DESIGN, SYNTHESIS, AND EVALUATION OF IRREVERSIBLE PEPTIDYL INHIBITORS FOR CLAN CA AND CLAN CD CYSTEINE PROTEASES

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DESIGN, SYNTHESIS, AND EVALUATION OF IRREVERSIBLE PEPTIDYL
INHIBITORS FOR CLAN CA AND CLAN CD CYSTEINE PROTEASES

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DEDICATION

To my parents, Dagmar and Helmut Götz.

To my grandparents, Margot and Johann Walch.

To Colin Heyes, my future husband.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

AA	amino acid residue
AAsn	aza-asparagine
AAsp	aza-aspartic acid
Ac	acetyl
Adp	adipic acid
Ala	alanine
AMC	7-amino-4-methylcoumarin
Arg	arginine
Asn	asparagine
AS	allyl sulfone
Asp	aspartic acid
Boc	*tert*-butoxycarbonyl
Brij	polyoxyethylenelaurylether
Bzl	benzyl, CH_2Ph
^{13}C NMR	carbon nuclear magnetic resonance
Cbz	benzyloxycarbonyl
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
Cys	cysteine
DEAD	diethyl azodicarboxylate
DCC	1,3-dicyclohexylcarbodiimide
DCU	1,3-dicyclohexylurea

DMAP	4-dimethylaminopyridine
DMF	*N,N*-dimethylformamide
DMSO	dimethylsulfoxide
DMSO-d$_6$	dimthylsulfoxide-d$_6$ deuterated
DPPI	dipeptidyl peptidase I
DTT	dithiothreitol
E-64	L-*trans*-epoxysuccinyl-leucylamido(4-guanidino)butane
EDC	1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride
EDTA	ethylenediaminetetraacetic acid
EP	epoxide
Eps	epoxysuccinyl
ESI-MS	electrospray ionization mass spectrometry
Et$_3$N	triethylamine
EtOAc	ethyl acetate
FAB	fast atom bombardment
Fla	fluoresceine amine
FMK	fluoromethyl ketone
FT-IR	Fourier transform infrared spectroscopy
Fum	fumarate
Gln	glutamine
Glu	glutamic acid
Gly	glycine
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid

His	histidine
HOBt	*N*-hydroxybenzotriazole
Hph	homophenylalanine
HRMS	high resolution mass spectrometry
^{1}H-NMR	proton nuclear magnetic resonance
I	inhibitor
IC	inhibitory concentration
IR	infrared spectroscopy
IBCF	isobutyl chloroformate
Ile	isoleucine
k	rate constant
k_{cat}	catalytic constant (s^{-1})
Ki	inhibition constant
K_m	Michaelis constant (M)
k_{obs}	observed rate constant
Leu	leucine
Lys	lysine
M	molarity (moles/liter)
MeOH	methanol
Met	methionine
MHC	major histocompatability complex
MHz	megahertz
min	minutes

mM	millimolar
ml	milliliter
MS	mass spectrometry
NaAc	sodium acetate
NI	no inhibition
Nle	norleucine
NMM	*N*-methylmorpholine
Nva	norvaline
OBzl	benzyloxy
Orn	ornithine
Pd	palladium
Phe	phenylalanine
PhPr	phenyl propyl carbonyl
PLP	pyridoxal phosphate
*p*Na	*p*-nitroanilide
Pro	proline
ppm	parts per million
RFU	relative fluorescence unit
s	second
SAR	structure activity relationships
Ser	serine
SmAE	*Schistosoma mansoni* asparaginyl endopeptidase
Sm32	*Schistosoma mansoni* asparaginyl endopeptidase

S_N2	secondary nucleophilic substitution
Suc	succinyl
t	time
TFA	trifluoroacetic acid
THF	tetrahydrofuran
Thr	threonine
TLC	thin layer chromatography
Trp	tryptophan
Tyr	tyrosine
UV	ultraviolet
v	velocity
Val	valine
VS	vinyl sulfone

SUMMARY

Cysteine proteases are a class of proteolytic enzymes, which are involved in a series of metabolic and catabolic processes, such as protein turnover, digestion, blood coagulation, apoptosis, fertilization and cell differentiation, and the immune response system. The development of novel potent and selective inhibitors for cysteine proteases has therefore gained increasing attention among medicinal chemists. In this thesis we have reported the design, synthesis, and evaluation of several peptidyl inhibitors for clan CA and clan CD cysteine proteases.

We have continued the investigation of dipeptidyl vinyl sulfones as potent and selective inhibitors for dipeptidyl peptidase I (DPPI), a lysosomal cysteine protease, which is involved in the processing of intracellular proteases, such as granzymes. We have found that DPPI tolerates negatively charged amino acid residues in the P2 position with inhibition rates of 7,600 $M^{-1}s^{-1}$. Dipeptidyl vinyl sulfones with positively charged amino acid residues at the P1 position, however, do not inhibit DPPI at all.

A second project focused on the epoxidation of the double bond of the vinyl sulfone moiety of the dipeptidyl vinyl sulfones. Instead of epoxidizing the double bond, we found that an isomerization had occurred. The newly formed compounds were determined to be allyl sulfones. We tested this new class of inhibitors with clan CA proteases and obtained inhibition rates of 560 $M^{-1}s^{-1}$ for Cbz-Leu-Phe-AS-Ph with calpain I.

Two new classes of compounds for the clan CD protease *S. mansoni* legumain were designed, synthesized, and evaluated. Aza-peptidyl epoxides were found to be

potent and selective inhibitors of *S. mansoni* legumain with IC_{50}'s as low as 45 nM. Aza-peptide Michael acceptors were derived from the aza-peptide epoxide design and synthesized in an analogous fashion. The aza-peptide Michael acceptors inhibited *S. mansoni* legumain with even lower IC_{50}'s, as low as 10 nM. However, the aza-peptide Michael acceptors react with thioalkylating agents contained in the buffer, such as DTT. The rates of degradation were determined spectroscopically, and half-lives of 3 to 20 minutes were measured. The degradation was also monitored in an ^{1}H NMR study, and the site of attack of the thiol group on the double bond could be observed. This observation gave us some insights into the enzymatic mechanism and lead us to conclude that the carbon of the double bond warhead which is closest to the P1 position is the point of attack for the legumain active site cysteine thiol.

CHAPTER 1

BACKGROUND

PROTEASES

Proteases are proteolytic enzymes, which catalyze the hydrolysis of peptide bonds. Proteases are found in plants, bacteria, protozoa, fungi, and mammals, and are essential for viral replication, and are involved in a series of essential metabolic and catabolic processes, such as protein turnover, digestion, blood coagulation, apoptosis, fertilization and cell differentiation, and the immune response system. However proteases are involved in a number of disease states and thus have gained tremendous attention as therapeutic targets with regards to viral and parasitic infections, stroke, cancer, Alzheimer's disease, neuronal cell death, and arthritis. They are designated either as endo- or exopeptidases, and cleave peptide bonds within a protein or peptide and remove amino acids from the N- or the C-terminus respectively. Depending on the catalytic residue responsible for peptide hydrolysis, the proteases have been divided into serine, cysteine, aspartic, and metallo- proteases[1]. However due to the growing number of proteases which are being discovered, a more in depth classification has become necessary. Barrett and Rawlings have therefore established a classification system, which organizes the various proteases within their individual catalytic category into evolutionary families and clans [2,3], and which forms a comprehensive and continuously expanding catalog of proteases: the MEROPS database. An individual protease is

1

assigned to a particular clan based on the three-dimensional structure, the arrangement of

catalytic residues in the polypeptide chains and limited similarities in amino acid

sequence around the catalytic amino acid residues. The name of the clan is derived from

the first letter of the catalytic type followed by a second arbitrary letter[2,3], giving rise to

clans SX, CX, AX and MX.

Proteases bind the substrate peptide chain along the active site cleft. The enzyme

subsites bind the peptide chain on either side of the site of hydrolysis, the scissile peptide

bond.   The subsites are specific for the particular side chains or the peptide backbone of

the peptide substrate. Berger and Schechter have developed a nomenclature for the

individual enzyme subsites and the complementary amino acid residues within the

peptide substrate (Figure 1.1)[4]. The subsites and the amino acid residues are assigned S1,

S2, etc. and P1, P2, etc. respectively towards the N-terminus of the substrate away from

the scissile peptide bond. In symmetry to the scissile bond the S1', S2', etc. subsites and

the P1', P2', etc. are directed towards the C-terminus.
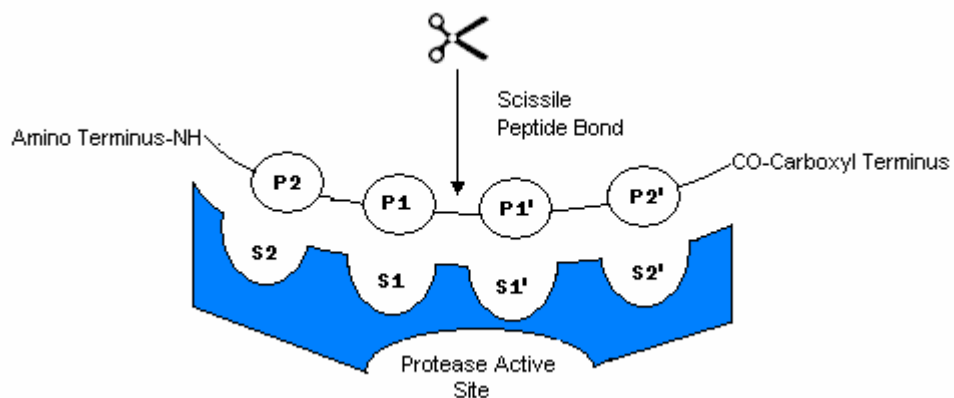
Figure 1.1. Nomenclature for Protease Subsite Specificity.

Cysteine proteases employ the sulfhydryl group of the side chain of a cysteine residue as the catalytic nucleophile. Together with the imidazole side chain of a histidine residue a catalytic dyad is formed between the thiol nucleophile and a proton donor/general base, as the imidazole group of the catalytic His polarizes and deprotonates the Cys sulphydryl group. Some families use a third residue to ensure proper orientation of the imidazolium ring of the catalytic His, thus forming a catalytic triad. In the attack of the active site cysteine on the scissile amide bond of the peptide backbone a tetrahedral intermediate is formed. This tetrahedral intermediate is stabilized by the oxyanion hole, which is found to be more rigid in serine proteases and more flexible in cysteine proteases. The intermediate collapses to release the first product and the resulting acyl enzyme then undergoes hydrolysis.

Aspartate Proteases

Metallo-Proteases

Serine Proteases

Cysteine Proteases

Clan CA
C1 Papain
   Cathepsins
   Cruzain
C2 Calpains

Clan CB
Human Rhinovirus
3C Protease

Clan CD
C14 Caspases
C13 Legumains
C25 Gingipains
C11 Clostripain
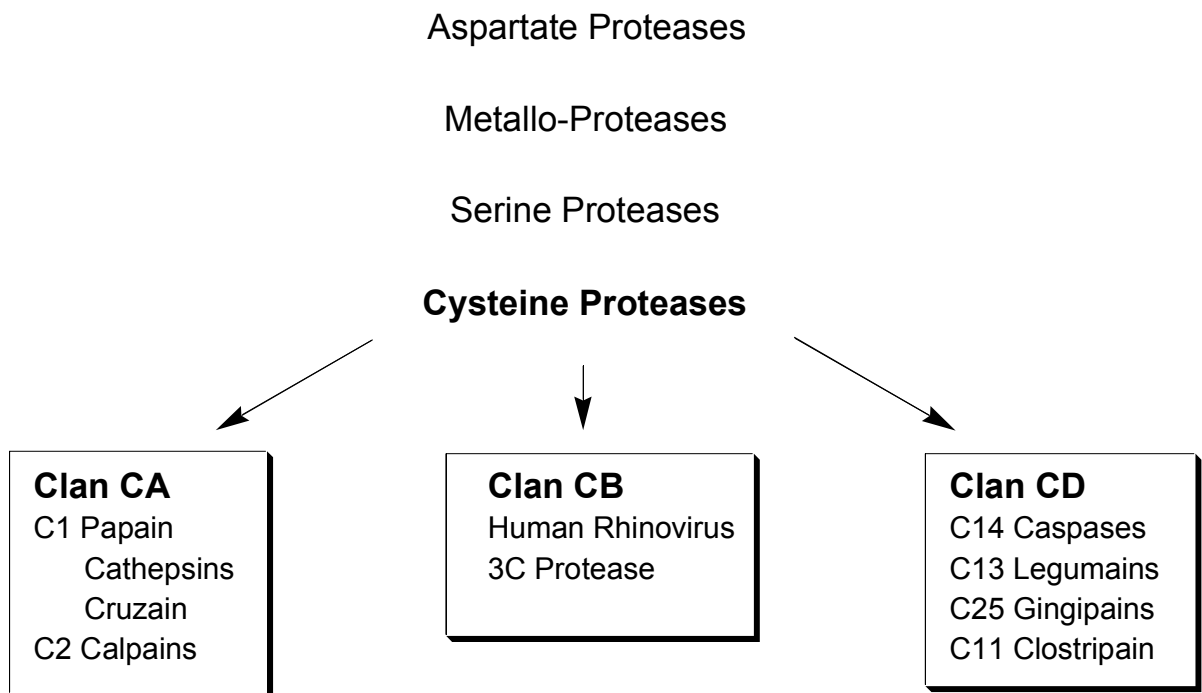
Figure 1.2. Classification of Cysteine Proteases into Families and Clans.

The majority of cysteine proteases, such as papain, calpains, cathepsins, and cruzain belong to the clan CA (Figure 1.2). According to the crystal structure of papain, Clan CA proteases are unique for their catalytic triad formed by Cys, His, and Asn. The oxyanion hole is created by a preceding Gln residue. Clan CA enzymes are inhibited by the natural product E-64 and cystatin. Clan CB is formed by a group of viral cysteine proteases, such as the human rhinovirus 3C protease, which are believed to have evolved from an ancestor also related to the serine protease chymotrypsin due to similarities in their three-dimensional structure and their amino acid sequence. Even though clan CD is the smallest of the clans, it contains some very important enzymes. Among them are caspases, legumains, gingipains, and clostripain. Clan CD enzymes are unique for their lack of inhibition by E-64, a natural inhibitor of cysteine proteases, and their specificity for the P1 amino acid residue. There are several other clans among the cysteine proteases, however, for our research we will only focus on clan CA and clan CD enzymes.

SUBSTRATE SPECIFICITY

The specificity for substrates varies greatly between the individual families and clans of cysteine proteases. The difference in electrostatic character and size of the enzyme subsites is responsible for the substrate specificity. In order to determine the nature of the enzyme subsites the preparation of a comprehensive substrate library is very useful, especially when the crystal structure of an enzyme is difficult to obtain.

Clan CA. *Papain.* Papain is a cysteine protease found in the latex of tropical papaya fruit. It is the most extensively studied protease, and also the first enzyme to have its crystal structure determined[5]. The substrate specificity of clan CA enzymes is primarily controlled by the S2 subsite. Papain is considered to have a very broad specificity compared to other clan CA proteases. Berger and Schechter have found that it can accommodate up to seven amino acid residues[6]. It prefers a bulky, non-polar side chain at P2 (e.g. Phe)[6], whereas the S1 subsite is less selective, but some preference for Arg and Lys over other residues such as Val has been observed[7]. The S' subsite specificities of papain are very broad[8]. The oxyanion hole is formed by the Cys 25 backbone and the Asn-19 side chain.

Cathepsin B. Cathepsin B is a lysosomal cysteine protease, which was first identified by its ability in cattle spleen to deamidate Bz-Arg-NH$_2$[9]. It possesses both endopeptidase and exopeptidase activities. The substrate specificity of cathepsin B is like papain also controlled by the restrictions of the S2 subsite. Unlike papain however, the location of the Glu 245 residue within the S2 subsite, also allows an arginine side chain at the P2 position in addition to large hydrophobic residues[10]. The S3 subsite prefers large hydrophobic residues and the S1 subsite a small residue or Arg. On the P1' side large hydrophobic residues were found to be optimal in cathepsin B substrates.

Dipeptidyl Peptidase I. Dipeptidyl peptidase I (DPPI) is a clan CA exopeptidase, which is found in the granules of cytotoxic lymphocytes, where it cleaves dipeptides from the N-termini of various zymogens in order to activate them. Its crystal structure was recently determined by Turk *et al*[11]. DPPI shows an absolute requirement for a free N-terminus because of its N-terminal Asp 1, which locks the substrate in position, as will be

further described in chapter 2. It prefers dipeptides over tripeptides and does not tolerate basic residues at the P2 position. The optimal AMC (4-aminomethyl coumarin) assay substrates used are free N-terminal dipeptides, such as Gly-Phe or Gly-Arg[12].

Calpains. Calpains are a unique familiy of cysteine proteases within the clan CA, as they require calcium for their activation. Their activity was first described as a calcium dependent neutral protease activity in rat brain by Guroff[13]. They are located in the cytosol of mammalian cells and play an important role in physiological processes that control the degradation of the cytoskeleton as well as hormone receptors[14,15]. The specificity of calpains is not fully understood, but it has been determined that hydrophobic residues (Tyr, Met, Leu, Val) as well as Arg are preferred by the S2 pocket[16]. Overall the substrate specificity is very restricted, and most oligopeptides are not cleaved.

