymphocyte granules at low pH at which they are inactive. Granzymes are delivered via the granules, which are exocyted at the site where killer and target cell surfaces interact (Peters et al., 1991).

Throughout this work, the nomenclature proposed by Schechter and Berger (Schechter and Berger, 1967) is used to define the position of the amino acids (P4, P3, P2, P1, P1', P2', P3') in the peptides with respect to the scissile bond and the corresponding positions in the active site of the enzyme (S4, S3, S2, S1, S1', S2', S3') (Figure 1.1). The purpose of this research was to study the substrate specificity of one of the granule enzymes, granzyme M. Commonly known as metase or Metase-1, this enzyme preferentially cleaves after methionine, leucine, and norleucine in synthetic substrates (Smyth et al., 1992; Smyth et al., 1995a; Kelly et al., 1996). Presence of granzyme M was first noticed during a study of granule-mediated cell lysis. Hydrolysis of the thioenzyl ester Boc-Ala-Ala-Met-SBzl was observed in assays of rat natural killer (RNK) leukemia cells (Hudig et al., 1991). The 30 kDa glycosylated enzyme (core
protein is 24 kDa without glycosylation (Smyth et al., 1992), found up to 35 kDa with heavy glycosylation (Smyth et al., 1996b), was first isolated from the granules of RNK-16 leukemia cells by Smyth et al. using recombinant complimentary DNA techniques (Smyth et al., 1992). The enzyme was named RNK-Met-1 and found to possess Met-ase activity. The mouse and human recombinant forms of granzyme M share a similar substrate profile as well as a large variety of structural characteristics with the enzyme obtained from mtt NK cells (Smyth et al., 1993; Kelly et al., 1996; Smyth et al., 1996b).

Granzyme M has several conserved characteristics that make it similar to other granzymes, and serine proteases in general. Like other members of the granzyme subfamily, granzyme M has unusual synthetic substrate preferences with respect to the P1 position (Odake et al., 1991). Granzyme M mimics other granzymes by being processed as a preproenzyme, of which the prepropeptide must be removed for full activity (Masson and Tschopp, 1987; Caputo et al., 1993; Smyth et al., 1995b). The enzyme has the characteristic Ile-Ile-Gly-Gly at the N-terminus of the mature enzyme shared by the rest of the granzyme subfamily (Smyth et al., 1992). In addition, granzyme M has the
conserved active site residues His\textsuperscript{57}, Asp\textsuperscript{102}, and Ser\textsuperscript{195} (chymotrypsin numbering), which make up the catalytic triad shared by all serine proteases (Smyth \textit{et al.}, 1992). Granzyme M contains the conserved Asp\textsuperscript{194}-Ser\textsuperscript{195}-Gly\textsuperscript{196}-Gly\textsuperscript{197}-Pro\textsuperscript{198} amino acid sequence found in the majority of serine proteases around the active site serine (Smyth \textit{et al.}, 1992). The enzyme has six conserved cysteine residues that form disulfide bridges found in other serine proteases. However, granzyme M also has a fourth potential disulfide bridge found only in two other serine proteases, α-chymotrypsin (Le Trong \textit{et al.}, 1987a; Le Trong \textit{et al.}, 1987b) and trypsin-like serine protease A (Gershenfeld \textit{et al.}, 1988). This disulfide linkage helps to bridge the active site in α-chymotrypsin and may do the same for granzyme M. The enzyme has one or more potential glycosylation sites at Asn residues, like in other granzymes (Griffiths and Isaaz, 1993). The murine, rat, and human granzyme M all share a conserved Asn\textsuperscript{166}, while the rat and human enzymes also have a second site at Asn\textsuperscript{179} and Asn\textsuperscript{196}, respectively, which could potentially be glycosylated.

Granzyme M has several unique features that separate it from the rest of the granzyme subfamily. It is the only known serine protease to have a Thr residue on the C-terminal side of the active site (Smyth \textit{et al.}, 1992). Unlike granzymes A and B, and a variety of other granule serine proteases, which have a dipeptide propeptide, granzyme M has a hexapeptide (McGuire \textit{et al.}, 1993; Smyth \textit{et al.}, 1993; Kelly \textit{et al.}, 1996). Deletion of the hexapeptide from the recombinant form of the human granzyme M results in generation of the active enzyme without the need for further processing (Smyth \textit{et al.}, 1996b). The sequence of residues between 202 and 221, some of which are involved in substrate specificity, is identical in each of the mouse, rat, and human granzyme M, but differs from all other granzymes (Kelly \textit{et al.}, 1996; Smyth \textit{et al.}, 1996a).
The limited expression pattern of granzyme M also differentiates it from the other granzymes. Substantial expression of the active enzyme appears to be localized in mature NK cells, while lower amounts are found in CD3+, CD56+ T Cells, and γδT cells (Smyth et al., 1992; Smyth et al., 1993; Smyth et al., 1995a; Kelly et al., 1996; Sayers et al., 2001). Minute amounts of inactive variants can be found in other cells (Davies et al., 1998; Taniguchi et al., 1999). Although the actual biological function of granzyme M is unknown, the limited expression of the enzyme is unique among the granzymes and suggests a specialized function for this protease. The enzyme may have evolved for a specific task in NK cells and for other immune responses (Kelly et al., 1996). Other roles suggested for granzyme M include cell growth and/or death, processing or discarding of cell membrane receptors, or chemotactic activities (Pilat et al., 1994; Smyth et al., 1996a).
RESULTS AND DISCUSSION

In this study, a series of new peptide thiobenzyl esters was synthesized with the structure $\text{Z-AA}_1$-$\text{AA}_2$-$\text{Met-SBzI}$ (where $\text{AA} =$ various amino acids) and employed to explore the P3 and P2 specificities of murine and human granzyme M. While human granzyme M hydrolyzes substrates containing Leu, Nle, and Met in the P1 position at similar rates, murine granzyme M prefers Nle or Met over Leu by 3- and 4-fold, respectively (Powers and Smyth, unpublished results). In addition, the starting material, Boc-Met-OH, used in the synthesis was inexpensive and readily available in the lab. Therefore, Met was chosen as the P1 amino acid in these studies instead of Nle or Leu. Peptidyl thiobenzyl esters are highly reactive and selective substrates useful for monitoring enzymes with low hydrolytic activities (Powers and Kam, 1995). The hydrolysis is observed spectrophotometrically in the presence of DTNB, which detects the benzyl thiol as it is cleaved from the substrate (Figure 1.2) (Farmer and Hageman, 1975). In addition, we used the substrates to look at similarities and differences between the amino acid preferences of granzyme M and $\alpha$-chymotrypsin.

Synthesis. Seventeen peptidyl thiobenzyl esters were synthesized in this project. The HCH-H-Met-SBzI was synthesized as illustrated in Scheme 1.1. The remainder of the peptide portions of the substrates were synthesized as illustrated in Scheme 1.2 except the tetrapeptide and Asp peptidyl derivatives. Their syntheses are illustrated in Schemes 1.3 and 1.4.

The methionine thiobenzyl ester was synthesized by reacting Boc-Met-OH with benzyl mercaptan using a DCC activated ester coupling (Scheme 1.1). The thioester was
Figure 1.2 Typical Binding Mode of a Peptide Thiobenzyl Ester with Granzyme M, its Hydrolysis, and its Reaction with DTNB to give a Spectrophotometrically Observable Species

Scheme 1.1 Synthesis of HCl-H-Met-SBzl
deprotected to give HCl-H-Met-SBzl, which was coupled to the remainder of the peptides.

To make the first series of peptides (Scheme 1.2), in which the P2 position was altered and the P3 position was alanine, Z-Ala-OH was reacted using the IBCF mixed anhydride coupling method with the various esters of each amino acid, HCl-H-AA-OCH₂, to give Z-Ala-AA-OCH₂. The ester of the dipeptide was deprotected to yield Z-Ala-AA-OH. The dipeptide acids were reacted with HCl-H-Met-SBzl using the DCC activated ester coupling method to yield Z-Ala-AA-Met-SBzl (1), as the final compounds. In the cases of AA being Lys(Boc) and Ser(tBu), the sidechains were deprotected to yield the deblocked tripeptides, giving two more final compounds, 2a and 2b, respectively.

To make the second series of peptides (Scheme 1.2), in which the P2 position was proline and the P3 position was altered, the ester HCl-H-Pro-OCH₂ was reacted using the IBCF mixed anhydride coupling method with the various N-protected acids of each amino acid, Z-AA-OH, to give Z-AA-Pro-OCH₂. The ester of the dipeptide was deprotected to yield Z-AA-Pro-OH. The dipeptide acid was reacted with HCl-H-Met-SBzl using the DCC activated ester coupling method to yield Z-AA-Pro-Met-SBzl (3), as the final compounds. In the cases of AA being Lys(Boc) and Ser(tBu), the sidechains were deprotected to yield the deblocked tripeptides, giving two more final compounds, 4a and 4b, respectively.

The compounds Z-Ala-Asp(tBu)-Met-SBzl and Z-Ala-Asp-Met-SBzl were prepared differently than the other substrates because coupling with the Asp amino acid is difficult in synthesis. The synthesis of these tripeptides is illustrated in Scheme 1.3.
Synthesis of Series 1

\[
\begin{align*}
Z-H & \text{+ HOCH-Amino Acid OCH}_3 \\
\xrightarrow{\text{IBCF, NMM, -20 °C/CH}_2Cl_2 \text{or THF}} & Z-N-H-Amino Acid OCH}_3 \\
\xrightarrow{1) \text{NaOH, CH}_3OH \text{, } 2) \text{HCl, 0 °C}} & Z-N-H-Amino Acid OCH}_3
\end{align*}
\]

\[
\begin{align*}
\xrightarrow{\text{HCHH-Met-SBzl, NMM, DCC/ HOBr, CH}_2Cl_2 \text{or THF, 0 °C}} & Z-N-H-N-Amino Acid OCH}_3 \text{S-phenyl} \\
\xrightarrow{TFA \text{or HCl/ EtOAc, 0 °C}} & Z-N-H-N-Amino Acid OCH}_3 \text{S-phenyl} \\
\xrightarrow{\text{if AA = Lys(Boc) or Ser(FBu)}} & Z-N-H-N-Amino Acid OCH}_3 \text{S-phenyl} \\
\end{align*}
\]

2a AA = Lys
2b AA = Ser

Synthesis of Series 2

\[
\begin{align*}
\xrightarrow{\text{HCHHN, Z-AA-OH, IBCF, NMM/CH}_2Cl_2 \text{or THF, 0 °C}} & \xrightarrow{\text{Z-NN-N}} \text{OCH}_3 \text{OCH}_3 \\
\xrightarrow{1) \text{NaOH, CH}_3OH \text{, } 2) \text{HCl, 0 °C}} & \xrightarrow{0) \text{HCl, 0 °C}} \xrightarrow{\text{TFA or HCl/EtOAc, 0 °C}} \xrightarrow{\text{if AA = Lys(Boc) or Ser(FBu)}} \\
\end{align*}
\]

3

AA = Ala, Phe, Pro, Lys(Boc), Lys, Ser(FBu), Ser

4a AA = Lys
4b AA = Ser

Scheme 1.2 Synthesis of Thiobenzyl Esters
Scheme 1.3 Synthesis of Z-Ala-Asp-Met-Sbd
The acid Z-Ala-OH was reacted using the DCC activated ester coupling method with pentafluorophenol to yield the ester Z-Ala-OPFP. The activated PFP ester was combined with H-Asp(Bu)-OH to give the dipeptide acid Z-Ala-Asp(Bu)-OH. The dipeptide was reacted with HCl·H-Met-SBzl using the DCC activated ester coupling method to yield one final product, Z-Ala-Asp(Bu)-Met-SBzl. The Asp sidechain of the tripeptide was deprotected to give another final product, Z-Ala-Asp-Met-SBzl.

Illustrated in Scheme 1.4 is the synthesis of the compounds Z-Asp(Bu)-Pro-Met-SBzl, Z-Asp-Pro-Met-SBzl, and Z-Ala-Ala-Pro-Met-SBzl. These compounds also had difficulties with their syntheses and were prepared differently than the other substrates. The acid Boc-Pro-OH was reacted with HCl·H-Met-SBzl using the IBCF activated anhydride coupling method to yield Boc-Pro-Met-SBzl. The dipeptide thioester was deprotected to give HCl·H-Pro-Met-SBzl. The thioester HCl·H-Pro-Met-SBzl was combined with Z-Asp(Bu)-OH or Z-Ala-Ala-OH using the DCC activated ester coupling method to give Z-Asp(Bu)-Pro-Met-SBzl and Z-Ala-Ala-Pro-Met-SBzl, respectively, as final products. The Asp sidechain of the tripeptide was deprotected to give the final product, Z-Asp-Pro-Met-SBzl. All of the final compounds were characterized by TLC, $^1$H NMR, HRMS, and elemental analysis (Table 1.1).

**Substrate Kinetics—Processing of Data.** The substrate kinetics were processed based on the Briggs and Haldane steady-state postulate which states that the rates of formation and breakdown of the enzyme-substrate complex are essentially equal, and, therefore, the enzyme-substrate complex concentration remains constant throughout the assay (Dixon and Webb, 1964; Cornish-Bowden, 1976). Substrate kinetics are based on the assumption that the enzyme first forms a complex with the substrate, and then has two
Scheme 4. Synthesis of Z-Asp(Bu)-Pro-Met-SBzl, Z-Asp-Pro-Met-SBzl, and Z-Asp-Ala-Pro-Met-SBzl
<table>
<thead>
<tr>
<th>Compound</th>
<th>Formula</th>
<th>Calculated</th>
<th>Found</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>H</td>
</tr>
<tr>
<td>Z-Ala-Ala-Met-SBzl</td>
<td>C$<em>{26}$H$</em>{32}$N$<em>{2}$O$</em>{2}$S$_{2}$</td>
<td>58.74</td>
<td>6.26</td>
</tr>
<tr>
<td>Z-Ala-Phe-Met-SBzl</td>
<td>C$<em>{26}$H$</em>{32}$N$<em>{2}$O$</em>{2}$S$_{2}$ • 0.50 DCU</td>
<td>64.22</td>
<td>6.86</td>
</tr>
<tr>
<td>Z-Ala-Pro-Met-SBzl</td>
<td>C$<em>{26}$H$</em>{32}$N$<em>{2}$O$</em>{2}$S$_{2}$</td>
<td>60.30</td>
<td>6.33</td>
</tr>
<tr>
<td>Z-Ala-Lys-Met-SBzl</td>
<td>C$<em>{26}$H$</em>{32}$N$<em>{2}$O$</em>{2}$S$<em>{2}$Cl • 0.25 H$</em>{2}$O</td>
<td>55.31</td>
<td>6.64</td>
</tr>
<tr>
<td>Z-Ala-Ser(tBu)-Met-SBzl</td>
<td>C$<em>{26}$H$</em>{32}$N$<em>{2}$O$</em>{2}$S$_{2}$</td>
<td>59.68</td>
<td>6.84</td>
</tr>
<tr>
<td>Z-Ala-Ser-Met-SBzl</td>
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<td>6.07</td>
</tr>
<tr>
<td>Z-Ala-Asp(tBu)-Met-SBzl</td>
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<td>6.54</td>
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<tr>
<td>Z-Ala-Asp-Met-SBzl</td>
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<td>56.33</td>
<td>5.78</td>
</tr>
<tr>
<td>Z-Phe-Pro-Met-SBzl</td>
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<td>6.20</td>
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<td>61.73</td>
<td>6.39</td>
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<td>Z-Ser(tBu)-Pro-Met-SBzl</td>
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<td>61.02</td>
<td>6.88</td>
</tr>
<tr>
<td>Z-Ser-Pro-Met-SBzl</td>
<td>C$<em>{26}$H$</em>{32}$N$<em>{2}$O$</em>{2}$S$_{2}$ • 0.05 DCU</td>
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<tr>
<td></td>
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<td>60.48</td>
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</tr>
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<td>----------------------</td>
<td>---------------------</td>
<td>-------</td>
<td>------</td>
</tr>
<tr>
<td>Z-Lys(Boc)-Pro-Met-SBzl</td>
<td>C&lt;sub&gt;3&lt;/sub&gt;H&lt;sub&gt;8&lt;/sub&gt;N&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;3&lt;/sub&gt;S&lt;sub&gt;2&lt;/sub&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Z-Lys-Pro-Met-SBzl</td>
<td>C&lt;sub&gt;3&lt;/sub&gt;H&lt;sub&gt;8&lt;/sub&gt;N&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;3&lt;/sub&gt;S&lt;sub&gt;2&lt;/sub&gt;Cl • 1.25 H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>55.26</td>
<td>6.81</td>
</tr>
<tr>
<td>Z-Asp(fBu)-Pro-Met-SBzl</td>
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<td>60.25</td>
<td>6.59</td>
</tr>
<tr>
<td>Z-Asp-Pro-Met-SBzl</td>
<td>C&lt;sub&gt;2&lt;/sub&gt;H&lt;sub&gt;12&lt;/sub&gt;N&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;3&lt;/sub&gt;S&lt;sub&gt;2&lt;/sub&gt; • 0.30 H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>57.36</td>
<td>5.91</td>
</tr>
<tr>
<td>Z-Ala-Ala-Pro-Met-SBzl</td>
<td>C&lt;sub&gt;3&lt;/sub&gt;H&lt;sub&gt;8&lt;/sub&gt;N&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;3&lt;/sub&gt;S&lt;sub&gt;2&lt;/sub&gt;</td>
<td>59.21</td>
<td>6.41</td>
</tr>
</tbody>
</table>
options. Either the enzyme and substrate will dissociate back to free enzyme and substrate, or the enzyme reacts with the substrate to give product.

\[
E + S \xrightarrow{k_1} ES \xrightarrow{k_2} E + P
\]

In this reaction equation, \(E\) is the enzyme, \(S\) is the substrate, \(ES\) is the enzyme-substrate complex, \(k_1\) is the rate constant for the formation of \(ES\), \(k_4\) is the rate constant of the breakdown of \(ES\) back to the free enzyme and substrate, \(P\) are the products formed, and \(k_3\) is the rate constant of the formation of those products from the \(ES\) complex. In the postulation, the substrate concentration is much greater than the enzyme concentration. Under this condition, the steady-state can be established, and the concentration of the \(ES\) complex will remain constant with time.

The following equation can be written to represent the steady-state assumption

\[
\frac{d[ES]}{dt} = k_1[E][S] - k_1[ES] - k_2[ES] = 0
\]

and the total enzyme concentration, \([E]_0\), can be represented by

\[
[E]_0 = [E] + [ES]
\]

Combining the two equations yields

\[
k_1[E]_0[S] - k_1[ES][S] - k_4[ES] - k_2[ES] = 0
\]

and simplification of this equation gives

\[
[ES] = \frac{[E]_0[S]}{[S] + (k_4 + k_2)/k_1}
\]
The term [ES] can not be measured experimentally, but the rate of product formation can be. Therefore, $dP/dt = v = k_3[ES]$, and the equation can be substituted and rewritten as

$$v = \frac{k_3[E][S]}{[S] + (k_1 + k_2)k_1}$$

The maximum velocity ($V_m$) is reached when the enzyme is saturated with substrate, or when $[ES] = [E]_o$. So, the maximum rate can be written as $V_m = k_3[ES] = k_3[E]_o$. The Michaelis constant ($K_m$), which is the substrate concentration at which the reaction velocity is at half of its maximum, can be written as $K_m = (k_1 + k_2)/k_1$. Then the above equation can be rewritten as

$$v = \frac{V_m[S]}{[S] + K_m}$$

which is the Michaelis-Menten equation. This can be converted to a linear equation, in the form of $y = mx + b$, by taking the reciprocal of the above equation to give

$$\frac{1}{v} = \left(\frac{K_m}{V_m}\right) \frac{1}{[S]} + \frac{1}{V_m}$$

This is the Lineweaver-Burk form of the equation. If $1/v$ is plotted against $1/[S]$, a straight line is obtained which corresponds to the reciprocal of the Michaelis-Menten equation. The straight line cuts through the baseline, or $x$-intercept, and is $-1/K_m$. The line also cuts the vertical axis, or $y$-intercept, at $1/V_m$, and has a slope of $K_m/V_m$. So, by using the slope, and determining the $V_m$ from the $y$-intercept, the $K_m$ can be found.

However, $V_m$ is not a fundamental property of the enzyme, meaning it can change depending on the concentration of the enzyme. Therefore, we need to identify a constant
that will reflect the maximum velocity of the reaction but be consistent regardless of enzyme concentration used. We can define $k_{cat}$, or the catalytic constant of the turnover of substrate to product per active site per unit time, as

$$k_{cat} = \frac{V_s}{[E]_0}$$

The $k_{cat}$ term is essentially equal to $k_2$ in the Michaelis-Menten equation, and so a substitution can be made for $k_2$. In the case where the substrate concentration is much less than the $K_m$ [$E] = [E]_0$ because very little of the enzyme-substrate complex forms compared to how fast the product is being generated. In addition, $[S]$ is negligible compared to $K_m$, so the Michaelis-Menten equation from above, modified with substitutions, becomes

$$v = \left( \frac{k_{cat}}{K_m} \right) [E][S]$$

where $k_{cat}/K_m$ is the apparent second-order rate constant of the enzymatic reaction. The $k_{cat}/K_m$ value is the measure of the catalytic efficiency of an enzyme to hydrolyze a particular substrate.

Although the preceding case assumes that $[S]$ is much less than $K_m$, it can be shown that this equation will hold true for any substrate concentration (Fersht, 1985). In some cases, $K_m$ can be treated as an apparent dissociation constant ($K_d$), as in the following equation

$$K_m = \frac{[E][S]}{\Sigma [ES]}$$
where $\Sigma[ES]$ is the sum of all of the bound enzyme species. Substituting this equation into the Michaelis-Menten equation and simplifying as follows gives

$$v = \frac{k_{cat}[E]_0[S]}{K_m + [S]} = \frac{k_{cat}[E]_0}{\frac{[E][S]}{\Sigma[ES]} + [S]} = \frac{k_{cat}[E]_0}{\Sigma[ES]} \frac{1}{[E] + \Sigma[ES]}$$

and because $[E]_0 = [E] + \Sigma[ES]$, then

$$v = \Sigma[ES]k_{cat} = \left(\frac{k_{cat}}{K_m}\right)[E][S]$$

which is the same equation presented above for the case where the substrate concentration is much less than $K_m$.

The value of $k_{cat}/K_m$ is especially useful when comparing the specificity of competing substrates (Fersht, 1985). Take, for example, two substrates, A and B, competing for the same catalytic site of an enzyme. The rates of each could be written as

$$v_A = \left(\frac{k_{cat}}{K_m}\right)_A [E][A]$$

and

$$v_B = \left(\frac{k_{cat}}{K_m}\right)_B [E][B]$$

which combine to give

$$\frac{v_A}{v_B} = \left(\frac{k_{cat}}{K_m}\right)_A \frac{[A]}{[B]}$$
This equation distinguishes the specificities of the different substrates with a particular enzyme. The ratio of the $k_{cat}/K_m$ of two different substrates can be used to determine which substrate the enzyme prefers.

To confirm the calculated $k_{cat}/K_m$ obtained from the above equations, the value can be recalculated using a second method which does not rely on needing to know $K_m$ or $V_s$. The above equations

$$\frac{k_{cat}}{K_m} = \frac{V_s}{[E]_0} \quad \text{and} \quad \frac{k_{cat}}{V_s} = \text{slope of the line}$$

can be combined and rewritten as

$$\frac{k_{cat}}{K_m} = \frac{1}{\text{slope of the line} \cdot [E]_0}$$

which, again, is the ratio that measures the catalytic efficiency of the enzyme with a particular substrate, as stated above. Thus, $k_{cat}/K_m$ can be determined without having to know either the $K_m$ or the $V_s$. When this method was used to calculate the $k_{cat}/K_m$ values for the thiobenzyl esters with murine granzyme M, human granzyme M, and $\alpha$-chymotrypsin, the results were the same as those obtained when determining $K_m$ and $V_s$ independently.

The enzymes used in these assays were human granzyme M, murine granzyme M, and $\alpha$-chymotrypsin. The substrates were the peptide thiobenzyl esters. The substrates were hydrolyzed by the enzymes, releasing benzyl mercaptan, which reacted with DTNB in the buffer solution to give 2-nitro-5-thio-benzoic acid. The 2-nitro-5-thio-benzoic acid absorbs light at 405 nm, which can be monitored and is a direct correlation of the rate of
substrate hydrolysis. An example Lineweaver-Burk plot is shown in Figure 1.3 for the substrate Z-Asp-Pro-Met-SBzI and human granzyme M.

![Lineweaver-Burk Plot of Z-Asp-Pro-Met-SBzI and Human Granzyme M](image)

Figure 1.3 Lineweaver-Burk Plot of Z-Asp-Pro-Met-SBzI and Human Granzyme M

**Amino acid preferences of murine and human granzyme M.** Like other members of the granzyme subfamily, granzyme M has unusual synthetic substrate preferences with respect to the P1 position. Similar to other chymotrypsin-like serine proteases, granzyme M has an Ala in the bottom of its S1 subsite, suggesting that the enzyme would prefer hydrophobic residues (Kelly et al., 1996). In addition, the enzyme has an unique Ser-Phe-Ser stretch across this subsite that helps define substrate specificity, which suggests long, narrow, hydrophobic residues (Smyth et al., 1992). These structural features explain the preference of granzyme M for the amino acids Met, Leu, and Nle in the P1 position (Odake et al., 1991).
The work done here has helped to define which amino acids are preferred in the P2 and P3 positions of granzyme M substrates. The results in Table 1.2 show that both the murine and human granzyme M prefer Pro at the P2 position (Z-Ala-Pro-Met-SBzl, \( k_{cat}/K_M = 38,600 \pm 3,760 \text{ M}^{-1}\text{s}^{-1} \) for human, 55,900 ± 592 M\(^{-1}\)s\(^{-1}\) for murine), followed by Ala (Z-Ala-Ala-Met-SBzl, \( k_{cat}/K_M = 17,100 \pm 94 \text{ M}^{-1}\text{s}^{-1} \) for human, 55,100 ± 4,030 M\(^{-1}\)s\(^{-1}\) for murine) with kinetic values approximately 50% of that obtained with Pro at P2 for human granzyme M. When Pro is in the P2 position, Ala, Ser, and Asp are preferred at the P3 position. Kinetically, a comparison between Z-Ala-Pro-Met-SBzl (\( k_{cat}/K_M = 38,600 \pm 3,760 \text{ M}^{-1}\text{s}^{-1} \) for human, 55,900 ± 592 M\(^{-1}\)s\(^{-1}\) for murine), Z-Ser-Pro-Met-SBzl (\( k_{cat}/K_M = 38,300 \text{ M}^{-1}\text{s}^{-1} \pm 2,200 \) for human, 56,600 ± 3,490 M\(^{-1}\)s\(^{-1}\) for murine), and Z-Asp-Pro-Met-SBzl (\( k_{cat}/K_M = 41,400 \pm 2,301 \text{ M}^{-1}\text{s}^{-1} \) for human, 65,800 ± 370 M\(^{-1}\)s\(^{-1}\) for murine) shows that Ala, Ser, and Asp are approximately equivalently preferred at P3. This could potentially be due to a shallow pocket with a positively charged residue(s) that interacts with the Ser or Asp of the substrate. Another possibility is that the S3 pocket is shallow, hydrophobic, and near the surface of the enzyme. This would allow for small hydrophobic amino acids, like Ala, to fit into the pocket, but also allow smaller charged amino acids to interact favorably with the solvent. The best substrates for granzyme M appear to be those with amino acids Asp, Ser, or Ala at P3, and a Pro at P2. We also tested one tetrapeptide, Z-Ala-Ala-Pro-Met-SBzl, containing P1 through P3 residues preferred by granzyme M. I would like to graciously thank Brad W. Bolton, an undergraduate who worked under my direction, who synthesized and kinetically tested the tetrapeptide. The tetrapeptide had improved \( k_{cat}/K_M \) values for both human (\( k_{cat}/K_M = 69,900 \pm 1,770 \text{ M}^{-1}\text{s}^{-1} \)) and murine (\( k_{cat}/K_M = 68,200 \pm 660 \text{ M}^{-1}\text{s}^{-1} \)) granzyme M over the
Table 1.2  Kinetic Constants for Thiobenzyl Ester Hydrolysis by Granzyme M and α-Chymotrypsin