Clan CD. The members of the clan CD enzymes distinguish themselves from the clan CA enzymes by their α/β protein fold. Unlike clan CA cysteine proteases, clan CD enzymes employ a catalytic dyad (His-Cys) for substrate hydrolysis. Their specificity is controlled by the S1 subsite, and varies for each enzyme within the clan.

Caspases. More than 15 enzymes belong to the only recently discovered family of caspases. They are involved in cytokine processing, apoptosis, and inflammation, where they either act directly on the particular process or are remotely involved by facilitating the maturation of procaspases. Caspases derive their name from their absolute specificity for an asparagine residue at the P1 position of their substrate (*c*ysteinyl *asp*artate specific prote*ase*). Their family can be further divided into groups according to the particular proteolytic function. Group I (1, 4, 5, 11, 12, 13, and 14)

includes caspases which are mediators of inflammatory processes. Group II (2, 3, and 7) contains caspases, which are late phase effectors of apoptosis. Group III (6, 8, 9, and 10) caspases are responsible for upstream processing of the effector caspases. In addition to the strict specificity of the S1 subsite for an Asp residue, caspases prefer four amino acid residues on the non-prime side of the scissile peptide bond. The S4 subsite provides additional control for the specificity within the family of the caspases. The optimal sequence for the group I caspases is Trp-Glu-His-Asp, Asp-Glu-X-Asp for group II, and Val- or Leu-Glu-X-Asp for group III. The oxyanion hole is formed by the backbone amide protons of Gly 238 and Cys 285.

Clostripain. Clostripain is a cysteine protease, which was first isolated in 1938 from the Gram-positive bacterium *Clostridium histolyticum*. This bacterium has been related to the gas gangrene syndrome[17]. Clostripain requires calcium ions and DTT for its activation. Like the other members of clan CD, clostripain is highly restricted in the S1 subsite. Similar to the serine protease trypsin, it cleaves only after Arg or Lys at the P1 position, with some preference for Arg over Lys[18]. Clostripain has a broad specificity on the P' side of the scissile bond, but will not accept Asp or Glu[19]. Unlike other peptidases it also accepts a proline residue in the P1' position. The dipeptide Z-Phe-Arg-AMC was determined to be the optimal substrate for enzymatic assays[20].

Gingipains. Gingipains were first described in 1984 as enzymes produced by *porphyromonas gingivalis*, the oral pathogen, which causes gingivitis. They require calcium ions and DTT for its activation along with a buffer medium at pH 8, similar to the periodontal cavity[21]. The active site is formed by a catalytic triad, unlike other clan CD proteases. According to the sequence homology and the crystal structure gingipains

7

show some relation to caspase-1 and caspase-3, but there are no similarities with any other cysteine proteases[22]. Gingipain K cleaves exclusively on the C-terminal side of lysine residues and has some preference for hydrophobic residues at P2 and P3, whereas gingipain R only accepts arginine residues in the P1 position and has little specificity for the P2 positon[21,23].

INHIBITOR DESIGN

Since the early 1970's inhibitors have been a useful tool in the characterization of binding sites, catalytic functional groups and transition-state geometries of cysteine proteases. In addition they can be used as active site titrants to determine a more accurate quantity of active enzyme. The design of highly selective inhibitors makes it possible to investigate the physiological function of a particular class of enzymes within an organism. Finally, cysteine protease inhibitors have great medicinal potential in the treatment of various diseases.

The design of irreversible inhibitors for proteases generally involves a peptide chain with a so-called warhead replacing the scissile peptide bond. The optimal peptide sequence for the inhibitor is derived from the best peptide substrate sequence, which can be determined through enzyme subsite mapping using a peptide library. There is considerable variation in the sequence specificity between proteases between the clans and even within a particular clan. As described earlier, the various enzyme subsites impose different requirements on their peptide substrates. Clan CD for example is highly specific for the residue in the P1 position, whereas clan CA is more selective for the P2

position. The length of the peptide portion also plays an important role for the specificity. It is therefore possible to design inhibitors specific for a particular enzyme. The inhibitor can then be used to investigate the physiological significance of the target enzyme.

The warhead consists of a reactive functionality that is attacked by the enzyme's catalytic nucleophile. A covalent bond is formed between the inhibitor and the catalytic residue and hence the enzyme is irreversibly inactivated. A variety of irreversible warheads have been developed so far. Powers *et al* have extensively reviewed the irreversible inhibitors for serine, cysteine, and threonine proteases reported up to date[24]. Alkylating agents include halomethyl ketones, diazomethyl ketones, acyloxymethyl ketones, epoxides, aziridines, vinyl sulfones, and azodicarboxamides. Among the acylating agents, aza-peptides, carbamates, acyl hydroxamates, β-lactams and a variety of heterocyclic inhibitors have been reported.

Once a peptide sequence and warhead are determined, a strategic SAR study is performed to optimize the parent compound in its inhibitory potency. With the rise of X-ray crystallography, the concept of the structure-based design has become a useful tool for the optimization of lead compounds. The binding of an inhibitor in the active site of an enzyme can be visualized with the crystal structure of an enzyme-inhibitor complex.

IRREVERSIBLE KINETICS

Irreversible inhibitors react with the target enzyme via an intermediate non-covalent enzyme-inhibitor complex E·I to form a covalent enzyme-inhibitor complex E-I.

$$E + I \xrightleftharpoons{\quad K_i \quad} E \bullet I \xrightarrow{\quad k_2 \quad} E\text{-}I,$$

where K_i is the dissociation constant for the intermediate complex and k_2 is the first-order

rate constant for the formation of E-I. Kitz and Wilson developed a method to determine

the values of K_i and k_2 by incubating enzyme and a large excess of inhibitor prior to

diluting the sample into a buffer solution containing a good substrate[25]. The residual

enzyme activity v_t can then be measured at time t of the incubation. This method is

referred to as the incubation method and described by the equation

$$\ln (v_t/v_0) = -k_{obs}t,$$

where v_0 is the initial rate of hydrolysis without the presence of the inhibitor and k_{obs} is

the pseudo-first-order rate constant for inactivation, which is described by

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

or in double reciprocal form:

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

$$K_i = [E][I]/[E\cdot I]$$

Pseudo-first-order kinetics are however only accurate, if the inhibitor

concentration is at least ten fold in excess of the enzyme concentration. If the inhibitor

concentration drops below, then second-order kinetics have to be applied using

$$k_{2nd}t = [1/(i - e)]\ln[e(i - x)/i(e - x)],$$

where i is the initial inhibitor concentration and e - x is the residual enzyme

concentration. The initial enzyme concentration is a requirement for second-order

kinetics.

For very fast inhibitors, where very low inhibitor concentrations are required, Tian and Tsou have developed an assay method, which monitors the substrate hydrolysis upon adding enzyme to a mixture of substrate and inhibitor[26]. This method is referred to as the progress curve method. It follows an exponential time course, where all the enzyme is inactivated and the product approaches a limited concentration $[P]_\infty$:

$$[P]_t = [P]_\infty(1 - e^{-tk_{obs}}),$$

which can be rewritten in logarithmic form

$$\ln ([P]_\infty - [P]_t) = \ln [P]_\infty - k_{obs}t$$

and values of k_{obs} can be obtained from the slope of the plots $\ln ([P]_\infty - [P]_t)$ versus t. However, this method is somewhat unreliable, since it requires an accurate estimate of $[P]_\infty$ and that parameter is only given by an asymptotic approach. However, the progress curve method is very practical for enzymes like calpain, which has low activity and therefore requires high enzyme concentrations. In addition the assays are more convenient and less time consuming.

The determination of the concentration of inhibitor required to decrease the activity of the enzyme by 50% (IC_{50}) is the standard method to initially screen a large number of inhibitors. However, the data only gives a crude comparison between the inhibitors, and does not allow to draw conclusions as to the reversibility of the inhibition mechanism. The incubation time is an important factor for IC_{50} assays of irreversible inhibitors, and can be used to establish a mathematical relationship between the pseudo-first order inactivation rate k_{obs} and IC_{50} values:

$$k_{obs} = \ln 2/t_{1/2} = 0.693/t_{assay}$$

$$k_{obs}/[I] = 0.693/(t_{assay} \times IC_{50})$$

For irreversible inhibition a longer incubation time is reflected in a lower IC_{50} value.

        The methods developed by Kitz and Wilson and Tian and Tsou efficiently analyze kinetic data. However not all mechanisms of inhibition follow this simple model, but require a more complex treatment of the assay data. Mechanism-based inactivators for example undergo a rearrangement before forming a covalent enzyme-inhibitor complex. Therefore kinetic data can also help to investigate a mechanism of inhibition.

REFERENCES

(1)   Barrett, A. *Ciba Foundation Symposium* **1980**, *75*, 1-13.

(2)   Barrett, A. J.; Rawlings, N. D. *Biol. Chem.* **2001**, *382*, 727-33.

(3)   Barrett, A. J.; Rawlings, N. D.; O'Brien, E. A. *J. Struct. Biol.* **2001**, *134*, 95-102.

(4)   Berger, A.; Schechter, I. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **1970**, *257*, 249-64.

(5)   Drenth, J.; Jansonius, J. N.; Koekoek, R.; Swen, H. M.; Wolthers, B. G. *Nature* **1968**, *218*, 929-32.

(6)   Schechter, I.; Berger, A. *Biochem. Biophys. Res. Commun.* **1967**, *27*, 157-162.

(7)   Kimmel, J. R.; Smith, E. L. *Adv. Enzymol. Relat. Subj. Biochem.* **1957**, *19*, 267-334.

(8)   Menard, R.; Carmona, E.; Plouffe, C.; Bromme, D.; Konishi, Y.; Lefebvre, J.; Storer, A. C. *FEBS Lett.* **1993**, *328*, 107-10.

(9)   Fruton, J. S.; Irving, G. W.; Bergmann, M. *J. Biol. Chem.* **1941**, *141*, 763-774.

(10)   Hasnain, S.; Hirama, T.; Huber, C. P.; Mason, P.; Mort, J. S. *J. Biol. Chem.* **1993**, *268*, 235-40.

(11)   Turk, D.; Janjic, V.; Stern, I.; Podobnik, M.; Lamba, D.; Dahl, S. W.; Lauritzen, C.; Pedersen, J.; Turk, V.; Turk, B. *Embo. J.* **2001**, *20*, 6570-82.

(12)   Kirschke, H.; Barrett, A. J.; Rawlings, N. D. *Protein Profile* **1995**, *2*, 1581-643.

(13)   Guroff, G. *J. Biol. Chem.* **1964**, *239*, 149-55.

(14)   Nixon, R. A. *Ann. N. Y. Acad. Sci.* **1989**, *568*, 198-208.

(15) Melloni, E.; Pontremoli, S. *Trends in Neurological Sciences* **1989**, *12*, 438-444.

(16) Mellgren, R. L.; Murachi, T. *Intracellular calcium-dependent proteolysis*; CRC Press: Boca Raton, FL, 1990.

(17) Kochalaty, W.; Weil, L.; Smith, L. *Biochem. J.* **1938**, *32*.

(18) Ogle, J. D.; Tytell, A. A. *Arch. Biochem. Biophys.* **1953**, *42*, 327-336.

(19) Ullmann, D.; Jakubke, H. D. *Eur. J. Biochem.* **1994**, *223*, 865-72.

(20) Kembhavi, A. A.; Buttle, D. J.; Rauber, P.; Barrett, A. J. *FEBS Lett.* **1991**, *283*, 277-80.

(21) Pike, R. N.; Potempa, J.; McGraw, W.; Coetzer, T. H.; Travis, J. *J. Bacteriol.* **1996**, *178*, 2876-82.

(22) Banbula, A.; Potempa, J.; Travis, J.; Bode, W.; Medrano, F. J. *Protein Sci.* **1998**, *7*, 1259-61.

(23) Mayrand, D.; Holt, S. C. *Microbiol. Rev.* **1988**, *52*, 134-52.

(24) Powers, J. C.; Asgian, J. L.; Ekici, O. D.; James, K. E. *Chem. Rev.* **2002**, *102*, 4639-750.

(25) Kitz, R.; Wilson, I. B. *J. Biol. Chem.* **1962**, *237*, 3245-3249.

(26) Tian, W.-X.; Tsou, C.-L. *Biochemistry* **1992**, *21*, 1028-1032.

CHAPTER 2

DESIGN AND EVALUATION OF INHIBITORS FOR

DIPEPTIDYL PEPTIDASE I

INTRODUCTION

Dipeptidyl peptidase I (DPPI, cathepsin C, EC 3.4.14.1) is a lysosomal cysteine protease. It can sequentially remove dipeptides from the N-termini of various peptides and proteins[1,2]. DPPI and other cysteine proteases such as cathepsin B, H, and L were found to degrade intracellular proteins[2]. DPPI is widely distributed in a variety of tissues[3] and is present in high levels in cytotoxic lymphocytes and mature myeloid cells[4]. DPPI and granzymes are found in the granules of cytotoxic lymphocytes[4] and DPPI is responsible for processing and activating progranzymes (Figure 2.1).

signal peptide———AA$_2$-AA$_1$———Ile-Ile-Gly-Gly--------CO$_2^-$

↓ signal peptidase

AA$_2$-AA$_1$———Ile-Ile-Gly-Gly-------- CO$_2^-$

progranzymes (sequence shown)
or zymogens of other serine proteases (procathepsin G)

↓ DPPI

Ile-Ile-Gly-Gly-------- CO$_2^-$
granzymes
cathepsin G
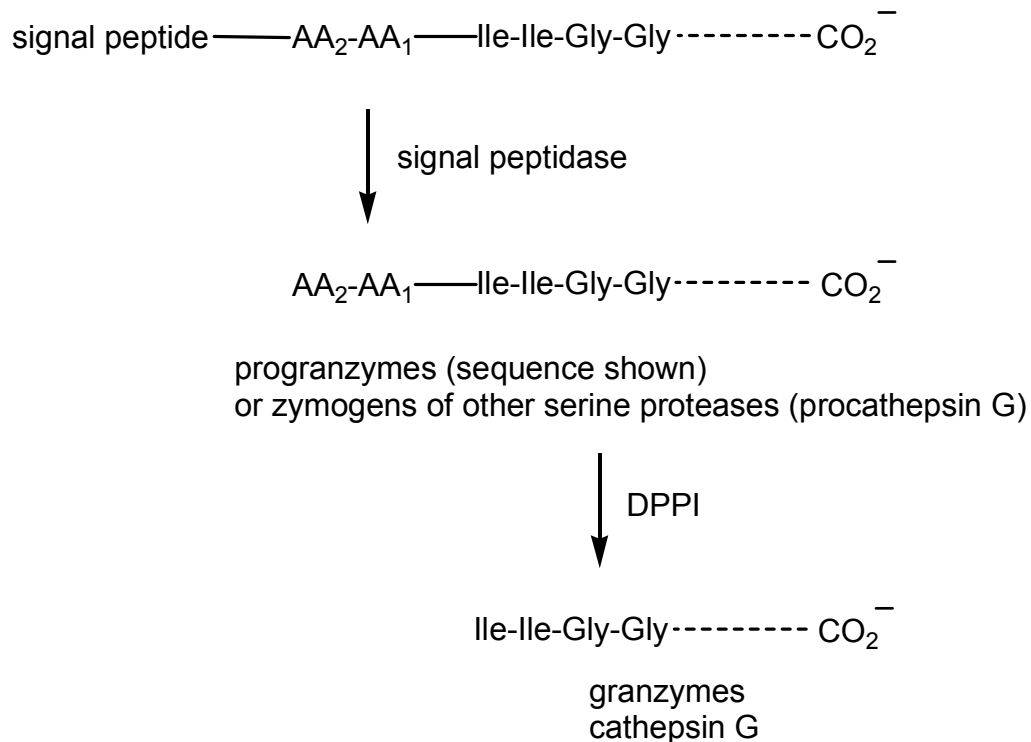
Figure 2.1. Processing of Progranzymes by DPPI to form Active Granzymes.

Granzymes, which are named for granule serine proteases, are found in the granules of activated cytotoxic T lymphocytes and natural killer cells. These enzymes may play a functional role in cellular lytic activity[5]. Lymphocytes cultured with a diazomethyl ketone DPPI inhibitor, Gly-PheCHN$_2$, lack granzyme A activity[4], have lower granzyme B activity[6] and reduced cytolytic function[7]. DPPI has been reported to activate the progranzymes A, B, and mast cell prochymase by removal of dipeptides from the N-terminus of their zymogens[8-10]. DPPI has been shown to process and activate progranzymes A and B *in vivo*[11].

DPPI has been purified from several species such as human, rat, bovine, porcine and goat[12-15]. DPPI is initially synthesized as a 55 kDa proenzyme and is converted to

the mature enzyme as a two chain form, a heavy chain of 25 kDa and a light chain of <10

kDa. These two chains have high similarity (30-40% sequence homology) to the heavy

and light chains of papain-like cysteine proteases[16]. Unlike other cysteine proteases,

which are monomeric proteins with a molecular mass of 20-30 kDa, DPPI exists as an

oligomeric structure with a molecular mass of 200 kDa[2]. It has been shown that human

DPPI consists of 4 identical subunits and each subunit is composed of three different

polypeptide chains, a heavy chain, a light chain, and a propeptide. The heavy chain and

the light chain are linked by disulfide bonds, and the pro region of the enzyme remains

bound to the mature enzyme. The regulation of processing and assembly of DPPI is still

unknown, but the x-ray structure of DPPI has recently been determined[17]. DPPI has four

active site clefts. The catalytic residues Cys 234 and His 381 are situated above the

oxyanion hole as expected. The S2 binding site has the shape of a deep pocket and is

formed by the side chains of Ile 429, Pro 279, Tyr 323 and Phe 278.   A chloride ion is

located on the bottom of this pocket.