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Kinetic Constants</th>
<th>Human Granzyme M&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Murine Granzyme M&lt;sup&gt;b&lt;/sup&gt; without BSA</th>
<th>Murine Granzyme M&lt;sup&gt;b&lt;/sup&gt; with BSA</th>
<th>Bovine α-Chymotrypsin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z-Ala-Ala-Met-SBzl</td>
<td>$k_{cat}$</td>
<td>10.8 ± 1.0</td>
<td>2.46 ± 0.5</td>
<td>4.35 ± 0.65</td>
<td>14.4 ± 0.86</td>
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<tr>
<td></td>
<td>$K_M$</td>
<td>635 ± 27</td>
<td>184 ± 45</td>
<td>79 ± 1.8</td>
<td>70.9 ± 2.2</td>
</tr>
<tr>
<td></td>
<td>$k_{cat}/K_M$</td>
<td>17,100 ± 94</td>
<td>13,400 ± 594</td>
<td>55,100 ± 4,030</td>
<td>203,000 ± 5,820</td>
</tr>
<tr>
<td>Z-Ala-Phe-Met-SBzl</td>
<td>$k_{cat}$</td>
<td></td>
<td></td>
<td></td>
<td>2.69 ± 0.12</td>
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<td></td>
<td>$K_M$</td>
<td></td>
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<td></td>
<td>661 ± 2.9</td>
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<td></td>
<td>$k_{cat}/K_M$</td>
<td></td>
<td></td>
<td></td>
<td>NH&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>Z-Ala-Pro-Met-SBzl</td>
<td>$k_{cat}$</td>
<td>10.6 ± 1.2</td>
<td>18.1 ± 2.8</td>
<td>12.3 ± 0.66</td>
<td>28.7 ± 3.6</td>
</tr>
<tr>
<td></td>
<td>$K_M$</td>
<td>275 ± 27.1</td>
<td>694 ± 99</td>
<td>220 ± 1.4</td>
<td>33.7 ± 5.8</td>
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<tr>
<td></td>
<td>$k_{cat}/K_M$</td>
<td>38,600 ± 3,760</td>
<td>26,000 ± 2,030</td>
<td>55,900 ± 592</td>
<td>852,000 ± 45,200</td>
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<tr>
<td>Z-Ala-Lys-Met-SBzl</td>
<td>$k_{cat}$</td>
<td>9.68 ± 0.39</td>
<td>6.07 ± 0.60&lt;sup&gt;d&lt;/sup&gt;</td>
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<td>19.2 ± 3.3</td>
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<td>Inhibitor</td>
<td>( k_{cat} )</td>
<td>( K_M )</td>
<td>( k_{cat}/K_M )</td>
<td>( k_{cat}/K_M )</td>
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<td>------------------</td>
</tr>
<tr>
<td>Z-Ala-Ser(tBu)-Met-SBzl</td>
<td>1.68 ± 0.21</td>
<td>1,300 ± 158</td>
<td>1,300 ± 52</td>
<td>1,300 ± 52</td>
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<td></td>
<td>12.8 ± 2.0</td>
<td>2,660 ± 41</td>
<td>4,800 ± 226</td>
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<tr>
<td></td>
<td>3.56 ± 0.78</td>
<td>210 ± 60</td>
<td>17,000 ± 1,580</td>
<td>17,000 ± 1,580</td>
<td>17,000 ± 1,580</td>
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<tr>
<td></td>
<td>494 ± 35</td>
<td>1,300 ± 111</td>
<td>379,000 ± 23,900</td>
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<tr>
<td>Z-Ala-Asp(tBu)-Met-SBzl</td>
<td>2.86 ± 0.18</td>
<td>29.4 ± 1.1</td>
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<td>97,200 ± 2,390</td>
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<tr>
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<td>0.90 ± 0.02</td>
<td>0.438 ± 0.01</td>
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<td>7.18 ± 0.03</td>
<td>7.18 ± 0.03</td>
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<tr>
<td></td>
<td>$K_M$</td>
<td>$k_{cat}/K_M$</td>
<td>$k_{cat}$</td>
<td>$k_{cat}/K_M$</td>
<td></td>
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<tr>
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<td></td>
</tr>
<tr>
<td>Z-Phe-Pro-Met-SBzl</td>
<td>695 ± 19</td>
<td>130 ± 0.5</td>
<td>ND</td>
<td>1,640 ± 119</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>0.0784 ± 0.001</td>
<td>0.634 ± 0.02</td>
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<td></td>
<td></td>
<td>0.0200 ± 0.0004</td>
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</tr>
<tr>
<td>Z-Pro-Pro-Met-SBzl</td>
<td>36.0 ± 1.5</td>
<td>2,180 ± 55.4</td>
<td>ND</td>
<td>3,510 ± 318</td>
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<tr>
<td></td>
<td>0.00785 ± 0.0003</td>
<td></td>
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<td>0.973 ± 0.03</td>
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<td>3.29 ± 0.85</td>
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<tr>
<td>Z-Ser(fBu)-Pro-Met-SBzl</td>
<td>42.3 ± 2.1</td>
<td>2,170 ± 206</td>
<td>ND</td>
<td>4,400 ± 23.8</td>
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<td>0.0917 ± 0.0056</td>
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<td>0.161 ± 0.03</td>
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<td>0.613 ± 0.06</td>
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<tr>
<td>Z-Ser-Pro-Met-SBzl</td>
<td>255 ± 55</td>
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<td>52.1 ± 7.6</td>
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<td>44.6 ± 2.2</td>
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<tr>
<td></td>
<td>$K_M$</td>
<td>$k_{cat}$</td>
<td>$k_{cat}/K_M$</td>
<td>$K_M$</td>
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<tr>
<td>Z-Lys(Boc)-Pro-Met-SBzl</td>
<td>6,650 ± 128</td>
<td>38,300 ± 2,200</td>
<td>ND</td>
<td>152 ± 8.2</td>
<td>920 ± 14</td>
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<td>26.3 ± 2.1</td>
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<td>150 ± 35.7</td>
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<td>894 ± 84</td>
<td>ND</td>
<td>59.0 ± 1.5</td>
<td>361 ± 14</td>
<td>14,700 ± 183</td>
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<tr>
<td>Z-Asp(Bu)-Pro-Met-SBzl</td>
<td>33.4 ± 1.7</td>
<td>1,260 ± 53.4</td>
<td>ND</td>
<td>32.9 ± 2.4</td>
<td>129 ± 22.8</td>
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<tr>
<td>Z-Asp-Pro-Met-SBzl</td>
<td>17.3 ± 0.55</td>
<td>29.0 ± 4.59</td>
<td>13.2 ± 0.124</td>
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Table 1.2 (cont’d)

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<th>$K_M$</th>
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<td>418 ± 15.7</td>
<td>41,400 ± 2,310</td>
<td>13.1 ± 0.51</td>
<td>188 ± 12.1</td>
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<td>441 ± 67.3</td>
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<td>30.0 ± 6.27</td>
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<td>29,200 ± 179</td>
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<td>361 ± 0.12</td>
<td>65,800 ± 370</td>
<td>16.1 ± 3.1</td>
<td>236 ± 47.5</td>
<td>68,200 ± 660</td>
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<td>366,000 ± 4,460</td>
<td>13.3 ± 1.15</td>
<td>50.5 ± 4.56</td>
<td>265,000 ± 1,020</td>
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</table>

*The units for $k_{cat}$, $K_M$, and $k_{cat}/K_M$ are s$^{-1}$, μM, and M$^{-1}$s$^{-1}$, respectively. *Kinetic rates for all three enzymes were carried out in a 0.1 M Hapes, 0.5 M NaCl, 9% DMSO, pH 7.5 buffer at 23 °C. 'NH = No hydrolysis detected after 30 min. 'Average of two different assays. ND = Not determined. *Average of four different assays.
tripeptides. However, the increased rates were less significant with murine granzyme M than with human granzyme M. Improvement in hydrolysis rates is expected with longer peptide chains due to the increased number of H-bonds likely to form between the enzyme and the substrate. In addition, the tetrapeptide may more closely resemble the natural substrate of the enzyme. Thus, its binding, hydrolysis, and release from the enzyme may occur more quickly.

As can be seen from the data in Table 1.2, substrates tested with the murine granzyme M with and without BSA had dramatically different results. The granzyme M stock solution containing BSA had significantly higher rates of hydrolysis with the thiobenzyl ester substrates. In addition, the values of $k_{cat}/K_M$ of substrates tested with murine granzyme M containing BSA were substantially higher (approximately 2- to 4-fold) than those found for assays lacking BSA. This type of activity profile suggests that BSA stabilized the murine granzyme M and that the enzyme was less degraded when tested with the substrates. Granzyme M is difficult to isolate in large quantities. A supply shortage of both human and murine granzyme M prohibited titration of the enzymes to determine the actual concentration of active enzyme in each batch.

Thiobenzyl ester substrates and $\alpha$-chymotrypsin. I would like to graciously thank Sudah Natarajan, an undergraduate who worked under my direction, who did P2 substrate kinetic work with $\alpha$-chymotrypsin. The results in Table 1.2 for $\alpha$-chymotrypsin show that this enzyme prefers Pro at the P2 position, as granzyme M does. Combined with the results of a sequence alignment of granzyme M and $\alpha$-chymotrypsin, which showed a high degree of homology (identity of 33-35%, positives of 48-49%) between the two, this suggests that granzyme M and $\alpha$-chymotrypsin may be structurally similar.

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at the S2 subsite. However, α-chymotrypsin also has a comparable preference for Lys at this position, which was not seen with granzyme M. When Pro is in the P2 position, α-chymotrypsin has the strongest preference for Ala and Lys at P3. The preference for Lys at P3 by α-chymotrypsin is in contrast to the preference granzyme M has for small, negatively charged or hydrophobic amino acids at P5.

Comparison of the subsites of granzyme M and α-chymotrypsin. No crystal structure of granzyme M exists yet. However, a molecular modeling study done with the crystal structure of α-chymotrypsin and the amino acid sequence of human granzyme M shows that residues Lys179 (Met192 in chymotrypsinogen) and Ser201 (Gly216 in chymotrypsinogen) may play a critical role in S1 substrate specificity of granzyme M (Smyth et al., 1996b). Mutation of these residues in human granzyme M to Met and Gly, respectively, led to a loss of Met-ase activity and a gain of chymase activity (hydrolyzes after Phe). Smyth et al. suggest that the Lys179 and Ser201 may potentially have ionic H-bonding interactions across the S1 binding pocket that give granzyme M its unique substrate specificity (Smyth et al., 1996a). Lys179 and Ser201 are conserved in both the rat and mouse versions of granzyme M.

The amino acid composition of the S2 subsite of α-chymotrypsin has been known for some time in the literature (Segal, 1972; Blevins and Tulinsky, 1985a; Schellenberger et al., 1991). The residues Ile39, Trp75, and the active site His57 surround the S2 subsite and give the pocket its specificity for bulky, nonpolar residues. In addition, the crystal structure of the enzyme has been investigated a multitude of times (Freer et al., 1970; Blevins and Tulinsky, 1985b; Tsukada and Blow, 1985; Tulinsky and Blevins, 1987). With respect to both amino acid sequences and conserved cysteine residues, α-
chymotrypsin is more homologous (identity of 33-35%) than any other serine protease to granzyme M (Smyth et al., 1996b). If two different proteins, one of which has a known tertiary structure, have a sequence identity of 25% or greater, the protein with the unknown structure may adopt a fold similar to that of the known protein (Doolittle, 1986; Sander and Schneider, 1991). Therefore, it is possible that the tertiary structure of granzyme M is similar to that of α-chymotrypsin. We took a closer look at this by aligning the amino acid sequences of murine and human granzyme M with the primary amino acid sequence of α-chymotrypsin. These results were compared to the crystal structure of bovine α-chymotrypsin to investigate the amino acid residues that likely surround the S2 subsite of granzyme M. The sequences of human granzyme M (SWISS-PROT P51124) and murine granzyme M (TrEMBL O08643) used in the comparative alignment were obtained by Smyth et al. (Smyth et al., 1993; Kelly et al., 1996). The crystal structure of bovine α-chymotrypsin (Protein Data Bank 4CHA) used in this analysis was procured by Tsukada and Blow (Tsukada and Blow, 1985).

Comparative sequence alignment of the two species of granzyme M with α-chymotrypsin was performed with NCBI’s BLAST 2 program (NCBI). The BLAST program works by using an algorithm to perform local alignments of query and subject amino acid sequences. It looks for common functional domains, motifs, and shorter stretches of similar amino acid sequences that are often repeated in proteins of different species. This is different than a global alignment, which tries to match the entire sequence, and may result in the detection of fewer similarities between sequences being compared. When BLAST is given a sequence query, the program breaks it up into subsequences and uses them to look for similarities between the query sequence and
subject sequences. Once all of the subsequences of the query have been compared, BLAST reassembles the subsequences in the best possible alignment for each query-subject sequence pair. The BLAST results are then displayed, as shown in Figure 1.4, with the best sequence alignment for the query and subject or subjects, along with some statistical data. The 'Score' value is called a bit score. This gives an indication of how

alpha-Chymotrypsin

Murine Granzyme M

Query: 14
IWWKDIHGFFVQVSLSTMGFGFLLHMKVIVDVEIEDY
Query:
Seq: 72
SKEFQGDHGFGVYFGLHFGFLNAMGFLEFLVYEY

Figure 1.4 Amino Acid Sequence Alignment of Murine Granzyme M and alpha-Chymotrypsin

good an alignment is between a query and subject sequence. The higher this number is, the better. The bit score number is calculated from values assigned for similar and identical residues, any gaps that are present in the sequences, the length of the query and subject sequences, and a variety of other factors. The number in the parenthesis after the bit score is the raw data before it is processed with the above factors. The 'Expect' value is the probability that the queried sequence will randomly match any other sequence. It takes into account the database size as well as the bit score. The closer this value is to zero, the less likely that the alignments found between the query and subject sequences

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happened by statistical chance, and the more likely that the similarities between the sequences have some biological significance. The 'Identities' value is a measure of the number of exact residue matches at given positions throughout the query and subject sequences. The 'Positives' value is a combination of both the exact residue matches and residues which are considered conservative substitutions (amino acids of similar nature) at the same position of the sequences. Lastly, the 'Gaps' value represents spaces added in the query and subject sequence in order to align the matched sections of the sequences. This is necessary because one of the sequences has more residues in a given section of its peptide chain than does the comparable section in the other sequence, which would cause misalignment of the matched sections if the gaps were not inserted to compensate.

The comparative alignment of murine granzyme M and α-chymotrypsin amino acid sequences showed a high degree of sequence homology, giving a Score of 117 bits (294), an Expect of 2e-25, an Identity of 82/234 (35%), Positives of 115/234 (49%), and Gaps of 14/234 (5%). The sequence alignment of human granzyme M with α-chymotrypsin gave similar results with a Score of 117 bits (294), an Expect of 2e-25, an Identity of 77/232 (33%), Positives of 113/232 (48%), and Gaps of 15/232 (5%).

Unsurprisingly, the two granzyme M sequences gave the best homology, giving a Score of 335 bits (860), an Expect of 5e-91, an Identity of 168/241 (69%), Positives of 194/241 (79%), and Gaps of 7/241 (2%).

The comparative alignment shows that Ile^{30} of α-chymotrypsin has been replaced with Leu^{33} in human granzyme M, allowing for similar hydrophobic interactions in the S2 subsite. The comparison also shows that the Trp^{215} of α-chymotrypsin has been substitute for a Phe^{200} in human granzyme M. Murine granzyme M shows this same
substitution of Phe for Trp at residue 201. Because these residue substitutions are all conservative, the amino acid specificity of the S2 pocket of murine and human granzyme M and α-chymotrypsin may not be that different. However, the murine granzyme M also has a Tyr in place of the Ile in α-chymotrypsin. This is not a conservative residue substitution, and therefore could alter the S2 amino acid specificity of murine granzyme M compared to human granzyme M and α-chymotrypsin. However, there was no difference in which amino acids human and murine granzyme M preferred at S2 (both preferred Pro). As a confirmation of our results from the BLAST 2 program, we compared the alignment of amino acid sequences of granzyme M and bovine α-chymotrypsin that we found were compared to those published in papers by Smyth et al. (Kelly et al., 1996; Smyth et al., 1996a). That work shows the same amino acid substitutions that we found.

A Phe in the S2 pocket of granzyme M, as predicted, could result in favorable ring stacking when Pro is in the P2 position of a substrate. The Leu in human granzyme M may also play the same role as Ile in α-chymotrypsin by creating a hydrophobic environment favorable to Pro at P2. The substitution of Ile in α-chymotrypsin by Tyr in murine granzyme M may promote ring stacking and create enough of a hydrophobic environment to make Pro a favored P2 residue in substrates. In essence, the predicted structure of the S2 subsite of granzyme M appears to be very similar to that of α-chymotrypsin, which would explain the similar preferences for Pro at P2 for both enzymes. However, crystal structures of granzyme M would be instrumental in determining if these speculations are correct.
A series of new and sensitive thiobenzyl ester substrates has been designed for the study of the subsite preference of granzyme M. This work focused on peptide thiobenzyl ester substrates containing Met at the P1 position. The substrates described here are useful probes into the subsite preferences of the enzyme. The substrate specificity of murine and human granzyme M has been compared. Although their specificities are the same, human granzyme M has a higher rate of hydrolysis than the equivalent murine granzyme M (in the absence of BSA). In addition, the substrate specificity of granzyme M has been compared to the closely related bovine α-chymotrypsin. While granzyme M and α-chymotrypsin will both accept Pro in the P2 position, α-chymotrypsin will also readily accept Lys, which granzyme M was unable to accommodate. This suggests that, despite the similarities between the enzymes, their S2 binding pockets have significant structural differences. Analogous results were seen with the S3 binding pocket of the enzymes. Granzyme M readily accepts the negatively charged Asp and Ser residues in S3, while α-chymotrypsin had dramatically lower rates with those substrates compared to those containing Ala at P3. The differences in the P2 and P3 substrate specificities between granzyme M and α-chymotrypsin suggests very different cellular functions. In vivo, α-chymotrypsin is produced in the pancreas and plays the role of protein digestion in the mammalian intestinal tract. The actual biological function of granzyme M is unknown. However, its limited expression in NK cells suggests that it may have evolved for a specific immunological task. In conclusion, this study has elucidated the optimal P2 and P3 substrate preferences of granzyme M. The preferred residues of Pro at P2 and a
short, negatively charged amino acid or alanine at P3 may lead to the design of potent inhibitors for this enzyme. Specific and potent inhibitors would be useful for understanding the biological roles granzyme M plays in vivo. In addition, an interesting future project would be evaluating the activity of thiobenzyl ester derivatives containing Nle or Leu at P1 with granzyme M.
**Experimental**

**Materials and Methods.** Benzyl mercaptan, 1,3-dicyclohexylearbodiimide (DCC), 5,5'-dithiobiocse(2-nitrobenzoic acid (DTT)), N-hydroxybenzotriazole (HOBt), N-methylmorpholine (NMM), iso-butylchloroformate (IBC), DCDB, DMSO-<sub>d6</sub>, and all common reagents and solvents were purchased from Aldrich Chemical Co. (Milwaukee, Wl) or Fischer Scientific Chemicals (Fair Banks, NJ). The protected amino acid derivatives Boc-Met-OH, Z-Ala-OH, Z-Phe-OH, Z-Pro-OH, Z-Ser(Thr)-OH, Z-Lys(Boc)-OH, Z-Asp(Bu)-OH, HCl-H-Ala-OMe, HCl-H-Pro-OMe, HCl-H-Phe-OMe, HCl-H-Lys(Boc)-OMe, HCl-H-Ser(Thr)-OMe, and H-Asp(Bu)-OH were purchased from BACHEM Bioscience, Inc. (King of Prussia, PA). Flash chromatography silica gel (particle size 32-63 μm) and thin-layer chromatography plates that were precoated with 250 μm of silica gel (F-254) were obtained from Scientific Adsorbents, Inc. (Atlanta, GA). The bovine pancreatic α-chymotrypsin was purchased from Sigma Chemical Co. (St. Louis, MO). The 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) was obtained from Research Organics, Inc. (Cleveland, OH). The Smyth labs provided marine, rat, and human granzyme M. Kinetic data was gathered on a Molecular Devices Thermomax Microplate Reader (Molecular Devices Corporation, Menlo Park, CA). ³¹H NMR spectra were obtained from a Varian Mercury 300 instrument. Mass spectra, both nominal and HRMS, were collected on a VG Instruments 70SE (FAB<sup>+</sup>). The elemental analyses were performed by Atlantic Microlabs (Atlanta, GA). All elemental analyses were within ± 0.3% of the calculated values for the formulas shown for each compound.
N-(t-Butyloxycarbonyl)alanylalanylthreonine thiopeptide ester (Boc-Ala-Ala-Met-SBzl). The synthesis of the substrate used as a standard in the kinetic analysis, Boc-Ala-Ala-Met-SBzl, was previously discussed (Harper et al., 1984). The remainder of the substrates used in the P2 and P3 enzyme mapping assays were synthesized as described below.

Methionine thiopeptide ester hydrochloride (HCl-H-Met-SBzl). Boc-Met-OH (7.480 g, 30.0 mmol) was dissolved in dry THF (100 ml) and cooled to 0 °C. Benzyl mercaptan (3.726 g, 30.0 mmol, 3.52 ml) was added dropwise to the solution, followed by HOBT (2.027 g, 15.0 mmol). The solution was kept at 0 °C and stirred for 15 min. After the allotted time, DCC (1.2 eq, 7.428 g, 36.0 mmol) was added, and the solution allowed to stir overnight at 4 °C. A white precipitate (DCU) formed and was removed from the solution by filtration. The THF was evaporated, giving a light yellow, clear oil. The oil was redissolved in ethyl acetate (50 ml). The organic layer was washed with a 4% NaHCO₃ solution (3 x 50 ml), 1 N HCl (3 x 50 ml), and a saturated NaCl solution (3 x 50 ml). The organic layer was dried over MgSO₄, filtered, and evaporated, giving a light-yellow oil. The crude product was purified by silica gel chromatography using 5% CH₃OH in CH₂Cl₂ as the eluent. The eluent was evaporated to give a yellow-white solid.

Trituration in hexanes for 1 h gave a fluffy, white product (Boc-Met-SBzl, yield 71%). Removal of the Boc group from Boc-Met-SBzl (4.809 g, 13.5 mmol) was carried out by the literature procedure (Odake et al., 1991) involving a saturated solution of HCl in ethyl acetate, giving a white solid: yield 92%; one spot on the TLC, Rf = 0.73 (10% CH₃OH in CH₂Cl₂). ¹H NMR (DMSO-d₆) δ 8.92-8.68 (s, 2H), 7.40-7.25 (m, 5H), 4.38 (s, 1H), 4.26
N-Benzylxycarbonylalanyllalaninyloethionine thiobenzyl ester (Z-Ala-Ala-Met-SBzl)-General Procedure for Peptide Thiobenzyl Ester Synthesis. A solution of Z-Ala-OH (4.688 g, 21.0 mmol) and NMM (2.124 g, 21.0 mmol) dissolved in dry THF (50 ml) or CH₂Cl₂ (75 ml) was cooled to −15 °C. IBCF (2.868 g, 21.0 mmol) was added dropwise and the solution stirred at −15 °C for 15 min. A solution of HCl-H-Ala-OMe (2.931 g, 21.0 mmol) and NMM (2.124 g, 21.0 mmol) dissolved in THF (75 ml) or CH₂Cl₂ (75 ml) was cooled to −15 °C and stirred for 15 min. After the allotted time, the solutions were combined and allowed to stir at rt overnight. When the reaction was run in THF, the THF was evaporated, and the resulting material redissolved in CH₂Cl₂ before proceeding to the next step. The organic layer was washed with a 4% NaHCO₃ solution (3 x 100 ml), 1 N HCl (3 x 100 ml), and a saturated NaCl solution (3 x 100 ml). The organic layer was dried over MgSO₄, filtered, and evaporated, giving a white solid (Z-Ala-Ala-OMe, yield 66%). The solid was dissolved in CH₂OH (35 ml) with 1.5 eq NaOH and stirred at rt for 4 h. The solution was cooled to 0 °C, and 3.5 N HCl was added dropwise until the solution was pH 2. A white precipitate formed (Z-Ala-Ala-OH, yield 38%), which was collected and washed with ice cold distilled water and ether.

To make Z-Ala-Ala-Met-SBzl, a solution containing HCl-H-Met-SBzl (0.814 g, 2.79 mmol) and NMM (0.282 g, 2.79 mmol) dissolved in CH₂Cl₂ (20 ml) was cooled to 0 °C for 15 min. To this solution, DCC (0.576 g, 2.79 mmol), HOBT (0.189 g, 1.40 mmol), and Z-Ala-Ala-OH (0.824 g, 2.79 mmol) were added. The solution was allowed to stir at 4 °C overnight. A white precipitate (DCU) formed and was removed from the solution.
by filtration. The filtrate was washed with a 4% NaHCO₃ solution (3 x 20 ml), 1 N HCl (3 x 20 ml), and a saturated NaCl solution (3 x 20 ml). The organic layer was dried over MgSO₄, filtered, and evaporated, giving a white solid. The crude product was purified by silica gel chromatography using 2% CH₂OH in CH₂Cl₂ as the eluent. The eluent was evaporated to give the purified product as a white solid: yield 33%; one spot on the TLC, \( R_f = 0.58 \) (10% CH₂OH in CH₂Cl₂). ¹H NMR (CDCl₃) \( \delta 7.36 \) and 7.26 (s and s, 10H), 6.72 (d, 1H), 5.38 (d, 1H), 5.12 (s, 2H), 4.82-4.72 (m, 1H), 4.56-4.48 (m, 1H), 4.28-4.20 (m, 1H), 4.08 (s, 2H), 2.56-2.40 (m, 2H), 2.28-2.12 (m, 1H), 2.06 (s, 3H), 1.96 (s, 2H), 1.44-1.32 (m, 6H). HRMS (FAB⁺) \( m/z \) calculated for C₂₉H₃₂N₂O₃S₂ (M + 1) 552.19399, found 532.19421. Anal. (C₂₉H₳₂N₂O₃S₂) C, H, N.

N-Benzoylcarbonylalanylphenylmethionine thiobenzyl ester (Z-Ala-Phe-Met-SBzl). Z-Ala-Phe-OH moiety was synthesized as described for Z-Ala-Ala-OH in the synthesis of Z-Ala-Ala-Met-SBzl. Z-Ala-Phe-OH (1.852 g, 5.0 mmol) was reacted with HCH-Met-SBzl (1.459 g, 5.0 mmol), NMM (0.506 g, 5.0 mmol), DCC (1.035 g, 5.0 mmol), and HOBT (0.338 g, 2.5 mmol) in CH₂Cl₂ (40 ml). The workup was similar to that used to synthesize the compound Z-Ala-Ala-Met-SBzl. The product was crystallized from hexanes/CH₂Cl₂ (1/1) to yield a white powder: yield 35%; one spot on TLC, \( R_f = 0.61 \) (10% CH₂OH in CH₂Cl₂). ¹H NMR (CDCl₃) \( \delta 7.44-7.08 \) (m, 15H), 6.92 (d, 1H), 5.12-5.04 (m, 2H), 4.98 (d, 1H), 4.20-4.04 (m, 3H), 3.08 (d, 2H), 2.42-2.30 (m, 2H), 2.20-2.05 (m, 1H), 2.03 (d, 1H), 2.00-1.84 (m, 1H), 1.34-1.24 (d, 3H). HRMS (FAB⁺) \( m/z \) calculated for C₃₂H₃₇N₂O₅S₂ (M + 1) 608.22529, found 698.22618. Anal. (C₃₂H₃₇N₂O₅S₂ • 0.25 DCU) C, H, N.
N-Benzylxoycarbonylalaninprolymethionine thiobenzyl ester (Z-Ala-Pro-Met-SBzl). Z-Ala-Pro-OH moiety was synthesized as described for Z-Ala-Ala-OH in the synthesis of Z-Ala-Ala-Met-SBzl. The reaction of Z-Ala-Pro-OH (1.602 g, 5.0 mmol) with HCl•H-Met-SBzl (1.459 g, 5.0 mmol), NMM (0.506 g, 5.0 mmol), DCC (1.032 g, 5.0 mmol), and HOBr (0.338 g, 2.5 mmol) was performed in CH₂Cl₂ (20 ml). The workup was similar to that used to synthesize the compound Z-Ala-Ala-Met-SBzl. The product was crystallized from diethyl ether/petroleum ether (3/2) to yield a white powder: yield 71%; one spot on TLC, Rf = 0.16 (2% CH₃OH in CH₂Cl₂). ¹H NMR (CDCl₃) δ 7.48 (s, 1H), 7.36-7.14 (d, 10H), 5.60 (s, 1H), 5.20-5.02 (s, 2H), 4.82 (s, 1H), 4.66-4.44 (m, 2H), 4.12 (s, 2H), 3.72-3.48 (m, 1H), 2.56-2.32 (s, 3H), 2.24-1.88 (m, 8H), 1.80-1.60 (s, 2H), 1.44-1.24 (d, 3H). HRMS (FAB⁺) m/z calculated for C₂₉H₃₁N₃O₇S₂ (M + 1) 558.20964, found 558.20981. Anal. (C₃₀H₂₉N₃O₇S₂) C, H, N.