In an effort to characterize the substrate specificity of DPPI, the Powers

laboratory has recently reported kinetic data for a series of dipeptide substrates for

DPPI[18]. It was found that the free amino group at the N-terminus of DPPI substrates

plays an important role. Turk *et al* have shown that the carboxylic acid group of Asp 1

locks this N-terminal amino group of the substrate into position. It was also found that

DPPI was unable to cleave substrates with a P1 Ile. The enzyme generally prefers large

hydrophobic or aromatic sidechains at the P1 residue, and small less bulky substituents at

the P2 position.

DPPI was inhibited by several general inhibitors of cysteine proteases[13] and by

E-64 slowly[19]. The most potent inhibitor of DPPI found up to date was Gly-PheCHN$_2$[20].

However, due to the high reactivity of the diazomethyl ketone group, more stable dipeptide inhibitors are needed.

Chih-Min Kam has previously synthesized a series of highly potent dipeptidyl vinyl sulfone inhibitors with the general formula AA_2-AA_1-VS-R in our laboratory. Similar to the synthetic substrates by Tran *et al*, the better inhibitors have large hydrophobic or aromatic residues at the P1 position, a free N-terminus and amino acids with small side chains at the P2 position.

Some of the vinyl sulfones were found non-toxic to cells and effective toward intracellular DPPI[6]. Thus, these vinyl sulfones are suitable for functional studies of DPPI.

Table 2.1. Inhibition of Intracellular DPPI in RNK-16 Cells by Dipeptide Vinyl Sulfones.

Inhibitors	%viability[a]	ID_{50} (μM)[b]
Gly-Phe-VS-Ph	47% (toxic)	NI
Ala-Phe-VS-Ph	77% (toxic)	15
Val-Phe-VS-Ph	>90%	6
Leu-Phe-VS-Ph	40% (toxic)	NI
Lys(Z)-Phe-VS-Ph	0% (toxic)	NI
Glu-Glu-VS-Ph	>80%	>250
Thr-Glu-VS-Ph	>90%	45
Ala-Hph-VS-Ph	>90%	0.1-1
Nva-Hph-VS-Ph	>90%	0.1-1

Ala-Lys(Fla-Adp)-VS-Ph	>90%	~100
Gly-Phe-VS-CH$_3$	>90%	21
Ala-Phe-VS-CH$_3$	ND	ND
Val-Phe-VS-CH$_3$	>90%	11
Leu-Phe-VS-CH$_3$	>90%	4.7
Boc-Gly-Phe-VS-Ph	61% (toxic)	330

[a]Viability was assessed by trypan blue exclusion after culture of RNK-16 cells with 100 μM or greater concentration of inhibitor for at least 20 hours.
[b]ID$_{50}$ was determined to be the concentration of inhibitor required to inhibit cellular DPPI by 50% after treatment of RNK-16 cells in culture for at least 20 hours and is representative of at least two experiments.

Dipeptide vinyl sulfones inhibited the intracellular DPPI of RNK-16 cells (Table 2.1)[6].

For example, Ala-Hph-VS-Ph, Nva-Hph-VS-Ph, Val-Phe-VS-Ph, and Leu-Phe-VS-CH$_3$

inhibited intracellular DPPI with ID$_{50}$'s of micromolar or sub-micromolar range.

The tolerance of the enzyme S2 subsite towards charged amino acid residues has

so far not been investigated. In order to explore the tolerance of the active site towards

charged amino acid residues in the P2 position of the dipeptidyl vinyl sulfone inhibitors

four vinyl sulfone inhibitors with Arg and Glu residues at P2 were synthesized and tested

for their potency with DPPI.

CHEMISTRY

Dipeptide vinyl sulfones (Figure 2.2) are prepared by coupling of Boc-AA-OH

with the hydrochloride salt of the amino acid vinyl sulfone[21]. Deblocking of the Boc

group or side chain protecting group of dipeptide vinyl sulfones with acid gives the

desired dipeptide vinyl sulfones. The amino acid vinyl sulfone derivative **6** is prepared from Boc-AA-OH via formation of the N,O-dimethylhydroxamate **1**, reduction to the aldehyde **2**, coupling with sulfonylphosphonate **4** using a Horner-Wittig reaction[22] and final removal of the Boc group with acid. Subsequent coupling with Boc-AA$_2$-OH and removal of the Boc group provided the dipeptidyl vinyl sulfone **7**.
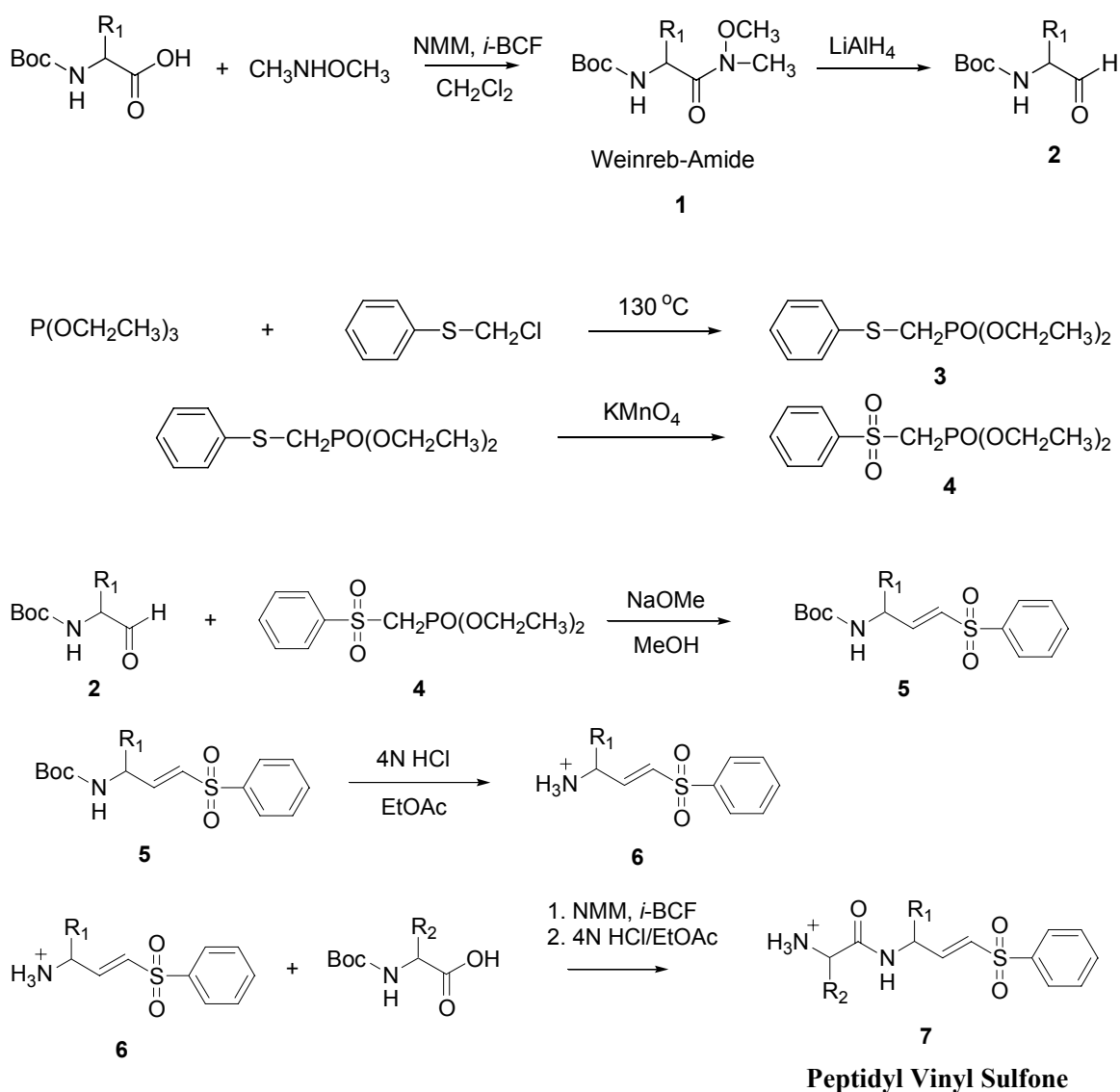
Figure 2.2. General Preparation of the Dipeptidyl Vinyl Sulfone.

RESULTS AND DISCUSSION

A variety of inhibitors including aldehydes[23,24], ketoesters, ketoamides[25,26], nitriles[27], diazomethyl ketones[20,28,29], halomethyl ketones[30-33], acyloxymethyl ketones[34-36], vinyl sulfones[21,37], and epoxysuccinyl derivatives[19,38,39] have been synthesized for cysteine proteases[40,41]. However, only a few synthetic inhibitors have been reported for DPPI[20,27]. Leupeptin and E-64 inhibit DPPI at high inhibitor concentration[19,40]. A reversible inhibitor Gly-NHCH(CH$_2$Ph)CN inhibits DPPI with a K_I value of 2.7 μM and Gly-Phe-VS-CH$_3$ inhibits DPPI with k_2/K_I of 56 M^{-1}s^{-1}[27]. The most effective inhibitor is Gly-Phe-CHN$_2$ with k_{obs}/[I] of 10^4 M^{-1}s^{-1}[20]. However the stability of the diazomethyl ketone functional group is limited due to its high reactivity. Therefore new types of inhibitors for DPPI are needed.

Inhibition Studies. Peptide vinyl sulfones are mechanism-based inhibitors for various cysteine proteases, the inhibitory potency of these compounds depends on their peptide sequences and the substituent on the vinyl sulfones[21,37]. The vinyl sulfones are quite selective toward cysteine proteases since they do not inhibit serine proteases. However, it has recently been reported that these compounds also react with the active site Thr of the catalytic β subunit of the proteasome[42]. The vinyl sulfone inhibited cysteine protease is quite stable, enzyme activity has not regained after a few days[21]. Most of the vinyl sulfones inhibit DPPI moderately and the inhibitory potency can be improved by varying the dipeptide sequence and substituent on the sulfones. The best inhibitors, Ala-Hph-VS-Ph and Nva-Hph-VS-Ph, were derived from the sequences of the best AMC substrates[18]. The vinyl sulfones containing a 4-chlorophenyl or methyl group,

21

as found by Chih-Min Kam, are less potent than those with a phenyl group indicating that the chloride on the phenyl group may interfere with the binding to the enzyme, and the methyl group lacks the interaction with the enzyme. Dipeptide vinyl sulfones with a large hydrophobic group such as Hph or Lys(Fla-Adp) at the P1 site inhibited DPPI more potently than other corresponding vinyl sulfones with a P1 Phe. The weak inhibition of the Lys derivative with a blocked side chain at the P2 site is consistent with the substrate studies confirming that DPPI does not prefer P2 bulky residues. The x-ray structure of DPPI shows that the S2 pocket is quite deep and can accommodate various hydrophobic residues[17]. However, it also indicates that the S1 site is located on the surface of the protein structure, thus various residues can fit in. Inhibition of DPPI by a series of dipeptide vinyl sulfones synthesized by Chih-Min Kam is shown in Table 2.2.

Table 2.2. Inhibition of Bovine Spleen DPPI by Dipeptide Vinyl Sulfones.

Inhibitors	[I] (μM)	$k_{obs}/[I]$ $(M^{-1}s^{-1})$ (k_2/K_I)
Gly-Phe-VS-Ph	83	150 ± 5
Ala-Phe-VS-Ph	17	430 ± 48
Val-Phe-VS-Ph	8.3	1600 ± 110
Leu-Phe-VS-Ph	8.3	1700 ± 110
Lys(Z)-Phe-VS-Ph	420	0.22 ± 0.02
Lys(Fla-Adp)-Phe-VS-Ph	420	0.67 ± 0.02
Ala-Hph-VS-Ph	0.004-0.043	$(2,000,000)[a]$
Nva-Hph-VS-Ph	0.004-0.043	$(890,000)[a]$
Ala-Lys(Fla-Adp)-VS-Ph	0.005-0.043	$(850,000)[a]$
Glu-Glu-VS-Ph	17	710 ± 36
Gly-Glu-VS-Ph	17	490 ± 19
Thr-Glu-VS-Ph	8.3	2900 ± 260
Gly-Phe-VS-CH$_3$	42	72 ± 4.0
Ala-Phe-VS-CH$_3$	42	450 ± 36
Val-Phe-VS-CH$_3$	8.3	340 ± 26
Leu-Phe-VS-CH$_3$	8.3	210 ± 18
Val-Phe-VS-Ph-Cl	42	8.7
Leu-Phe-VS-Ph-Cl	42	6.2
Boc-Gly-Phe-VS-Ph	420	NI[b]
Boc-Thr(OtBu)-Glu-VS-Ph	420	NI[b]

| Phe-VS-Ph | 420 | 32%[c] |

Most of these dipeptides which contain the activation sequence of granzymes (Gly-Glu, Glu-Glu, or Thr-Glu[43]) or Phe at the P1 site inhibit DPPI moderately with $k_{obs}/[I]$ values of 10^2-10^3 $M^{-1}s^{-1}$. The best inhibitor Ala-Hph-VS-Ph, which is derived from the best dipeptide AMC sequence, has a k_2/K_I value of 2,000,000 $M^{-1}s^{-1}$[18]. Two other dipeptides with an Ala-Lys(Fla-Adp) or Nva-Hph sequence are also potent inhibitors for DPPI with k_2/K_I values of (8.5-8.9) x 10^5 $M^{-1}s^{-1}$. The inhibition constants (k_2/K_I) of these three inhibitors were obtained by progress curve method instead of incubation method since DPPI was completely inhibited when the condition of incubation method was performed. Vinyl sulfones with a bulky blocking group at the P2 site such as Lys(Z) or Lys(Fla-Adp) showed very poor inhibition, which is consistent with the study of AMC substrates. Substitution of the phenyl group on the sulfones with a methyl or 4-chlorophenyl group decreased the inhibitory potency. The blocked dipeptide vinyl sulfones do not inhibit DPPI while the amino acid derivatives inhibit this enzyme weakly. Several dipeptide vinyl sulfones were also tested with other lysosomal cysteine proteases by Mrs. Chih-Min Kam (Table 2.3).

The best DPPI inhibitor Ala-Hph-VS-Ph inhibited DPPI more potently than cathepsins B, L, and H by at least over 6600 folds. Although Nva-Hph-VS-Ph inhibited DPPI potently, it also inhibited cathepsins L and H moderately. Other dipeptide vinyl sulfones also inhibited DPPI more selectively. The N-terminal blocked dipeptide vinyl

sulfones do not inhibit DPPI, indicating the importance of the free amino group at the N-terminus, which is also shown in the dipeptide substrates[18].

Table 2.3. Inhibition of Various Cysteine Proteases by Dipeptide Vinyl Sulfones.

Inhibitors	$k_{obs}/[I]$ $(M^{-1}s^{-1})$			
	DPPI[a]	Cat B[b]	Cat L[c]	Cat H[d]
Ala-Hph-VS-Ph	2,000,000[e]	58	304	96
Nva-Hph-VS-Ph	890,000[e]	23	1420	4700
Val-Phe-VS-Ph	1600	0.1	21	8.3
Gly-Phe-VS-CH$_3$	72	1.3	1.0	2.7
Val-Phe-VS-CH$_3$	340	0.3	21	NI[f]
Leu-Phe-VS-CH$_3$	210	NI	106	NI

[a]Inhibition constants were measured by incubation method in 50 mM NaAc, 30 mM NaCl, 1 mM EDTA, 1mM DTT, pH 5.5 buffer and at 23 ˚C. Gly-Phe-AMC (0.091 mM) was used as the substrate.
[b]Inhibition constants were measured in 0.1 M KHPO$_4$, 1.25 mM EDTA, 0.01% Brij, pH 6.0 buffer and at 23 ˚C. Z-Arg-Arg-AMC (0.4 mM) was used as the substrate.
[c]Inhibition constants were measured in 50 mM Mes, 2.5 mM EDTA, 1mM DTT, 0.01% Brij, pH 5.5 buffer and at 23 ˚C. Z-Phe-Arg-AMC (0.005 mM) was used as the substrate.
[d]Inhibition constants were measured in 50 mM Pipes, 2.5 mM EDTA, 1mM DTT, 0.01% Brij, pH 6.8 buffer and at 23 ˚C. Arg-AMC (0.25 mM) was used as the substrate.
[e]The k_2/K_I value was measured by progress curve method.
[f]No inhibition after 20 min incubation of enzyme with inhibitor in the buffer.

It was well established by Chih-Min Kam, that DPPI prefers a Hph or Phe residue at the P1 position. The tolerance of the S2 site towards charged residues could therefore be explored with dipeptidyl vinyl sulfones bearing a Hph or Phe in the P1 position and a Glu or Arg in the P2 position. The kinetic data showed that DPPI tolerates the negative charge of the Glu side chain, but does not improve the potency (Table 2.4). Clearly,

small, uncharged residues at the P2 position can be better accommodated by the S2 pocket. The positively charged Arg side chain has a detrimental effect on the potency. The complete lack of inhibition can most likely be attributed to the affinity of the negatively charged Asp 1 at the entry of the S2 pocket. We assume that the inhibitor does not even enter the active site, but merely binds to the entry of the pocket.