N-Benzylxoycarbonylalaninlysylmethionine thiobenzyl ester (Z-Ala-Lys-Met-SBzl). The Z-Ala-Lys(Boc)-OH moiety was synthesized similar to that described for Z-Ala-Ala-OH in the synthesis of Z-Ala-Ala-Met-SBzl. Z-Ala-Lys(Boc)-OH (1.129 g, 2.5 mmol) was added to a solution of HCl•H-Met-SBzl (0.730 g, 2.5 mmol), NMM (0.253 g, 2.5 mmol), DCC (0.516 g, 2.5 mmol), and HOBr (0.169 g, 1.25 mmol) in CH₂Cl₂ (120 ml). The workup was similar to that used to synthesize the compound Z-Ala-Ala-Met-SBzl. The crude product was purified by silica gel chromatography using 5% CH₃OH in CH₂Cl₂ as the eluent, giving a white powder (Z-Ala-Lys(Boc)-Met-SBzl, yield 56%). Removal of the Boc group from Z-Ala-Lys(Boc)-Met-SBzl (0.952 g, 1.38 mmol) was carried out by the literature procedure (Odaie et al., 1991) involving a saturated solution of HCl in ethyl acetate, giving a sticky, white solid. The product was treated with diethyl
ether and dried on the vacuum pump to yield a white powder: yield 89%; one spot on TLC, Rf = 0.02 (10% CH₂OH in CH₂Cl₂). ¹H NMR (DMSO-d₆) δ 8.64 (d, 1H), 8.06 (d, 1H), 8.00-7.84 (s, 2H), 7.48 (d, 1H), 7.40-7.24 (m, 10H), 5.08-4.96 (q, 2H), 4.56-4.48 (m, 1H), 4.32-4.24 (m, 1H), 4.12-4.00 (m, 3H), 2.76-2.68 (s, 2H), 2.60-2.36 (m, 2H), 2.04-1.80 (m, 5H), 1.79-1.68 (m, 1H), 1.67-1.44 (m, 3H), 1.40-1.24 (m, 2H), 1.23-1.12 (d, 3H). HRMS (FAB⁺) m/z calculated for C₂₀H₂₄N₂O₈S₂ (M + 1) 589.25184, found 589.25562. Anal. (C₂₀H₂₄N₂O₈S₂ • 0.25 H₂O) C, H, N.

N-Benzylxocarbonylalaninyl(N-(β-I-butylseryl))methionine thiobenzyl ester
(Z-Ala-Ser(Bu)-Met-SBzl). The Z-Ala-Ser(Bu)-OH moiety was synthesized similar to that as described for Z-Ala-Ala-OH in the synthesis of Z-Ala-Ala-Met-SBzl. To a solution containing HCl-H-Met-SBzl (1.459 g, 5.0 mmol) and NMM (0.506 g, 5.0 mmol) in CH₂Cl₂ (100 ml), Z-Ala-Ser(Bu)-OH (1.832 g, 5.0 mmol), DCC (1.032 g, 5.0 mmol), and HOBT (0.338 g, 2.5 mmol) were added. The workup was similar to that used to synthesize the compound Z-Ala-Ala-Met-SBzl. The crude product was purified by silica gel chromatography using 5% CH₂OH in CH₂Cl₂ as the eluent, followed by crystallization from CH₂Cl₂/hexanes (1/1), followed by silica gel chromatography using ethyl acetate CH₂Cl₂ (1/1) as the eluent to give a white powder: yield 59%; one spot on TLC, Rf = 0.68 (10% CH₂OH in CH₂Cl₂). ¹H NMR (CDCl₃) δ 7.50 (d, 1H), 7.40-7.16 (m, 10H), 6.88 (m, 1H), 5.24 (m, 1H), 5.20-5.00 (m, 2H), 4.88-4.78 (m, 1H), 4.52-4.44, (s, 1H), 4.28-4.20 (s, 1H), 4.16-4.07 (m, 2H), 3.90-3.84 (d, 1H), 3.44-3.36 (t, 1H), 2.60-2.40 (m, 2H), 2.30-2.16 (m, 1H), 2.06 (s, 3H), 2.04-1.90 (m, 1H), 1.44-1.36 (m, 3H), 1.24-1.10 (d, 9H). HRMS (FAB⁺) m/z calculated for C₂₀H₂₄N₂O₈S₂ (M + 1) 604.25151, found 604.25162. Anal. (C₂₀H₂₄N₂O₈S₂) C, H, N.
N-Benzylxoycarbonylanalyserylmethionine thiobenzyl ester (Z-Ala-Ser-Met-SBzl). The synthesis of this compound is a continuation of Z-Ala-Ser(Bu)-Met-SBzl. To Z-Ala-Ser(Bu)-Met-SBzl (0.746 g, 1.24 mmol) cooled to 0 °C, refrigerated (4 °C) TFA (10 ml) was added dropwise until the material dissolved to form a clear, colorless solution. The solution was stirred for 45 min at 0 °C, and then allowed to come to rt. The reaction was followed by TLC. When none of the starting material could be detected by TLC, the TFA was evaporated, giving a clear, colorless oil. The crude product was purified by silica gel chromatography using 10% CH3OH in CH2Cl2 as the eluent. The eluent was evaporated to leave behind a white, sticky solid. Treatment with diethyl ether yielded the product as a fine, white powder; yield 33%; one spot on TLC, Rf = 0.50 (10% CH3OH in CH2Cl2). 1H NMR (DMSO-d6) δ 8.44 (s, 1H), 7.96 (s, 1H), 7.48 (s, 1H), 7.40-7.20 (m, 10H), 5.01 (d, 2H), 5.00-4.88 (m, 1H), 4.60-4.50 (m, 1H), 4.40-4.24 (m, 1H), 4.16-4.00 (m, 3H), 3.76-3.56 (m, 2H), 2.48-2.34 (m, 1H), 2.06-1.94 (m, 4H), 1.92-1.80 (m, 1H), 1.26-1.14 (d, 3H). HRMS (FAB+) m/z calculated for C26H33N3O8S2 (M + 1) 548.18890, found 548.18960. Anal. (C26H33N3O8S2) C, H, N.

N-Benzylxoycarbonylanalyserylmethionine thiobenzyl ester (Z-Ala-Asp(Bu)-Met-SBzl). Z-Ala-OH (1.228 g, 5.5 mmol) was dissolved in dry THF (15 ml) and cooled to 0 °C. Pentafluorophenol (FFP) (1.114 g, 6.05 mmol) was added dropwise to the solution, at which point a white precipitate began to form. DCC (1.248 g, 6.05 mmol) was added to the solution, and the solution allowed to stir at 0 °C for 1 h. More white precipitate formed (DCU), which was filtered, and the THF evaporated, giving a white solid. The crude material was triturated in hexanes for 1 h, which resulted in a white powder (Z-Ala-OPFP, yield 81%). Although the TLC showed
a faint spot in addition to the product spot, it was deemed pure enough to continue with the next step of the synthesis.

To synthesize the Z-Ala-Asp(Bu)-OH, Z-Ala-OPFP (1.714 g, 4.40 mmol) was added to a slurry of H-Asp(Bu)-OH (0.842 g, 4.45 mmol) and DMF (40 ml) at rt. NMM (0.450 g, 4.45 mmol) was added dropwise to the slurry and the solution stirred overnight at rt. The DMF was evaporated leaving a clear, yellow oil. The crude product was purified by silica gel chromatography using 2% CH$_3$OH in CH$_2$Cl$_2$ as the eluent to elute the unreacted PFP, and ramping up to 10% CH$_3$OH in CH$_2$Cl$_2$ to elute the product, which became a white gum when concentrated and vacuum dried. Treatment with hexanes/petroleum ether/diethyl ether (1/1/1) and evaporation of these organic liquids gave a white powder (Z-Ala-Asp(Bu)-OH, yield 48%).

To make Z-Ala-Asp(Bu)-Met-SBzl, a solution containing HCl-H-Mec-SBzl (0.619 g, 2.12 mmol) and NMM (0.214 g, 2.12 mmol) dissolved in CH$_2$Cl$_2$ (30 ml) was cooled to 0 °C for 15 min. To this solution, DCC (0.437 g, 2.12 mmol), HOBt (0.143 g, 1.06 mmol), and Z-Ala-Asp(Bu)-OH (0.836 g, 2.12 mmol) were added. The workup was similar to that used to synthesize the compound Z-Ala-Ala-Met-SBzl. The crude product was purified by silica gel chromatography using 5% CH$_3$OH in CH$_2$Cl$_2$ as the eluent, followed by another column using ethyl acetate/CH$_2$Cl$_2$ (1/1) as the eluent, and treatment with ether to yield the product as a white powder; yield 44%; one spot on TLC, Rf = 0.71 (10% CH$_3$OH in CH$_2$Cl$_2$). $^1$H NMR (CDCl$_3$) δ 7.52-7.41 (m, 1H), 7.40-7.20 (m, 10H), 5.22 (d, 1H), 5.12 (s, 2H), 4.81-4.71 (m, 2H), 4.24-4.12 (m, 1H), 4.09 (s, 2H), 2.66-2.40 (m, 3H), 2.28-2.12 (m, 1H), 2.06 (s, 3H), 2.00-1.92 (m, 2H), 1.48-1.36 (m, 42
N-Benzoylcarbonylalanylspartylmethionine thiobenzyl ester (Z-Ala-Asp-Met-SBzl). The synthesis of this compound is a continuation of Z-Ala-Asp(Bu)-Met-SBzl. To Z-Ala-Asp(But)-Met-SBzl (0.502 g, 0.79 mmol) cooled to 0 °C, refrigerated (4 °C) TFA (10 ml) was added dropwise until the material dissolved to form a clear, colorless solution. The remainder of the workup was similar to that used to synthesize Z-Ala-Ser-Met-SBzl. The crude product was purified by silica gel chromatography using 10% CH$_2$OH in CH$_2$Cl$_2$ as the eluent, and after being concentrated, gave a white powder: yield 48%; one spot on TLC, $R_f$ = 0.25 (10% CH$_2$OH in CH$_2$Cl$_2$). $^1$H NMR (DMSO-d$_6$) $\delta$ 8.40 (d, 1H), 8.24 (d, 1H), 7.52 (d, 1H), 7.40-7.20 (m, 10H), 5.08-4.92 (q, 2H), 4.62-4.48 (m, 2H), 4.12-3.94 (m, 3H), 2.80-2.66 (m, 2H), 2.60-2.34 (m, 2H), 2.00 (s, 3H), 1.92-1.80 (m, 1H), 1.28-1.12 (d, 3H). HRMS (FAB$^+$) m/z calculated for C$_{32}$H$_{44}$N$_{10}$O$_{15}$S$_2$ (M + 1) 576.18382, found 576.18417. Anal. (C$_{32}$H$_{44}$N$_{10}$O$_{15}$S$_2$) C, H, N.

N-Benzoylcarboxyphenylprolylmethionine thiobenzyl ester (Z-Phe-Pro-Met-SBzl). Z-Phe-Pro-OH moiety was synthesized as described for Z-Ala-Ala-OH in the synthesis of Z-Ala-Ala-Met-SBzl. The reaction of Z-Phe-Pro-OH (0.757 g, 1.91 mmol) with HCl·H-Met-SBzl (0.559 g, 1.91 mmol), NMM (0.194 g, 1.91 mmol), DCC (0.394 g, 1.91 mmol), and HOBT (0.129 g, 0.96 mmol) was performed in CH$_2$Cl$_2$ (40 ml). The workup was similar to that used to synthesize the compound Z-Ala-Ala-Met-SBzl. The crude product was purified by silica gel chromatography using 5% CH$_3$CH$_2$OH in CH$_2$Cl$_2$. Evaporating the eluent gave a clear, colorless, sticky gel as the final product: yield 80%; one spot on TLC, $R_f$ = 0.81 (10% CH$_3$OH in CH$_2$Cl$_2$). $^1$H NMR (CDCl$_3$) $\delta$
N-Benzoyloxy carbonylprolylprolinethionine thiobenzyl ester (Z-Pro-Pro-Met-SBzl). Z-Pro-Pro-OH moiety was synthesized as described for Z-Ala-Ala-OH in the synthesis of Z-Ala-Ala-Met-SBzl. The reaction of Z-Pro-Pro-OH (1.039 g, 3.0 mmol) with HCl·H-Met-SBzl (0.876 g, 3.0 mmol), NMM (0.303 g, 3.0 mmol), DCC (0.619 g, 3.0 mmol), and HOObt (0.203 g, 1.5 mmol) was performed in dry THF (34 ml) and CH₂Cl₂ (50 ml). The workup was similar to that used to synthesize the compound Z-Ala-Ala-Met-SBzl. The crude product was purified by silica gel chromatography using 5% CH₂OH in CH₂Cl₂. Evaporating the eluent gave a white, sticky solid as the final product: yield 60%; one spot on TLC, Rf = 0.53 (5% CH₂OH in CH₂Cl₂). ¹H NMR (CDCl₃) δ 8.76 (d, 1H), 7.64 (m, 1H), 7.40-7.08 (m, 10H), 5.22-4.92 (m, 2H), 4.80-4.64, (m, 1H), 4.59-4.34 (m, 2H), 4.08-3.96 (d, 2H), 3.80-3.20 (m, 4H), 2.68-2.33 (m, 2H), 2.32-1.68 (m, 15H). HRMS (FAB⁺) m/z calculated for C₂₅H₂₅N₂O₅S₂ (M + 1) 584.2259, found 584.22774. Anal. (C₂₅H₂₅N₂O₅S₂) C, H, N.

N-Benzoyloxy carbonyl(N-(ß-tert-butyloxycarbonyl))prolinethionine thiobenzyl ester (Z-Ser(Bu)-Pro-Met-SBzl). Z-Ser(Bu)-Pro-OH moiety was synthesized as described for Z-Ala-Ala-OH in the synthesis of Z-Ala-Ala-Met-SBzl. To a solution containing HCl·H-Met-SBzl (0.876 g, 3.0 mmol) and NMM (0.303 g, 3.0 mmol) dissolved in CH₂Cl₂ (20 ml), DCC (0.619 g, 3.0 mmol), HOObt (0.203 g, 1.5 mmol), and Z-Ser(Bu)-Pro-OH (1.177 g, 3.0 mmol) were added. The workup was similar to that used to
synthesize the compound Z-Ala-Ala-Met-SBzI. The crude product was purified by silica gel chromatography using 5% CH3OH in CH2Cl2. Evaporating the eluent and drying the material on the vacuum pump gave a sticky, yellow-white solid as the final product: yield 82%; one spot on TLC, Rf = 0.69 (5% CH3OH in CH2Cl2). 1H NMR (CDCl3) δ 7.44 (d, 1H), 7.40-7.16 (m, 10H), 5.60 (d, 1H), 5.08 (s, 2H), 4.66-4.44 (m, 3H), 4.10 (s, 2H), 3.92-3.80 (m, 1H), 3.70-3.40 (m, 3H), 2.60-2.45 (m, 2H), 2.44-2.29 (m, 1H), 2.28-2.09 (m, 2H), 2.08-1.88 (m, 7H), 1.20-1.04 (m, 9H). HRMS (FAB+) m/z calculated for C32H34N3O4S2 (M + 1) 630.26716, found 630.2676. Anal. (C32H34N3O4S2) C, H, N.

N-Benzoxycarbonylserylpromylmethionine thiobenzyl ester (Z-Ser-Pro-Met-SBzI). The synthesis of this compound is a continuation of Z-Ser(tBu)-Pro-Met-SBzI.

To Z-Ser(tBu)-Pro-Met-SBzI (0.778 g, 1.24 mmol) cooled to 0 °C, refrigerated (4 °C) TFA (10 ml) was added dropwise until the material dissolved to form a clear, light-yellow solution. The remainder of the workup was similar to that used to synthesize Z-Ala-Ser-Met-SBzI. The crude product was partially purified by silica gel chromatography using 5% CH3OH in CH2Cl2 as the eluent, and after being concentrated, gave a dark-green oil. The material was dissolved in CH2Cl2 (20 ml) washed with distilled water (3 x 25 ml) and saturated NaCl solution (3 x 25 ml). The organic layer was dried over MgSO4, and after concentrating, resulted in an amber oil as the final product; yield 33%; one spot on TLC, Rf = 0.44 (5% CH3OH in CH2Cl2). 1H NMR (CDCl3) δ 7.40-7.12 (m, 10H), 5.76 (d, 1H), 5.08 (s, 2H), 4.80-4.73 (m, 1H), 4.72-4.62 (m, 1H), 4.60-4.54 (m, 1H), 4.08 (s, 2H), 3.96-3.90 (m, 1H), 3.88-3.78 (m, 1H), 3.76-3.60 (m, 2H), 2.52-2.44 (m, 2H), 2.40-2.32 (m, 4H), 2.31-1.90 (m, 6H). HRMS (FAB+)
m/z calculated for C_{33}H_{39}N_{3}O_{8}S_{2} (M + 1) 574.20455, found 574.20490. Anal. (C_{33}H_{33}N_{3}O_{8}S_{2} · 0.65 DCU) C, H, N.

N-Benzzyloxy carbonyl (N-(c-t-butyloxycarboxy) benzyllsyl) prolyl methionine thiobenzyl ester (Z-Lys(Boc)-Pro-Met-SBzl). Z-Lys(Boc)-Pro-OH moiety was synthesized as described for Z-Ala-Ala-OH in the synthesis of Z-Ala-Ala-Met-SBzl. To a solution containing HCI·H-Met-SBzl (0.876 g, 3.0 mmol) and NMM (0.303 g, 3.0 mmol) dissolved in CH_{2}Cl_{2} (20 ml), DCC (0.619 g, 3.0 mmol), HOBT (0.203 g, 1.5 mmol), and Z-Lys(Boc)-Pro-OH (1.433 g, 3.0 mmol) were added. The workup was similar to that used to synthesize the compound Z-Ala-Ala-Met-SBzl. The crude product was purified by silica gel chromatography using 5% CH_{3}OH in CH_{2}Cl_{2}. Evaporating the eluent and drying the material on the vacuum pump gave a fluffy, white solid as the final product: yield 55%; one spot on TLC, R_{f} = 0.80 (10% CH_{3}OH in CH_{2}Cl_{2}). 1H NMR (CDCl_{3}) δ 8.30 (d, 1H), 7.44-7.14 (m, 10H), 5.59 (d, 1H), 5.08 (s, 2H), 4.88-4.70 (m, 1H), 4.60-4.40 (m, 2H), 4.10 (s, 2H), 3.76-3.62 (m, 2H), 3.26-3.00 (s, 2H), 2.50-2.41 (m, 2H), 2.40-2.30 (m, 1H), 2.29-2.10 (m, 2H), 2.09-2.00 (m, 3H), 1.99-1.88 (m, 3H), 1.80-1.56 (m, 2H), 1.55-1.30 (s, 9H). HRMS (FAB^+) m/z calculated for C_{33}H_{39}N_{3}O_{8}S_{2} (M + 1) 715.31992, found 715.31613. Anal. (C_{33}H_{33}N_{3}O_{8}S_{2} · 0.65 DCU) C, H, N.

N-Benzzyloxy carbonyl prolyl methionine thiobenzyl ester (Z-Lys-Pro-Met-SBzl). The synthesis of Z-Lys-Pro-Met-SBzl is a continuation of Z-Lys(Boc)-Pro-Met-SBzl. Removal of the Boc group from Z-Lys(Boc)-Pro-Met-SBzl (0.586 g, 0.82 mmol) was carried out by the literature procedure (Odake et al., 1991) involving a saturated solution of HCl in ethyl acetate, giving a clear, sticky, colorless gel. The product was redissolved in CH_{2}Cl_{2} (10 ml), concentrated and dried on the vacuum pump to yield a
fine, white solid: yield 71%; one spot on TLC, \( R_f = 0.20 \) (10% CH\(_3\)OH in CH\(_2\)Cl\(_2\)). \(^1\)H NMR (CDCl\(_3\)) \& 8.24-8.80 (s, 2H), 7.46 (d, 1H), 7.40-7.10 (m, 10H), 6.28 (d, 1H), 5.20-4.88 (m, 2H), 4.80-4.60 (m, 1H), 4.54-4.40 (m, 2H), 4.12-3.99 (m, 2H), 3.80-3.52 (m, 2H), 3.50-3.20 (m, 1H), 3.04-2.80 (m, 2H), 2.64-2.36 (m, 2H), 2.28-1.84 (m, 9H), 1.83-1.30 (m, 6H). HRMS (FAB\(^+\)) \( m/z \) calculated for C\(_{34}\)H\(_{43}\)N\(_4\)O\(_8\)S\(_2\) (M + 1) 615.26749, found 615.26600. Anal. (C\(_{31}\)H\(_{29}\)N\(_4\)O\(_8\)S\(_2\)Cl \& 1.25 H\(_2\)O) C, H, N.

**N-Benzylxoxycarbonyl(N-\(\gamma\)-butylaspartyl)prolinemethionine thiobenzyl ester (Z-Asp(Bu)-Pro-Met-SBz).** To a solution containing HCl-H-Met-SBz (1.459 g, 5.0 mmol) and NMM (0.506 g, 5.0 mmol) dissolved in CH\(_2\)Cl\(_2\) (10 ml), a solution containing NMM (0.506 g, 5 mmol), IBCF (0.683 g, 5.0 mmol), and Boc-Pro-OH (1.076 g, 5.0 mmol) in CH\(_2\)Cl\(_2\) (35 ml) were added. The workup was similar to the IBCF coupling used in the synthesis of Z-Ala-Ala-OH. The Boc-Pro-Met-SBz was purified by silica gel chromatography using 5% CH\(_3\)OH in CH\(_2\)Cl\(_2\). Evaporating the eluent and drying the material on the vacuum pump gave a clear, yellow oil. Treatment with diethyl ether gave a white solid (Boc-Pro-Met-SBz, yield 66%). Removal of the Boc group from Boc-Pro-Met-SBz (1.498 g, 3.31 mmol) was carried out by the literature procedure (Odake et al., 1991) involving a saturated solution of HCl in ethyl acetate, giving a white, sticky solid (HCl-H-Pro-Met-SBz, yield 86%).

To make Z-Asp(Bu)-Pro-Met-SBz, a solution containing HCl-H-Pro-Met-SBz (0.778 g, 2.0 mmol) and NMM (0.202 g, 2.0 mmol) dissolved in CH\(_2\)Cl\(_2\) (15 ml) was cooled to 0 °C for 15 min. To this solution, DCC (0.413 g, 2.0 mmol), HOBT (0.135 g, 1.0 mmol), and Z-Asp(Bu)-OH (0.647 g, 2.0 mmol) were added. The workup was similar to that used to synthesize the compound Z-Ala-Ala-Met-SBz. The crude product
was purified by silica gel chromatography using 5% CH$_3$OH in CH$_2$Cl$_2$ as the eluent, followed by another column using ethyl acetate/hexanes (2/1) as the eluent.

Recrystallization from THF/CH$_2$Cl$_2$ (1/1) and hexanes yielded the product as a white solid: yield 57%; one spot on TLC, $R_f = 0.61$ (5% CH$_3$OH in CH$_2$Cl$_2$). $^1$H NMR (CDCl$_3$) δ 7.52 (d, 1H), 7.40-7.12 (m, 10H), 5.46 (d, iH), 5.16-5.00 (q, 2H), 4.86-4.74 (m, 1H), 4.73-4.62 (m, 2H), 4.02 (s, 2H), 3.80-3.64 (m, 2H), 2.96-2.84 (m, 1H), 2.60-2.14 (m, 5H), 2.06-1.88 (m, 7H), 1.42-1.32 (m, 9H). HRMS (FAB$^+$) m/z calculated for C$_{33}$H$_{33}$N$_3$O$_7$S$_2$ (M + 1) 658.26207, found 658.26126. Anal. (C$_{33}$H$_{33}$N$_3$O$_7$S$_2$) C, H, N.

N-Benzylxycarbonylaspartylprolylmethionine thiobenzyl ester (Z-Asp-Pro-Met-SBzl). The synthesis of this compound is a continuation of Z-Asp(tBu)-Pro-Met-SBzl. To Z-Asp(tBu)-Pro-Met-SBzl (0.143 g, 0.22 mmol) cooled to 0 °C, refrigerated (4 °C) TFA (4 ml) was added dropwise until the material dissolved to form a clear, light-yellow solution. The remainder of the workup was similar to that used to synthesize Z-Ala-Ser-Met-SBzl. The crude product was purified by silica gel chromatography using 10% CH$_3$OH in CH$_2$Cl$_2$ as the eluent. The solvent was evaporated, giving a clear, colorless gel as the final product: yield 68%; one spot on TLC, $R_f = 0.38$ (10% CH$_3$OH in CH$_2$Cl$_2$). $^1$H NMR (CDCl$_3$) δ 7.42 (d, 1H), 7.40-7.16 (m, 10H), 5.68 (d, 1H), 5.16-5.00 (q, 2H), 4.96-4.84 (m, 1H), 4.72-4.56 (m, 2H), 4.06 (s, 2H), 3.92-3.72 (m, 2H), 3.02-2.88 (dd, 1H), 2.80-2.68 (dd, 1H), 2.56-2.40 (m, 2H), 2.39-2.24 (m, 1H), 2.20-2.10 (m, 1H), 2.04 (s, 3H), 2.01-1.80 (m, 4H). HRMS (FAB$^+$) m/z calculated for C$_{39}$H$_{32}$N$_3$O$_7$S$_2$ (M + 1) 602.19947, found 602.19962. Anal. (C$_{39}$H$_{32}$N$_3$O$_7$S$_2$ • 0.3 H$_2$O) C, H, N.

N-Benzylxycarbonylasglycylalanylprolylmethionine thiobenzyl ester (Z-Ala-Ala-Pro-Met-SBzl). Z-Ala-Ala-OH moiety was synthesized as described above in the
synthesis of Z-Ala-Ala-Met-SBzl. The HCl·H-Pro-Met-SBzl moiety was synthesized as described for Z-Asp(tBu)-Pro-Met-SBzl. To make Z-Ala-Ala-Pro-Met-SBzl, a solution containing HCl·H-Pro-Met-SBzl (0.620 g, 1.59 mmol) and NMM (0.161 g, 1.59 mmol) dissolved in THF (27 ml) was cooled to 0 °C for 15 min. To this solution, DCC (0.328 g, 1.59 mmol), HOBT (0.107 g, 0.795 mmol), and Z-Ala-Ala-OH (0.468 g, 1.59 mmol) were added. The workup was similar to that used to synthesize the compound Z-Ala-Ala-Met-SBzl. The crude product was purified by silica gel chromatography using 10% CH3OH in CH2Cl2 as the eluent. Recrystallization from THF/CH2Cl2 (1/1) and hexanes yielded the product as a white solid: yield 51%; one spot on TLC, \( R_f = 0.63 \) (10% CH3OH in CH2Cl2). 1H NMR (CDCl3): δ 7.55-7.45 (m, 2H), 7.40-7.16 (m, 10H), 5.45 (d, 1H), 5.10 (s, 2H), 4.83-4.73 (m, 2H), 4.58 (d, 1H), 4.45-4.35 (m, 1H), 4.10 (s, 2H), 3.80-3.55 (m, 3H), 2.47-2.30 (m, 3H), 2.29-2.10 (m, 3H), 2.02 (s, 3H), 1.90-1.63 (m, 1H), 1.40-1.20 (m, 6H). HRMS (FAB+) m/z calculated for C31H49N5O6S2 (M + 1) 629.24675, found 629.24421. Anal. (C31H49N5O6S2) C, H, N.

**Granzyme M Storage Concerns.** Storing dilute murine granzyme M resulted in a loss of enzymatic activity, especially when left at room temperature for extended periods. When the enzyme is more concentrated and stored at 4 °C, granzyme M retains consistent hydrolytic activity for months. One of the stock solutions of murine granzyme M used was diluted, and BSA was added to stabilize it. This resulted in diluted murine granzyme M that gave consistent hydrolytic activity over several months of storage. However, adding BSA to the enzyme had some significant consequences. As can be seen from the data in Table 1.2, substrates tested with the murine granzyme M with and without BSA had dramatically different results. The granzyme M stock solution

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containing BSA had significantly higher hydrolytic activity with the thio benzyl ester substrates. This suggests that BSA stabilized the murine granzyme M, and the enzyme was less degraded than in samples of enzyme lacking BSA. A supply shortage of both human and murine granzyme M prohibited titration of these enzymes.

**Enzyme Assays-General Method.** The thiobenzyl ester substrate hydrolysis was followed by measuring the increase in absorbance at 405 nm due to the reaction between the thiobenzyl group released from the peptide and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) to produce 2-nitro-5-thio-benzoic acid (ε₄₁₂ = 13,260 M⁻¹cm⁻¹) (Ellman, 1959; Riddles et al., 1983). A Molecular Devices Thermomax Microplate Reader was used to spectrophotometrically obtain the kinetic measurements. The assays were carried out in a buffer of 0.1 M Hepes, 0.5 M NaCl, at pH 7.5 (assay buffer) at 23 °C. The stock substrate solutions were made up in DMSO and used immediately. The DTNB stock solution was similarly made up in DMSO. Both human and murine granzyme M (M₀ = 30,000) were stored in a solution of 0.1 M Hepes, 0.5 M NaCl, at pH 7.5 at 4 °C, and kept at 0 °C when used in the assays. Dilutions of the enzymes were made with the buffer solution. One stock solution of the murine enzyme also contained BSA (1 mg/ml), which had a significant effect on the kinetic results (Table 1.2). No human or rat granzyme M stock solution contained BSA. The bovine α-chymotrypsin (M₀ = 25,000) solution was made up in and diluted with 1 mM HCl, and stored at -20 °C. A single active site center was assumed for all the enzymes. The assays were conducted with 200 µl assay buffer, 10 µl of DTNB (5 mM) in DMSO, 10 µl of substrate stock solution in DMSO, and 10 µl of enzyme stock solution at 23 °C. Substrate concentrations were between 4.35 µM and 217 µM. Human and murine granzyme M concentrations were
between 28.7 nM and 143 nM. The α-chymotrypsin concentrations were between 2.17 nM and 870 nM. To compensate for the rates of background hydrolysis, the procedure above was carried out, except 10 μL of assay buffer was used in place of the enzyme stock solution. All substrates showed low hydrolysis in aqueous buffer.

For all the substrate assays, at least four, and usually five to eight, different substrate concentrations were tested. Duplicate runs at each concentration were performed. Initial hydrolysis rates were used with Lineweaver-Burk plots to obtain the data. The kinetic values were obtained from a computer-determined linear regression fit of the data. Correlation coefficients for the Lineweaver-Burk plots were usually greater than 0.98, but never below 0.92.

Negative controls were also run for both murine and human granzyme M. Active site serine to alanine granzyme M mutants were mixed with all of the thiobenzyl ester substrates under the same assay conditions described above for 30 min. No enzymatic activity could be detected.
REFERENCES


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CHAPTER 2
AZA-PEPTIDE MICHAEL ACCEPTORS AS INHIBITORS FOR CLOSTRIPAIN AND GINGIPAIN

INTRODUCTION

Cysteine Proteases. Cysteine proteases are found in protozoa, bacteria, fungi, and mammals, both those inherent in the species, and those introduced by infecting viruses (Barrett, 1986). These enzymes participate in the hydrolysis of a variety of proteins and play major roles both intracellularly (protein processing, degradation, and turnover) and extracellularly (bacterial enzymes secreted to degrade host defenses). There are currently seven clans of cysteine proteases (Barrett and Rawlings, 2001). The majority of cysteine proteases belong to clan CA, which includes calpain I, the cathepsins, and papain. Papain is likely the best known and most studied cysteine protease. Most of what is known about this class of enzymes comes from research done on papain and the other clan CA enzymes.

All cysteine proteases have a highly conserved catalytic triad consisting of the amino acid residues Cys, His, and Asn (Berti and Storer, 1995). The cysteine and histidine residues of the active site are thought to play the major role. The active site asparagine residue has been speculated to help stabilize and aid in the hydrolytic activity of the catalytic pair through hydrogen bonding (Kirschke and Wiederanders, 1994). The normal state of the enzymatic residue appears to be a negatively charged thiol group in conjunction with a protonated histidine (Lewis et al., 1976; Polgar and Halasz, 1982). The histidine donates its proton to the substrate while the highly nucleophilic cysteine
thiolate hydrolizes proteins through an acyl thioester intermediate formed upon attack of
the peptide scissile bond (Fink and Angelides, 1976; Plapp, 1982; Dufour et al., 1995).
In cysteine proteases, a highly conserved region called the oxyanion hole, composed of a
 glutamine residue and the backbone hydrogen of the active site cysteine residue,
stabilizes the tetrahedral intermediate during substrate hydrolysis (Ménard et al., 1991;
Ménard et al., 1995).

The extended binding site of cysteine proteases are frequently able to recognize
peptide residues from the S4 subsite to the S3' subsite (Schechter and Berger, 1967). The
cysteine proteases vary greatly in which subsite determines the primary substrate
specificity of the enzymes. For example, the most important substrate-peptide interaction
for the clan CA enzymes is at the S2 subsite (Baker and Drenth, 1987; Gour-Salin et al.,
1994), while for clan CD it is the S1 subsite (Ogle and Tveten, 1953; Sleath et al., 1990;
Howard et al., 1991; Pike et al., 1994). The remainder of the subsites vary greatly in
depth, size, and residues lining the substrate binding pocket, which accounts for the
specificity difference found for substrates and inhibitors between the varying cysteine
proteases.

**Clan CD.** A small but particularly important group of cysteine proteases are
those in clan CD, which include clostripain, gingipains K and R, the caspases, legumain,
and separase. They are thought to share a similar catalytic site design and substrate
binding domain, implying a common ancestry (Chen et al., 1998). The caspase family,
which contains greater than 15 members, is involved in apoptosis, or programmed cell
death. They are associated with a variety of disorders and illnesses including stroke,
Alzheimer's disease, and Parkinson's disease. Legumain is involved with antigen

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processing and immune disorders. Very little is known about separase, other than its involvement in eukaryotic cell division and separation of chromosomes in meiosis of yeasts (Buonomo et al., 2000; Uhmann et al., 2000). The focus of this project were the clan CD enzymes, clostripain, gingipain K, and gingipain R, three of the few bacterial cysteine proteases known.

Clostripain is a cysteine protease isolated from the growth culture of *Clostridium histolyticum*, an anaerobic Gram-positive bacterium involved in gas gangrene syndrome (Kochalaty et al., 1938; Kochalaty and Krejci, 1948; Lepow et al., 1952; Mitchell and Harrington, 1970). The existence of clostripain has been known for over sixty years (Kochalaty et al., 1938; Maschmann, 1938; Van Heyningen, 1960). However, its amino acid preference at S1 was unknown until 1953 (Ogle and Tytell, 1953). Clostripain prefers to cleave at the carboxy terminus of Arg and Lys, much like trypsin (Labouesse and Gros, 1960). However, unlike trypsin, clostripain has a stronger preference for Arg over Lys in the P1 position of substrates, but will not cleave after any other natural amino acids (Ogle and Tytell, 1953; Gros and Labouesse, 1960; Nordwig and Stauchi, 1983; Porter et al., 1971). The preference for Arg residues over Lys at P1 has been attributed to higher *Km* and lower *kcat* values of Lys substrate analogs with clostripain (Cole et al., 1971). This preference is much less pronounced in inhibitors tested with clostripain (Wikstrom et al., 1989; Kenbhavi et al., 1991).

Clostripain has little amino acid preference in the P1' position, but will not accept Asp and Glu (Ullmann and Jakubke, 1994). However, the enzyme will readily accept Pro at S1'. The Arg-Pro peptide bond is generally resistant to hydrolysis (Ando et al., 1959). The ability of clostripain to cleave this peptide linkage gives it an important role

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in peptide and protein sequence analysis, as well as making it a useful transpeptidase for synthetic peptide formation with an Arg-Pro linkage (Labouesse and Gros, 1960; Mitchell and Harrington, 1968; Haniu et al., 1982; Andersen, 1985; Fortier and MacKenzie, 1986; Yagisawa et al., 1990; Günther et al., 2000).

The enzyme displays esterase (hydrolyzes esters to acids and alcohols), amidase (hydrolyzes amide bonds), and endopeptidase (hydrolyzes peptide bonds in the interior of the chain) activity with approximately equivalent rates of hydrolysis, suggesting a highly efficient catalytic mechanism for hydrolyzing amide bonds (Mitchell and Harrington, 1970; Cole et al., 1971; Kembhavi et al., 1991). The hydrolytic activity of the enzyme relies on the presence of calcium ions which are believed to enhance the esterase activity, as well as stabilize the enzyme against thermal inactivation (Labouesse and Gros, 1960). However, other metal ions and EDTA often act as inhibitors (Mitchell and Harrington, 1968). Because of the active site cysteine, a thiol reducing agent is necessary to activate clostripain (Labouesse and Gros, 1960; Mitchell and Harrington, 1968).

Mature clostripain is made up of two non-covalently bonded peptide chains; a heavy one (43 kDa), which contains the active site cysteine (Cys\(^{44}\)), and a light one (15.4 kDa). Surprisingly, the peptide chains are not held together by the disulfide bridges present in the enzyme (Gilles et al., 1979; Gilles et al., 1983). However, in the pro-form of the enzyme, a peptide linker containing Arg residues at either end holds the enzyme together (Dargatz et al., 1993; Witte et al., 1994). This suggests that the enzyme may be able to autolyse this linkage to give the mature enzyme, a rare ability among enzymes. Clostripain shows no primary homology with any other cysteine proteinase or any known protein structure (Gilles et al., 1983; Dargatz et al., 1993). Due to the lack of structural
homology with other enzymes, clostripain was thought to have a converging evolution of active sites or conservation of a primitive active site during divergent evolution (Gilles et al., 1983; Gilles et al., 1984). However, it is now thought to have evolved from a distinct evolutionary line (Kembhavi et al., 1991).

Gingipains K and R are cysteine proteases isolated from cultures of Porphyromonas gingivalis, a Gram-negative anaerobic rod-shaped bacterium associated with the initiation and progression of periodontal disease (White and Mayrand, 1981; Zambon et al., 1981; Moore et al., 1982; Slots et al., 1986; Mayrand and Holt, 1988). The disease is characterized by lesions of the gingival connective tissue, resorption of the alveolar bone, and excessive leukocyte infiltration (White and Mayrand, 1981). In the periodontal cavities associated with this disease, gingipains K and R make up two of at least eight different proteases and other cofactors present and recognized as important virulent factors of the bacteria (Greiner and Mayrand, 1987; Marsh et al., 1989; Smalley et al., 1989). The hydrolysis by the gingipains comprises about 85% of the total proteolytic activity surrounding Porphyromonas gingivalis (Pavloff et al., 1995; Okamoto et al., 1996). The enzymes hydrolyze a variety of proteins, including collagen (Bekedal-Hansen et al., 1988), fibronectin (Wikstrom and Linde, 1986; Uitto et al., 1989), fibrinogen (Lantz et al., 1986), and immunoglobulins (Sundqvist et al., 1985; Kadowaki et al., 1994). They can also lyse erythrocytes and iron-binding proteins, providing amino acids and iron as nutrition for the bacterium, which is unable to utilize saccharides as an energy source (Carlsson et al., 1984; Shah and Gharbia, 1989; Nishikata and Yoshinura, 1991). More recently, both gingipains were found to degrade the proinflammatory cytokines tumor necrosis factor-α and interleukins-1B, -8, and -6.
suggesting that the enzymes may play a role in disrupting the cytokine activity surrounding the bacterium (Calkins et al., 1998; Fletcher et al., 1998; Mikolajczyk-Pawlinska et al., 1998; Banbula et al., 1999). In addition, gingipain R can activate the plasma coagulation cascade and could potentially contribute to cardiovascular diseases, which are extraordinarily prevalent in chronic periodontitis patients (Imamura et al., 1997).

The gingipains (Porphyromonas gingivalis clostripain) have many features in common. Being cysteine proteases, both gingipains need a thiol reducing agent, such as DTT, for activation (Birkedal-Hansen et al., 1988). The enzymes use calcium ions for stabilization and prevention of autolysis, although they do not require it for activity (Birkedal-Hansen et al., 1988; Kadowaki et al., 1994). Both gingipains are associated with bacterial outer membranes and vesicles, as well as being secreted in a soluble form into the culture medium where most of the proteolytic activity is found (Smalley et al., 1989; Smalley and Birr, 1991; Okamoto et al., 1996). Studies have shown the enzymes to be active at pH 8.0, the same pH as the periodontal cavity (Kadowaki et al., 1994; Pike et al., 1994). The gingipains also play a role in activating cell surface proteins of the bacterium to proliferate bacterial colonization. Other than a small amount of sequence homology with each other, and the conserved catalytic triad, neither enzyme has primary sequence homology with any other cysteine protease (Okamoto et al., 1995; Okamoto et al., 1996). A comparison of the crystal structure of gingipain R with those of caspase-1 and -3 shows some topological homology (Banbula et al., 1998; Eichinger et al., 1999). However, unlike caspase-1 and -3, gingipain R has a unique fold not found in any other known crystallized enzyme.
A variety of differences between gingipain K and gingipain R make them unique. The major difference between the two types of gingipains is their P1 specificitiyer. Gingipain K cleaves specifically after Lys and will not hydrolyze peptides or proteins after any other natural amino acids (Grenier and McBride, 1987; Otska et al., 1987; Pike et al., 1995). The enzyme has little specificity for the P2 position, except it will not tolerate Arg or Lys residues (Pike et al., 1994). Gingipain K, analogously to clostripain, can effectively cleave the Lys-Pro peptide bond, which is usually resistant to proteolytic hydrolysis (Pike et al., 1994).

Gingipain R has a strict specificity for Arg in the P1 position of substrates and will not cleave after any other natural amino acids (Chen et al., 1992). In contrast to gingipain K, gingipain R prefers hydrophobic residues at P2 and P3 (Kadowaki et al., 1994). Gingipain R has increased activity in assays when the dipeptide Gly-Gly is added to the buffer, an observation which has not been seen with any other enzymes (Chen et al., 1991). Unlike gingipain K, gingipain K is inhibited when Gly-Gly is added to the assay buffer (Pike et al., 1994). Gingipain R can undergo autolysis in its propeptide form (Okamoto et al., 1995). On the other hand, gingipain K is not autoprotoleolytic, but is thought to be processed by gingipain R. (Okamoto et al., 1996; Kadowaki et al., 1998). Finally, in crude fractions of intact Porphyromonas gingivalis cells, gingipain R is found at a 3-fold higher concentration than gingipain K (Potempsa et al., 1997).

Cysteine Protease Inhibitors. A variety of inhibitors have been tested against cysteine proteases, particularly clan CA cysteine proteases. Only a few inhibitors have been reported for clostripain and gingipains K and R. Almost none have been designed specifically with these clan CD enzymes in mind. One class of inhibitors is based on the...
potent epoxysuccinyl cysteine protease inhibitor E-64 (L-\textit{trans}\textendash epoxysuccinyl-leucylamido(4-guanidino)butane) isolated from \textit{Aspergillus japonicus}, illustrated in Figure 2.1 (Hanada \textit{et al.}, 1978). For most cysteine proteases, especially those in the papain super family (clan CA), E-64 and its derivatives are selective, irreversible inhibitors with no activity towards trypsin, chymotrypsin, and a variety of other enzymes (Barrett \textit{et al.}, 1982). However, E-64 and its derivatives are reversible inhibitors of clostripain \((K_i = 10-20 \text{ mM})\), probably acting as substrate analogs (Barrett \textit{et al.}, 1982). E-64 will not inactivate gingipain \(K\), but inhibits gingipain \(R\) at several thousand-fold molar excess over the enzyme (Chen \textit{et al.}, 1992; Pike \textit{et al.}, 1994).

![Figure 2.1 Structure of E-64](image)

As analog to epoxysuccinyl inhibitors is the aziridinyl peptide class (Figure 2.2). Although being potent, irreversible inhibitors of the papain superfamily, the aziridinyl peptides act as reversible inhibitors for clostripain (Schirmeister and Peric, 2000). The ester aziridinyl derivatives were found to be the most potent inhibitors of clostripain in the series tested, with the best being \(\text{EtO-Azi-Leu-OBzl} (K_i = 56.0 \pm 4.3 \text{ mM})\) (Schirmeister and Peric, 2000).

Several other reversible inhibitors have been tested with cysteine proteases for
their inactivation potency, including the natural inhibitors leupeptin (Ac-Leu-Leu-Arg-H) and histatin 5 (a salivary protein). Although they proved to be rather potent inhibitors towards clostripain ($K_i = 60 \text{nM}$ and $K_i = 10 \text{nM}$, respectively), neither is specific for the enzyme (Guinan et al., 2001). Gingipain R is also sensitive to leupeptin, while gingipain K seems to be resistant to it (Pike et al., 1994).

A variety of irreversible inhibitors have been tested with cysteine proteases as well. These include peptidyl chloromethyl (CMK) and chloroethyl ketones (CEK), acyloxymethyl ketones, diazomethyl ketones, sulfoxium methyl ketones, and aza-peptide active esters. All of these inhibitor classes are potent, irreversible inhibitors that work essentially the same, inhibiting the enzyme by acylating the active site cysteine residue.

The peptidyl chloromethyl and chloroethyl ketones can inhibit both cysteine and serine proteases, and therefore do not distinguish between the enzyme classes (Rauber et al., 1986; Wikstrom et al., 1989; Albeck et al., 1996). While peptidyl chloroethyl ketones reduce side reactions in a cellular environment (Wikstrom et al., 1989), both types are chemically reactive toward simple thiols and non-protectolytic enzymes, which can result in toxic side effects in vivo (Rauber et al., 1986; Babine and Bender, 1997).

Clostripain is rapidly and selectively inactivated by α-N-tosyl-L-lysine chloromethyl ketone (TLMK) with a second order inactivation constant of 87,000 M$^{-1}$s$^{-1}$ (Porter et al., 1994).
Unlike other cysteine proteases, clostripain is actually more susceptible to peptidyl chloromethyl ketones (Phe-Ala-Lys-Arg-CEK, \( k_{3rd} = 10,100,000 \text{ M}^{-1}\text{s}^{-1} \)) than the corresponding peptidyl chloromethyl ketones (Phe-Ala-Lys-Arg-CMK, \( k_{3rd} = 1,860,000 \text{ M}^{-1}\text{s}^{-1} \)) (Wikstrom et al., 1989). Two of the most potent chloromethyl ketones with gingipain \( R \), and among the fastest of any known inhibitors, include D-Phe-Pro-Arg- CMK \( k_{3rd}/[I] = 22,220,000 \text{ M}^{-1}\text{s}^{-1} \) and D-Phe-Phe-Arg-CMK \( k_{3rd}/[I] = 47,659,000 \text{ M}^{-1}\text{s}^{-1} \) (Pike et al., 1994). They also rapidly inhibit gingipain \( K \) with \( k_{3rd}/[I] \) values of 1,108,000 \text{ M}^{-1}\text{s}^{-1} and 1,050,000 \text{ M}^{-1}\text{s}^{-1}, respectively (Potempa et al., 1997).

Peptidyl acyloxymethyl ketone inhibitors are very similar to the chloromethyl ketones, but have a reduced activity due to a larger leaving group which is only weakly nucleophilic (Smith et al., 1988). Acyloxymethyl ketones vary greatly because a wide variety of leaving groups can be used that span the S’ subsites of cysteine proteases (Brömme et al., 1994). This allows these inhibitors to be highly selective and have controlled reactivity toward cysteine proteases. Peptidyl acyloxymethyl ketones are very selective toward cysteine proteases and can be made chemically inert, when compared to chloromethyl ketones, toward most non-enzymatic nucleophiles (Smith et al., 1988). The acyloxymethyl ketone, Z-Phe-Lys-CH_2OCOPh(2,4,6-(CH_3)), was tested by Potempa et al. with gingipain \( K \) (Potempa et al., 1997). The inhibitor potently inactivated the enzyme with a rate constant of \( k_{3rd} = 4,200,000 \text{ M}^{-1}\text{s}^{-1} \). Gingipain \( R \) was also inactivated by this inhibitor with a \( k_{3rd}/[I] \) value of 1,920 \text{ M}^{-1}\text{s}^{-1}.

Peptidyl diazomethyl ketones are selective for cysteine proteases, but not serine or metalloproteases, even with amino acid specificity designed for those enzymes (Leary et al., 1977; Watanabe et al., 1979; Green and Shaw, 1981b; Kirshke and Shaw, 1981;
Zumbrunn et al., 1988). They are stable towards simple thiols. However, their greatest
drawback is the difficulty of their synthesis due to their instability in acids (Green and
Shaw, 1981b). A potent peptidyl diazomethane found to inactivate clostripain is Z-Phe-
Arg-CHN₂, which has a second-order inactivation constant of 86,000 M⁻¹s⁻¹ (Zumbrunn et
al., 1988).

Peptidyl sulfonium methyl ketones are haloketone analogs containing a sulfide
leaving group. While these inhibitors irreversibly inactivate cysteine proteases, they have
mixed results with serine proteases (Rauber et al., 1988). Several potent sulfonium
methyl ketones have been made for clostripain (Rauber et al., 1988; Kembhavi et al.,
1991). One of the more potent ones is Z-Phe-Lys-CH₂S(C₆H₅)₂ (kₙd = 1,400,000 M⁻¹s⁻¹)
(Kembhavi et al., 1991).

Aza-peptide active esters are those in which the P1 amino acid has a nitrogen in
place of the α-carbon on the peptide backbone. The effect of this is to have no
stereospecificity at the aza-α amino acid due to the triagonal planer configuration of the
nitrogen atom. This can decrease the likelihood of an enzyme hydrolyzing the inhibitor
due to the lack of an L configuration. Aza-peptide esters were originally designed as
active site titrants and inhibitors for serine proteases (Powers et al., 1984), but were later
found to irreversibly inhibit cysteine proteases as well (Magar and Abeles, 1992). The
inhibitory potency of the aza-peptide active esters increases with more electronegative
leaving groups (Xing and Henzlik, 1998).

Michael Acceptor. Michael acceptor derivatives are another class of
irreversible inhibitors for cysteine proteases, and include vinyl sulfones and α,β-
unsaturated carbonyl derivatives. While the class of inhibitors generally irreversibly
inhibits cysteine proteases, they usually do not inhibit serine proteases (Palmer et al., 1995).

The first $\alpha,\beta$-unsaturated carbonyl inhibitor was a fumarate derivative of E-64c (an E-64 derivative) designed to inactivate cathepsin B, shown in Figure 2.3 (Barrett et al., 1982). This lead to the synthesis of a series of $\alpha,\beta$-unsaturated carbonyl derivatives

![Figure 2.3 Structure of E-64c and its Fumarate Derivative](image)

specific for papain (Hantzlick and Thompson, 1984; Thompson et al., 1986). Both the cis- and trans- derivatives were found to inhibit cathepsin B and papain (Govanjian and Abeles, 1996).

Vinyl sulfones are Michael acceptors which contain a double bond activated by an electron withdrawing sulfone, and are highly potent, specific, irreversible cysteine protease inhibitors (Palmer et al., 1995). Peptidyl vinyl sulfone inhibitors are stable, unreactive towards nucleophiles, and need the catalytic machinery of the cysteine protease for activation. In addition, they can be modified on both the $P$ and $P'$ sides of the molecule to allow selectivity and reactivity toward target enzymes. Peptidyl vinyl sulfones are more widely used than the $\alpha,\beta$-unsaturated carbonyl derivatives. However, neither type of Michael acceptor inhibits clan CD enzymes.
Currently, no specific, potent, selective inhibitors exist for either of the gingipains or clostripain. The goal of this project was to develop inhibitors based on the substrate specificity of these enzymes. Effective inhibitors for gingipain K and R are important for understanding the roles the enzymes play for Porphyromonas gingivalis, as well as their interaction with human proteins and the progression of periodontal disease. Specific inhibitors for clostripain may help expand the understanding of this unique enzyme and give insight into its proteolytic mechanism. We have developed two such inhibitors that are selective for clan CD, and specific for gingipain K and clostripain.
**RESULTS AND DISCUSSION**

**Design of Aza-peptide Michael Acceptors.** Our initial interest with aza-peptide Michael acceptors arose from our results with aza-peptide epoxides (Asgian *et al.*, 2002). The aza-peptide epoxides proved to be especially potent towards the clan CD enzymes, caspases and legumain. The caspases prefer to cleave on the carboxyl side of Asp residues, while legumain prefers Asn in P1. The aza-Asp and aza-Asn epoxide analogs were specific for their respective enzymes. We were interested in seeing how changing the reactive moiety would effect the inhibition rates. One reactive moiety we decided to try was an α,β unsaturated carbonyl. By combining the Michael acceptor inhibitor class with aza-peptide active esters, we hoped to generate a new class of inhibitors capable of being potent inactivators of clan CD enzymes. The aza-peptide Michael acceptors represent a novel class of potent, specific cysteine protease inhibitors.

The inhibitors were designed with the clan CD enzymes, clostripain, gingipain K, and gingipain R, in mind. Clostripain prefers Arg at P1 in substrates, but almost equivalently accepts Lys at P1 in inhibitors. Gingipain K cleaves only after a Lys residue in substrates. Gingipain R will cleave only after Arg at the P1 position of a substrate, but studies have shown that it can accept Lys at P1 in inhibitors (Potempa *et al.*, 1997). We designed our inhibitors as substrate analogs to take advantage of these amino acid preferences. A standard peptide substrate in the enzymatic binding site can be seen in Figure 2.4. Because of difficulties in synthesis with arginine, lysine was the obvious choice for the P1 position. In addition, ornithine, an analog of lysine containing one less methylene group in the side chain, was used to see if a shortened chain could

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Figure 2.4 Evolution of the Aza-peptide Michael Acceptors
significantly effect the reactivity or selectivity of the inhibitors towards the enzymes. For the P2 position, leucine was chosen, as small hydrophobic residues are about equivalently accept in the S2 subsites of all three enzymes.

As mentioned earlier, an aza-peptide is one in which the α-carbon of an amino acid of the peptide chain has been replaced by a nitrogen. Therefore, it is less likely to be hydrolyzed by an enzyme due to its triagonal planar orientation. With this in mind, the α-carbon of the P1 lysine and ornithine amino acid residues were replaced with a nitrogen atom, creating the aza-amine acid.

The last step of the design of our inhibitors was the replacement of the scissile peptide bond with an α,β-unsaturated moiety. The evolution of this type of inhibitor started with E-64 (Figure 2.4), a potent cysteine protease inactivator (Hanada et al., 1978). Its effectiveness towards cysteine proteases inspired the synthesis of a variety of analogs, including the epoxysuccinyl inhibitor E-64c, and its fumarate analog, illustrated in Figure 2.4 (Barrett et al., 1982). This lead to the design of peptidyl vinyl sulfones, which are activated α,β-unsaturated carboxyls, and potent, specific inhibitors of cysteine proteases (Palmer et al., 1995). In an attempt to mimic the activity seen between vinyl sulfones and cysteine proteases, we added a c,β-unsaturated double bond to our inhibitors where normally the scissile bond would be in a substrate. This gave us our aza-peptide Michael acceptors with the structure PhPr-L.a-Aaa-CH=CH-CO₂Et (Aaa = aza-Lys or aza-Orn), illustrated in Figure 2.4.

Synthesis. Two aza-peptide Michael acceptor inhibitors were synthesized in this project. The inhibitors were prepared in the following manner, and are illustrated in Scheme 2.1. The aza-peptide synthesis began by reacting Boc-NH(CH₂)₂OH (1a) or
Scheme 2.1 Synthesis of aza-Orn and aza-Lys Michael Acceptors
Boc-NH(CH₂)₄OH (1b) with iodooxo benzolic acid (IBX) in an oxidation reaction, which resulted in an aldehyde or a cyclic compound, respectively. The resulting aldehyde (2a) or dihydropyrrrole (2b) was reacted with the peptide hydrazide as described below.

The ester HC•H-Leu-OMe was coupled directly to hydrocinnamic acid using the IBCF mixed anhydride coupling method. The PhPr-Leu-OMe intermediate was combined with hydrazine in a condensation reaction to form PhPr-Leu-NHNH₂ (3). The hydrazine was combined with Boc-NH(CH₂)₄CHO (2a) or 1-(tert-butoxycarbonyl)-3,4-dihydropyrrrole (2b) to form the corresponding hydrazones through a condensation reaction. The hydrazones were reduced to give PhPr-Leu-NHNH(CH₂)₄-NH-Boc (4a) or PhPr-Leu-NHNH(CH₂)₄-NH-Boc (4b). Fumaric acid monoethyl ester was combined with PhPr-Leu-NHNH(CH₂)₄-NH-Boc (4a) or PhPr-Leu-NHNH(CH₂)₄-NH-Boc (4b) to form PhPr-Leu-A0rn(Boc)-CH=CHCO₂Et (5a) and PhPr-Leu-A1.lys(Boc)-CH=CHCO₂Et (5b), respectively. Deprotecting both compounds yielded the final azapeptide Michael acceptors, PhPr-Leu-A0rn-CH=CHCO₂Et (6a) and PhPr-Leu-A0rn-CH=CHCO₂Et (6b). The final compounds were characterized by TLC, ¹H NMR, HRMS, and elemental analysis.

**Inhibition Kinetics.** Inhibitors of enzymes fall into two general types, reversible and irreversible (Roberts, 1977). When an enzyme is combined with a reversible inhibitor, a chemical equilibrium develops between them. Reversible inactivation exhibits a certain degree of inhibition, depending on the inhibitor concentration. After the system reaches maximum inhibition, which usually happens fairly quickly, the interaction between the enzyme and inhibitor is independent of time (Dixon and Webb, 1964). Removing the inhibitor, either physically (dialysis) or by a chemical reaction, will
restore the activity of the enzyme. On the other hand, an irreversible inhibitor will completely inactivate the enzyme after a period of time, even if the inhibitor is at a low concentration, provided that it is at a higher concentration than the enzyme (Dixon and Webb, 1964). Therefore, this type of inhibition is time-dependent, meaning the degree of inhibition increases over time as the enzyme is in contact with the inhibitor. No enzymatic activity can be recovered through physical means, although chemically it may be restored. With PhPr-Leu-AOmn-CH=CHCO₂Et, only irreversible inhibition was observed. However, with PhPr-Leu-ALys-CH=CHCO₂Et, both types of inhibitory activity were observed for different enzymes.

As with other α,β unsaturated inhibitors, the aza-peptide Michael acceptors were expected to be irreversible inhibitors of the cysteine proteases (Hanzlick and Thompson, 1984). This proved to be the case. When gingipain K was incubated with either PhPr-Leu-ALys-CH=CHCO₂Et or PhPr-Leu-AOmn-CH=CHCO₂Et, and the rate of substrate hydrolysis observed for 1 hour, no recovery of any former activity was observed. The same was found to be true with clostripain inhibited by PhPr-Leu-ALys-CH=CHCO₂Et over a 2 hour period. However, clostripain, inhibited by PhPr-Leu-AOmn-CH=CHCO₂Et, began to regain activity within fifteen to twenty minutes. An explanation of this is covered in the Stability of the Inhibitors section below. The aza-peptide Michael acceptors are irreversible inhibitors with cysteine proteases.