Table 2.4. Inhibition of DPPI by Dipeptidyl Vinyl Sulfones with a Charged P1 Amino Acid.

Inhibitors	[I] (μM)	$k_{obs/[I]}$ ($M^{-1}s^{-1}$)
Glu-Hph-VS-Ph	5	7680 ± 220
Glu-Phe-VS-Ph	156	95 ± 4
Arg-Phe-VS-Ph	420	NI[a]
Arg-Hph-VS-Ph	420	NI[a]

[a] NI = No inhibition after incubating for 1 hour.

Structure and Mechanism. The x-ray structure of DPPI indicates that the side chain carboxyl group of the N-terminal Asp 1 is close to the S2 site[17]. This carboxyl group may act as an anchor for the N-terminal amino group of dipeptide substrates or inhibitors. The proposed mechanism for the inhibition of DPPI by vinyl sulfones is shown in Figure 2.3[21].
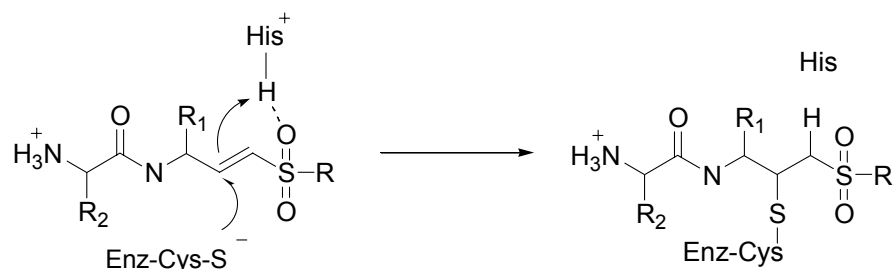
Figure 2.3. DPPI Mechanism of Inhibition by Dipeptidyl Vinyl Sulfones.

The thiol group of the active site Cys 234 can attack the carbon on the vinyl group to give a covalently linked enzyme-inhibitor derivative. The oxygen of the sulfonyl group interacts by hydrogen bonding with His 381 at the active site, which enhances the binding between the enzyme and inhibitor. According to Turk *et al.* the S2 site controls the binding with the two negative charges of Asp 1 at the entry of the pocket and the chloride ion situated at the bottom of the deep hydrophobic pocket[17]. This suggests that positively charged P2 residues do not even enter the pocket but merely bind to the Asp 1 at the entry of the pocket, which is in agreement with the kinetic data of Arg-Phe-VS-Ph as well as Arg-Hph-VS-Ph.

Stability of Vinyl Sulfones. The unblocked dipeptide inhibitors with a free N-terminal amine group have a tendency to form a stable seven-membered ring structure, therefore these dipeptides are less stable than their corresponding blocked dipeptides. The vinyl sulfone functional group is stable under acidic conditions, however it will decompose when exposed to nucleophiles in the neutral or basic buffer. For example, the unblocked dipeptide vinyl sulfones are quite stable in the pH 5.5 buffer ($t_{1/2} > 318$ min), where the enzymatic activities are measured, but decompose in the pH 7.5 buffer by buffer catalysis ($t_{1/2} = 7$-110 min) except for Ala-Lys(Fla-Adp)-VS-Ph (Table 2.6). At pH 7.5, the stability of these compounds was also affected by the size of the P2 amino

acid, for example the unblocked dipeptide with P2 Gly or Ala have a shorter half life than those with Val, Nva, or Leu. Therefore, the bulky group at P2 will hinder the cyclization of the amino group.

Table 2.6. Stability of Dipeptide Acyloxymethyl Ketones and Vinyl Sulfones.

Inhibitor	$t_{1/2}$ (min)		
	pH 7.5 50 mM Hepes (100 mM)	pH 7.5 50 mM Phosphate (100 mM)	pH 5.5 50 mM Acetate
Gly-Phe-CH$_2$OCOPh	13000		2200
Gly-Phe-VS-Ph	13 (8.4)	14 (8.5)	540
Ala-Phe-VS-Ph	31 (15)	37 (18)	1400
Val-Phe-VS-Ph	90 (42)	93 (50)	980
Leu-Phe-VS-Ph	60 (26)	62 (29)	1200
Glu-Glu-VS-Ph	36		723
Thr-Glu-VS-Ph	60 (33)	59 (24)	1000
Boc-Gly-Phe-VS-Ph	NR[a]		NR[b]
Gly-Phe-VS-Ph-4-Cl	7.3	7.8	480
Leu-Phe-VS-Ph-4-Cl	19	13	510
Boc-Gly-Phe-VS-Ph-4-Cl	NR[b]		NR[b]
Gly-Phe-VS-CH$_3$	50	53	580
Val-Phe-VS-CH$_3$	103		672

| Leu-Phe-VS-CH$_3$ | 97 | 110 | 1300 |

[a]No reaction in 24 hours.

[b]No reaction in 2 hours.

Cellular Efficacy of Vinyl Sulfones. Inhibition of intracellular DPPI by many vinyl sulfones was performed by the Hudig lab using RNK-16 cells (Table 2.1). The cellular efficacy of these compounds (ID$_{50}$) toward DPPI not only depends on their inhibitory potency toward the enzyme, but also on their ability to enter the cell and their stability in the cell medium. Six vinyl sulfones had an ID$_{50}$ of less than 25 μM, where Ala-Hph-VS-Ph and Nva-Hph-VS-Ph are the most potent inhibitors. This is due to their high inhibitory potency toward DPPI, although Ala-Hph-VS-Ph is less stable at either pH 7.5 or 5.5 when compared to others. Both Glu-Glu-VS-Ph and Thr-Glu-VS-Ph have moderate inhibition, but they contain a charged amino acid at pH 5.5. They are not good inhibitors of intracellular DPPI.

SUMMARY AND PERSPECTIVE

Comparison of the inhibition of DPPI by various inhibitors indicates that the vinyl sulfones with Ala-Hph or Nva-Hph are the best inhibitors for DPPI. These compounds are non-toxic to cells and also effective toward intracellular DPPI, thus they are suitable for functional studies of DPPI. Gly-Phe-CHN$_2$ is also a potent inhibitor of DPPI. However, due to the high reactivity of the diazomethyl ketone functional group, this compound is easily decomposed under the acidic conditions required for the measurement of cysteine protease activities. Dipeptide acyloxymethyl ketones are

limited by their stability and weak inhibition toward DPPI, although these compounds are effective and selective toward intracellular DPPI.

EXPERIMENTAL

Materials and Methods. DPPI from human spleen and rat mast cell lines were purified by a previous procedure[13]. DPPI from bovine spleen was obtained from Calbiochem, San Diego, CA. Materials were obtained from Acros, Bachem Bioscience Inc., or Sigma Aldrich and used without further purification. The purity of each compound was confirmed by TLC, ^{1}H NMR, MS, and elemental analysis. Chemical shifts are reported in ppm relative to an internal standard (trimethylsilane). TLC was performed on Sorbent Technologies (250 μm) silica gel plates. The ^{1}H NMR spectra were obtained on a Varian Gemini 300 MHz spectrometer. Electrospray ionization (ESI), fast-atom-bombardment (FAB) and high-resolution mass spectrometry were obtained using Micromass Quattro LC and VG Analytical 70-SE instruments. Elemental analysis was carried out by Atlantic Microlab Inc., Norcross, GA.

Enzyme Assay. Enzymatic hydrolysis rates of dipeptide AMC substrates by bovine spleen DPPI were measured in 50 mM NaOAc, 30 mM NaCl, pH 5.5 buffer containing 1 mM EDTA, 1 mM DTT and at 23 °C. The stock solution of substrate Gly-Phe-AMC (5 mM) was prepared in Me$_2$SO and stored at -20 °C. Enzymatic hydrolysis rates were measured fluorometrically (λ_{ex} = 360 nm, λ_{em} = 460 nm) using a Tecan fluorescence plate reader when 10 μL of an enzyme stock solution (0.22 - 2.2 μM) was added to a well containing 0.2 mL of buffer and 10 μl of substrate. Enzymatic activities of cathepsin B were measured in 0.1 M KHPO$_4$, 1.25 mM EDTA, 0.01% Brij, pH 6.0

buffer and at 23 °C. Z-Arg-Arg-AMC (0.4 mM) was used as the substrate. Enzymatic

activities of cathepsin L were measured in 50 mM Mes, 2.5 mM EDTA, 1mM DTT,

0.01% Brij, pH 5.5 buffer and at 23 °C. Z-Phe-Arg-AMC (0.005 mM) was used as the

substrate. Enzymatic activities of cathepsin H were measured in 50 mM Pipes, 2.5 mM

EDTA, 1mM DTT, 0.01% Brij, pH 6.8 buffer and at 23 °C. Arg-AMC (0.25 mM) was

used as the substrate.

Inhibition Kinetics: Incubation method. The inactivation reaction was initiated

at 23 °C by addition of a 25 μL (or 10 μL) aliquot of inhibitor in Me_2SO to 0.25 mL (or

0.1 mL) of buffered solution and 25 μL (or 10 μL) of enzyme (2.2 μM). Aliquots (10

μL) were withdrawn at various time intervals, diluted into 0.48 mL of buffer solution and

10 μL of 20 mM Gly-Phe-AMC (0.4 mM). Enzymatic hydrolysis rates were measured

fluorometrically using a Spex fluoremeter. When using the Tecan microplate reader,

aliquots (10 μl) were withdrawn and added to the well containing 0.2 mL of buffer

solution and 10 μL of 5 mM Gly-Phe-AMC. Inhibition of cathepsins B, L, and H were

measured similarly. First order inactivation rate constants (k_{obs}) were obtained from

plots of ln v_t/v_o vs time and had correlation coefficients greater than 0.98. Inactivation

rate constants were the average of duplicate experiments.

Diethyl Phenylsulfonylmethanephosphonate ($Ph-SO_2-CH_2-PO(OEt)_2$). A

mixture of chloromethyl phenyl sulfide (3.2 g, 20 mmole) and triethyl phosphite (3.3 g,

20 mmole) was heated at 130 - 140 °C for 5 h[22]. The resultant mixture was distilled

under reduced pressure, and the starting materials were distilled out. The oily residue was

diethyl phenylmercaptomethanephosphonate (yield 65%). ^{1}H-NMR ($CDCl_3$) 1.2-1.3 (t,

6H, 2 x CH_3), 3.1-3.2 (d, 2H, SO_2-CH_2), 4-4.2 (m, 4H, CH_2CH_3), 7.2-7.4 (m, 5H, Ph).

Diethyl phenylsulfonylmethanephosphonate was prepared by oxidation of diethyl phenylmercaptomethanephosphonate with potassium permanganate[22] (55% yield). ^{1}H-NMR (CDCl$_3$): 1.2-1.3 (t, 6H, 2 x CH$_3$), 3.7-3.8 (d, 2H, SO$_2$-CH$_2$), 4.1-4.2 (m, 4H, 2 x CH_2CH$_3$), 7.5-7.7 (m, 3H, Ph), 7.9-8.0 (d, 2H, Ph).   MS m/z 293 (M + 1).

Boc-Phe-H. Boc-Phe-N(OCH$_3$)CH$_3$ was prepared from Boc-Phe-OH and N,O-dimethylhydroxylamine hydrochloride using standard mixed anhydride coupling procedure (93% yield). ^{1}H-NMR (CDCl$_3$) δ 1.4 (s, 9H, Boc), 2.8-2.9 (m, 1H, CH$_2$-Phe), 3.0-3.1 (m, 1H, CH$_2$-Phe), 3.2 (s, 3H, N-CH$_3$), 3.6 (s, 3H, O-CH$_3$), 4.9 (m, 1H, α-C), 5.2 (b, 1H, NH), 7.1-7.3 (m, 5H, Ph). MS (FAB $^+$) m/z 309 (M + 1, 30 %), 253 (M – tBu + 1, 100 %). Reduction of Boc-Phe-N(OCH$_3$)CH$_3$ with lithium aluminum hydride according to a previously described method[44] gave Boc-Phe-H (88% yield). ^{1}H-NMR (CDCl$_3$) δ 1.4 (s, 9H, Boc), 3.1 (d, 2H, CH$_2$-Phe), 4.4 (m, 1H, α-C), 5.0 (b, 1H, NH), 7.1-7.3 (m, 5H, Ph), 9.6 (s, 1H, CHO). MS (FAB$^+$) m/z 250 (M + 1, 15 %), 150 (M – Boc + 1, 100 %).

Phe-VS-Ph·HCl. Boc-Phe-VS-Ph was prepared by reaction of Boc-Phe-H with diethyl phenylsulfonylmethanephosphonate in the presence of 2 N sodium methoxide[22] (85% yield). ^{1}H-NMR (CDCl$_3$) δ 1.3-1.4 (s, 9H, Boc), 2.9 (d, 2H, CH$_2$-Phe), 4.4-4.5 (b, 1H, α-H), 4.6-4.7 (b, 1H, NH), 6.3 (d, 1H, CH=CH), 6.9-7.0 (dd, 1H, CH=CH), 7.1-7.3 (m, 5H, Ph), 7.5-7.8 (m, 5H, Ph). MS (FAB$^+$) m/z 388 (M + 1, 15 %), 288 (M – Boc + 1, 100 %). Boc-Phe-VS-Ph was deblocked with 6.7 N HCl in EtOAc to give Phe-VS-Ph·HCl (88% yield). ^{1}H-NMR (DMSO-d$_6$) δ 2.9-3.0 (m, 1H, CH$_2$-Phe), 3.1-3.2 (m, 1H, CH$_2$-Phe), 4.2 (b, 1H, α-H), 6.7-6.8 (m, 2H, CH=CH), 7.1-7.3 (m, 6H, Ph), 7.6-7.8 (m, 5H, Ph), 8.6-8.8 (b, 2H, Ph).   MS (FAB$^+$) m/z 288 (M - Cl, 100 %).

Hph-VS-Ph·HCl. Boc-Hph-VS-Ph was prepared by reaction of Boc-Hph-H with diethyl phenylsulfonylmethanephosphonate in the presence of 2 N sodium methoxide (75% yield). ^{1}H-NMR (CDCl$_3$) δ 1.4 (s, 9H, Boc), 1.7-2.0 (m, 2H, CH$_2$-Hph), 2.6-2.7 (m, 2H, CH$_2$-Hph), 4.3-4.4 (m, 1H, α-H), 4.4-4.5 (m, 1H, α-H), 6.4 (d, 1H, CH=CH), 6.8-6.9 (dd, 1H, CH=CH), 7.1-7.3 (m, 5H, Ph), 7.5-7.7 (m, 3H, Ph), 7.8-7.9 (d, 2H, Ph). MS (FAB$^+$) *m/z* 302 (M – Boc + 1, 100 %). Boc-Hph-VS-Ph was deblocked with 6.7 N HCl in EtOAc to give Hph-VS-Ph·HCl (90% yield).

Glu-Phe-VS-Ph·HCl. Boc-Glu-Phe-VS-Ph was prepared from Boc-Glu(O*t*Bu)-OH and Phe-VS-Ph·HCl using the standard mixed anhydride coupling method (76 % yield). ^{1}H-NMR (CDCl$_3$) δ 1.4 (s, 18H, Boc and *t*-Bu), 1.8 (m, 2H, Glu-CH$_2$), 2.0 (m, 2H, Glu-CH$_2$), 2.7 (m, 2H, Phe-CH$_2$), 4.0 (m, 1H, α-H), 4.7 (b, 1H, α-H), 5.2 (m, 1H, NH), 6.5 (d, 1H, CH=CH), 6.9 (dd, 1H, CH=CH), 7.1-7.3 (m, 5H, Ph), 7.5-7.7 (m, 3H, Ph), 7.8-7.9 (d, 2H, Ph). MS (FAB$^+$) m/z 572 (M + 1, 100%). Boc-Glu-Phe-VS-Ph was deblocked with 6.7 N HCl in EtOAc gave Glu-Phe-VS-Ph·HCl (82 %yield). ^{1}H-NMR (DMSO-d$_6$) δ 1.8-2.0 (m, 2H, Glu-CH$_2$), 2.1-2.2 (m, 2H, Glu-CH$_2$), 2.9 (d, 2H, Phe-CH$_2$), 4.0 (m, 1H, α-H), 5.7 (m, 1H, α-H), 6.8 (d, 1H, CH=CH), 6.8-7.0 (dd, 1H, CH=CH), 7.1-7.3 (m, 5H, Ph), 7.5-7.8 (m, 5H, Ph), 8.1 (b, 2H, NH$_2$), 8.9 (b, 1H, NH), 12.2 (b, 1H, COOH). MS (FAB$^+$) *m/z* 417 (M - Cl, 100%). HRMS calcd. for C$_{21}$H$_{25}$N$_2$O$_5$S: 417.14842. Obsd. 417.14864. Anal. calcd. for C$_{21}$H$_{25}$N$_2$O$_5$SCl·0.5H$_2$O: C, 54.60, H, 5.67, N, 6.06. Found: C, 54.30, H, 5.88, N, 6.12.