The rates of irreversible inhibition of PhPr-Leu-ALys-CH=CHCO₂Et and PhPr-Leu-AOmn-CH=CHCO₂Et with clostripain, gingipains K and R, cathepsin B, and calpain I were measured using the incubation method. With this technique, the enzyme and the inhibitor are incubated in a mixture. In this case, the incubation mixture included the
assay buffer, the enzyme and the inhibitor. Aliquots of this mixture are taken at various time points and diluted in an assay solution containing the assay buffer and a fluorogenic or chromogenic substrate for the protease. Combining the aliquot with the assay buffer and substrate mixture essentially quenches the reaction, as the inhibitor and enzyme become so dilute that they are unlikely to interact with each other at any appreciable rate. Any enzymatic activity that remains after this quenching is measured by a change in fluorescence or absorbance due to the hydrolysis of the substrate by the enzyme.

The general reaction equation for the inactivation of an enzyme by an irreversible inhibitor is

\[ E + I \xrightleftharpoons{\kappa_1}{\kappa_2} E\cdot I \xrightarrow{k_2} EI \]

where E is the enzyme, I is the inhibitor, E·I is a reversible enzyme-inhibitor complex, which normally takes place very quickly, \( \kappa_1 \) and \( \kappa_2 \) are the rates of formation and breakdown of the enzyme-inhibitor complex, E·I is covalently bound complex, which usually happens at a slower rate, and \( k_2 \) is the inactivation rate constant of the formation of the covalently bound complex. Typically, irreversible inhibition kinetics are done under pseudo-first order conditions, in which the inhibitor concentration is at least 10-fold higher than the enzyme concentration. The total enzyme concentration in the assay is a sum of all enzyme species present, and can be represented by the equation (Kitz and Wilson, 1962)

\[ [E]_0 = [E] + [E\cdot I] + [E-I] \]

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The sum of the total active enzyme species, or those not covalently bound to the inhibitor, and thus can still hydrolyze substrate, can be represented by the equation

\[ e = [E] + [E*I] \]

if the enzyme-inhibitor solution is diluted before the assay. The rate of decrease of active enzyme left in the assay can be determined from the following equation

\[ \frac{de}{dt} = -k_2[E*I] \]

Substituting for [E*I] and rearranging the rate equation gives:

\[ \frac{de}{e} = -k_2 \left( 1 - \frac{[E]}{e} \right) \, dt \]

Integrating both sides of the equation, the left hand side from \( e_0 \) to \( e \), and the right hand side from time 0 to \( t \), the equation becomes

\[ \ln \left( \frac{e}{e_0} \right) = -k_2 t \left( 1 - \frac{[E]}{e} \right) \]

where \( e_0 \) is the active enzyme concentration at time 0, and \( e \) is the active enzyme at time \( t \). Substituting for \( e \) on the right hand side of the equation and rearranging gives

\[ \ln \left( \frac{e}{e_0} \right) = -k_2 t \left( \frac{[E*I]}{[E] + [E*I]} \right) \]

The formation and breakdown of the enzyme-inhibitor complex can be represented by the dissociation constant, \( K_i \), and are related to this term by the equation
\[ K_1 = \frac{[I][E]}{[E+I]} \]

Substituting this relationship into the above equation for [E+I], simplifying and rearranging gives

\[ \ln \left( \frac{e}{e_0} \right) = -k_2 t \left( \frac{[I]/K_1}{1 + [I]/K_1} \right) = \frac{-k_2[I]}{K_1 + [I]} \]

As stated earlier

\[ [E]_o = e + [E-I] = [E] + [E-I] + [I] \]

and, at \( t = 0 \), \([E+I] = 0 \) and \([E-I] = 0 \). So, \( e_0 = [E] = [E]_o \) which makes the above equation

\[ \ln \left( \frac{e}{[E]_o} \right) = \frac{-k_2[I]}{K_1 + [I]} \]

Over the course of the assay, the concentration of active enzyme still present (\( e \), or \([E] \) and \([E+I] \)) cannot be experimentally measured. However, the rate at which free enzyme can hydrolyze a fluorogenic or chromogenic substrate can be measured easily. This rate is directly proportional to the quantity of viable enzyme present. By replacing the enzyme concentrations above with their respective rates of substrate hydrolysis, the equation can be rewritten as

\[ \ln \left( \frac{v}{v_0} \right) = \frac{-k_2[I]}{K_1 + [I]} \]

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where $v_i$ is the rate of hydrolysis of substrate at time $t$ with inhibitor present, and $v_o$ is the rate of hydrolysis of substrate in the control assay (no inhibitor present).

In our case, $[I] \gg [I]_{th}$ and we can define an apparent rate constant as

$$k_{obs} = \frac{k_2[I]}{K_i + [I]}$$

where $k_{obs}$ is the pseudo-first order rate constant. Substituting this into the equation gives

$$\ln \left( \frac{v}{v_o} \right) = -k_{obs}t$$

The $k_{obs}$ is unaffected by the type of substrate. The equation uses a ratio of the rates of hydrolysis of the substrate by the enzyme, with and without inhibitor (the control). This ratio should always be the same for any type of substrate, and therefore $k_{obs}$ is constant with respect to substrate type used. However, inhibitor concentration can affect the $k_{obs}$.

The second order rate constant is given by the term $k_{obs}[I]$, which is a measure of the potency of an inhibitor. These values can be used to compare inhibitor potency towards enzymes.

A plot of the natural log of the residual enzyme activity, $\ln (v/v_o)$, versus time gives the observed rate of inactivation ($k_{obs}$) as the slope. An example of this type of plot can be seen in Figure 2.5. Taking the pseudo-first order rate inhibition constant and dividing it by the inhibitor concentration will give $k_{obs}/[I]$. The $k_{obs}/[I]$ values are then used to compare the potency of different inhibitors with the same enzyme.

As can be seen from Table 2.1, the inhibitor PhPr-Leu-OMe-CH=CHCO$_2$Et had lower inhibitory activity toward clostripain (rate of 788 ± 169 M$^{-1}$s$^{-1}$) than other types of
inhibitors, like peptidyl chloromethyl ketones (Wikstrom et al., 1989). However, unlike the chloromethyl ketone inhibitors, PhPr-Leu-AOrn-CH=CHCO₂Et is selective for the clan CD enzymes, clostripain and gingipain K. With gingipain K, PhPr-Leu-ALys-CH=CHCO₂Et and PhPr-Leu-AOrn-CH=CHCO₂Et were potent inhibitors with rates of 3,280,000 ± 652,000 M⁻¹s⁻¹ and 927,000 ± 59,000 M⁻¹s⁻¹, respectively. These numbers are comparable to the values of other inhibitors tested with gingipain K. In addition, the inhibitors are selective for gingipain K and clostripain. Clan CA cysteine proteases papain and calpain I were not inhibited at all by PhPr-Leu-ALys-CH=CHCO₂Et or PhPr-Leu-AOrn-CH=CHCO₂Et. Cathepsin B showed only weak inhibition with PhPr-Leu-ALys-CH=CHCO₂Et (kₐ₀/[I] = 9.09 M⁻¹s⁻¹) and PhPr-Leu-AOrn-CH=CHCO₂Et (kₐ₀/[I])
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>PhPr-Leu-ALys-CH=CHCO₂Et</th>
<th>PhPr-Leu-AMor-CH=CHCO₂Et</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clostripain</td>
<td>40,700 ± 7,580°</td>
<td>788 ± 169°</td>
</tr>
<tr>
<td>Gingipain K</td>
<td>3,280,000 ± 652,000°</td>
<td>927,000 ± 59,000°</td>
</tr>
<tr>
<td>Gingipain R</td>
<td>289 ± 84</td>
<td>32.0 ± 0.5</td>
</tr>
<tr>
<td>Papain</td>
<td>NI°</td>
<td>NI</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>Caspase-6</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>Caspase-8</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>Cathepsin B</td>
<td>9.09</td>
<td>2.97</td>
</tr>
<tr>
<td>Calpain</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>Trypsin</td>
<td>17.0 ± 4.7°</td>
<td>NI</td>
</tr>
</tbody>
</table>

°Inhibition kinetics were carried out in a 20 mM Tris/HCl, 10 mM CaCl₂, 0.005% Brij 35, 2 mM DTT, 13% DMSO, pH 7.6 buffer. ‡Second order rate constant (k_{ord}), enzyme to inhibitor ratio was 1:1. †Kinetic rate constant for inhibition (k) taking into account the rate of reaction of DTT with the inhibitor. †Inhibition kinetics were carried out in a 0.2 M Tris/HCl, 0.1 M NaCl, 5 mM CaCl₂, 2 mM DTT, 13% DMSO, pH 8.0 buffer. †Inhibition kinetics were carried out in a 0.2 M Tris/HCl, 0.1 M NaCl, 5 mM CaCl₂, 2 mM DTT, 9% DMSO, pH 7.5 buffer. †Inhibition kinetics were carried out in a 50 mM Hepes, 3 mM DTT, 2.5 mM EDTA, 9% DMSO, pH 7.5 buffer. NI = no
Table 2.1 (cont’d)

inhibition. aInhibition kinetics for all three caspases were carried out in a 20 mM Pipes, 0.1% Chaps, 10% sucrose, 100 mM NaCl, 1 mM EDTA, 10 mM DTT, 10% DMSO, pH 7.2 buffer. bInhibition kinetics were carried out in a 0.1 M potassium phosphate, 1.25 mM EDTA, 0.01% Brij 35, 9% DMSO, pH 6.0 buffer. cInhibition kinetics were carried out in a 50 mM Hepes, 0.5 M CaCl₂, 0.5 M cysteine, 4% DMSO, pH 7.5 buffer. dInhibition kinetics were carried out in a 0.1 M Hepes, 0.01 M CaCl₂, 5% DMSO, pH 7.5 buffer. eThis is a reversible inhibition rate constant (K_i) given in units of μM.
= 2.97 M⁻¹·s⁻¹). Even within clan CD itself, the inhibitors were selective. Gingipain R was weakly inhibited by PhPr-Leu-ALys-CH=CHCO₂Et and PhPr-Leu-AOrn-CH=CHCO₂Et with $k_{inh}$/[I] values of $289 \pm 84$ M⁻¹·s⁻¹ and $32.0 \pm 0.5$ M⁻¹·s⁻¹, respectively. Neither PhPr-Leu-ALys-CH=CHCO₂Et nor PhPr-Leu-AOrn-CH=CHCO₂Et showed any inhibitor activity towards the caspases-3, -6, and -8.

We found that the inhibitor PhPr-Leu-ALys-CH=CHCO₂Et, when combined with clostripain, inactivated the enzyme too quickly for pseudo-first order kinetics. In this case, we needed to drop the inhibitor concentration until it was the same as that of the enzyme. When the inhibitor to enzyme ratio is less than 10 to 1, second order kinetics must be used to solve for the inhibition rate constant ($k_{2nd}$). The $k_{2nd}$ can be determined from the equation

$$\ln \left( \frac{[E]/[E]_0}{[I]/[I]_0} \right) = ([E]_0 - [I]_0)k_{2nd} \cdot t$$

where $[E]$ is the concentration of the enzyme as it changes over the course of the assay, $[E]_0$ is the initial concentration of the enzyme, $[I]$ is the concentration of the inhibitor as it changes over the course of the assay, and $[I]_0$ is the initial concentration of the inhibitor. The values obtained for $[E]/[E]_0$ and $[I]/[I]_0$ represent the residual amount of enzyme and inhibitor, respectively, still in the incubation mixture. The $k_{2nd}$ was obtained from the slope of a plot of $\ln((([E]/[E]_0)/([I]/[I]_0)))$ versus time. The inhibition plot of PhPr-Leu-ALys-CH=CHCO₂Et inactivating clostripain can be seen in Figure 2.6.

As shown in Table 2.1, PhPr-Leu-ALys-CH=CHCO₂Et has moderate inhibitory activity toward clostripain ($k_{2nd} = 40,700 \pm 7,580$ M⁻¹·s⁻¹) compared to other inhibitors
Second Order Kinetic Inhibition of Clostripain by PhPr-Leu-ALys-CH=CHCO₂Et

\[ y = -2.1845 \times 10^{-5}x - 3.0618 \times 10^{-6} \]
\[ R^2 = 0.984725 \]

![Graph showing second order kinetic inhibition](image)

Figure 2.6 Second Order Kinetic Inhibition of Clostripain by PhPr-Leu-ALys-CH=CHCO₂Et

tested with it, like peptidyl sulfonium methyl ketones Z-Phe-Lys-CH₂S(CH₃)₂ (k_{sod} = 1,400,000 M⁻¹s⁻¹) or chloromethyl ketones (Phe-Ala-Lys-Arg-CEK, k_{sod} = 10,100,000 M⁻¹s⁻¹) (Wikstrom et al., 1989; Kembhavi et al., 1991). However, unlike either the sulfonium methyl ketones or chloromethyl ketones, PhPr-Leu-ALys-CH=CHCO₂Et is selective for the clan CD enzymes, clostripain and gingipain K.

The inhibition of trypsin by PhPr-Leu-ALys-CH=CHCO₂Et was observed to be reversible. Reversible inhibition can be further divided into more categories, which include competitive, non-competitive, uncompetitive inhibition, and mixed inhibition. Competitive inhibition is a reversible inhibition in which the inhibitor and substrate both compete for the active site of the enzyme (Roberts, 1977). Non-competitive inhibition is
characterized by the inhibitor binding to the enzyme at some site other than the active site, and does not interfere with the enzyme binding with the substrate (Roberts, 1977). In this type of inhibition, the inhibitor makes a 'deac-endo' enzyme-inhibitor complex in which either the enzyme will not hydrolyze the substrate, even though the substrate can still bind in the proteolytic site, or the hydrolysis rate of the substrate will slow compared to the enzyme and substrate complexed alone. Uncompetitive inhibition is characterized by the inhibitor only binding with the enzyme-substrate complex (Roberts, 1977). This means that there is no binding site on the enzyme for the inhibitor to interact with until the substrate is bound into the active site of the enzyme. Mixed inhibition is a combination of both competitive and non-competitive effects.

These four types of reversible inhibition can be distinguished by the visual outcome of plotting the inhibition rates on a Dixon plot. A competitive inhibitor will give lines, due to rates of substrate hydrolysis at different substrate concentrations, which cross in the fourth quadrant (i.e. $-x, +y$) of a graph. A non-competitive inhibitor will give a graph in which the rates of hydrolysis at each substrate concentration will intersect at the x-axis in between the third and fourth quadrant (i.e. $-x, y = 0$). Uncompetitive inhibition will give a Dixon plot in which the rates of hydrolysis at each substrate concentration will be parallel to each other, and never intersect. Mixed inhibition will give a graph in which the rates of hydrolysis at each substrate concentration will intersect in the fourth quadrant (i.e. $-x, +y$) of a graph. Therefore, competitive and mixed inhibitions can be distinguished from non-competitive and uncompetitive, but not from each other. These can be resolved visually through a Lineweaver-Burk plot of the data (Dixon and Webb, 1964; Roberts, 1977). A competitive inhibition plot will have lines

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that intersect at the y-axis, while a mixed inhibition plot will have lines that cross in the fourth quadrant (i.e. -x, +y). The inhibitor PhPr-Leu-ALys-CH=CHCO2Et was observed to be a competitive reversible inhibitor of trypsin. The mathematics of this type of inhibition is described below.

The model for competitive inhibition is given by the reaction equations

\[
\begin{align*}
E + S & \xrightarrow{k_1} ES \xrightarrow{k_2} E + P \\
+ & \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad
\end{align*}
\]

where E is the enzyme, S is the substrate, ES is the enzyme-substrate complex, P is the products formed, \(k_1\) is the velocity constant for the formation of the enzyme-substrate complex, \(k_1\) is velocity constant for the breakdown of the complex into free enzyme and substrate, \(k_2\) is the rate constant of product formation, I is the inhibitor, \(K_i\) is the inhibition constant, and EI is the enzyme-inhibitor complex. The inhibition constant, \(K_i\), is an equilibrium constant between the enzyme-inhibitor complex, and free enzyme and inhibitor or

\[
K_i = [E][I]/[EI]
\]

where \([E]\) is the free enzyme concentration in the solution, \([I]\) is the free inhibitor concentration in the solution, and \([EI]\) is the concentration of the enzyme-inhibitor complex in the solution. The \(K_i\) can be found from the Michaelis-Menten equation for a competitive system

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\[ v = \frac{V_s}{K_m(1 + [I]/K_i) + [S]} \]

where \( v \) is the reaction velocity, \( K_m \) is the Michaelis constant, \([S]\) is the substrate concentration in solution, and \( V_s \) is the maximum velocity obtained at high substrate concentrations. However, Dixon simplified the work involved to solve for \( K_i \), which previously involved calculating the effect on the velocity of the reaction by varying both the \([S]\) and \([I]\) independently (Dixon, 1953). By plotting \(1/v\) against \([I]\) while keeping \([S]\) constant, a straight line will be obtained. By doing this at two or more different substrate concentrations, the value of \( K_i \) can be approximated from the equation

\[ \frac{1}{v} = \frac{1}{V_s} + \frac{K_m}{V_s} \left( 1 + \frac{[I]}{K_i} \right) \frac{1}{[S]} \]

which represents the reciprocal of the Michaelis equation for a competitive system. A plot of \(1/v\) versus \([I]\) at several different substrate concentrations gives straight lines that cross in the fourth quadrant (i.e. \(-x, +y\)) where \([I] = -K_i \). The intersection can be shown to be \(-K_i \) by setting the equations of the lines for two different substrate concentrations equal to each other, or

\[ \left( 1 + \frac{[I]}{K_i} \right) \frac{1}{[S]_1} = \left( 1 + \frac{[I]}{K_i} \right) \frac{1}{[S]_2} \]

where \([S]_1\) and \([S]_2\) are the two different substrate concentrations for the same enzymatic assay. The only way this equation can be true is if \([S]_1 = [S]_2\), which obviously is not the case, or if \([I] = -K_i \). The \( K_i \) for PHPr-Leu-ALys-CH-CHCO_2Et with trypsin was determined from three lines at three different substrate concentrations, each containing

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five inhibitor concentrations. To find the value of $K_i$ from the x-axis, the equation of each line was set equal to each of the other two lines and solved for $x$. This gave three values, which were averaged to give the $K_i$ listed in Table 2.1 for trypsin and PhPr-Leu-ALys-CH=CHCO$_2$Et. Figure 2.7 shows the Dixon plot of trypsin being competitively

![Dixon Plot](image)

Figure 2.7 Dixon Plot of the Data with Trypsin Inhibited by PhPr-Leu-ALys-CH=CHCO$_2$Et

and reversibly inhibited by PhPr-Leu-ALys-CH=CHCO$_2$Et. The Lineweaver-Burk plot, showing that PhPr-Leu-ALys-CH=CHCO$_2$Et is indeed inactivating trypsin as a competitive reversible inhibitor, and not as a mixed inhibitor, is shown in Figure 2.8. It should be noted that, although the lines at varying concentrations do not intersect directly on the y-axis in the Lineweaver-Burk plot, as is predicted for a competitive inhibitor, the
Figure 2.8 Lineweaver-Burk Plot with Trypsin Inhibited by PhPr-Leu-ALys-CH=CHCO₂Et

points do line up very near x = 0. The reason for the slight discrepancy may be the lack of enough data points in each line to get a truly accurate plot. However, it is clear that the lines do not cross in the fourth quadrant (i.e. -x, +y) as would happen if PhPr-Leu-ALys-CH=CHCO₂Et was inactivating trypsin as a mixed inhibitor. PhPr-Leu-ALys-CH=CHCO₂Et inhibits trypsin with a $K_i = 17.0 \pm 4.7 \mu M$.

Specificity. The aza-peptide Michael acceptors are selective irreversible inhibitors for the enzymes gingipain K and clostripain. As can be seen in the Table 2.1, PhPr-Leu-ALys-CH=CHCO₂Et and PhPr-Leu-AOmn-CH=CHCO₂Et are completely ineffective with the cysteine proteases, papain and calpain I. Cathepsin B, a clan CA cysteine protease, was weakly inhibited by both compounds. Within clan CD, the inhibitors were unable to inactivate any of the caspases (-3, -6, and -8), and only weakly inhibited gingipain R. Trypsin, a serine protease, was reversibly inhibited by only PhPr-
Leu-ALys-CH=CHCO₂Et. The PhPr-Leu-AOrn-CH=CHCO₂Et had no inhibitory activity at all with trypsin. This suggests that the aza-peptide Michael acceptors can not only distinguish between cysteine and serine proteases, but also between the cysteine protease clans. Within clan CD, the inhibitors are able to differentiate between the members. Table 2.2 lists a variety of inhibitors with the enzymes papain, cathepsin B, calpain I, caspases-3, -6, -8, and gingipain K. Many of the inhibitors in the list are very potent for the enzyme listed, but may react with other enzymes as well. However, as shown in the table, PhPr-Leu-ALys-CH=CHCO₂Et and PhPr-Leu-AOrn-CH=CHCO₂Et show little activity towards any of the enzymes in the list.

Papain is a plant cysteine protease from the latex of the Carica papaya tree. It is likely the most extensively studied cysteine protease. The most important binding subsite of papain, as in most clan CA enzymes, is the S2 position. Papain prefers hydrophobic residues, such as Phe, at the P2 position of substrates (Otto and Schirmeister, 1997). Although the S1 pocket is less defined, it will accept Arg, Lys, and a variety of other amino acids. The peptidyl epoxysuccinyl, HO-(2S,3S)-Eps-Leu-NH-(CH₂)₇-NH₂, the peptidyl diazomethyl ketone, Z-Leu-Val-Gly-CHN₂, and the peptidyl vinyl sulfone, Z-Phe-HpH-CH=CH-SO₂-OPh are all potent, irreversible inhibitors of papain, while the aza-peptide Michael acceptors do not inhibit it at all (Table 2.2). It is peculiar that neither PhPr-Leu-ALys-CH=CHCO₂Et nor PhPr-Leu-AOrn-CH=CHCO₂Et inhibited papain, as the enzyme will accept Lys at P1. This suggests the active site of papain may have a catalytic architecture different from that of the clan CD enzymes. The peptide folding structure and active site design of the clan CA and clan CD enzymes are very different.
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Inhibitor</th>
<th>$k_{obs}/[I]$</th>
<th>$k_2/K_i$</th>
<th>$k_{2nd}$</th>
<th>$K_i$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(M⁻¹s⁻¹)</td>
<td>(M⁻¹s⁻¹)</td>
<td>(M⁻¹s⁻¹)</td>
<td>(μM)</td>
<td></td>
</tr>
<tr>
<td>Papain</td>
<td>HO-(2S,3S)-Eps-Leu-NH-(CH₂)₃-NH₂⁻</td>
<td>874,000</td>
<td></td>
<td></td>
<td></td>
<td>(Barrett <em>et al.</em>, 1982)</td>
</tr>
<tr>
<td></td>
<td>Z-Leu-Val-Gly-CHN₂</td>
<td>600,000</td>
<td></td>
<td></td>
<td></td>
<td>(Abrahamson <em>et al.</em>, 1991)</td>
</tr>
<tr>
<td></td>
<td>Z-Phe-Hph-CH=CH-SO₂-OPh</td>
<td>325,000</td>
<td></td>
<td></td>
<td></td>
<td>(Roush <em>et al.</em>, 2001)</td>
</tr>
<tr>
<td></td>
<td>PhPr-Leu-ALys-CH=CHCO₂Et</td>
<td>Niᵇ</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PhPr-Leu-AOrn-CH=CHCO₂Et</td>
<td>Ni</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cat B</td>
<td>Pro-Phe-Arg-CH₂Cl</td>
<td>5,100,000</td>
<td></td>
<td></td>
<td></td>
<td>(Barrett <em>et al.</em>, 1982)</td>
</tr>
<tr>
<td></td>
<td>Z-Val-Lys-CH₂-OCOPh(2,6-(CF₃)₃)</td>
<td>2,000,000</td>
<td></td>
<td></td>
<td></td>
<td>(Pliura <em>et al.</em>, 1992)</td>
</tr>
<tr>
<td></td>
<td>Mu-Phe-Lys-CH=CHSO₂Ph·HBr⁺</td>
<td>11,300</td>
<td></td>
<td></td>
<td></td>
<td>(Palmer <em>et al.</em>, 1995)</td>
</tr>
<tr>
<td></td>
<td>PhPr-Leu-ALys-CH=CHCO₂Et</td>
<td>9.09</td>
<td></td>
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<tr>
<td></td>
<td>PhPr-Leu-AOrn-CH=CHCO₂Et</td>
<td>2.97</td>
<td></td>
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<tr>
<td>Cat I</td>
<td>Z-Leu-Phe-CH₂F</td>
<td>136,000</td>
<td></td>
<td></td>
<td></td>
<td>(Chatterjee <em>et al.</em>, 1996)</td>
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<tr>
<td></td>
<td>Z-Leu-Leu-Tyr-CHN₂</td>
<td>230,000</td>
<td></td>
<td></td>
<td></td>
<td>(Crawford <em>et al.</em>, 1988)</td>
</tr>
<tr>
<td>Enzyme</td>
<td>Peptide</td>
<td>SI</td>
<td>References</td>
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<tr>
<td>Trypsin</td>
<td>D-Phe-Pro-Arg-CH$_2$Cl</td>
<td>3,500,000</td>
<td>(Lijnen et al., 1984)</td>
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<td></td>
<td>D-Phe($\alpha$-Me)-Pro-Arg-NH$_2$</td>
<td>2.3</td>
<td>(Brady et al., 1995)</td>
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<tr>
<td></td>
<td>1-Naphthyl-Arg-CF$_3$</td>
<td>0.2</td>
<td>(Kam et al., 1995)</td>
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<tr>
<td></td>
<td>PhPr-Leu-ALys-CH=CHCO$_2$Et</td>
<td>17.0 ± 4.7</td>
<td></td>
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<td></td>
<td>PhPr-Leu-ALOm-CH=CHCO$_2$Et</td>
<td>NI</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Caspase 3</td>
<td>Z-Val-Ala-Asp-CH$_3$F</td>
<td>16,000</td>
<td>(Garcia-Calvo et al., 1998)</td>
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<td></td>
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<tr>
<td></td>
<td>Z-Asp-Glu-Val-AAasp-Eps(2S,3S)-OEt</td>
<td>205,300</td>
<td>(Asgian et al., 2002)</td>
<td></td>
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<tr>
<td></td>
<td>Ac-Asp-Glu-Val-Asp-H</td>
<td>2.3 x 10$^4$</td>
<td>(Garcia-Calvo et al., 1998)</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>PhPr-Leu-ALys-CH=CHCO$_2$Et</td>
<td>NI</td>
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<tr>
<td></td>
<td>PhPr-Leu-ALOm-CH=CHCO$_2$Et</td>
<td>NI</td>
<td></td>
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<tr>
<td>Caspase 6</td>
<td>Z-Val-Ala-Asp-CH$_3$F</td>
<td>7,100</td>
<td>(Garcia-Calvo et al., 1998)</td>
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<tr>
<td>Compound</td>
<td>Value</td>
<td>Source</td>
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<tr>
<td>Z-Asp-Glu-Val-AAsp-Eps(2S,3S)-OEt</td>
<td>7,100</td>
<td>(Asgian et al., 2002)</td>
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<tr>
<td>Boc-Ile-Glu-Thr-Asp-H</td>
<td>5.6 x 10^3</td>
<td>(Garcia-Calvo et al., 1998)</td>
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<tr>
<td>PhPr-Leu-Lys-CH=CHCO_2Et</td>
<td>NI</td>
<td></td>
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<tr>
<td>PhPr-Leu-ACOM-CH=CHCO_2Et</td>
<td>NI</td>
<td></td>
<td></td>
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<tr>
<td>Casp-8</td>
<td>280,000</td>
<td>(Garcia-Calvo et al., 1998)</td>
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<tr>
<td>Z-Asp-Glu-Val-AAsp-Eps(2S,3S)-OEt</td>
<td>6,600</td>
<td>(Asgian et al., 2002)</td>
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<tr>
<td>Ac-Asp-Glu-Val-Asp-H</td>
<td>9.2 x 10^4</td>
<td>(Garcia-Calvo et al., 1998)</td>
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<tr>
<td>PhPr-Leu-Lys-CH=CHCO_2Et</td>
<td>NI</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>PhPr-Leu-ACOM-CH=CHCO_2Et</td>
<td>NI</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Ging R</td>
<td>22,220,080</td>
<td>(Pike et al., 1994)</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>D-Phe-Pro-Arg-CH_2Cl</td>
<td>47,650,000</td>
<td>(Pike et al., 1994)</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Z-Phe-Lys-CH_2OCOPh(2,4,6-(CH_3)_3)</td>
<td>1,920</td>
<td>(Potempa et al., 1997)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PhPr-Leu-Lys-CH=CHCO_2Et</td>
<td>289 ± 84</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>PhPr-Leu-ACOM-CH=CHCO_2Et</td>
<td>32.0 ± 0.5</td>
<td></td>
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</tr>
</tbody>
</table>

*Eps = epoxysucinyl. *NI = no inhibition. *Mu = morpholine-CO-.
(Schröder et al., 1993; Wilson et al., 1994; Rotonda et al., 1996; Eichinger et al., 1999; Watt et al., 1999). The clan CD enzymes have a more open, shallow proteolytic binding site than that of papain, which is crowded and deep. As proposed for the aza-peptide epoxides by Powers et al., the aza-peptide Michael acceptors may be too rigid to properly align with the catalytic residues of papain (Asgian et al., 2002). Or perhaps the presence of the aza-amino acid, with its triagonal geometry, is prohibiting the proper alignment of the inhibitors, preventing the catalytic cysteine residue from attacking the reactive moiety of the aza-peptide Michael acceptors. As was seen with the aza-peptide epoxides, the aza-peptide Michael acceptors do not work well with papain (Asgian et al., 2002).