Arg-Phe-VS-Ph·2HCl. Boc-Arg(Boc)$_2$-Phe-VS-Ph was prepared from Boc-Arg(Boc)$_2$-OH and Phe-VS-Ph·HCl using standard mixed anhydride coupling method, yield 100 %. ^{1}H-NMR (CDCl$_3$) δ 1.4-1.6 (m & s, 29H, 3 x Boc and Arg-CH$_2$), 1.6-1.7

(m, 2H, Arg-CH$_2$), 2.9-3.0 (m, 2H, Phe-CH$_2$), 3.5 (m, 2H, Arg-CH$_2$), 4.7 (b, 1H, α-H), 4.9

(m, 1H, α-H), 6.5 (d, 1H, CH=CH), 6.9-7.0 (dd, 1H, CH=CH), 7.0-7.3 (m, 5H, Ph), 7.5-

7.7 (m, 3H, Ph), 7.8-7.9 (d, 2H, Ph). MS (FAB$^+$) m/z 744 (M + 1, 100 %). Boc-

Arg(Boc)$_2$-Phe-VS-Ph was deblocked with 6.7 N HCl in EtOAc gave Arg-Phe-VS-

Ph·2HCl (77% yield). ^{1}H-NMR (DMSO-d$_6$) δ 1.3-1.5 (m, 2H, Arg-CH$_2$), 1.6-1.8 (m, 2H,

Arg-CH$_2$), 2.9 (m, 2H, Phe-CH$_2$), 3.0-3.2 (m, 2H, Arg-CH$_2$), 3.8 (m, 1H, α-H), 4.8 (m,

1H, α-H), 6.7 (d, 1H, CH=CH), 6.8-7.0 (dd, 1H, CH=CH), 7.1-7.3 (m, 5H, Ph), 7.6-7.8

(m, 3H, Ph), 7.8-7.9 (d, 2H, Ph), 8.2-8.4 (b, 4H, 2 x NH$_2$), 9.0 (b, 3H, 3 x NH). MS

(FAB$^+$) m/z 444 (M – 2 Cl + 1, 100 %). HRMS calcd. for C$_{22}$H$_{30}$N$_5$O$_3$S: 444.20694.

Obsd. 444.20637. Anal. calcd. for C$_{22}$H$_{31}$N$_5$O$_3$SCl$_2$·0.91H$_2$O: C, 49.58, H, 6.21, N, 13.14.

Found: C, 49.28, H, 6.20, N, 13.45.

Glu-Hph-VS-Ph·HCl. Boc-Glu-Hph-VS-Ph was prepared from Boc-Glu-OH

and Hph-VS-Ph·HCl using standard mixed anhydride coupling method (100% yield). ^{1}H-

NMR (CDCl$_3$) δ 1.4 (s, 18H, Boc and *t*-Bu), 1.8 (m, 2H, Glu-CH$_2$), 2.0 (m, 2H, Glu-CH$_2$),

2.2 (m, 1H, Hph-CH$_2$), 2.4 (m, 1H, Hph-CH$_2$), 2.6-2.7 (m, 2H, Hph-CH$_2$), 4.0 (m, 1H, α-

H), 4.7 (b, 1H, α-H), 5.2 (m, 1H, NH), 6.5 (d, 1H, CH=CH), 6.9 (dd, 1H, CH=CH), 7.1-

7.3 (m, 5H, Ph), 7.5-7.7 (m, 3H, Ph), 7.8-7.9 (d, 2H, Ph). MS (FAB$^+$) m/z 588 (M + 1,

100 %). Boc-Glu-Hph-VS-Ph was deblocked with 6.7 N HCl in EtOAc to give Glu-Hph-

VS-Ph·HCl (86% yield). ^{1}H-NMR (DMSO-d$_6$) 1.7-1.8 (m, 2H, Glu-CH$_2$), 1.9-2.1 (m, 2H,

Glu-CH$_2$), 2.1-2.2 (m, 2H, Hph-CH$_2$), 2.9 (d, 2H, Hph-CH$_2$), 4.0 (m, 1H, α-H), 5.7 (m,

1H, α-H), 6.8 (d, 1H, CH=CH), 6.8-7.0 (dd, 1H, CH=CH), 7.1-7.3 (m, 5H, Ph), 7.5-7.8

(m, 5H, Ph), 8.1 (b, 2H, NH$_2$), 8.9 (b, 1H, NH), 12.2 (b, 1H, COOH). MS (FAB$^+$) *m/z*

431 (M - Cl, 100 %). HRMS calcd. for C$_{22}$H$_{27}$N$_2$O$_5$S: 431.16407. Obsd. 431.16652.

Anal. calcd. for $C_{22}H_{26}N_2O_5S \cdot HCl \cdot 0.4H_2O$: C, 55.73, H, 5.91, N, 5.90. Found: C, 55.85, H, 6.12, N, 5.71.

Arg-Hph-VS-Ph·2HCl. Boc-Arg(Boc)$_2$-Hph-VS-Ph was prepared from Boc-Arg(Boc)$_2$-OH and Hph-VS-Ph·HCl using standard mixed anhydride coupling method (85% yield). ^{1}H-NMR (CDCl$_3$) 1.4-1.6 (m & s, 29H, 3 x Boc and Arg-CH$_2$), 1.6-1.7 (m, 2H, Arg-CH$_2$), 1.8-2.0 (m, 2H, Hph-CH$_2$), 2.6-2.8 (m, 2H, Hph-CH$_2$), 3.9 (m, 2H, Arg-CH$_2$), 4.2 (m, 1H, α-H), 4.7 (b, 1H, α-H), 5.9 (b, 1H, NH), 6.4-6.8 (dd, 1H, CH=CH), 6.9-7.0 (dd, 1H, CH=CH), 7.0-7.3 (m, 5H, Ph), 7.5-7.7 (m, 3H, Ph), 7.8-7.9 (d, 2H, Ph), 9.2-9.4 (b, 2H, 2 x NH); Boc-Arg(Boc)$_2$-Hph-VS-Ph was deblocked with 6.7 N HCl in EtOAc to give Arg-Hph-VS-Ph·2HCl (77% yield). ^{1}H-NMR (DMSO-d$_6$) 1.3-1.5 (m, 2H, Arg-CH$_2$), 1.6-2.0 (m, 4H, 2 x Arg-CH$_2$), 2.5-2.6 (m, 2H, Hph-CH$_2$), 3.1-3.2 (m, 2H, Arg-CH$_2$), 3.9-4.0 (m, 1H, α-H), 4.4-4.6 (m, 1H, α-H), 6.8 (d, 1H, CH=CH), 6.9-7.0 (dd, 1H, CH=CH), 7.1-7.3 (m, 5H, Ph), 7.6-7.8 (m, 3H, Ph), 7.8-7.9 (d, 2H, Ph), 8.2-8.4 (b, 4H, 2 x NH$_2$), 8.8 (b, 1H, NH), 9.1 (b, 1H, NH). MS (FAB$^+$) *m/z* 458 (M − 2 Cl, 100 %). HRMS calcd. for $C_{23}H_{32}N_5O_3S$: 458.22259. Obsd. 458.22424. Anal. calcd. for $C_{23}H_{31}N_5O_3S \cdot 2HCl \cdot 1H_2O$: C, 50.36, H,6.43, N, 12.77. Found: C, 50.35, H, 6.48, N, 12.79.

REFERENCES

(1)   Coffey, J. W.; De Duve, C. *J. Biol. Chem.* **1968**, *243*, 3255-63.

(2)   McDonald, J. K.; Callahan, P. X.; Ellis, S. *Methods Enzymol.* **1972**, *25*, 272-281.

(3)   Kominami, E.; Ishido, K.; Muno, D.; Sato, N. *Biol. Chem. Hoppe Seyler* **1992**, *373*, 367-73.

(4)   McGuire, M. J.; Lipsky, P. E.; Thiele, D. L. *J. Biol. Chem.* **1993**, *268*, 2458-2467.

(5)   Hudig, D.; Allison, N. J.; Pickett, T. M.; Winkler, U.; Kam, C.-M.; Powers, J. C. *J. Immunol.* **1991**, *147*, 1360-1368.

(6)   Korver, G. E.; Kam, C. M.; Powers, J. C.; Hudig, D. *Int. Immunopharmacol.* **2001**, *1*, 21-32.

(7)   Thiele, D. L.; McGuire, M. J.; Lipsky, P. E. *J. Immnol.* **1997**, *158*, 5200-5210.

(8)   Smyth, M. J.; McGuire, M. J.; Thia, K. Y. *J. Immunol.* **1995**, *154*, 6299-6305.

(9)   Kummer, J. A.; Kamp, A. M.; Citarella, F.; Horrevoets, J. G.; Hack, C. E. *J. Biol. Chem.* **1996**, *271*, 9281-9286.

(10)   McEuen, A. R.; Ashworth, D. M.; Walls, A. F. *Eur. J. Biochem.* **1998**, *253*, 300-308.

(11)   Pham, C. T.; Ley, T. J. *Proc. Natl. Acad. Sci. U S A* **1999**, *96*, 8627-32.

(12)   Pal, S.; Raghav, N.; Kamboj, R. C.; Singh, H. *Neurochem. Int.* **1993**, *22*, 59-68.

(13)   McGuire, M. J.; Lipsky, P. E.; Thiele, D. L. *Arch. Biochem. Biophys.* **1992**, *295*, 280-288.

(14)   McDonald, J. K.; Callahan, P. X.; Zeitman, B. B.; Ellis, S. *J. Biol. Chem.* **1969**, *244*, 6199-6208.

(15)   Kuribayashi, M.; Yamada, H.; Ohmori, T.; Yanai, M.; Imoto, T. *J. Biochem.* **1993**, *113*, 441-449.

(16)   Ishidoh, K.; Muno, D.; Sato, N.; Kominami, E. *J. Biol. Chem.* **1991**, *266*, 16312-16317.

(17)   Turk, D.; Janjic, V.; Stern, I.; Podobnik, M.; Lamba, D.; Dahl, S. W.; Lauritzen, C.; Pedersen, J.; Turk, V.; Turk, B. *Embo. J.* **2001**, *20*, 6570-82.

(18) Tran, T. V.; Ellis, K. A.; Kam, C. M.; Hudig, D.; Powers, J. C. *Arch. Biochem. Biophys.* **2002**, *403*, 160-70.

(19) Barrett, A. J.; Kembhavi, A. A.; Brown, M. A.; Kirschke, H.; Knight, C. G.; Tama, M.; Hanada, K. *Biochem. J.* **1982**, *201*, 189-198.

(20) Green, G. D. J.; Shaw, E. *J. Biol. Chem.* **1981**, *256*, 1923-1928.

(21) Palmer, J. T.; Rasnik, D.; Klaus, J. L.; Brömme, D. *J. Med. Chem.* **1995**, *38*, 3193-3196.

(22) Shahak, I.; Almog, J. *Synthesis* **1969**, *4*, 170-172.

(23) Yasuma, T.; Oi, S.; Choh, N.; Nomura, T.; Furuyama, N.; Nishimura, A.; Fujisawa, Y.; Sohda, T. *J. Med. Chem.* **1998**, *41*, 4301-8.

(24) Woo, J.-T.; Sigeizumi, S.; Yamaguchi, K.; Sugimoto, K.; Kobori, T.; Tsuji, T.; Kondo, K. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 1501-1504.

(25) Hu, L.-Y.; Abeles, R. H. *Arch. Biochem. Biophys.* **1990**, *281*, 271-274.

(26) Li, Z.; Patil, G. S.; Golubski, Z. E.; Hori, H.; Tehrani, K.; Foreman, J. E.; Eveleth, D. D.; Bartus, R. T.; Powers, J. C. *J. Med. Chem.* **1993**, *36*, 3472-3480.

(27) Thompson, S. A.; Andrews, P. R.; Hanzlik, R. P. *J. Med. Chem.* **1986**, *29*, 104-11.

(28) Shaw, E.; Mohanty, S.; Colic, A.; Stioka, V.; Turk, V. *FEBS Lett.* **1993**, *334*, 340-342.

(29) Kirschke, H.; Shaw, E. *Biochem. Biophys. Res. Commun.* **1981**, *101*, 454-8.

(30) Ahmed, N. K.; Martin, L. A.; Watts, L. M.; Palmer, J.; Thornburg, L.; Prior, J.; Esser, R. E. *Biochem. Pharm.* **1992**, *44*, 1201-1207.

(31) Angliker, H.; Anagli, J.; Shaw, E. *J. Med. Chem.* **1992**, *35*, 216-20.

(32) Rauber, P.; Angliker, H.; Walker, B.; Shaw, E. *Biochem. J.* **1986**, *239*, 633-640.

(33) Shaw, E. *Meth. in Enzymol.* **1990**, *63*, 271-347.

(34) Paetzel, M.; Strynadka, N. C. *Protein. Sci.* **1999**, *8*, 2533-6.

(35) Pliura, D. H.; Bonaventura, B. J.; Smith, R. A.; Coles, P. J.; Krantz, A. *Biochem. J.* **1992**, *288*, 759-62.

(36) Brömme, D.; Smith, R. A.; Coles, P. J.; Kirschke, H.; Storer, A. C.; Krantz, A. *Biol. Chem. Hoppe-Seyler* **1994**, *375*.

(37) Bromme, D.; Klaus, J. L.; Okamoto, K.; Rasnick, D.; Palmer, J. T. *Biochem. J.* **1996**, *315 (Pt 1)*, 85-9.

(38) Schaschke, N.; Assfalg-Machleidt, I.; Machleidt, W.; Turk, D.; Moroder, L. *Bioorg. & Med. Chem. Lett.* **1997**, *5*, 1789-1797.

(39) Gour-Salin, B. J.; Lachance, P.; Plouffe, C.; Storer, A. C.; Menard, R. *J. Med. Chem.* **1993**, *36*, 720-725.

(40) Otto, H. H.; Schirmeister, T. *Chem. Rev.* **1997**, *97*, 133-172.

(41) Powers, J. C.; Asgian, J. L.; Ekici, O. D.; James, K. E. *Chem. Rev.* **2002**, *102*, 4639-750.

(42) Bogyo, M.; McMaster, J. S.; Gaczynska, M.; Tortorella, D.; Goldberg, A. L.; Ploegh, H. *Proc. Natl. Acad. Sci. U S A* **1997**, *94*, 6629-34.

(43) Dikov, M. M.; Springman, E. B.; Yeola, S.; Serafin, W. E. *J. Biol. Chem.* **1994**, *269*, 25897-904.

(44) Fehrentz, J.-A.; Castro, B. *Synthesis* **1983**, *8*, 676-678.

CHAPTER 3

PEPTIDYL ALLYL SULFONES: A NEW CLASS OF INHIBITORS FOR CLAN CA PROTEASES
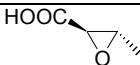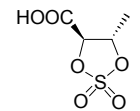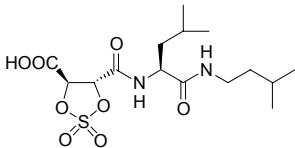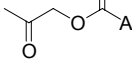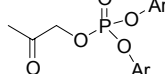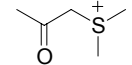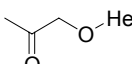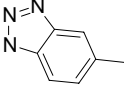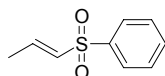
INTRODUCTION

Calpains. Calpains are a class of cysteine proteases, which are activated by calcium. They belong to the clan CA of cysteine proteases together with papain, DPPI, cruzains, and cathepsins. Several forms of this family of enzymes are known. So far eight homologs of human calpain have been identified, two of which have received increased attention: calpain I (or μ-calpain) and calpain II (or m-calpain). Their activity *in vitro* is dependent on the calcium concentration (micromolar amounts for calpain I and millimolar amounts for calpain II)[1-4]. They are present in the cytosol of mammalian cells and play an important role in physiological processes that control the degradation of the cytoskeleton as well as hormone receptors[5,6]. Enhanced calpain activity however has been related to cell injury due to ischemic stroke[7], physical damage[8], and hypoxia[9]. Thus, calpain is an attractive target for the development of inhibitors, which should be useful for the treatment of a variety of diseases.

Cysteine proteases are effectively inhibited by a variety of peptidic as well as nonpeptidic structures. Clan CA cysteine proteases are effectively inhibited by several classes of peptidyl inhibitors including transition state, reversible and irreversible

inhibitors[10]. Examples for reversible transition state inhibitors are peptide aldehydes[11-14], α-diketones[15-18], α-ketoesters[19,20], α-ketoamides[19,21] and α-ketoacids[19]. Clan CA proteases are irreversibly inhibited by peptidyl diazomethyl ketones and fluoromethyl ketones[22,23], peptidyl epoxides (E-64, E-64-c, E-64-d)[24], and vinyl sulfones[25] (Table 3.1). However the potency and specificity of the irreversible inhibitors have so far been limited.

Table 3.1. Irreversible Inhibitors for Calpain.