Cathepsin B is a lysosomal cysteine protease which also belongs to the papain superfamily. As papain, the primary specificity for cathepsin B is Phe at P2 (Green and Shaw, 1981a). At the P1 position, cathepsin B normally prefers small amino acids. However, the enzyme will also accept Arg at this position, as is demonstrated by the potent, irreversible chloromethyl ketone inhibitor Pro-Phe-Arg-CH₂Cl (k_{obs}/[I] = 5,100,000 M⁻¹s⁻¹) in Table 2.2 (Barrett et al., 1982; Nägler et al., 1999). Potent peptidyl acyloxymethyl ketones, including Z-Val-Lys-CH₂OCOPh(2,6-(CF₃)₂) (k₅/Kᵢ = 2,000,000 M⁻¹s⁻¹), have been designed for cathepsin B. This irreversible inhibitor indicates that the enzyme does not mind having Lys at P1, either. Although only a moderate inhibitor for cathepsin B, the vinyl sulfone, Mu-Phe-Lys-CH=CHSO₂Ph HB₃, demonstrates the same acceptance of Lys at P1. Again, as with papain, it is interesting that neither PhPr-Leu-ALys-CH=CHCO₂Et (k_{obs}/[I] = 9.09 M⁻¹s⁻¹) nor PhPr-Leu-Aom-CH=CHCO₂Et (k_{obs}/[I] = 2.97 M⁻¹s⁻¹) inhibited cathepsin B well. However, due to the similarities between cathepsin B and papain, the catalytic architecture may also be responsible in this case for
the lack of reactivity between the enzyme and the inhibitors. These results are similar that that observed with aza-peptide epoxides, in which the inhibitors showed little or no activity, suggesting a similarity in the binding mode of the inhibitors (Asgian et al., 2002). I would like to graciously thank Marion Götz for doing the kinetic work with the aza-peptide Michael acceptors and cathepsin B.

Calpain I is a cysteine protease closely related to papain. It plays a variety of biological roles throughout mammalian cells. The enzyme contains a calcium binding domain, and requires calcium ions for activity (Berti and Storer, 1995). The amino acid preference of calpain I at the P1 position is for Tyr, Met, Lys, or Arg (Mattis et al., 1977). At the P2 position, the enzyme prefers hydrophobic residues such as Leu and Val (Mattis et al., 1977). The irreversible inhibitors Z-Leu-Phe-CH2F, Z-Leu-Leu-Tyr-CHN2, and Z-Leu-Leu-Tyr-CH=CH-SO2-Ph are all potent inhibitors of calpain I (Table 2.2). However, even though PhPr-Leu-ALys-CH=CHCO2Et and PhPr-Leu-AOrrn-CH=CHCO2Et contain the amino acids preferred by calpain I, neither showed any inhibitory activity toward it. As suggested above for cathepsin B and papain, the inhibitors may not properly align in the proteolytic pocket to allow the active site cysteine of calpain I to attack the reactive moiety. I would like to graciously thank Marion Götz for doing the kinetic work with the aza-peptide Michael acceptors and calpain I.

Trypsin is a digestive serine protease found in the small intestines. It is the only serine protease we tried with these inhibitors. We chose this enzyme due to its amino acid specificity. The enzyme has a large S1 subsite containing an aspartic acid residue deep within the pocket. This gives trypsin a specificity for Lys or Arg at the P1 position of substrates (Babine and Bender, 1997). The S2 subsite of trypsin is not well defined,
and so will accept a variety of amino acids at this position without much specificity (Bode et al., 1990). As shown in Table 2.2, D-Phe(α-Me)-Pro-Arg-NH₂ is a potent reversible inhibitor of trypsin ($K_i = 2.3 \text{ } \mu M$). The peptidyl trifluoroketone, 1-naphthoyl-Arg-CF₃ is an even more potent inhibitor of trypsin ($K_i = 0.2 \text{ } \mu M$). While PhPr-Leu-ΑOrn-CH=CHCO₂Et did not inhibit trypsin at all, PhPr-Leu-ΑLys-CH=CHCO₂Et proved to be a reversible inhibitor of the enzyme with a $K_i = 17.0 \pm 4.7 \text{ } \mu M$. This is nearly 5-fold less potent than D-Phe(α-Me)-Pro-Arg-NH₂ and nearly 100-fold less potent than 1-naphthoyl-Arg-CF₃. The lack of inhibition of trypsin by PhPr-Leu-ΑOrn-CH=CHCO₂Et can be explained by the short aza-Orn side chain, which may not have been able to reach far enough into the P1 pocket to form favorable electrostatic and H-bonding interactions with the enzyme. While PhPr-Leu-ΑLys-CH=CHCO₂Et does reversibly inhibit trypsin, it does so only at the micromolar range, where better inhibitors reach into the sub-micromolar range.

Caspases-3, -6, and -8 are cysteine proteases of the clan CD involved in apoptosis, or cell death. The enzymes will cleave only after Asp at P1. At the P2 position, all three caspases will accept a variety of hydrophobic residues, but do have their favorites (Thornberry et al., 1997). Caspase-3 prefers Val at P2, while caspase-6 prefers His, and caspase-8 prefers Thr in this position. All three enzymes are inhibited irreversibly by the fluoromethyl ketone, Z-Val-Ala-Asp-CH₂F ($k_{obs}/[I] = 16,000 \text{ } M^{-1} s^{-1}$, 7,100 M⁻¹ s⁻¹, and 280,000 M⁻¹ s⁻¹, for caspase-3, -6, and -8, respectively). The three caspases are also irreversibly inhibited by the aza-peptide epoxide Z-Asp-Glu-Val-AAsp-Eps(2S,3S)-OEt ($k_{obs}/[I] = 205,300 \text{ } M^{-1} s^{-1}$, 7,100 M⁻¹ s⁻¹, and 6,600 M⁻¹ s⁻¹, for caspase-3, -6, and -8, respectively). In addition, caspases-3 and -8 were reversibly inhibited by the potent
peptide aldehyde Ac-Asp-Glu-Val-Asp-H ($K_i = 2.3 \times 10^{-4} \text{ M}$ and $9.2 \times 10^{-4} \text{ M}$, respectively), while caspase-6 was inhibited by Boc-Ile-Glu-Thr-Asp-H with a $K_i = 5.6 \times 10^{-3} \text{ M}$. Neither PhPr-Leu-ALys-CH=CHCO$_2$Et nor PhPr-Leu-AOrn-CH=CHCO$_2$Et showed any inhibitory activity toward any of the three caspases tested. The most obvious reason why the aza-peptide Michael acceptors did not inhibit the caspases is their strict specificity for Asp at P1.

Gingipain R, as stated earlier, is a cysteine protease of the clan CD synthesized by the bacterium Porphyromonas gingivalis. The enzyme has a strict specificity for Arg in the P1 position of substrates and will not cleave after any other natural amino acids (Chen et al., 1992). Gingipain R prefers hydrophobic residues at P2 and P3 (Kadowaki et al., 1994). As previously mentioned, we designed PhPr-Leu-ALys-CH=CHCO$_2$Et and PhPr-Leu-AOrn-CH=CHCO$_2$Et with the idea that they might also inhibit gingipain R. This turned out to be the case, with $k_{	ext{cat}}/K_{i}$ values of $289 \pm 84 \text{ M}^{-1}\text{s}^{-1}$ and $32.0 \pm 0.5 \text{ M}^{-1}\text{s}^{-1}$ for the aza-Lys and aza-Orn derivatives, respectively. However, the inhibition of gingipain R by the aza-peptide Michael acceptors was very poor compared to other inhibitors. For example, the chloromethyl ketones, D-Phe-Pro-Arg-CH$_2$Cl and D-Phe-Phe-Arg-CH$_2$Cl, inhibited gingipain R at $k_{	ext{cat}}/K_{i}$ values $22,220,000 \text{ M}^{-1}\text{s}^{-1}$ and $47,650,000 \text{ M}^{-1}\text{s}^{-1}$, respectively. These inhibitors have some of the fastest inactivation rates known to exist between inhibitor and enzyme (Pike et al., 1994). Clearly, the preference for Arg at P1 plays a large role in whether inhibitors will be potent inactivators or not for gingipain R.

The aza-peptide Michael acceptors inactivity toward such a wide variety of other enzymes, including trypsin, papain, and cathepsin B, which are usually much more generous with which amino acids they will accept in their subsites, is a novel feature of
these inhibitors. The ability to distinguish between the clan CD members is an interesting aspect as well, not found in many other classes of inhibitors. PhPr-Leu-ALys-CH=CHCO₂Et and PhPr-Leu-AOm-CH=CHCO₂Et are selective, irreversible inhibitors for gingipain K and clostripain, and represent a new class of inhibitor. By altering the P1 aza-amino acid, the aza-peptide Michael acceptors have the potential to generate effective and selective inactivators for all the clan CD enzymes.

Stability of the Inhibitors. PhPr-Leu-ALys-CH=CHCO₂Et and PhPr-Leu-AOm-CH=CHCO₂Et were effective inhibitors of gingipain K and clostripain. However, during the course of the assay, at the concentrations needed to get interpretable results, neither inhibitor was able to bring the rate of substrate hydrolysis to zero. This suggested that the inhibitors might be reacting with the enzyme but were forming only weak covalent bonds and were undergoing a reverse Michael reaction. Other explanations include the inhibitors being hydrolyzed by the enzyme, that they might be reacting with something in the buffer solution, or that they might be reversible inhibitors.

In a Michael reaction, a nucleophile attacks one of the carbons of an α,β-
unsaturated carbonyl compound, such as PhPr-Leu-ALys-CH=CHCO₂Et and PhPr-Leu-
AOm-CH=CHCO₂Et, to form a carbanion. The carbanion picks up a proton from a nearby protonated base or the buffer solution. However, the Michael reaction is reversible under conditions in which the proton can be abstracted from the newly formed methylene group to reform the carbanion. In our case, the environment is slightly basic with the buffers at pH 7.5 and 8.0, respectively, for clostripain and gingipain K. To study the possibility of the inhibitor undergoing a reverse Michael reaction, long-term enzymatic assays between clostripain and PhPr-Leu-ALys-CH=CHCO₂Et or PhPr-Leu-
AOm-CH=CHCO₂Et were monitored. The clostripain began to regain activity after about 15 to 20 min with the PhPr-Leu-AOm-CH=CHCO₂Et. However, even after 2 hr, clostripain incubated with PhPr-Leu-ALys-CH=CHCO₂Et had regained none of its former activity. A similar assay was run with both inhibitors and gingipain K. After 1 hr, gingipain K had regained none of its former activity with either inhibitor. A control for both enzymes was also run, replacing the inhibitor with DMSO, which continued hydrolyzing substrate at a consistent rate throughout the entire assay period. These results suggest that, at least with clostripain, the aza-Orn derivative did not form a strong covalent bond with the enzyme, and likely underwent a reverse Michael reaction. However, the aza-Lys derivative appears to have bound tightly with both enzymes, and was not sensitive toward a reverse Michael reaction. In addition, gingipain K seems to form a strong covalent bond with PhPr-Leu-AOm-CH=CHCO₂Et, as the inhibitor was insensitive toward a reverse Michael reaction with this enzyme.

The conditions are present for both inhibitors to undergo reverse Michael reactions with each enzyme. However, only the PhPr-Leu-AOm-CH=CHCO₂Et with clostripain is susceptible to the reverse reaction. A possible reason for this may be due to the short aza-Orn side chain. The side chain of aza-Orn does not reach as far into the S1 subsite of clostripain as that of aza-Lys. This may result in only weak electrostatic interactions between the inhibitor and the residues lining the S1 subsite of clostripain. The bonding between aza-Orn and the S1 subsite may not be strong enough to overcome the reverse Michael reaction. In contrast, gingipain K does seem able to form strong electrostatic interactions between PhPr-Leu-AOm-CH=CHCO₂Et and S1 subsite. The
covalent bond between the enzyme and PhPr-Leu-AOmr-CH=CHCO₂Et is able to maintain a more stable enzyme-inhibitor complex than in clostripain.

The inhibitors did not act as substrates for gingipain K and clostripain. If they had been, the enzymes would have regained activity over the 1-2 hour assays as the inhibitors were hydrolyzed, or may not have lost any hydrolytic activity in the first place. However, neither of these results was not observed.

Another possibility was that the inhibitors could potentially be reversible for gingipain K and clostripain. This seemed unlikely, as neither aza-peptide inhibitors, nor α,β-unsaturated carbonyl inhibitors are reversible with cysteine proteases. A reversible inhibitor is differentiated from irreversible inhibitors by its time-independent inactivation of the enzyme (Dixon and Webb, 1964). This means that, over time, and usually quite quickly, the inhibitor and enzyme will reach an equilibrium. A definite inactivation will be seen, depending on the inhibitor concentration, in comparison to the control. However, it will reach a point where the rate of substrate hydrolysis (i.e. residual enzyme activity) will no longer decline.

In contrast, irreversible inhibitors are time-dependent. This means that over a period of time, the inhibitor will continue to inactivate the enzyme until the residual enzymatic activity reaches zero, as long as the inhibitor is at a higher concentration than the enzyme. Our assays clearly showed a decrease in residual enzymatic activity over the time of the assay. However, they did reach a point at which residual activity was no longer declining. The results of this observation could be explained by two possibilities. One was that the inhibitors were reversible. The second was that PhPr-Leu-Alys-CH=CHCO₂Et and PhPr-Leu-AOmr-CH=CHCO₂Et were reacting with something in the
assay buffer. This would result in the depletion of viable inhibitor to a quantity less than that of the enzyme concentration in the enzymatic assay before it could completely inhibit the enzyme. Therefore, we looked at the stability of the inhibitors in the assay buffers being used.

A UV/Vis wavelength scan (190-380 nm) was performed on each inhibitor in both the gingipain K and clostripain assay buffers. The scan revealed that the degradation of the double bond of the reactive moiety could be observed at 250 nm (Figure 2.9). We suspected that the DTT in the buffers was degrading the inhibitors. A solution containing the same concentrations of DTT and inhibitor as those in the enzymatic assays was monitored at the 250 nm wavelength for both inhibitors. An immediate loss of the double bond could be seen at 250 nm. The assay was repeated with buffer lacking DTT. No degradation of the double bond was observed for either inhibitor over 45 min. Further, pH played a role in degradation of the inhibitor. Both PhPr-Leu-ALys-CH=CHCO2Et and PhPr-Leu-AOmr-CH=CHCO2Et degraded about twice as quickly in the gingipain K buffer (pH 8.0) as they did in the clostripain buffer (pH 7.5). With the gingipain K buffer, PhPr-Leu-ALys-CH=CHCO2Et had a half-life of about 3.8 min, while the half-life of PhPr-Leu-AOmr-CH=CHCO2Et was approximately 1.7 min. In the clostripain buffer, PhPr-Leu-ALys-CH=CHCO2Et had a half-life of about 6.1 min, while the half-life of PhPr-Leu-AOmr-CH=CHCO2Et was approximately 2.9 min. The rate of degradation of the inhibitors by DTT in the assay buffers was determined by pseudo-first order kinetics. The results can be seen in Table 2.3. The data suggests that the inability of either inhibitor to completely inactivate gingipain K or clostripain was due to the reaction with DTT in the assay buffer.
Other thiol reducing agents commonly used in cysteine protease assay buffers include cysteine and 2-mercaptoethanol. These thios were incubated with PhPr-Leu-ALys-CH=CHCO₂Et and PhPr-Leu-AOmn-CH=CHCO₂Et in the gingipain K buffer as well. As shown in Table 2.3, they also reacted with the inhibitors. Cysteine degraded the inhibitors at approximately twice the rate of DTT, while 2-mercaptoethanol reacted with the inhibitors approximately half as fast as the DTT.

The rates of degradation of PhPr-Leu-ALys-CH=CHCO₂Et and PhPr-Leu-AOmn-CH=CHCO₂Et by DTT in the assay buffers were used to correct the inactivation rates of the inhibitors with gingipain K and clostripain. We used the Topham (Topham, 1985)
Table 2.3 Rates of Reaction of Thiols with Aza-peptide Michael Acceptors

<table>
<thead>
<tr>
<th>Thiol</th>
<th>$k_2$ [Thiol] (M$^{-1}$s$^{-1}$)</th>
<th>PhPr-Leu-Lys-CH=CHCO$_2$Et</th>
<th>PhPr-Leu-AOM-CH=CHCO$_2$Et</th>
</tr>
</thead>
<tbody>
<tr>
<td>DTT$^a$</td>
<td>0.632 ± 0.14</td>
<td>1.36 ± 0.00</td>
<td></td>
</tr>
<tr>
<td>DTT$^b$</td>
<td>1.98 ± 0.03</td>
<td>3.84 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>Cysteine$^b$</td>
<td>4.73 ± 0.15</td>
<td>5.81 ± 0.12</td>
<td></td>
</tr>
<tr>
<td>Mercaptoethanol$^b$</td>
<td>0.37 ± 0.01</td>
<td>0.90 ± 0.05</td>
<td></td>
</tr>
</tbody>
</table>

$^a$Clostripain buffer, 20 mM Tris/HCl, 10 mM CaCl$_2$, 0.005% Brij 35, 2 mM DTT, 13% DMSO, pH 7.6. $^b$Gingipain K buffer, 0.2 M Tris/HCl, 0.1 M NaCl, 5 mM CaCl$_2$, 2 mM DTT or 2 mM cysteine or 2 mM 2-mercaptoethanol, 13% DMSO, pH 8.0.
modification of an equation presented by Purdie and Heggie (Purdie and Heggie, 1970) and Ashani et al. (Ashani et al., 1972), which describes kinetics with unstable inhibitors, to calculate the actual rates of inactivation of the inhibitors towards clostripain and gingipain K. This mathematical treatment of the kinetics can only be applied to pseudo-first order inhibition rates. The equation is

$$\ln \left( \frac{v_t}{v_0} \right) = -k_i [I]_0 \cdot \left[ (1-e^{-kt})/k_d \right]$$

where $v_t$ is the rate of hydrolysis of the substrate after the inhibitor is added at time $t$, $v_0$ is the rate of hydrolysis of the substrate of the control (no inhibitor), $[I]_0$ is the initial concentration of inhibitor, $k_d$ is the degradation rate of the inhibitor by DTT in the buffer, and $k_i$ is the inactivation constant of the inhibitor taking into account the rate of inhibitor degradation. This equation can be further simplified by replacing the bracketed term with $\tau$ to yield

$$\ln \left( \frac{v_t}{v_0} \right) = -k_i [I]_0 \cdot \tau$$

This equation is analogous to the equation that describes irreversible inhibition by stable inhibitors (Aldridge, 1950; Rakitizis, 1984)

$$\ln \left( \frac{v_t}{v_0} \right) = -k_{inact} [I]_0 \cdot \tau$$

A plot of $\ln \left( \frac{v_t}{v_0} \right)$ versus $\tau$ yields $k_i$, the actual rate of inactivation of the enzyme by the inhibitor if it was not being degraded by DTT in the buffer solution.

Before the rate of degradation of PhPr-Leu-ALys-CH=CHCO₂Et was applied, the inhibitor inactivated gingipain K with a $k_{inact}/[I]$ of 1,360,000 M⁻¹s⁻¹. After the DTT degradation correction was applied, the inactivation rate was found to be 3,280,000 M⁻¹s⁻¹, a value nearly three times higher. The inactivation rate of gingipain K by PhPr-Leu-AOrn-CH=CHCO₂Et was nearly five times as high after the DTT degradation
correction with rates of 260,000 M⁻¹s⁻¹ before and 927,000 M⁻¹s⁻¹ after. The inactivation rate before DTT degradation correction for clostripain and PhPr-Leu-AOm-CH=CH-CO₂Et was 125 M⁻¹s⁻¹, and after was 788 M⁻¹s⁻¹, more than six times higher than before the correction was applied. For the inactivation of clostripain by PhPr-Leu-ALys-CH=CH-CO₂Et, a DTT degradation correction could not be applied because the enzymatic assay was performed under second order kinetic conditions. The inactivation rate of PhPr-Leu-ALys-CH=CHCO₂Et and clostripain was 40,700 M⁻¹s⁻¹. No rate of inactivation with DTT degradation correction was determined.

**Mechanism.** The hydrolysis of a substrate by a cysteine protease begins via an attack by the active site cysteine on the scissile bond forming a covalent tetrahedral adduct (Figure 2.10). The oxyanion hole created by a glutamine residue and the backbone hydrogen of the active site cysteine residue helps to stabilize the adduct. The

![Diagram](image-url)

*Figure 2.10 Mechanism of Substrate Hydrolysis by Cysteine Proteases*
substrate is protonated immediately before or during the attack (Plapp, 1982; Dufour et al., 1995). Release of the amide portion of the substrate results in the acyl group of the substrate being bound in the active site in the form of a thioester. Finally, an attack by a water molecule on the carbonyl and the subsequent "reverse" reaction releases the newly cleaved peptide, returning the enzyme to its native state.

The aza-peptide Michael acceptors were designed to resembled natural peptide substrates for gingipain K and clostripain. The carbonyl nearest C-2 of unsaturated moiety of the aza-peptide inhibitor lines up directly with the peptide substrate scissile bond carbonyl (Figure 2.11). In theory, the active site cysteine could potentially attack the carbazate carbonyl of the inhibitor. However, this is unlikely with the highly electrophilic double bond nearby and lack of a good leaving group from this carbonyl. The other option is a nucleophilic attack by the active site cysteine residue on either of the electrophilic carbons, C-2 or C-3. Carboxyls adjacent to both of these carbons make a Michael addition by the cysteine residue at either carbon possible.

The C-2 carbon has several advantages that may make it more likely to be the point of attack. The C-2 is nearer the carbazate carbonyl and close enough to the active site cysteine residue to potentially be attacked (Figure 2.11, path a), as is the case with other irreversible inhibitors, like the peptidyl chloromethyl and diazomethyl ketones. This is also the mechanistic route observed for other α,β-unsaturated carbonyl derivatives and vinyl sulfones. Evidence that inhibition by other Michael acceptors proceeds via this path was obtained in an experiment using an α,β-unsaturated carbonyl derivative which replaced the hydrogen with a chlorine at C-2 (Govardhan and Abeles, 1996). A Michael addition at C-2 released a 1:1 stoichiometric amount of the chloride ion. Another
**Figure 2.11** Possible Mechanisms of Inhibition by Aza-peptide Michael

Acceptors
argument for a C-2 attack over a C-3 attack is that the ester (i.e. adjacent to C-3) is more electron withdrawing than the carbazate (i.e. adjacent to the C-2). Therefore, due to resonance, the C-2 carbon is more electropositive than the C-3 carbon.

The carbonyl moiety adjacent to C-3 is essential for reactivity of aza-peptide Michael acceptors. We have found that eliminating the ester moiety adjacent to C-3 and replacing it with an alkyl group causes total loss of activity of the aza-peptide Michael acceptors with caspases (Özlem Doğan, unpublished results). It is unclear why this would happen. One possibility is that hydrogen bond formation takes place between the ester carbonyl and a residue(s) within the proteolytic pocket to help align the α,β-unsaturated moiety into a position that the active site cysteine can attack it. Another possibility is that removing the ester alters the resonance of the double bond. The resonance would now flow in the direction of the carbazate, and the C-3 carbon would be more electropositive. Yet, there is no inactivation of the enzyme by these modified inhibitors. These arguments suggest that the carbon at C-2 more likely to be attacked.

However, there is strong experimental evidence that suggests the C-3 carbon may be the point of attack by the active site cysteine (Figure 2.11, path b). A crystal structure of caspase-1 inhibited by an aza-peptide epoxide shows that its C-2 carbon is attacked (Ron Rubin, unpublished results; Asgian et al., 2002). As seen in Figure 2.12, the active site cysteine residue of caspase-1 nucleophilically attacks the C-2 position of the epoxide ring, which is analogous to the C-3 position in the aza-peptide Michael acceptors. This suggests that there is more going on with the inhibition of enzymes by aza-peptide epoxides than convenience of a nearby target.

The basic structure of aza-peptide Michael acceptors and aza-peptide epoxides is

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very similar. They may bind in the enzyme active site in a comparable fashion. In addition, gingipains K and R, clostripain, and the caspases all belong to the cysteine protease clan CD. The inhibitors may have a similar binding mode in the active site and surrounding recognition subsites with these enzymes. In fact, work comparing the crystal structure of gingipain R with those of caspases-1 and -3 suggests that the proteolytic site is very similar between the enzymes (Banbula et al., 1998; Eichinger et al., 1999). This similarity may carry over to gingipain K and clostripain, as well. Perhaps the active site cysteine residue can reach much farther into the binding pocket than might be expected from the reaction of cysteine proteases with peptide substrates. Or perhaps the azapeptide Michael acceptors are aligned in the proteolytic site of clostripain and gingipain K that positions the C-3 carbon closer to the active site cysteine residue. A crystal structure of an azapeptide Michael acceptor with a clan CD enzyme would be instrumental in understanding this peculiar binding mechanism. However, despite the structural similarities between the caspases and gingipain R, the proposed mechanism for inhibition of clostripain and gingipains K with azapeptide Michael acceptors is a nucleophilic attack at C-2 of the unsaturated bond.
Comparison with other Gingipain K and Clostripain Inhibitors. A variety of inhibitors have been tested with clostripain and gingipain K. None have been designed specifically with these clan CD enzymes in mind. Several of these classes of inhibitors, along with some of their properties, can be seen in Table 2.4.

One class of inhibitors is the epoxysuccinyl inhibitors, which include E-64 (L-trans-epoxysuccinyl-leucylamide(4-guanidino)butane) and its derivatives. The epoxysuccinyl inhibitors are selective, irreversible inactivators of many cysteine proteases, especially those in the papain super family (clan CA) (Barrett et al., 1982). However, E-64 and its derivatives are moderate reversible inhibitors of clostripain ($K_i = 10-20$ μM), probably acting as a substrate analogs (Barrett et al., 1982). E-64 shows no inhibitory activity with gingipain K (Chen et al., 1992; Pike et al., 1994). An analog to epoxysuccinyl inhibitors is the aziridinyl peptide class. They are potent, irreversible inhibitors of the papain superfamily, but are not selective between them (Schirmeister and Peric, 2000). Aziridinyl peptides act as moderate reversible inhibitors of clostripain, with the most potent being the ester derivate EtO-Azi-Leu-OBzl ($K_i = 56.0 \pm 4.3$ μM) (Schirmeister and Peric, 2000).

Several natural reversible inhibitors have been tested with cysteine proteases, including the natural inhibitors leupeptin (Ac-Leu-Leu-Arg-H) and histatin 5 (a salivary protein). They are potent inhibitors towards clostripain ($K_i = 60$ nM and $K_i = 10$ nM, respectively), but gingipain K is resistant to them (Pike et al., 1994; Gusman et al., 2001). However, they are not selective between enzyme classes.