Warhead	Inhibitor	k_{obs} ($M^{-1}s^{-1}$) or IC_{50} (μM)	Reference
	Ep-475	7,450	26
		$IC_{50} = 0.3$	27
	Cbz-Leu-Leu-Tyr-CH=N$_2$	230,000	28
	Cbz-Leu-Leu-Phe-CH$_2$F	290,000	15
	Cbz-Leu-Leu-Phe-CH$_2$Cl	9,500	29
	Cbz-Leu-Phe-CH$_2$OCO-2,6-Cl$_2$-Ph	11,000	28
	Cbz-Leu-Phe-CH$_2$OPO(O-2-Me-Bzl)$_2$	365,000	30
	Cbz-Leu-Leu-Phe-CH$_2$S(Me)$_2$	200,000	31
		230,000	32
	Cbz-Leu-Leu-Tyr-VS-Ph	24,300	25

E-64 Inhibitors. Many potent inhibitors have also been inspired by mimicking

natural inhibitors. E-64 (*N*-(L-3-*trans*-carboxyoxiran-2-carbonyl)-L-leucyl)-amido(4-

guanido)butane) is one example for a natural irreversible inhibitor (Figure 1.3), which

was first isolated from *Asperigillus japonicus* by Hanada *et al* in 1978[33]. E-64 is a

universal inhibitor for clan CA proteases, such as papain (0.104 nM)[35], cathepsin B

(89,400 $M^{-1}s^{-1}$)[34], bromelain (0.110 nM for fruit bromelain and 0.025 nM for stem

bromelain)[35], ficin (0.084 nM)[35], cathepsin L (96,250 $M^{-1}s^{-1}$)[36], calpain II (7,500 $M^{-1}s^{-1}$)[37-39], and cruzain (70,600 $M^{-1}s^{-1}$)[40], but does not inhibit clan CD proteases such as

caspases[41], legumains[42], and gingipains[43], and only inhibits clostripain very slowly[44].
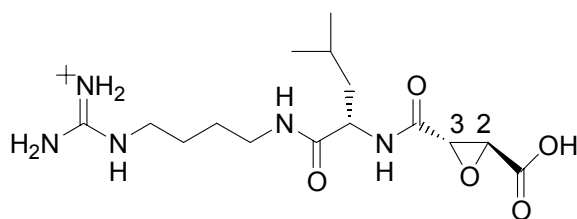
Figure 3.1. Structure of the Cysteine Protease Inhibitor E-64.

The *trans*-epoxy succinic acid (HOOC-(2*S*,3*S*)-EP-CO-) was found to be the

reactive moiety. The active site cysteine performs a nucleophilic attack on the C_2 or C_3

carbon of the oxirane ring depending on the enzyme, and the epoxide opens up resulting

in the formation of a hydroxyl group, and the enzyme is bound irreversibly. Several E-64

analogs, which retain the expoxy succinate moiety but vary the amino acid substituents,

have been analyzed for inhibitory potency with calpain. However, E-64 derivatives are

broad spectrum inhibitors, and exclusive selectivity for calpain inhibition has not been

achieved so far.

INHIBITOR DESIGN

In order to develop new functional groups suitable for irreversible inhibition of cysteine proteases, we attempted the synthesis of peptidyl epoxy sulfones. The design of the peptidyl epoxy sulfone was based on vinyl sulfones, which are a class of promising irreversible peptidyl inhibitors for clan CA cysteine proteases. Vinyl sulfones contain a Michael acceptor functional group and react irreversibly with the active site cysteine as described in chapter 2. Palmer *et al* have developed the vinyl sulfone warhead, and found that it was a potent inhibitor of calpain[25]. Another widely used warhead is the epoxide moiety of the natural product inhibitor E-64. Aza-peptide epoxides, for example are potent and selective inhibitors for clan CD cysteine proteases including caspases and legumain[45]. This class will be further described in chapter 4. We proposed that combining the reactivity of the E-64 epoxide warhead with the selectivity of the vinyl sulfone moiety will result in both a potent and specific new class of inhibitors for cysteine proteases (Figure 3.2).
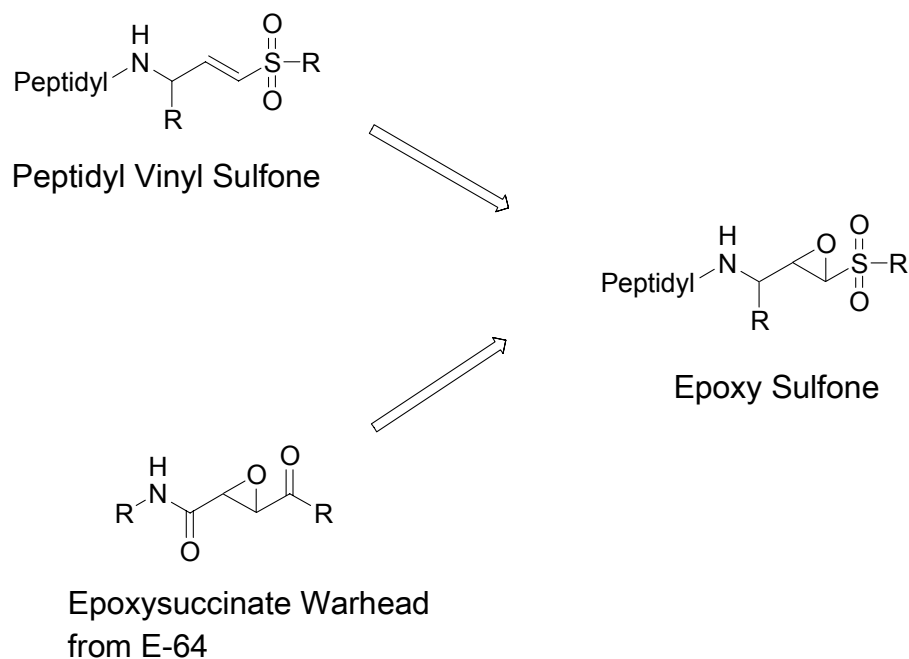
Figure 3.2. Hypothesis for a New Functional Group.

We focused on the clan CA cysteine proteases calpain and papain, and thus decided to attach this novel warhead to an optimal di- or tripeptide sequence for satisfactory calpain and papain inhibition. We chose Leu-Phe, Ala-Phe, and Val-Phe with the N-terminal Cbz-protecting group[15].

RESULTS AND DISCUSSION

Our strategy for the synthesis of epoxy sulfones began with the synthesis of the appropriate peptidyl vinyl sulfone (Figure 3.3). Vinyl sulfones have been previously synthesized and prepared by coupling the chiral amino acid aldehydes **1** and the corresponding sulfonyl phosphonate **2** using Wadsworth-Emmons chemistry (see chapter

2)[25]. The stereochemistry of the vinyl sulfone is primarily E, but the presence of small amounts of the Z isomer cannot be excluded[46]. The Boc-group was then removed using 4N HCl in ethyl acetate. Coupling of the salt **4** to a Cbz-protected amino acid **5** using standard mixed anhydride conditions furnished the dipeptidyl vinyl sulfone precursor **6**.
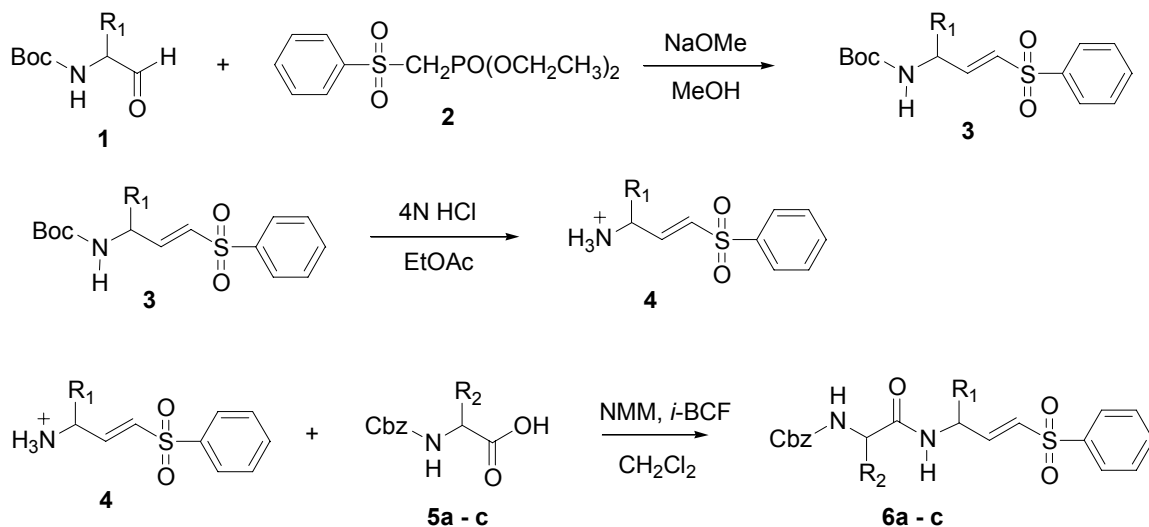
Figure 3.3. Preparation of Cbz-protected Vinyl Sulfones.

In order to synthesize the epoxy sulfone, we decided to epoxidize the double bond moiety in the peptidyl vinyl sulfone precursor using basic peroxidizing conditions. When we tried to epoxidize the vinyl sulfone using *t*BuLi and *n*BuOOH in freshly distilled THF at -78 °C, the formation of a new compound was observed by TLC. The vinyl sulfone starting material was consumed within 20 minutes. The new compound was purified by column chromatography and carefully characterized using several spectroscopic methods. We found that the conjugated double bond of the vinyl sulfone moiety was no longer present in the ^{1}H NMR. However according to the MS, the molecular weight of the

newly formed product was unchanged. Elemental analysis also confirmed that the new compound was a constitutional isomer of the vinyl sulfone starting material. The characteristic sulfone stretch was still present in the FT-IR spectrum. Since the new compound was formed by isomerization and not oxidation, the critical reagent was the *t*BuLi base and not the oxidizing *n*BuOOH. Treatment of the vinyl sulfone with *n*BuLi without the peroxide *t*BuOOH gave the same product. However, the reaction was much more sluggish, and required several hours at room temperature. We considered several possible isomeric structures for the *n*BuLi reaction product. A substituted oxazoline formed by cyclization or an allyl sulfone (AS), with the double bond isomerized toward the adjacent α-carbon were two likely possibilities (Figure 3.4).
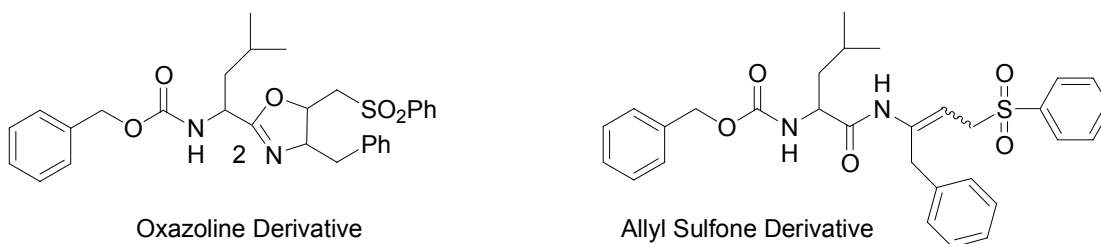
Figure 3.4. Possible Constitutional Isomers of the Vinyl Sulfone Starting Material.

Oxazolines have characteristic ^{13}C NMR signals at 170 ppm for the C-2 carbon atom[47]. Oxazolines have previously been synthesized, and we prepared the parent compound **11** (Figure 3.5) following the synthetic method described by Wipf *et al*[47]. This oxazoline could potentially acylate the active site cysteine of a cysteine protease, but the ^{1}H NMR spectrum was not identical to our isomerization product. The ^{13}C NMR spectrum of our product has a new peak at 106 ppm and not one at 170 ppm for the

oxazoline. In addition we observed the presence of an additional NH in the ^{1}H NMR. This ruled out the formation of the oxazoline in the isomerization reaction.
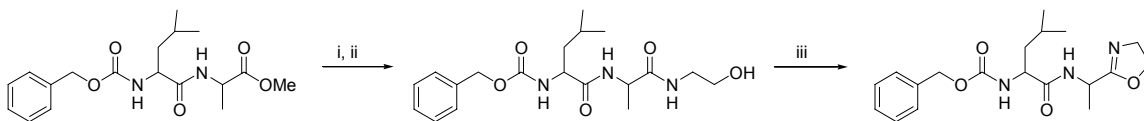
Figure 3.5. Preparation of the Peptidyl Oxazoline. *i*) 1) NaOH, MeOH; 2) HCl; *ii*) H$_2$N(CH$_2$)$_2$OH, NMM, IBCF, CH$_2$Cl$_2$; *iii*) PPh$_3$, DEAD, THF.

The allyl sulfone structure (**7**) was supported by all the spectroscopic data. The methylene of the phenylalanine side chain shifts substantially from 2.9 ppm to 3.8 ppm in the ^{1}H NMR spectrum and the shift is explained by the close proximity to the double bond in the newly formed isomerization product. The distinct triplet at 4.9 ppm is consistent with a vinylogous proton. Upon decoupling the vinyl proton triplet at 4.9 ppm, the methylene group next to the sulfone moiety at 3.83 ppm appears as a singlet, indicating that the protons involved are located on adjacent carbons. It has previously been reported that treatment of vinyl sulfones with strong base results in the formation of allyl sulfones (Figure 3.6), as allyl sulfones are thermodynamically more stable than vinyl sulfones[48,49]. The stereochemistry of the isomerized double bond was not determined, which leaves the E to Z ratio of the final compounds unknown. Both E and Z or an E/Z mixture have been obtained upon base catalyzed isomerization of vinyl sulfones [50,51].
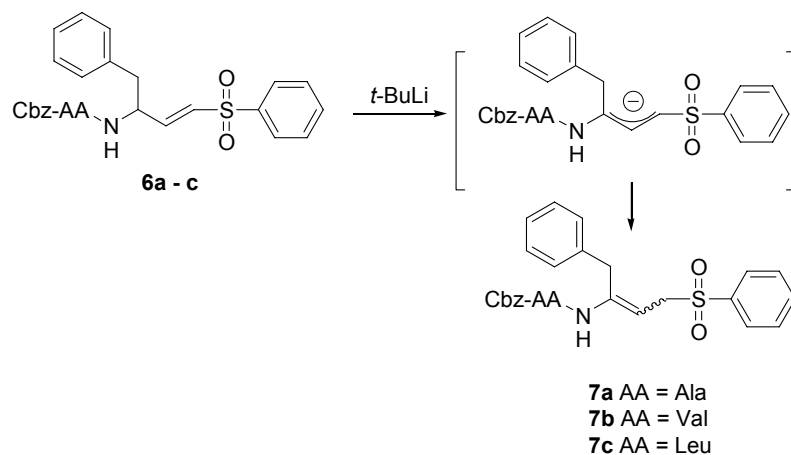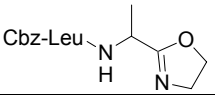
Figure 3.6. Isomerization of Dipeptidyl Vinyl Sulfones to Allyl Sulfones.

The novel, newly synthesized peptidyl allyl sulfones had the potential of being cysteine protease inhibitors and were tested along with the oxazoline **11** with papain, calpain I, and cathepsin B (Table 3.2). This new class of compounds shows moderate rates of calpain and papain inactivation. The oxazoline was also a slow inhibitor of calpain I. Kinetic inhibition assays were performed using calpain I from bovine erythrocytes and Suc-Leu-Tyr-AMC as the fluorogenic substrate[52]. Cathepsin B was tested using commercially available enzyme and Cbz-Arg-Arg-AMC as the substrate[53]. Papain inhibition was measured using commercially available papain and Cbz-Arg-Phe-pNA as the substrate[54]. The dipeptidyl allyl sulfones exhibit no inhibition with cathepsin B and limited inhibition with both calpain I and papain. Striking is the $k_{obs}/[I]$ value of 564 $M^{-1}s^{-1}$ for Cbz-Leu-Phe-AS-Ph (Isomer B) with calpain I. This dipeptidyl allyl sulfone was synthesized from the dipeptidyl vinyl sulfone precursor Cbz-Leu-D-Phe-VS-Ph. When the double bond isomerizes the original Z stereochemistry of the vinyl sulfone is lost and the product has an unknown ratio of E to Z isomers at the new allyl double

bond. There are several factors, which can contribute to a mixed ratio of isomers, such as the so-called "syn-effect"[50,51] and the chelating lithium metal. This explains the difference in inhibitor potency between Cbz-Leu-Phe-AS-Ph (Isomer A), which is derived from the vinyl sulfone precursor Cbz-Leu-L-Phe-VS-Ph, and Cbz-Leu-Phe-AS-Ph (Isomer B). The initial stereochemistry of the phenylalanine side chain probably plays a significant role in the final ratio of Z to E isomers in the allyl sulfone inhibitor.

Table 3.2. Inhibition of Various Cysteine Proteases by Dipeptidyl Allyl Sulfones.

		$k_{obs}/[I]$ $(M^{-1} s^{-1})^a$		
	Inhibitor	Calpain I[b]	Papain[c]	Cathepsin B[d]
7a	Cbz-Ala-Phe-AS-Ph	3 ± 0	9 ± 0	N.I.
7b	Cbz-Val-Phe-AS-Ph	3 ± 0	6 ± 1	N.I.
7c	Cbz-Leu-Phe-AS-Ph (Isomer A)[e]	23 ± 4	49 ± 6	N.I.
8	Cbz-Leu-Phe-AS-Ph (Isomer B)[f]	564 ± 32^g	15 ± 1	N.I.
6c	Cbz-Leu-Phe-VS-Ph	550 ± 2^g	$10 \pm$	N.I.
11		13 ± 1	N.I.	N.I.