The peptidyl chloromerchyl and chloroethyl ketones are potent inhibitors of both clostripain (Phe-Ala-Lys-Arg-CEK, $k_{in} = 10,100,000$ M$^{-1}$s$^{-1}$) and gingipain K (D-Phe-
<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-04 and its Derivatives</td>
<td>• potent, specific inhibitors for cysteine protease, especially clan CA</td>
</tr>
<tr>
<td></td>
<td>• not selective between the cysteine proteases</td>
</tr>
<tr>
<td></td>
<td>• reversible inhibitors of clostripain</td>
</tr>
<tr>
<td></td>
<td>• do not inhibit gingipain K</td>
</tr>
<tr>
<td>Aziridinyl Peptides</td>
<td>• potent, specific inhibitors for cysteine proteases, especially clan CA</td>
</tr>
<tr>
<td></td>
<td>• not selective between cysteine proteases</td>
</tr>
<tr>
<td></td>
<td>• inhibit clostripain reversibly</td>
</tr>
<tr>
<td>Leupeptin and Other Natural Inhibitors</td>
<td>• no enzymatic specificity; will react with other enzyme classes</td>
</tr>
<tr>
<td></td>
<td>• ineffective toward gingipain K</td>
</tr>
<tr>
<td>Chloromethyl and Chloroethyl Ketones</td>
<td>• reversible inhibitors of clostripain</td>
</tr>
<tr>
<td></td>
<td>• potently inhibit both serine and cysteine proteases</td>
</tr>
<tr>
<td></td>
<td>• high reactivity leads to toxic side effects <em>in vivo</em></td>
</tr>
<tr>
<td></td>
<td>• irreversible inhibitors of clostripain and gingipain K</td>
</tr>
<tr>
<td>Class</td>
<td>Properties</td>
</tr>
<tr>
<td>-----------------------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Acyloxymethyl Ketones</td>
<td>• reduced activity due to large leaving groups</td>
</tr>
<tr>
<td></td>
<td>• not selective between cysteine proteases</td>
</tr>
<tr>
<td></td>
<td>• may react with non-enzymatic nucleophiles</td>
</tr>
<tr>
<td>Diazomethyl Ketones</td>
<td>• difficult to synthesize; unstable in acids</td>
</tr>
<tr>
<td></td>
<td>• may act as substrates instead of as inhibitors for some enzymes</td>
</tr>
<tr>
<td></td>
<td>• may be toxic \textit{in vivo}</td>
</tr>
<tr>
<td>Sulfonium Methyl Ketones</td>
<td>• irreversibly inhibit clostripain</td>
</tr>
<tr>
<td></td>
<td>• irreversibly inhibit cysteine proteases</td>
</tr>
<tr>
<td></td>
<td>• mixed results with serine protease</td>
</tr>
<tr>
<td></td>
<td>• not selective between cysteine proteases</td>
</tr>
<tr>
<td></td>
<td>• irreversibly inhibit clostripain</td>
</tr>
<tr>
<td>Aza-peptide Active Esters</td>
<td>• inhibits serine and cysteine proteases</td>
</tr>
<tr>
<td></td>
<td>• weak inhibitors of cysteine proteases</td>
</tr>
<tr>
<td></td>
<td>• not selective between cysteine proteases</td>
</tr>
<tr>
<td>α,β-Unsaturated Carbonyl and Vinyl Sulfones</td>
<td>have the potential to be hydrolyzed</td>
</tr>
<tr>
<td></td>
<td>may react with nucleophilic groups other than the catalytic residue</td>
</tr>
<tr>
<td></td>
<td>irreversibly inhibit cysteine proteases</td>
</tr>
<tr>
<td></td>
<td>not selective between cysteine proteases</td>
</tr>
<tr>
<td></td>
<td>do not inhibit clostripain or gingipain K</td>
</tr>
</tbody>
</table>
Pro-Arg-CMK, $k_{\text{obs}}/I] = 1,108,800 \text{ M}^{-1}\text{s}^{-1}$ (Porter et al., 1971; Potempa et al., 1997). However, they are also potent inhibitors of other cysteine proteases as well as serine proteases (Rauber et al., 1986; Wikstrom et al., 1989; Albeck et al., 1996). The chloroketones can not distinguish between the enzyme classes. While peptidyl chloroethyl ketones reduce side reactions in a cellular environments (Wikstrom et al., 1989), both types are chemically reactive toward simple thiols and non-proteolytic enzymes, which can result in toxic side effects in vivo (Rauber et al., 1986; Babine and Bender, 1997).

Peptidyl acyloxymethyl ketone inhibitors are very similar to the chloromethyl ketones, but have a reduced activity due to a larger leaving group which is only weakly nucleophilic (Smith et al., 1988). Peptidyl acyloxymethyl ketones are very selective toward cysteine proteases, but not selective between them (Smith et al., 1988). They are potent inhibitors of gingipain K (Z-Phe-Lys-CH$_2$OCOPh(2,4,6-(CH$_3$)$_2$), $k_{\text{obs}} \approx 4,200,000 \text{ M}^{-1}\text{s}^{-1}$) (Potempa et al., 1997), but have not been tested with clostripain.

Peptidyl diazomethyl ketones are selective for cysteine proteases, but not serine or metalloproteases, even with amino acid specificity designed for those enzymes (Leary et al., 1977; Watanabe et al., 1979; Green and Shaw, 1981b; Kirschke and Shaw, 1981; Zumbrunn et al., 1988). Some have suggested that they might be toxic in vivo (Page, 1990), and recent work with embryos supports this theory (Atschosso and Harris, 1994). However, their greatest drawback is the difficulty of their synthesis due to their instability in acids (Green and Shaw, 1981b). They have been found to inhibit clostripain (Z-Phe-Arg-CHN$_2$, $k_{\text{obs}} = 86,000 \text{ M}^{-1}\text{s}^{-1}$) (Zumbrunn et al., 1988), but have not been tested with gingipain K.
Peptidyl sulfonium methyl ketones are chloroketone analogs that are irreversible inhibitors of cysteine proteases, but have mixed results with serine proteases (Rauber et al., 1988). In addition, they are not selective between cysteine proteases. Several potent sulfonium methyl ketones have been made for clostripain, one of the most potent being Z-Phe-Lys-CH$_2$S(CH$_3$)$_2$, with a second order rate of inactivation of 1,400,000 M$^{-1}$s$^{-1}$ (Rauber et al., 1988; Kembhavi et al., 1991). None have been tested with gingipain K.

Aza-peptide active esters were originally designed as active site titrants and inhibitors for serine proteases (Powers et al., 1984), but were later found to irreversibly inhibit cysteine proteases as well (Magarth and Abeles, 1992). They are not selective between the classes of inhibitors, and are weak inhibitors of cysteine proteases. In addition, they can potentially be hydrolyzed by enzymes that are less selective with their subsite preferences, and may react with nucleophiles other than proteolytic enzymes (Powers et al., 1984).

The α,β-unsaturated carbonyl derivatives and vinyl sulfones are Michael acceptors. They irreversibly inhibit cysteine proteases, but generally do not inhibit serine proteases (Palmer et al., 1995). However, they are not selective between cysteine proteases. The vinyl sulfone inhibitors are stable, unreactive towards nucleophiles, and need the catalytic machinery of the cysteine protease for activation. However, neither type of Michael acceptor inhibits clan CD enzymes.

The aza-peptide Michael acceptors, unlike many of the other inhibitor classes, can distinguish between serine and cysteine proteases. They are selective, irreversible inhibitors of gingipain K and clostripain, while PhPr-Leu-Lys-CH=CHCO$_2$Et acts as a weak, reversible inhibitor of trypsin ($K_i = 17.0 \pm 4.7$ μM) and PhPr-Leu-AOm-
CH=CHCO₂Et does not inhibit trypsin at all. In contrast to many of the other inhibitor types, the aza-peptide Michael acceptors are selective between the cysteine protease clans. While gingipain K and clostripain were potently inhibited by PhPr-Leu-ALys-CH=CHCO₂Et and PhPr-Leu-AOmn-CH=CHCO₂Et, cathepsin B was only weakly inactivated by both inhibitors \(k_{\text{depl}}/[I] = 9.09 \text{ M}^{-1}\text{s}^{-1} \) and \(2.97 \text{ M}^{-1}\text{s}^{-1}\), respectively), and papain and calpain I were not inhibited at all. Within the cysteine protease clan CD, the aza-peptide Michael acceptors are easily able to distinguish between the members, unlike any of the other inhibitor classes. Gingipain R is only weakly inhibited by both inhibitors \(k_{\text{depl}}/[I] = 289 \pm 84 \text{ M}^{-1}\text{s}^{-1}\) for PhPr-Leu-ALys-CH=CHCO₂Et, and \(k_{\text{depl}}/[I] = 32.0 \pm 0.5 \text{ M}^{-1}\text{s}^{-1}\) for PhPr-Leu-AOmn-CH=CHCO₂Et), while caspases-3, -6, and -8 showed no inactivation at all. The aza-peptide Michael acceptors, PhPr-Leu-ALys-CH=CHCO₂Et and PhPr-Leu-AOmn-CH=CHCO₂Et, are potent, selective inhibitors for both gingipain K \(k_i = 3,280,000 \pm 652,000 \text{ M}^{-1}\text{s}^{-1}\) and \(k_i = 927,000 \pm 59,000 \text{ M}^{-1}\text{s}^{-1}\), respectively) and clostripain \(k_{\text{at}} = 40,700 \pm 7,580 \text{ M}^{-1}\text{s}^{-1}\) and \(k_i = 788 \pm 169 \text{ M}^{-1}\text{s}^{-1}\), respectively).

Perspectives. The aza-peptide Michael acceptors were developed and synthesized based on the combination of aza-peptide and \(\alpha,\beta\)-unsaturated Michael acceptor peptide inhibitors of cysteine proteases, as well as previous results obtained by our lab with aza-peptide epoxide inhibitors. The aza-peptide Michael acceptors were designed to inactivate clostripain and gingipains K and R. As the data shows, they proved to be poor inhibitors with gingipain R. However, the inhibitors were effective with clostripain, and were especially potent inactivators of gingipain K. We also tested PhPr-Leu-ALys-CH=CHCO₂Et and PhPr-Leu-AOmn-CH=CHCO₂Et with a variety of other enzymes, including a serine protease, but they proved to have little or no inhibitor
activity toward those enzymes. The aza-peptide Michael acceptors represent a novel type of inhibitor that are selective, irreversible inhibitors of cysteine proteases and are specific for clan CD enzymes. Future work with these inhibitors include the following:

- Extending the non-primed side of the inhibitors to increase potency toward, and perhaps selectivity between, clostripain and gingipain K. This may also eliminate some or all of the activity seen with the other enzymes.

- It is possible to learn more about the specificities of the primed side subsite preferences of clostripain and gingipain K by extension of the P side of the inhibitors. This may eliminate activity with other enzymes as well.

- The design and synthesis of aza-Arg, aza-Asp, or aza-Asn derivatives of aza-peptide Michael acceptors could lead to the development of highly reactive and selective inhibitors for the other clan CD proteases.

- Selective inhibitors for separate would be especially useful to study its biological role and get a better understanding of its responsibilities in vivo.

- Extending the work to include aza-peptide vinyl sulfones may give similar or improved rates of inhibition with clan CD enzymes, and yet have the benefits of being inert toward other nucleophiles, like the thiol reducing agents, and being inactive toward serine proteases.
Before this work, no selective, potent inhibitors existed for either gingipain K or clostripain. The goal of this project was to develop inhibitors based on the substrate specificity of these enzymes. This was accomplished with the development of PhPr-Leu-Ala-CH=CHCO\textsubscript{2}Et and PhPr-Leu-Orn-CH=CHCO\textsubscript{2}Et, representatives of the aza-peptide Michael acceptors, a novel type of inactivator that are selective, irreversible inhibitors of cysteine proteases specific for clan CD enzymes. An effective inhibitor for gingipain K is especially important for understanding the roles the enzyme plays for Porphyromonas gingivalis. It is also important to learn how the enzyme interacts with human proteins and what functions it has in the progression of periodontal disease. Specific inhibitors for clostripain may help expand the understanding of this unique enzyme and give insight into its proteolytic mechanism. We have developed two such inhibitors that are selective for gingipain K and clostripain. In summary, the conclusions are:

- A new class of inhibitors for gingipain K and clostripain, the aza-peptide Michael acceptors, has been designed and synthesized.
- No inhibitors previously synthesized combine the triagonal planar attributes of the aza-peptide active esters and the highly reactive moiety of Michael acceptors.
- The novel aza-peptide Michael acceptors are highly reactive and specific for clan CD cysteine proteases gingipain K and clostripain, a clan resistant to many other types of common cysteine protease inhibitors.
• The aza-peptide Michael acceptors are weak, reversible inhibitors towards trypsin, and possibly other serine proteases as well. They may very well be specific irreversible inhibitors for clan CD cysteine proteases alone.

• Due the highly narrow P1 specificity of clan CD cysteine proteases, the aza-peptide Michael acceptors can distinguish between clan CD enzymes.
Materials and Methods. Hydrazine, potassium phosphate, piperazine-1,4-bis(2-ethanesulfonic acid), (Pipes), cysteine, hydrocinnamic acid, fumaric acid monomethyl ester, 33% HBr in glacial acetic acid, p-toluenesulfonic acid monohydrate (Tos-GH), 1,3-dicyclohexylurea (DCC), ethylenediaminetetraacetic acid (EDTA), threo-1,4-dimercapto-2,3-butane diol (DiT), 2-mercaptoethanol, polyoxyethylene (23) lauryl ether (Brij 35), N-hydroxybenzotriazole (HOBr), N-methylmorpholine, N,N-butyllchloroformate, 2-iodobenzoic acid, 2-amino-2-(hydroxymethyl)-1,3-propane diol (Tris), potassium bromate, sodium cyanoborohydride, 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC), solid sodium, CDCl₃, DMSO-d₆, and all common reagents and solvents were purchased from Aldrich Chemical Co. (Milwaukee, WI) or Fischer Scientific Chemicals (Fair Banks, NJ). The (--)-dichloro-d-tartrate was purchased from ACROS Organics (Morris Plains, NJ). The 3-(tert-butyloxy)carbonylamino)propanol and 4-(tert-butyloxy)carbonylamino)butanol were purchased from Fluka (through Aldrich Chemical Co. Milwaukee, WI). The protected amino acid HCl+H-Leu-OMe was purchased from BACHEM Bioscience, Inc. (King of Prussia, PA). Flash chromatography, silica gel (particle size 32-63 μm) and thin-layer chromatography plates that were precoated with 250 μm of silica gel (F-254) were obtained from Scientific Adsorbents, Inc. (Atlanta, GA). The Sue-Ala-Phe-Lys-AMC-TFA, Z-Phe-Arg-AMC, papain, clostripain, and trypsin were purchased from Sigma Chemical Co. (St. Louis, MO). Cathepsin B was purchased from Athens Research and Technology, Inc. (Athens, Georgia). Calpain 1 was purchased from Calbiochem (La Jolla, CA). The Ac-Asp-Glu-
Val-Asp-AMC substrate was obtained from BIORAL Research Laboratories (Plymouth Meeting, PA). The 4-(2-hydroxyethyl)-1-piperazinioethanesulfonic acid (Hepes) was obtained from Research Organics, Inc. (Cleveland, OH). Caspases-3, -6, and -8 were gifts from the Salvesen lab (Burnham Institute, La Jolla, CA). The Potempa lab provided the gingipains K and R (University of Georgia, Athens, GA). $^1$H NMR spectra were obtained with a Varian Mercury 300 instrument. Nominal mass spectra were collected on a VG Instruments 70SE (FAB$^+$) or on a Micromass Quattro LC (ESI$^+\prime$). High-resolution mass spectra were run on either the VG Instruments 70SE or an Applied Biosystems QSTAR XL (ESI$^+\prime$). The elemental analyses were performed by Atlantic Microlabs (Atlanta, GA). The kinetic data was gathered on a Tecan Spectrafluor Microplate Reader (Tecan US, Research Triangle Park, NC). All elemental analyses were within ±0.4% of the calculated values for the formulas shown for each compound.

Isoxoxybenzoic acid (IBX). This was synthesized based on the method described by Dess and Martin (Dess and Martin, 1983): yield 84%; product spot on TLC, Rf= 0.02 (10% CH$_3$OH in CH$_2$Cl$_2$). $^1$H NMR (DMSO-$d_6$) δ 8.16-8.12 (d, 1H), 8.94-7.92 (m, 2H), 7.86-7.80 (t, 1H). MS (FAB$^+$) m/z 281 (M+1).

PhPr-Leu-OMe. A solution of hydrocinnamic acid (3.003 g, 20.0 mmol) and CH$_2$Cl$_2$ (30 ml) was cooled to -15 °C. NMM (2.023 g, 20.0 mmol) and iBCF (2.738 g, 20.0 mmol) were added dropwise and the solution stirred at -15 °C for 15 min. A solution of HCl-H-Leu-OMe (3.633 g, 20.0 mmol) and NMM (2.023 g, 20.0 mmol) dissolved in CH$_2$Cl$_2$ (40 ml) was cooled to -15 °C and stirred for 15 min. After the allotted time, the solutions were combined and allowed to stir at rt overnight. The organic layer was washed with a 4% NaHCO$_3$ solution (3 x 100 ml), 1 N HCl (3 x
100 ml) and a saturated NaCl solution (3 x 75 ml). The organic layer was dried over MgSO₄, filtered, and evaporated, giving a clear, light-yellow oil, which was used without further purification; yield 92%; product spot on TLC, RF = 0.70 (10% CH₂OH in CH₂Cl₂). ¹H NMR (CDCl₃) δ 7.32-7.16 (m, 5H), 5.76-5.66 (d, 1H), 4.68-4.54 (m, 1H), 3.78-3.66 (s, 3H), 3.02-2.88 (t, 2H), 2.61-2.44 (m, 2H), 1.64-1.39 (m, 3H), 1.00-0.86 (dd, 6H). MS (ESI⁻) m/z 278 (M⁻+1).

PhFv-Leu-NHNH₂. To a solution of PhPr-Leu-OMe (5.110 g, 18.4 mmol) in CH₂OH (45 ml), hydrazine (10 eq. 5.897 g, 184 mmol, 5.776 ml) was added quickly, dropwise. The reaction mixture was allowed to stir at rt overnight. The solvent was evaporated, giving a fluffy, white solid. The crude product was purified by silice gel chromatography using 10% CH₂OH in CH₂Cl₂ as the eluent. The solvent was evaporated, giving a white solid; yield 79%; one spot on TLC, RF = 0.36 (10% CH₂OH in CH₂Cl₂). ¹H NMR (DMSO-d₆) δ 9.14 (s, 1H), 8.00-7.90 (d, 1H), 7.34-7.12 (m, 5H), 4.40-4.12 (m, 3H), 2.88-2.70 (t, 2H), 2.48-2.30 (m, 2H), 1.52-1.30 (m, 3H), 0.90-0.71 (dd, 6H). MS (ESI⁻) m/z 278 (M⁻+1).

Bec-NH-(CH₂)₄-CHO. To a solution of 3-(t-butyloxy carbonylamino)propanol (1.419 g, 8.1 mmol) dissolved in DMSO (15 ml), IBX (2 eq, 4.500 g, 16.2 mmol) was added to form a slurry. The reaction was allowed to stir at rt for 5 hr. The solid IBX dissolved slowly, but a white precipitate formed after approximately 1 hr of reaction time. Distilled water (80 ml) was added to the reaction mixture, and the resultant aqueous solution filtered and then extracted with diethyl ether (3 x 175 ml). The organic layers were combined, dried over MgSO₄, filtered, and evaporated. The crude oil was purified by silica gel chromatography using 10% CH₂OH in CH₂Cl₂ as the eluent. The
solvent was evaporated to leave a clear, colorless oil with a very pleasant flowery or candy-like scent; yield 67%: one spot on TLC, RF = 0.70 (10% CH₂OH in CH₂Cl₂). ¹H NMR (CDCl₃) δ 9.80 (s, 1H), 4.94 (s, 1H), 3.47-3.38 (m, 2H), 2.76-2.65 (t, 2H), 1.47 (s, 9H). MS (ESI⁺) m/z 174 (M+).

1-(t-Butyloxycarbonyl)-3,4-dihydropyrrole. To a solution of 4-(t-
butyloxycarbonylamino)butanol (1.540 g, 8.1 mmol) in DMSO (15 ml), IBX (2 eq, 4.500 g, 16.2 mmol) was added at rt and reacted for 5.7 hr. The remainder of the workup was similar to that used for Boc-NH-(CH₂)₃-CHO. The crude oil was purified by silica gel chromatography in 10% CH₂OH in CH₂Cl₂ and concentrated, leaving the product as a clear, faintly yellow oil; yield 58%; one spot on TLC, RF = 0.71 (10% CH₂OH in CH₂Cl₂). ¹H NMR (CDCl₃) δ 5.47 (s, 1H), 5.40 (s, 1H), 3.60-3.20 (m, 2H), 2.14-1.74 (m, 2H), 1.45 (s, 9H). MS (ESI⁺) m/z 170 (M+).

PhPr-Leu-NH=CH(CH₂)₂-NH-Boc. This procedure is based on the method
used by Gray and Parker (Gray and Parker, 1975) to prepare Boc-3-aminopropanol
benzyldihydrozone. To a solution of Boc-NH-(CH₂)₃CHO (0.942 g, 5.43 mmol)
dissolved in absolute ethanol (11 ml) at rt, PhPr-Leu-NNH₂ (1.498 g, 5.43 mmol) was
added to form a slurry. The reaction mixture was allowed to stir at rt overnight.
The solvent was evaporated and the crude product dissolved in CH₂Cl₂ (50 ml). The organic
layer was washed with a 10% citric acid solution (3 x 40 ml), a saturated NaHCO₃
solution (3 x 25 ml), and a saturated NaCl solution (3 x 25 ml). The organic layer was
dried over MgSO₄, filtered, and concentrated. The material was then purified by silica
gel chromatography using 10% CH₂OH in CH₂Cl₂ as the eluent. The eluent was
evaporated, giving a white, foamy solid that was used without further purification; yield

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82% yield; product spot by TLC, \( R_f = 0.56 \) (10% CH\(_3\)OH in CH\(_2\)Cl\(_2\)). \(^1\)H NMR (CDCl\(_3\)) \( \delta \) 10.05 (s, 1H), 8.90 (s, 1H), 7.40-7.08 (m, 5H), 6.30-6.16 (t, 1H), 5.52-5.36 (t, 1H), 4.52-4.40 (m, 1H), 3.44-3.28 (m, 2H), 3.00-2.86 (m, 2H), 2.57-2.44 (m, 4H), 1.71-1.44 (m, 3H), 1.40 (s, 9H), 0.98-0.82 (m, 6H). MS (EI\(^+\)) \( m/z \) 433 (M+1).

**PhPr-Leu-NH-N=CH(CH\(_2\))\(_2\)-NH-Boc.** This procedure is a modification of the method used by Gray and Parker (Gray and Parker, 1975) to prepare Boc-3-aminopropanal benzoylhydrazone. The 1-( tert-butloxyycarbonyl)-3,4-dihydropyrrole (0.805 g, 4.76 mmol) was reacted with PhPr-Leu-NH\(_2\) (1.320 g, 4.76 mmol) and p-toluene sulfonic acid monohydrate (0.3 eq, 0.272 g, 1.43 mmol) in absolute ethanol (12 ml) at rt. The remainder of the workup was similar to that used to synthesize PhPr-Leu-NH-N=CH(CH\(_2\))\(_2\)-NH-Boc. The crude product was purified by silica gel chromatography using 10% CH\(_3\)OH in CH\(_2\)Cl\(_2\) as the eluent. The eluent was evaporated to give a foamy white solid, which was used without further purification: yield 73%.

product spot by TLC, \( R_f = 0.55 \) (10% CH\(_3\)OH in CH\(_2\)Cl\(_2\)). \(^1\)H NMR (CDCl\(_3\)) \( \delta \) 9.77 (s, 1H), 8.80 (s, 1H), 7.43-7.13 (m, 5H), 6.07-5.89 (t, 1H), 5.45-5.34 (t, 1H), 4.48-4.31 (m, 1H), 3.20-3.09 (t, 2H), 3.00-2.87 (m, 2H), 2.59-2.42 (m, 2H), 2.40-2.28 (m, 2H), 2.08-1.96 (m, 2H), 1.81-1.64 (m, 3H), 1.42 (s, 9H), 1.00-0.78 (m, 6H). MS (FAB\(^+\)) \( m/z \) 447 (M+1).

**PhPr-Leu-NH-NH(CH\(_2\))\(_2\)-NH-Boc.** This procedure is based on the method used by Gallina et al. to synthesize a series of Z-hydrazones (Calabretta et al., 1995). PhPr-Leu-NH-N=CH(CH\(_2\))\(_2\)-NH-Boc (7.928 g, 4.46 mmol) and NaBH\(_3\)CN (Seq. 2.400 g, 22.3 mmol) were dissolved in anhydrous THF (23 ml). Glacial acetic acid (164 eq, 27.85 g, 464 mmol, 26.5 ml) was added dropwise to the solution, and the reaction mixture allowed
to stir at rt overnight. The solvents were evaporated and the crude oil dissolved in ethyl acetate (20 ml). Distilled water (20 ml) was added to the solution, which was vigorously stirred while enough solid NaHCO₃ was added to turn the pH of the aqueous layer basic. The organic layer was isolated and washed with a saturated NaCl solution (2 x 15 ml), a saturated NaHCO₃ solution (10 ml), and again with a saturated NaCl solution (10 ml). The organic layer was dried over MgSO₄, filtered, and evaporated. The crude solid was purified by silica gel chromatography using 1/1 ethyl acetate/hexanes as the eluent. The solvent was evaporated, leaving a white, foamy solid that was used without further purification: yield 59%; product spot on TLC, Rf = 0.43 (10% CH₂OH in CH₂Cl₂).

¹H NMR (CDCl₃) δ 9.83 (s, 1H), 9.54 (s, 1H), 7.30-7.13 (m, 5H), 6.94 (s, 1H), 6.60 (s, 1H), 4.60-4.47 (m, 1H), 3.25-3.06 (m, 2H), 3.04-2.93 (m, 4H), 2.60-2.51 (m, 2H), 1.87-1.72 (m, 3H), 1.70-1.60 (m, 2H), 1.42 (s, 9H), 0.98-0.82 (m, 6H). MS (ESI⁺) m/z 435 (M+1).

PhPr-Leu-NHNH(CH₂)₃-NH-Boc. This procedure is based on the method used by Gallina et al. to synthesize a series of Z-hydrazines (Calabretta et al., 1995). PhPr-Leu-NHNH(CH₂)₃-NH-Boc (1.543 g, 3.46 mmol) and NaBH₄CN (5 eq, 1.086 g, 17.3 mmol) dissolved in anhydrous THF (24 ml) were reacted with glacial acetic acid (104 eq, 21.608 g, 360 mmol, 20.6 ml). The remainder of the workup was similar to that used for PhPr-Leu-NHNH(CH₂)₃-NH-Boc. The crude product was purified by silica gel chromatography using 1/1 ethyl acetate/hexanes as the eluent. The solvent was evaporated to give a clear, colorless oil. Treatment with CH₂Cl₂, followed by diethyl ether and evaporation gave a white, foamy solid, which was used without further purification: yield 49%; product spot on TLC, Rf = 0.67 (10% CH₂OH in CH₂Cl₂).

¹H NMR (CDCl₃) δ 9.00 (s, 1H), 8.75 (s, 1H), 7.28-7.12 (m, 5H), 4.86 (s, 1H), 4.80 (s, 1H),
4.51-4.42 (m, 1H), 3.24-3.04 (m, 2H), 3.02-2.93 (m, 4H), 2.77-2.68 (m, 2H), 2.62-2.48 (m, 2H), 2.01-1.90 (m, 1H), 1.88-1.78 (m, 2H), 1.76-1.60 (m, 4H), 1.42 (s, 9H), 0.97-0.82 (m, 6H). MS (ESI) m/z 449 (M+1).

PhPr-Leu-Orn(Boc)-CH=CHCO2Et. PhPr-Leu-NH₂(CH₂)_3-NH-Boc (0.100 g, 0.23 mmol) was dissolved in chloroform (4 ml) at rt. To this solution, fumaric acid monoethyl ester (2 eq, 0.066 g, 0.46 mmol), HOBT (2.4 eq, 0.074 g, 0.55 mmol) and EDC (2.4 eq, 0.106 g, 0.55 mmol) were added. A white precipitate formed after approximately 15 min. The reaction mixture was allowed to stir at rt overnight. The reaction mixture was diluted with chloroform (up to 25 ml) and washed with a 10% citric acid solution (3 x 25 ml), a saturated NaN₂CO₃ solution (3 x 20 ml), and a saturated NaCl solution (3 x 20 ml). The organic layer was dried over MgSO₄, filtered, and evaporated. The crude product was purified by silica gel chromatography four separate times using 5% CH₂OH in CH₂Cl₂ as the eluent. The solvent was evaporated, the material treated with 1:1 diethyl ether/hexanes, and evaporated again to leave behind a sticky, white solid: yield 14%; one spot on TLC, Rf = 0.33 (5% CH₂OH in CH₂Cl₂). ¹H NMR (CDCl₃) δ 9.40 (s, 1H), 7.35-7.07 (m, 6H), 6.82-6.78 (d, 1H), 5.87 (s, 1H), 5.05 (s, 1H), 4.53-4.41 (m, 1H), 4.27-4.14 (q, 2H), 3.20-3.04 (m, 2H), 3.01-2.94 (t, 2H), 2.71-2.47 (m, 2H), 1.80-1.60 (m, 6H), 1.42 (s, 9H), 1.37-1.24 (t, 3H), 0.98-0.84 (m, 6H). MS (ESI) m/z 561 (M+1).

PhPr-Leu-Lys(Boc)-CH=CHCO₂Et. PhPr-Leu-NH₂(CH₂)_3-NH-Boc (0.100 g, 0.22 mmol) fumaric acid monoethyl ester (2 eq, 0.063 g, 0.44 mmol), HOBT (2.4 eq, 0.072 g, 0.53 mmol), and EDC (2.4 eq, 0.102 g, 0.53 mmol) were reacted in DMF (5 ml) at rt. The workup was similar to that used for PhPr-Leu-Orn(Boc)-CH=CH-CO₂Et.

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The crude material was purified by silica gel chromatography three separate times using 5% CH₃OH in CH₂Cl₂ as the eluent. The solvent was evaporated, the material treated with hexanes, and evaporated again, giving a sticky, white solid: yield 21%; one spot on TLC, Rf = 0.30 (5% CH₃OH in CH₂Cl₂). ¹H NMR (CDCl₃) δ 9.48 (s, 1H), 7.30-7.09 (m, 6H), 6.82-6.78 (d, 1H), 5.89 (s, 1H), 4.78 (s, 1H), 4.54-4.44 (m, 1H), 4.28-4.14 (q, 2H), 3.75-3.57 (m, 2H), 3.14-3.06 (m, 2H), 3.01-2.95 (m 2H), 2.70-2.52 (m, 2H), 1.76-1.62 (m, 5H), 1.60-1.49 (m, 2H), 1.42 (s, 9H), 1.38-1.20 (q, 3H), 0.98-0.84 (m, 6H). MS (ESI⁺) m/z 575 (M+1).