[a]k_{obs} is the pseudo first order rate constant obtained from plots of $\ln v_t/v_0$ vs time unless indicated otherwise. [b]Calpain I assay conditions: Irreversible kinetic assays were performed by the incubation method with calpain I from porcine erythrocytes. Enzymatic activities of calpain I were measured at 23 °C in 50 mM Hepes buffer (pH 7.5) containing 10 mM cysteine and 5 mM $CaCl_2$. [c]Papain assay conditions: Incubation kinetics were measured using an enzyme stock solution for the papain assays, which was freshly prepared from 330 μL of enzyme storage solution (1.19 mg/ml) diluted with 645 μL papain buffer (50 mM Hepes, and 2.5 mM EDTA at pH 7.5) and 25 μL of DTT (0.1 M). [d]Cathepsin B assay conditions: Enzymatic activities of cathepsin B were measured by the incubation method in 0.1 M $KHPO_4$, 1.25 mM EDTA, 0.01% Brij, pH 6.0 buffer and at 23 °C. [e]Allyl sulfone is derived from the L-isomer of phenylalanine. [f]Allyl sulfone is derived from the D-isomer of phenylalanine. [g]k_{obs} is obtained by the progress curve method and corrected for substrate, as the inhibitor concentration is too low to be measured by the incubation method.

Mechanism of Inhibition. One mechanism for the inhibition reaction could involve re-isomerisation of the peptidyl allyl sulfone to the vinyl sulfone. This isomerization could be catalyzed by the active site histidine and would be reflected by a higher inhibition rate ($k_{obs}/$[I]) for the parent vinyl sulfone inhibitor. However, as the parent vinyl sulfone Cbz-Leu-Phe-VS-Ph ($k_{obs}/$[I] = 10 $M^{-1}s^{-1}$) was five times less potent with papain than the corresponding allyl sulfone Cbz-Leu-Phe-AS-Ph (Isomer A) ($k_{obs}/$[I] = 49 $M^{-1}s^{-1}$), we conclude that the mechanism of inhibition does not involve re-isomerisation of the allyl sulfone to the vinyl sulfone with a subsequent attack of the cysteine thiol on the Michael acceptor double bond. With calpain I it is possible, that the isomerization mechanism is occurring, since the vinyl sulfone is a much more effective inhibitor of calpain.

Two other mechanisms can be envisaged for enzyme inhibition by allyl sulfones (Figure 3.7). The active site cysteine could directly displace phenyl sulfinic acid in an S_N2 reaction (pathway b) or could attack the allylic double bond with loss of phenyl sulfinic acid (pathway a). Both pathways result in alkylation of the active site cysteine residue. It has previously been reported that allyl sulfones when reacted with nucleophiles undergo a tosyl elimination or displacement process, in which the allyl sulfone moiety undergoes alkylation at either the α- or γ-carbon[49,55,56]. An alternative possibility for enzyme inactivation follows a mechanism-based pathway, which is initiated by removal of the amide nitrogen proton by a base or the active site histidine, followed by the elimination of phenyl sulfinic acid and the formation of the imine **9**. The active site cysteine then reacts with **9** in a Michael addition, which also irreversibly alkylates the enzyme. Several PLP-dependent enzymes are irreversibly inhibited by

mechanism-based inactivators, where the pathway also follows displacement and β-elimination reactions. A vinylic imine forms as an intermediate before undergoing attack by a nucleophile in the active site of the enzyme[57].
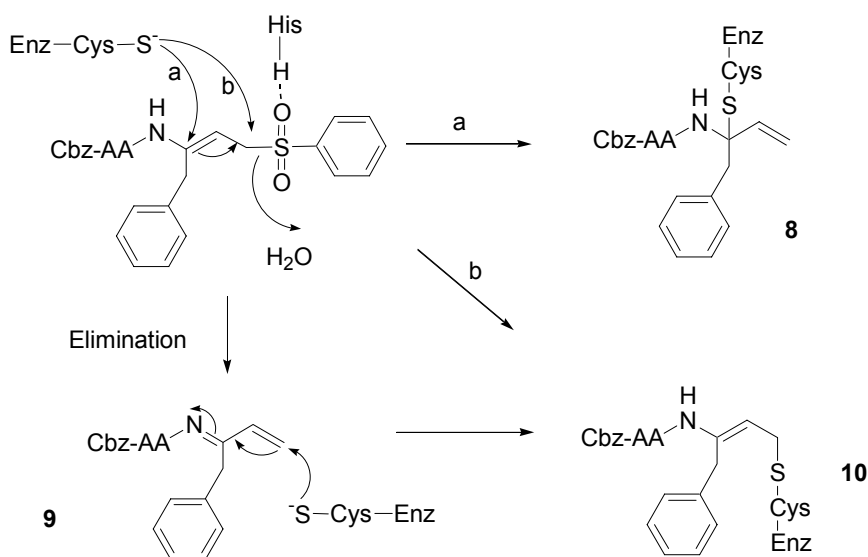
Figure 3.7. Proposed Enzyme Mechanisms of Inhibition.

CONCLUSIONS

In summary we report, that in an effort to synthesize epoxy sulfone inhibitors using basic oxidative conditions we unexpectedly produced dipeptidyl allyl sulfones, a new class of inhibitors, by isomerization of the double bond. This new class of inhibitors contains a novel cysteine protease warhead and shows moderate rates of calpain and papain inactivation. Since it has been reported in the literature that inhibitors, which target calpain, are also potent inhibitors of cruzain and cathepsin L and cathepsin S, the allyl sulfones will also be tested with those particular enzymes. Additionally, it would be

of interest to extend the allyl sulfone warhead to other cysteine proteases and investigate the effect of the stereochemistry of the double bond on the potency of the inhibitor. It would also be of interest to determine the mechanism of inhibition since allyl sulfones are likely mechanism-based inhibitors.

EXPERIMENTAL

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

Papain and Cathepsin B Assays. The incubation method was used to measure the irreversible inhibition of papain and cathepsin B. With cathepsin B, 30 μL of a stock inhibitor solution was added to 300 μL of a 0.1 M potassium phosphate buffer containing 1.25 mM EDTA, 0.01% Brij 35 at pH 6.0, followed by the addition of 30 μL of a freshly prepared cathepsin B solution (approximate concentration 6.98×10^{-3} μg/μL) in the same potassium phosphate buffer containing 1 mM DTT (freshly prepared). Aliquots (50 μL) from the inhibition mixture were withdrawn at various time intervals and added to 200

51

µL of a 0.1 M potassium phosphate buffer containing 1.25 mM EDTA, 0.01% Brij 35 at pH 6.0, and the substrate Cbz-Arg-Arg-AMC (499 µM). The release of 7-amino-4-methylcoumarin was monitored (λ_{ex} = 360 nm, λ_{em} = 465 nm) using a Tecan Spectra Fluor microplate reader. Pseudo first-order inactivation rate constants were obtained from plots of ln v_t/v_o versus time.

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

Calpain Assays. 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 CaCl$_2$, 0.5 M cysteine, at pH 7.5 (calpain I buffer), 6.5 µl of Suc-Leu-Tyr-AMC substrate solution 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 10 min at 465 nm. The k_2 values were obtained by non-linear regression analysis and corrected for substrate (1 + [S]/K_m = 1.274).

Diethyl Phenylsulfonylmethanephosphonate (2, PhSO$_2$CH$_2$PO(OEt)$_2$). A mixture of chloromethyl phenyl sulfide (3.2 g, 20 mmole) and triethyl phosphite (3.3 g, 20 mmole) was heated at 130 - 140 °C for 5 h. The resultant mixture was distilled under reduced pressure, and the starting materials were distilled out. The oily residue was

diethyl phenylmercaptomethanephosphonate, yield 3.4 g (65%). ^{1}H-NMR (CDCl$_3$) δ

1.2-1.3 (t, 6H, 2 x CH$_3$), 3.1-3.2 (d, 2H, S-CH$_2$), 4.0-4.2 (m, 4H, 2 x CH$_2$), 7.2-7.4 (m,

5H, Ph). Diethyl phenylsulfonylmethanephosphonate was prepared by oxidation of

diethyl phenylmercaptomethanephosphonate with potassium permanganate, yield 55%.

^{1}H-NMR (CDCl$_3$) δ 1.2-1.3 (t, 6H, 2 x CH$_3$), 3.7-3.8 (d, 2H, SO$_2$-CH$_2$), 4.1-4.2 (m, 4H, 2

x CH$_2$), 7.5-7.7 (m, 3H, Ph), 7.9-8.0 (d, 2H, Ph). MS m/z 293 (M + 1).

2-(*tert*-Butoxycarbonylamino)-3-phenylpropionaldehyde (1, Boc-Phe-H).

Boc-Phe-N(OCH$_3$)CH$_3$ was prepared from Boc-Phe-OH and *N,O*-

dimethylhydroxylamine hydrochloride using standard mixed anhydride coupling

procedure, yield 93%. ^{1}H-NMR (CDCl$_3$) δ 1.4 (s, 9H, Boc), 2.8-2.9 (m, 1H, CH$_2$-Phe),

3.0-3.1 (m, 1H, CH$_2$-Phe), 3.2 (s, 3H, N-CH$_3$), 3.6 (s, 3H, O-CH$_3$), 4.9 (m, 1H, α-H), 5.2

(b, 1H, NH), 7.1-7.3 (m, 5H, Ph). MS (FAB$^+$) m/z 309 (M + 1, 30%), 253 (M − *t*Bu + 1,

100%). Reduction of Boc-Phe-N(OCH$_3$)CH$_3$ with lithium aluminum hydride according

to a previously described method gave Boc-Phe-H, yield 88%. ^{1}H-NMR (CDCl$_3$) δ 1.4

(s, 9H, Boc), 3.1 (d, 2H, CH$_2$-Phe), 4.4 (m, 1H, α-H), 5.0 (b, 1H, NH), 7.1-7.3 (m, 5H,

Ph), 9.6 (s, 1H, CHO). MS (FAB$^+$) m/z 250 (M + 1, 15%), 150 (M − Boc + 1, 100%).

Phenyl-(*3S*)-3-amino-4-phenylbut-1-enyl Sulfone Hydrochloride (4, Phe-VS-

Ph·HCl). Boc-Phe-VS-Ph was prepared by reaction of Boc-Phe-H with diethyl

phenylsulfonylmethanephosphonate in the presence of 2 N sodium methoxide, yield 85%.

^{1}H-NMR (CDCl$_3$) δ 1.3-1.4 (s, 9H, Boc), 2.9 (d, 2H, CH$_2$-Phe), 4.4-4.5 (b, 1H, α-H), 4.6-

4.7 (b, 1H, NH), 6.3 (d, 1H, CH=), 6.9-7.0 (dd, 1H, CH=), 7.1-7.3 (m, 5H, Ph), 7.5-7.8

(m, 5H, SO$_2$-Ph). MS (FAB$^+$) m/z 388 (M + 1, 15%), 288 (M − Boc + 1, 100%). Boc-

Phe-VS-Ph was deblocked with 6.7 N HCl in EtOAc to give Phe-VS-Ph·HCl, yield 88%.

^{1}H-NMR (DMSO-d$_6$) δ 2.9-3.0 (m, 1H, CH$_2$-Phe), 3.1-3.2 (m, 1H, CH$_2$-Phe), 4.2 (b, 1H, α-H), 6.7-6.8 (m, 2H, CH=), 7.1-7.3 (m, 6H, CH= and Ph), 7.6-7.8 (m, 5H, SO$_2$-Ph), 8.6-8.8 (b, 2H, NH$_2$). MS (FAB$^+$) *m/z* 288 (M - Cl, 100%).

Phenyl-(3R)-3-amino-4-phenylbut-1-enyl Sulfone Hydrochloride (D-Phe-VS-Ph·HCl). Boc-D-Phe-VS-Ph was prepared by reaction of Boc-D-Phe-H with diethyl phenylsulfonylmethanephosphonate in the presence of 2 N sodium methoxide, yield 85%. ^{1}H-NMR (CDCl$_3$) δ 1.3-1.4 (s, 9H, Boc), 2.9 (d, 2H, CH$_2$-Phe), 4.4-4.5 (b, 1H, α-H), 4.6-4.7 (b, 1H, NH), 6.3 (d, 1H, CH=), 6.9-7.0 (dd, 1H, CH=), 7.1-7.3 (m, 5H, Ph), 7.5-7.8 (m, 5H, SO$_2$-Ph). MS (FAB$^+$) *m/z* 388 (M + 1, 15%), 288 (M – Boc + 1, 100%). Boc-D-Phe-VS-Ph was deblocked with 6.7 N HCl in EtOAc to give D-Phe-VS-Ph·HCl, yield 88%. ^{1}H-NMR (DMSO-d$_6$) δ 2.9-3.0 (m, 1H, CH$_2$-Phe), 3.1-3.2 (m, 1H), 4.2 (b, 1H, α-H), 6.7-6.8 (m, 2H, CH=), 7.1-7.3 (m, 6H, CH= and Ph), 7.6-7.8 (m, 5H, SO$_2$-Ph), 8.6-8.8 (b, 2H, NH$_2$). MS (FAB$^+$) *m/z* 288 (M - Cl, 100%).

Phenyl-(3S)-3-(N-carbobenzyloxyleucyl)amino-4-phenylbut-1-enyl Sulfone (6c, Cbz-Leu-Phe-VS-Ph). Cbz-Leu-Phe-VS-Ph was prepared from Cbz-Leu-OH and Phe-VS-Ph·HCl using standard mixed anhydride coupling method, yield 82%. ^{1}H-NMR (CDCl$_3$) δ 0.8-0.9 (2d, 6H, 2 x Leu-CH$_3$), 1.4-1.6 (m, 2H, Leu-CH$_2$), 2.06 (m, 1H, Leu-CH), 2.9-3.0 (m, 2H, CH$_2$-Phe), 3.9-4.0 (m, 1H, α-H), 4.8-4.9 (b, 1H, NH), 4.9-5.0 (m, 1H, α-H), 5.1 (m, 2H, Cbz) 6.3-6.4 (d & b, 2H, NH and CH=), 6.9-7.0 (dd, 1H, CH=), 7.1-7.4 (m, 10H, 2 x Ph), 7.5-7.7 (m, 5H, SO$_2$-Ph). MS (ESI) *m/z* 535.

Phenyl-3-(N-carbobenzyloxyleucyl)amino-4-phenylbut-2-enyl Sulfone (7c, Cbz-Leu-Phe-AS-Ph). Butyllithium (3.63 ml, 6.17 mmol, 1.7 M in pentane) was added dropwise to a solution of tert-butylhydroperoxide (2.55 ml, 8.42 mmol, 3.3 M in toluene)

in freshly distilled THF (80 ml) at –78 ˚C under argon. A solution of Cbz-Leu-Phe-VS-Ph (3.00 g, 5.61 mmol) in dry THF (30 ml) was added dropwise. The reaction was continued to stir at -20 ˚C for 45 minutes (TLC Hex/EtOAc 1:1). The reaction was quenched with saturated aqueous ammonium chloride (50 ml) and allowed to warm to room temperature. The organic layer was separated, and the aqueous layer was extracted with ethyl acetate (3 x 30 ml). The extracts were then washed with aqueous sodium sulfite (10 %, 3 x 20 ml). The combined organic layers were dried (MgSO$_4$) and evaporated to give Cbz-Leu-Phe-AS-Ph as a white powder, yield 63 %. ^{1}H-NMR (CDCl$_3$) δ 0.8-0.9 (2d, 6H, 2 x Leu-CH$_3$), 1.4-1.6 (m, 2H, Leu-CH$_2$), 3.8 (s, 2H, CH$_2$-Phe), 3.9 (d, 2H, CH$_2$-SO$_2$), 4.1 (m, 1H, α-H), 4.8 (t, 1H, CH=), 4.9-5.0 (b, 1H, NH), 5.1 (m, 2H, Cbz), 7.1-7.4 (m, 10H, 2 x Ph), 7.5-7.7 (m, 5H, SO$_2$-Ph), 8.4 (b, 1H, NH). ^{13}C NMR (400 MHz, CDCl$_3$) δ 172.1, 156.3, 144.4, 140.8, 135.7, 134.2, 133.6, 131.2, 129.5, 129.4 x 2, 129.3, 128.8 x 2, 128.7, 128.6, 128.4, 128.3, 127.8, 127.3, 126.9, 106.3, 67.6, 56.0, 55.6, 41.7, 40.4, 22.1, 21.0, 18.4. MS (FAB$^+$) *m/z* 535 (M + 1, 100 %). Anal. Calcd. for C$_{30}$H$_{34}$N$_2$O$_5$S: C, 67.39, H, 6.56, N, 5.43. Found: C, 67.41, H, 6.56, N, 5.43.

Phenyl-(3*R*)-3-(N-carbobenzyloxyleucyl)amino-4-phenylbut-1-enyl Sulfone (6c, Cbz-Leu-D-Phe-VS-Ph). Cbz-Leu-D-Phe-VS-Ph was prepared from Cbz-Leu-OH and D-Phe-VS-Ph·HCl using standard mixed anhydride coupling method, yield 81%. ^{1}H-NMR (CDCl$_3$) δ 0.8-0.9 (2d, 6H, 2 x Leu-CH$_3$), 1.4-1.6 (m, 2H, Leu-CH$_2$), 2.06 (m, 1H, Leu-CH), 2.9-3.0 (m, 2H, CH$_2$-Phe), 3.9-4.0 (m, 1H, α-H), 4.8-4.9 (b, 1H, α-H), 4.9-5.0 (m, 1H, NH), 5.1 (m, 2H, Cbz), 6.3-6.4 (d & b, 2H, NH and CH=), 6.9-7.0 (dd, 1H, CH=), 7.1-7.4 (m, 10H, 2 x Ph), 7.5-7.7 (m, 5H, SO$_2$-Ph). MS (ESI) *m/z* 535.