N¹,3-Aminopropyl-N¹-trans-(3-ethoxycarbonylacycloxy) N²-(3-phenylpropanoyl)leucylhydrazine (PhPr-Leu-AMn-CH=CHCO₂Et). To PhPr-Leu-AMn(Boc)-CH=CH-CO₂Et (0.025 g, 0.045 mmol) dissolved in CH₂Cl₂ (2 ml) and cooled to 0 °C, refrigerated (4 °C) TFA (1.5 ml) was added dropwise to form a clear, faintly yellow solution. The reaction mixture stirred for 1 hr at 0 °C, and then allowed to warm to rt to react another 2.75 hr. The solvents were evaporated, and the material treated with diethyl ether (4 x 1 ml). The diethyl ether was decanted after each treatment until the material was a foamy white solid: yield 93%; one spot by TLC, Rf = 0.02 (5% CH₃OH in CH₂Cl₂). ¹H NMR (CDCl₃) δ 10.57 (s, 1H), 8.01 (s, 2H), 7.30-7.11 (m, 6H), 6.82-6.78 (d, 1H), 4.49-4.39 (m, 1H), 4.27-4.14 (q, 2H), 3.84-3.44 (m, 2H), 3.17-3.01 (m, 2H), 3.00-2.84 (m, 2H), 2.71-2.47 (m, 2H), 2.00-1.65 (m, 3H), 1.61-1.42 (m, 2H), 1.39-1.21 (t, 3H), 0.98-0.84 (m, 6H). HRMS (ESI⁺) m/z calculated for C₂₃H₃₂N₄O₇ (M+1) 461.276395, found 461.276729. Anal. Formula: C₂₃H₃₂N₄O₇ • 0.75 H₂O. Calculated: C, 53.09; H, 6.60; N, 9.53. Found: C, 53.04; H, 6.51; N, 9.29.
N*-4-Aminobutyli-N*-trans-(3-ethoxy carbonyl acryloyl)-N'- (3-phenylpropanoyl leucyl) hydrazine (PhPr-Leu-ALys-CH=CHCO2Et). PhPr-Leu-ALys(Boc)-CH=CHCO2Et (0.021 g, 0.037 mmol) and refrigerated at 4 °C (TFA (1.5 ml) dissolved in CH2Cl2 (2 ml) were reacted at 0 °C. The remainder of the workup was similar to that used to synthesize PhPr-Leu-AOm-CH=CHCO2Et. The material was treated with diethyl ether (2 x 1 ml); the ether being decanted after each treatment, giving a foamy, white solid: yield 95%; one spot by TLC, Rf= 0.02 (5% CH3OH in CH2Cl2).

1H NMR (CDCl3) δ 10.22 (s, 1H), 8.01 (s, 2H), 7.30-7.11 (m, 6H), 6.82-6.78 (d, 1H), 6.39 (s, 1H), 4.48-4.40 (m, 1H), 4.28-4.14 (q, 2H), 3.78-3.61 (m, 2H), 3.14-2.89 (m, 4H), 2.70-2.52 (m, 2H), 1.91-1.60 (m, 5H), 1.59-1.42 (m, 2H), 1.39-1.21 (q, 3H), 0.98-0.82 (m, 6H). HRMS (ESI+) m/z calculated for C29H34N4O2 (M+1) 475.292046, found 475.291718. Anal. C29H34N4O2 • 0.25 TFA. Calculated: C, 53.52; H, 6.44; N, 9.08. Found: C, 53.84; H, 6.71; N, 8.76.

Enzyme Assays-General Methods. The effectiveness of the inhibitors toward each enzyme was measured by following the decrease in rate of hydrolysis of synthetic substrates (through fluorescence or absorbance) sensitive toward each particular enzyme. A Tecan Spectrafluor Microplate Reader (Tecan US, Research Triangle Park, NC) was used to spectrophotometrically obtain the kinetic measurements. The enzymatic assays were carried out in a variety of buffer solutions. Unless otherwise noted, the enzymes were stored in their respective buffers at -20 °C (clostripain, gingipains K and R, papain, cathepsin B, calpain I, and trypsin), or -72 °C (caspases-3, -6, and -8) prior to use, and kept at 0 °C when being used for the assays. Dilutions of the enzymes were made with the same buffer solutions. A single active site was assumed for all of the enzymes. The
substrates used in the enzymatic assays were either peptide 7-amino-4-methylcoumarin derivatives (AMC, excitation λ = 360 nm, emission λ = 465 nm) or peptide p-nitroanilide derivatives (pNA, absorbance = 405 nm). The stock substrate solutions for each enzyme were made up in DMSO.

**Inhibition Kinetics-Incubation Method.** Inhibition rates with clostripain, gingipains K and R, cathepsin B, and calpain I were all measured by the incubation method. This method involved mixing enzyme and inhibitor at time zero. After various time intervals, aliquots are removed from the inhibition incubation mixture, added to a substrate solution, and assayed for residual activity.

Clostripain was purchased from Sigma Chemical Co. (St. Louis, MO) as a solid which was dissolved in an activation solution of 8 mM DTT at a concentration of 5.962 μM and stored at -20 °C prior to use. The inhibition of clostripain began with the addition of 50 μl of stock inhibitor solution (1.2 μM for PhPr-Leu-ALys-CH=CHCO₂Et, and 78 μM for PhPr-Leu-AOrn-CH=CHCO₂Et) in DMSO to a solution of 500 μl of 20 mM Tris/HCl, 10 mM CaCl₂, 0.005% Brij 35, 2 mM DTT buffer at pH 7.6 (clostripain buffer) and 10 μl of the stock enzyme solution. Aliquots (50 μl) of this incubation mixture were taken at various time points and added to a solution containing 200 μl of the clostripain buffer and 10 μl 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 data obtained for PhPr-Leu-ALys-CH=CHCO₂Et and clostripain was processed using second order kinetics. The data obtained for PhPr-Leu-AOrn-CH=CHCO₂Et with clostripain was processed by pseudo-first order kinetics.
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 μM, which was stored at -20 °C prior to use. Before using the enzyme, an aliquot (1 μl) 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 50 μl of stock inhibitor solution (80 nM for PhPr-Leu-ALys-CH=CHCO₂Et, and 600 nM for PhPr-Leu-AOrm-CH=CHCO₂Et) in DMSO to 488 μl of the diluted enzyme solution (4.61 nM) in gingipain K buffer warmed to rt. Aliquots (50 μl) of this incubation mixture were taken at various time points and added to a solution containing 200 μl of the gingipain K buffer and 10 μl of the substrate Suc-Ala-Phe-Lys-AMC • TFA stock solution (0.910 mM) in DMSO. The enzymatic activity was monitored by following the change in fluorescence at 465 nm. The data for the inhibitors PhPr-Leu-ALys-CH=CHCO₂Et and PhPr-Leu-AOrm-CH=CHCO₂Et with gingipain K was processed by pseudo-first order kinetics.

The gingipain R 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 7.5 at a concentration of 40 μM, which was stored at -20 °C prior to use. During the kinetic assay, the enzyme was kept at 0 °C. The inhibition of gingipain R began with the addition of 25 μl of stock inhibitor solution (156 μM for PhPr-Leu-ALys-CH=CHCO₂Et, and 1.25 nM for PhPr-Leu-AOrm-CH=CHCO₂Et) in DMSO to a solution of 244 μl of 0.2 M Tris/HCl, 0.1 M NaCl, 5 mM CaCl₂, 2 mM DTT
buffer at pH 7.5 (gingipain R buffer) and 1 μl of the stock enzyme solution. Aliquots (25 μl) of this incubation mixture were taken at various time points and added to a solution containing 100 μl of the gingipain R buffer and 5 μl of Z-Phe-Arg-AMC substrate solution (1 mM stock) in DMSO. The enzymatic activity was monitored by following the change in fluorescence at 465 nm. The data for the inhibitors PhPr-Leu-ALys-CH=CHCO₂Et and PhPr-Leu-AOrn-CH=CHCO₂Et with gingipain K was processed by pseudo-first order kinetics.

The cathepsin B was purchased from Athens Research and Technology, Inc. (Athens, Georgia) in a solution of 20 mM NaAc, 1 mM EDTA, at pH 5.0 at a concentration of 31.2 μM and stored at -20 °C prior to use. Before being used, the stock cathepsin B solution was diluted 100-fold by mixing with a solution of 0.1 M potassium phosphate, 1.25 mM EDTA, 0.01% Brij 35, and 1 mM DTT at pH 6.0 (cathepsin B buffer). A solution of 10 ml cathepsin B buffer and 25 μl of Z-Arg-Arg-AMC substrate solution (2 M stock) in DMSO was also prepared. The inhibition of cathepsin B began with the addition of 30 μl of stock inhibitor (2.5 mM for both PhPr-Leu-ALys-CH=CHCO₂Et and PhPr-Leu-AOrn-CH=CHCO₂Et) to 300 μl of the cathepsin B buffer and 30 μl of the diluted cathepsin B solution (0.312 μM). Aliquots (50 μl) of this were taken at various time points and added to a solution containing 200 μl of the cathepsin B buffer/substrate solution. The enzymatic activity was monitored by following the change in fluorescence at 465 nm. The data for the inhibitors PhPr-Leu-ALys-CH=CHCO₂Et and PhPr-Leu-AOrn-CH=CHCO₂Et with cathepsin B was processed by pseudo-first order kinetics.
Inhibition Kinetics-Progress Curve Method. Papain and caspases-3, -6, and -8 were studied by the method described by Tian and Tsou (Tian and Tsou, 1982). This method involved continuous measurement of enzymatic activity through the hydrolysis of a synthetic substrate in the presence of inhibitor.

Papain, purchased from Sigma Chemical Co. (St. Louis, MO), was stored at -20 °C in a solution of 50 mM Hepes, 3 mM DTT, 2.5 mM EDTA at pH 7.5 (papain buffer) at a concentration of 51.7 μM prior to use. Before being used, the papain stock solution was diluted 100-fold by mixing 10 μl of stock papain, 39 μl of 0.1 M DTT, and 951 μl of papain buffer. The papain assay was conducted with 224 μl of papain buffer, 18 μl of Z-Phe-Arg-pNA substrate solution (7 mM stock) in DMSO, 4.2 μl of inhibitor solution (10 mM for both PhPr-Leu-ALys-CH=CHCOCH3 and PhPr-Leu-AOM-CH=CHCOCH3) in DMSO, and 4.2 μl of the diluted papain (0.517 μM) solution at 23 °C. The enzymatic activity was monitored by following the change in absorbance for 20 min at 405 nm.

Neither PhPr-Leu-ALys-CH=CHCOCH3 nor PhPr-Leu-AOM-CH=CHCOCH3 showed any activity towards papain when compared to the control.

All three caspases (caspase-3, caspase-6, and caspase-8) were obtained from Guy Salvesen's lab (Burnham Institute, La Jolla, CA) and stored at -72 °C prior to use. Before being used, the caspases were diluted in a solution of 20 mM Pipes, 0.1% Chaps, 10% sucrose, 100 mM NaCl, 1 mM EDTA, 10 mM DTT at pH 7.2 (caspase buffer) to concentrations of 2 nM for caspase-3, 20 nM for caspase-6, and 100 nM for caspase-8. The enzymatic assays of all three caspases were performed the same way. The caspase assay was conducted with 40 μl of the caspase buffer, 5 μl of Ac-Asp-Glu-Val-Asp-AMC substrate solution (2 mM stock) in DMSO, 5 μl of inhibitor solution (10 mM for both
PhPr-Leu-ALys-CH=CHCO2Et and PhPr-Leu-AOmr-CH=CHCO2Et) in DMSO, and 50 μl of the diluted enzyme solution. The enzymatic activity was monitored by following the change in fluorescence for 20 min at 465 nm. Neither PhPr-Leu-ALys-CH=CHCO2Et nor PhPr-Leu-AOmr-CH=CHCO2Et showed any activity towards any of the caspases when compared to the controls.

Calpain I was purchased from Calbiochem (La Jolla, CA) in a solution of 30% glycerol at a concentration of 6.96 μM and stored at -20 °C prior to use. The calpain I assay was conducted with 235 μl of a solution of 50 mM Hepes, 0.5 M CaCl₂, 0.5 M cysteine, at pH 7.5 (calpain I buffer), 6.5 μl of Sue-Leu-Tyr-AMC substrate solution (50 mM) in DMSO, 4.2 μl of stock inhibitor solution (10 mM for both PhPr-Leu-ALys-CH=CHCO2Et and PhPr-Leu-AOmr-CH=CHCO2Et) in DMSO, and 4.2 μl of the enzyme solution (6.96 μM) at 23 °C. The enzymatic activity was monitored by following the change in fluorescence for 20 min at 465 nm. Neither PhPr-Leu-ALys-CH=CHCO2Et nor PhPr-Leu-AOmr-CH=CHCO2Et showed any activity towards calpain I when compared to the control.

Inhibition Kinetics-Reversible Inhibition. Trypsin was purchased from Sigma Chemical Co. (St. Louis, MO) as a solid which was dissolved in a solution of 1 mM HCl at a concentration of 1 μM, kept at 0 °C, and used immediately. The trypsin assay was conducted with 224 μl of a solution of 0.1 M Hepes, 0.01 M CaCl₂, at pH 7.5 (trypsin buffer), 9 μl of Z-Phe-Arg-AMC substrate solution (1 mM, 2 mM, and 4 mM stock) in DMSO, 4.2 μl of inhibitor solution (1.25 mM, 2.5 mM, 5 mM, 7.5 mM, and 10 mM for PhPr-Leu-ALys-CH=CHCO2Et, and 2.5 mM, 5 mM, and 7.5 mM for PhPr-Leu-AOmr-CH=CHCO2Et) in DMSO, and 4.2 μl of the trypsin stock solution (1 μM) at 23 °C. The
enzymatic activity was monitored by following the change in fluorescence at 465 nm. The enzymatic assay was followed for 10 min with the 2.5 mM stock inhibitor solution and 1 mM stock substrate solution for both PhPr-Leu-ALys-CH=CHCO₂Et and PhPr-Leu-AOmn-CH=CHCO₂Et. The hydrolysis rate of the substrate by trypsin when combined with PhPr-Leu-ALys-CH=CHCO₂Et was linear over 10 min, but lower than the control rate of hydrolysis of the substrate, suggesting that it was inhibiting reversibly. The assay times were dropped to 3 min. Trypsin was assayed with PhPr-Leu-ALys-CH=CHCO₂Et at five concentrations (1.25 mM, 2.5 mM, 5 mM, 7.5 mM, 10 mM stock solutions) with each substrate concentration (1 mM, 2 mM, 4 mM stock solutions) as described above. The hydrolysis rate of the substrate by trypsin when combined with PhPr-Leu-AOmn-CH=CHCO₂Et was linear over 10 min, but the equivalent to the control rate of hydrolysis of the substrate, suggesting that it was not inhibiting trypsin. The assay was repeated at the 5 mM and 7.5 mM concentrations if PhPr-Leu-AOmn-CH=CHCO₂Et. The results were the same. It was concluded that PhPr-Leu-AOmn-CH=CHCO₂Et did not inhibit trypsin. The data for PhPr-Leu-ALys-CH=CHCO₂Et with trypsin was processed as a competitive reversible inhibitor by a Dixon plot. PhPr-Leu-AOmn-CH=CHCO₂Et showed no inhibitory activity towards trypsin when compared to the control.

**Inhibition Kinetics—Processing of Data.** In the clostripain, gingipains K and R, and cathepsin B assays, in which the ratio of inhibitor to enzyme concentrations was 10:1 or greater, the enzymatic reaction was assumed to be pseudo-first order. The inactivation constant ($k_{obs}$) can be obtained from the following equation

$$\ln \left( \frac{n}{n_0} \right) = -k_{obs} \cdot t$$

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where \( v_1 \) is the rate of hydrolysis of the substrate after the inhibitor is added, and \( v_0 \) is the rate of hydrolysis of the substrate of the control (no inhibitor). The \( k_{obs} \) can be found from the slope of a plot of \( \ln (v/v_0) \) versus time. The \( k_{obs} \) for PhPr-Leu-\( \text{AOrn-CH}=\text{CHCO}_2\text{Et} \) with clostripain was determined from five data points over two half-lives. The assays were run in triplicate. The \( k_{obs} \) for PhPr-Leu-\( \text{AOrn-CH}=\text{CHCO}_2\text{Et} \) with gingipain K was determined from four data points over two half-lives. The assays were run in triplicate. The \( k_{obs} \) for PhPr-Leu-\( \text{Alys-CH}=\text{CHCO}_2\text{Et} \) with gingipain K was determined from four data points over two half-lives. The assays were run in quadruplicate. The \( k_{obs} \) for PhPr-Leu-\( \text{AOrn-CH}=\text{CHCO}_2\text{Et} \) with gingipain R was determined from four data points over two half-lives. The assays were run in duplicate. The \( k_{obs} \) for PhPr-Leu-\( \text{Alys-CH}=\text{CHCO}_2\text{Et} \) with gingipain R was determined from four or five data points over two half-lives. The assays were run in duplicate. The \( k_{obs} \) for PhPr-Leu-\( \text{AOrn-CH}=\text{CHCO}_2\text{Et} \) with cathepsin B was determined from five data points over three half-lives. No duplicate assays were run. The \( k_{obs} \) for PhPr-Leu-\( \text{Alys-CH}=\text{CHCO}_2\text{Et} \) with cathepsin B was determined from six data points over three half-lives. No duplicate assays were run. The correlation coefficients for the inhibition plots were generally greater than 0.98, but never lower than 0.92. Control experiments were performed as the enzymatic assays listed above for each enzyme, but with an appropriate amount of DMSO in place of the inhibitor solution. The substrate hydrolysis rates of the controls were observed for 15 to 45 min, depending on the enzyme. Over this time period, the hydrolysis rates were stable for each enzyme. The substrate hydrolysis rate for the control was used as the \( v_0 \) for the calculations of the inhibition rate constants.
In the clostripain assay with PhPr-Leu-ALys-CH=CHCO₂Et, in which the inhibitor to enzyme ratio was less than 10:1, second order kinetic analysis was necessary to interpret the data. The inactivation constant \( k_{2nd} \) can be obtained from the equation

\[
\ln \left( \frac{[E]/[E]₀}{[I]/[I]₀} \right) = (\ln I) [I]₀ k_{2nd} \cdot t
\]

where \([E]\) is the concentration of the enzyme as it changes over the course of the assay, \([E]₀\) is the initial concentration of the enzyme, \([I]\) is the concentration of the inhibitor as it changes over the course of the assay, and \([I]₀\) is the initial concentration of the inhibitor. The \( k_{2nd} \) was obtained from the slope of a plot of \( \ln([E]/[E]₀)/(I)/[I]₀) \) versus time. The \( k_{2nd} \) for PhPr-Leu-ALys-CH=CHCO₂Et with clostripain was determined from at least six time points over three half-lives. The assays were run in triplicate. The correlation coefficients for the plots were between 0.98 and 0.96. A control experiment was performed as the enzymatic assay listed above, but with an appropriate amount of DMSO in place of the inhibitor solution. The control substrate hydrolysis rate was observed for 35 min. Over this time period, the hydrolysis rate was stable.

In the trypsin assay with PhPr-Leu-ALys-CH=CHCO₂Et, in which the inhibitor inactivated the enzyme as a competitive reversible inhibitor, pseudo-first order reverse inhibitor kinetic analysis via a Dixon plot was necessary to interpret the data. A competitive inhibitor is one that binds to the same site in the enzyme as the substrate, but forms a non-productive complex. This means that enzyme can not hydrolyze the inhibitor, nor can it form a covalent bond, and the only possible outcome is for the inhibitor to be released back into the solution. The inhibition constant, \( K_i \), is an equilibrium constant between the enzyme-inhibitor complex, and free enzyme and inhibitor or
\[ K_i = [E][I]/[EI] \]

where [E] is the free enzyme concentration in the solution, [I] is the free inhibitor concentration in the solution, and [EI] is the enzyme-inhibitor complex in the solution.

The value of \( K_i \) can be approximated by an equation presented by Dixon (Dixon, 1953)

\[
\frac{1}{v} = \frac{1}{V_s} + \frac{K_m}{V_s} \left( 1 + \frac{[I]}{K_i} \right) \frac{1}{[S]}
\]

which represents the reciprocal of the Michaelis equation for a competitive system

\[
v = \frac{V_s}{K_m(1+[I]/K_i) + [S]}
\]

where \( v \) is the reaction velocity, \( K_m \) is the Michaelis constant, \([S]\) is the substrate concentration in solution, and \( V_s \) is the maximum velocity obtained at high substrate concentrations. A plot of \( 1/v \) versus \([I]\) at several different substrate concentrations gives straight lines that cross where \([I] = -K_i\). This can be seen by setting the equations of the lines for two different substrate concentrations equal to each other, or

\[
\left( 1 + \frac{[I]}{K_i} \right) \frac{1}{[S_1]} = \left( 1 + \frac{[I]}{K_i} \right) \frac{1}{[S_2]}
\]

where \([S_1]\) and \([S_2]\) are the two different substrate concentrations for the same enzymatic assay. The only way this equation can be true is if \([S_1] = [S_2]\), which obviously is not the case, or if \([I] = -K_i\). The \( K_i \) for PhPr-Leu-Al-ys-CH=CHCO_2Et with trypsin was determined from three lines at three different substrate concentrations, each containing five inhibitor concentrations. To find the value of \( K_i \), from the \( x \)-axis, the equation of each line was set equal to each of the other two lines and solved for \( x \). This gave three values, which were averaged to give the \( K_i \) listed in Table 2.1 for trypsin and PhPr-Leu-
ALys-CH=CHCO₂Et. The correlation coefficients for the plots were between 0.98 and 0.96. The assays were run in duplicate and a control experiment was performed as the enzymatic assay listed above, but with an appropriate amount of DMSO in place of the inhibitor solution. The control substrate hydrolysis rate was observed for 10 min. Over this time period, the hydrolysis rate was stable.

**Inhibition Kinetics-Stability of the Enzyme-Inhibitor Complex.** To determine whether there was any recovery of activity by either gingipain K or clostripain after they were inhibited with enzyme, the enzymatic assays were monitored for 1-2 hours. The enzymatic assays were run with incubation kinetics as listed above for each enzyme. However, the aliquots from the incubation solution were taken much further apart over a much longer time frame. The clostripain began to regain activity after about 15 to 20 min with the PhPr-Leu-Aom-CH=CHCO₂Et. However, even after 2 hr, clostripain incubated with PhPr-Leu-ALys-CH=CHCO₂Et had regained none of its former activity, but was still hydrolyzing substrate at the same rate. A control was also run containing no inhibitor, which continued hydrolyzing substrate at a consistent rate throughout this extended assay period. This suggested that the aza-Om derivative did not bind as tightly into the active site of clostripain as the aza-Lys derivative. Gingipain K did not regain any of its former activity after 1 hour when incubated with either PhPr-Leu-ALys-CH=CHCO₂Et or PhPr-Leu-Aom-CH=CHCO₂Et.

**Inhibition Kinetics-Reaction of Inhibitors with DTT.** For the inactivation rates of the inhibitors with clostripain and gingipain K, with which the inhibitors proved to be especially effective, the rate of degradation of the inhibitor due to its interaction with DTT was determined. A UV/Vis wavelength scan of PhPr-Leu-ALys-CH=CHCO₂Et and
PhPr-Leu-AOmr-CH=CHCO₂Et revealed that the double bond of the reactive moiety could be observed at 250 nm. To measure the rate of degradation of the inhibitor in the DTT buffer, a solution containing the inhibitor and DTT buffer were incubated together. The concentrations used were identical to those used in the inhibition assays. The solution was monitored at 250 nm until no further loss of absorbance was detected (usually three to twelve minutes). All assays were run in duplicate. Background absorbance was determined by using the assay buffer, but replacing the inhibitor with DMSO. Control experiments were run as described above, except the buffer used lacked DTT. With no DTT present, no degradation of the inhibitor was observed over 45 min. The same assays were run with cysteine and 2-mercaptoethanol replacing the DTT, and their rates were observed.

The ratio of DTT to inhibitor concentrations was 10:1, so the rate of degradation \( k_d \) of the inhibitor by DTT could be determined using pseudo-first order kinetics. The value of \( k_d \) was determined by plotting the \( \ln ([I]) \) versus time, where \([I]\) is the concentration of unreacted PhPr-Leu-ALys-CH=CHCO₂Et or PhPr-Leu-AOmr-CH=CHCO₂Et in the well at various times. The observed change in absorbance was converted to concentration using the Beer-Lambert law. The extinction coefficient for each inhibitor was determined in each enzyme buffer solution by measuring the absorbance of five known concentrations of the inhibitor in buffer solution (less DTT) at 250 nm. For each inhibitor in each buffer solution, the average of the five extinction coefficients was calculated. The extinction coefficient for PhPr-Leu-ALys-CH=CHCO₂Et in the gingipain K buffer was 4,358 M⁻¹cm⁻¹, and in the clostripain buffer
was 4,250 M⁻¹cm⁻¹. The extinction coefficient for PhPr-Leu-\text{AOm}-\text{CH}=\text{CHCO}_2\text{Et} in the
gingipain K buffer was 4,802 M⁻¹cm⁻¹, and in the clostripain buffer was 4,706 M⁻¹cm⁻¹.

The degradation of the inhibitors by DTT was also monitored by a wavelength
scan (190-380 nm) until no further change in absorbance at 250 nm was observed.
Although the degradation of the inhibitors had gone to completion, the absorbance did
not reach zero at 250 nm. This suggested that the absorbance at this wavelength was not
entirely due to the double bond of the reactive moiety of the inhibitors. The extinction
coefficients listed above do not reflect this. To compensate for the difference, the change
in extinction coefficient ($\Delta\varepsilon_{250}$) between 100% inhibitor present in the incubation mixture
and no inhibitor present in the incubation mixture was determined. The $\Delta\varepsilon_{250}$ for PhPr-
Leu-\text{ALys}-\text{CH}=\text{CHCO}_2\text{Et} in the gingipain K buffer was 3,093 M⁻¹cm⁻¹, and in the
clostripain buffer was 3,207 M⁻¹cm⁻¹. The $\Delta\varepsilon_{250}$ for PhPr-Leu-\text{AOm}-\text{CH}=\text{CHCO}_2\text{Et}
in the gingipain K buffer was 2,929 M⁻¹cm⁻¹, and in the clostripain buffer was 3,591
M⁻¹cm⁻¹. These $\Delta\varepsilon_{250}$ values, rather than the previously calculated extinction coefficients,
were used to convert the measured absorbances of PhPr-Leu-\text{ALys}-\text{CH}=\text{CHCO}_2\text{Et} or
PhPr-Leu-\text{AOm}-\text{CH}=\text{CHCO}_2\text{Et} to their respective concentrations in the DTT degradation
assays.

**Inhibition Kinetics-Unstable Inhibitors.** Once determined, the $k_d$ value can be
placed into the equation first discussed by Purdie and Heggie (Purdie and Heggie, 1970)
and by Ashani \textit{et al.} (Ashani \textit{et al.}, 1972), and simplified by Topham (Topham, 1985)
which deals with unstable inhibitors and their hydrolysis by the enzymes they are trying
to inhibit. The same rate expressions can be applied here, where an inhibitor is being
degraded by a component of the buffer solution while simultaneously inhibiting the

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enzyme. The ratio of inhibitor to enzyme, as well as the DTT to inhibitor, must be 10:1 for these equations to be valid. The equation is

$$\ln \left( \frac{v}{v_0} \right) = -k_i [I]_0 \cdot \left[ 1 - \left( 1 - e^{-k_d t} \right) \right]$$

where $v$ is the rate of hydrolysis of the substrate after the inhibitor is added at time $t$, $v_0$ is the rate of hydrolysis of the substrate of the control (no inhibitor), $[I]_0$ is the initial concentration of inhibitor, $k_d$ is the degradation rate of the inhibitor by DTT in the buffer, and $k_i$ is the inactivation constant of the inhibitor taking into account the rate of inhibitor degradation. This equation can be further simplified by replacing the bracketed term with $t$ to yield

$$\ln \left( \frac{v}{v_0} \right) = -k_i [I]_0 \cdot t$$

This equation is analogous to the equation that describes irreversible inhibition by stable inhibitors (Aldridge, 1950; Rakitzis, 1984)

$$\ln \left( \frac{v}{v_0} \right) = -k_{obs} [I]_0 \cdot t$$

A plot of $\ln \left( \frac{v}{v_0} \right)$ versus $t$ yields $k_i$, the actual rate of inactivation of the enzyme by the inhibitor if it was not being degraded by DTT in the buffer solution. The inactivation rates for clostripain and gingipain K listed in Table 2.1 are those with the DTT degradation correction already applied except for that with PhPr-Leu-ALys-CH=CH-CO$_2$Et and clostripain. The conditions under which this enzymatic assay was performed, in which the PhPr-Leu-ALys-CH=CH-CO$_2$Et and clostripain were in 1:1 ratio, forced the data to be calculated with second order kinetics. Therefore, no rate of inactivation of clostripain by PhPr-Leu-ALys-CH=CH-CO$_2$Et with DTT degradation correction was determined.
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