Phenyl-3-(N-carbobenzyloxyleucyl)amino-4-phenylbut-2-enyl Sulfone (7c,

Cbz-Leu-Phe-AS-Ph) (Isomer B). Cbz-Leu-D-Phe-VS-Ph was treated with

butyllithium and tert-butylhydroperoxide in freshly distilled THF as described with Cbz-

Leu-Phe-VS-Ph above to give Cbz-Leu-Phe-AS-Ph (Isomer B) as a white powder, yield

15 %. ^{1}H-NMR (CDCl$_3$) δ 0.8-0.9 (2d, 6H, 2 x Leu-CH$_3$), 1.4-1.6 (m, 2H, Leu-CH$_2$),

2.06 (m, 1H, Leu-CH), 3.8 (s, 2H, CH$_2$-Phe), 3.9 (d, 2H, CH$_2$-SO$_2$), 4.1 (m, 1H, α-H), 4.8

(t, 1H, CH=), 4.9-5.0 (b, 1H, NH), 5.1 (m, 2H, Cbz), 7.1-7.4 (m, 10H, 2 x Ph), 7.5-7.7

(m, 5H, SO$_2$-Ph); 8.4 (b, 1H, NH). MS (ESI) *m/z* 535 (M + 1, 100 %). Anal. calcd. for

C$_{30}$H$_{34}$N$_2$O$_5$S: C, 67.39, H, 6.56, N, 5.43. Found: C, 67.12, H, 6.61, N, 5.33.

Phenyl-(3*S*)-3-(N-carbobenzyloxyvalyl)amino-4-phenylbut-1-enyl Sulfone

(6b, Cbz-Val-Phe-VS-Ph). Cbz-Val-Phe-VS-Ph was prepared from Cbz-Val-OH and

Phe-VS-Ph·HCl using standard mixed anhydride coupling method, yield 98%. ^{1}H-NMR

(CDCl$_3$) δ 0.70-0.84 (2d, 6H, Val-CH$_3$), 2.06 (m, 1H, Val-CH), 2.91 (m, 2H, CH$_2$-Phe),

3.82 (m, 1H, α-H), 4.80-4.90 (m, 1H, α-H), 5.10 (s, 2H, Cbz), 5.96 (d, 1H, NH) 6.34 (d,

1H, CH=), 6.9-7.0 (dd, 1H, CH=), 7.1-7.4 (m, 10H, 2 x Ph), 7.5-7.7 (m, 5H, SO$_2$-Ph).

MS (ESI) *m/z* 521 (M + 1, 100 %).

Phenyl-3-(N-carbobenzyloxyvalyl)amino-4-phenylbut-2-enyl Sulfone (7b,

Cbz-Val-Phe-AS-Ph). Cbz-Val-Phe-VS-Ph was treated with butyllithium and tert-

butylhydroperoxide in freshly distilled THF as described with Cbz-Leu-Phe-VS-Ph above

to give Cbz-Val-Phe-AS-Ph as a white powder, yield 21 %. ^{1}H-NMR (CDCl$_3$) δ 0.70-

0.85 (2d, 6H, Val-CH$_3$), 2.1 (m, 1H, Val-CH), 3.80 (d, 2H, CH$_2$-Phe), 3.95 (m, 3H, CH$_2$-

SO$_2$ and α-H), 4.90 (t, 1H, CH=), 5.12 (m, 3H, NH and Cbz), 7.1-7.4 (m, 10H, 2 x Ph),

7.55 (t, 2H, SO$_2$-Ph), 7.65 (t, 1H, SO$_2$-Ph), 7.91 (d, 2H, SO$_2$-Ph), 8.42 (b, 1H, NH). ^{13}C

NMR (300 MHz, CDCl$_3$) δ 172.1, 156.3, 144.4, 140.8, 135.7, 134.2, 133.6, 131.2, 129.5, 129.4 x 2, 129.3, 128.8 x 2, 128.7, 128.6, 128.4, 128.3, 127.8, 127.3, 126.9, 106.3, 67.6, 56.0, 55.6, 41.7, 22.0, 21.0, 18.4. MS (FAB$^+$) m/z 521 (M + 1, 100 %). Anal. calcd. for C$_{29}$H$_{32}$N$_2$O$_5$S·1/10 H$_2$O: C, 66.67, H, 6.21, N, 5.36. Found: C, 66.31, H, 6.23, N, 5.26.

Phenyl-(3S)-3-(N-carbobenzyloxyalanyl)amino-4-phenylbut-1-enyl Sulfone (6a, Cbz-Ala-Phe-VS-Ph). Cbz-Ala-Phe-VS-Ph was prepared from Cbz-Ala-OH and Phe-VS-Ph·HCl using standard mixed anhydride coupling method, yield 88%. ^{1}H-NMR (CDCl$_3$) δ 1.25 (d, 3H, Ala-CH$_3$), 2.91 (dq, 2H, CH$_2$-Phe), 4.10 (m, 1H, α-H), 4.80-4.90 (m, 1H, α-H), 5.10 (s, 2H, Cbz), 6.34 (b, 1H, NH), 6.39 (d, 1H, CH=), 6.90-6.98 (dd, 1H, CH=), 7.10-7.39 (m, 10H, 2 x Ph), 7.55 (t, 2H, SO$_2$-Ph), 7.65 (t, 1H, SO$_2$-Ph), 7.91 (d, 2H, SO$_2$-Ph). ^{13}C NMR (400 MHz, CDCl$_3$) δ 172.1, 156.3, 144.4, 140.8, 135.7, 134.2, 133.6, 131.2, 129.5, 129.4 x 3, 129.3, 128.8 x 2, 128.7, 128.6, 128.4, 128.3, 127.8, 127.3, 126.9, 67.6, 51.7, 51.6, 40.2, 19.4.

Phenyl-3-(N-carbobenzyloxyalanyl)amino-4-phenylbut-2-enyl Sulfone (7a, Cbz-Ala-Phe-AS-Ph). Cbz-Ala-Phe-VS-Ph was treated with butyllithium and tert-butylhydroperoxide in freshly distilled THF as described with Cbz-Leu-Phe-VS-Ph above to give Cbz-Ala-Phe-AS-Ph as a white powder, yield 93 %. ^{1}H-NMR (CDCl$_3$) δ 1.21 (d, 3H, Ala-CH$_3$), 3.80 (m, 4H, CH$_2$-Phe and CH$_2$-SO$_2$), 4.12 (q, 1H, α-H), 4.88 (t, 1H, CH=), 5.11 (m, 3H, NH and Cbz), 7.30 (m, 10H, 2 x Ph), 7.50 (t, 2H, SO$_2$-Ph), 7.64 (t, 1H, SO$_2$-Ph), 7.83 (d, 2H, SO$_2$-Ph), 8.40 (b, 1H, NH). ^{13}C NMR (400 MHz, CDCl$_3$) δ 172.1, 156.3, 144.4, 140.8, 135.7, 134.2, 133.6, 131.2, 129.5, 129.4 x 2, 129.3, 128.8 x 2, 128.7, 128.6, 128.4, 128.3, 127.8, 127.3, 126.9, 107.2, 67.6, 51.7, 51.6, 40.2, 19.4. MS (FAB$^+$) m/z 493 (M + 1, 100 %). HRMS calcd. for C$_{27}$H$_{29}$N$_2$O$_5$S: 493.17972. Obsd.

493.17663. Anal. calcd. for $C_{27}H_{28}N_2O_5S$: C, 65.83, H, 5.73, N, 5.69. Found: C, 65.73, H, 5.82, N, 5.68.

N-Benzyloxycarbonylleucylalanine Methyl Ester (Cbz-Leu-Ala-OMe). Cbz-Leu-Ala-OMe was prepared from Cbz-Leu-OH and Ala-OMe using standard mixed anhydride coupling, yield 93 %. ^{1}H-NMR (CDCl$_3$) δ 0.90 (d, 6H, Leu-CH$_3$), 1.41 (d, 3H, Ala-CH$_3$), 1.58 (m, 1H, Leu-CH), 1.62 (m, 2H, Leu-CH$_2$), 3.88 (s, 3H, OCH$_3$), 4.21 (m, 1H, α-H), 4.59 (m, 1H, α-H), 5.11 (s, 2H, Cbz), 5.23 (b, 1H, NH), 6.45 (b, 1H, NH), 7.31 (m, 5H, Ph).

N-Benzyloxycarbonylleucylalanine (Cbz-Leu-Ala-OH). Cbz-Leu-Ala-OMe (4.23 g, 12.2 mmol) was suspended in methanol at room temperature. Aqueous sodium hydroxide (14.69 ml, 1N, 14.69 mmol) was added. The reaction was stirred for 1 hour (TLC Hex/EtOAc 1:1). The reaction was cooled to 0 ºC and acidified with 1N HCl (pH 2). The mixture was extracted with ethyl acetate (3 x 50 ml). The organic layer was washed with water (3 x 20 ml) and brine (3 x 20 ml) and dried (MgSO$_4$). The solvent was evaporated to give Cbz-Leu-Ala-OH as a white powder, yield 85 %. ^{1}H-NMR (CDCl$_3$) δ 0.90 (d, 6H, Leu-CH$_3$), 1.41 (d, 3H, Ala-CH$_3$), 1.59 (m, 1H, Leu-CH), 1.63 (m, 2H, Leu-CH$_2$), 4.30 (m, 1H, α-H), 4.59 (m, 1H, α-H), 5.11 (s, 2H, Cbz), 5.60 (b, 1H, NH), 6.97 (b, 1H, NH), 7.31 (m, 5H, Ph).

N-(N-Benzyloxycarbonylleucylalanyl)-N-2-ethanolamine (Cbz-Leu-Ala-NH(CH$_2$)$_2$OH). Cbz-Leu-Ala-OH was coupled to ethyl hydroxyl amine using standard mixed anhydride coupling to give crude Cbz-Leu-Ala-NH(CH$_2$)$_2$OH, which was recrystallized from cold ethyl acetate to give a white powder, yield 63 %. ^{1}H-NMR (CDCl$_3$) δ 0.90 (d, 6H, Leu-CH$_3$), 1.41 (d, 3H, Ala-CH$_3$), 1.58 (m, 1H, Leu-CH), 1.62

(m, 2H, Leu-CH$_2$), 2.92 (b, 1H, OH), 3.38 (m, 1H, NH*CH$_2$*), 3.41 (m, 1H, NH*CH$_2$*), 3.69 (d, 2H, *CH$_2$*OH), 4.13 (m, 1H, α-H), 4.43 (m, 1H, α-H), 5.11 (s, 2H, Cbz), 5.19 (b, 1H, NH), 6.45 (b, 1H, NH), 6.79 (b, 1H, NH), 7.31 (m, 5H, Ph).

N-(N-Benzyloxycarbonylleucyl)-N-2-(4,5-dihydrooxazol-2-yl)ethylamine (11, Cbz-Leu-Ala-oxazoline). Cbz-Leu-Ala-NH(CH$_2$)$_2$OH (1.15 g, 3.02 mmol) was dissolved in THF (20 ml). Triphenyl phosphine (1.19 g, 4.54 mmol) was added followed by dropwise addition of diisopropylazadicarboxylate (0.92 g, 4.54 mmol). The reaction was stirred for 2 hours at room temperature. The solvent was evaporated, and the crude oil was subjected to column chromatography (silica, MeOH/ CH$_2$Cl$_2$ 7 %) to give Cbz-Leu-Ala-oxazoline as a white powder, yield 75 %. ^{1}H-NMR (CDCl$_3$) δ 0.90 (d, 6H, Leu-CH$_3$), 1.41 (d, 3H, Ala-CH$_3$), 1.58 (m, 1H, Leu-CH), 1.69 (m, 2H, Leu-CH$_2$), 3.83 (t, 2H, NCH$_2$), 4.19 (m, 1H, α-H), 4.36 (t, 2H, OCH$_2$), 4.62 (m, 1H, α-H), 5.11 (s, 2H, Cbz), 5.19 (b, 1H, NH), 6.62 (b, 1H, NH), 7.36 (m, 5H, Ph). MS (FAB$^+$) *m/z* 362 (M + 1, 100 %). HRMS calcd. for C$_{19}$H$_{28}$N$_3$O$_4$: 362.20813. Obsd. 362.20798. Anal. calcd. for C$_{19}$H$_{27}$N$_3$O$_4$·1/4 H$_2$O: C, 62.36, H, 7.57, N, 11.48. Found: C, 62.22, H, 7.55, N, 11.64.

REFERENCES

(1)   Johnson, P. *Int. J. Biochem.* **1990**, *22*, 811-822.

(2)   Mellgren, R. L.; Rozanov, C. B. *Biochem. Biophys. Res. Commun.* **1990**, *168*, 589-95.

(3)   Mellgren, R. L.; Murachi, T. *Intracellular calcium-dependent proteolysis*; CRC Press: Boca Raton, FL, 1990.

(4)   Melloni, E.; Pontremoli, S. *Trends in Neurological Sciences* **1989**, *12*, 438-444.

(5)   Nixon, R. A. *Ann. N. Y. Acad. Sci.* **1989**, *568*, 198-208.

(6)   Melloni, E.; Salamino, F.; Sparatore, B. *Biochimie* **1992**, *74*, 217-23.

(7)   Bartus, R. T.; Elliott, P. J.; Hayward, N. J.; Dean, R. L.; Harbeson, S.; Straub, J. A.; Li, Z.; Powers, J. C. *Neurological Research* **1995**, *17*, 249-258.

(8)   Seubert, P.; Ivy, G.; Larson, J.; Lee, J.; Shahi, K.; Baudry, M.; Lynch, G. *Brain Res.* **1988**, *459*, 226-32.

(9)   Arai, A.; Vanderklish, P.; Kessler, M.; Lee, K.; Lynch, G. *Brain Res.* **1991**, *555*, 276-80.

(10)   Powers, J. C.; Asgian, J. L.; Ekici, O. D.; James, K. E. *Chem. Rev.* **2002**, *102*, 4639-750.

(11)   Tsujinaka, T.; Kajiwara, Y.; Kambayashi, J.; Sakon, M.; Higuchi, N.; Tanaka, T.; Mori, T. *Biochem. Biophys. Res. Commun.* **1988**, *153*, 1201-1208.

(12)   Woo, J.-T.; Sigeizumi, S.; Yamaguchi, K.; Sugimoto, K.; Kobori, T.; Tsuji, T.; Kondo, K. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 1501-1504.

(13)   Sasaki, T.; Kishi, M.; Saito, M.; Tanaka, T.; Higuchi, N.; Kominami, E.; Katunuma, N.; Murachi, T. *J. Enzyme Inhib.* **1990**, *3*, 195-201.

(14) Iqbal, M.; Messina, P. A.; Freed, B.; Das, M.; Chatterjee, S.; Tripathy, R.; Tao, M.; Josef, K. A.; Dembofsky, B.; Dunn, D.; Griffith, E.; Siman, R.; Senadhi, S. E.; Biazzo, W.; Bozyczko-Coyne, D.; Meyer, S. L.; Ator, M. A.; Bihovsky, R. *Bioorganic & Medicinal Chemistry Letters* **1997**, *7*, 539-544.

(15) Chatterjee, S.; Ator, M. A.; Bozyczko-Coyne, D.; Josef, K.; Wells, G.; Tripathy, R.; Iqbal, M.; Bihovsky, R.; Senadhi, S. E.; Mallya, S.; O'Kane, T. M.; McKenna, B. A.; Siman, R.; Mallamo, J. P. *J. Med. Chem.* **1997**, *40*, 3820-8.

(16) Chatterjee, S.; Iqbal, M.; Mallya, S.; Senadhi, S. E.; O'Kane, T. M.; McKenna, B. A.; Bozyczko-Coyne, D.; Kauer, J. C.; Siman, R.; Mallamo, J. P. *Bioorg. Med. Chem.* **1998**, *6*, 509-22.

(17) Chatterjee, S.; Gu, Z. Q.; Dunn, D.; Tao, M.; Josef, K.; Tripathy, R.; Bihovsky, R.; Senadhi, S. E.; O'Kane, T. M.; McKenna, B. A.; Mallya, S.; Ator, M. A.; Bozyczko-Coyne, D.; Siman, R.; Mallamo, J. P. *J. Med. Chem.* **1998**, *41*, 2663-6.

(18) Azzo, W.; Woessner, J. F., Jr. *J. Biol. Chem.* **1986**, *261*, 5434-41.

(19) Li, Z.; Patil, G. S.; Golubski, Z. E.; Hori, H.; Tehrani, K.; Foreman, J. E.; Eveleth, D. D.; Bartus, R. T.; Powers, J. C. *J. Med. Chem.* **1993**, *36*, 3472-3480.

(20) Angelastro, M. R.; Mehdi, S.; Burkhart, J. P.; Peet, N. P.; Bey, P. *J. Med. Chem.* **1990**, *33*, 11-13.

(21) Li, Z.; Ortega-Vilain, A.-C.; Patil, G. S.; Chu, D.-L.; Foreman, J. E.; Eveleth, D. D.; Powers, J. C. *J. Med. Chem.* **1996**, *39*, 4089-4098.

(22) Angliker, H.; Anagli, J.; Shaw, E. *J. Med. Chem.* **1992**, *35*, 216-20.

(23) Chatterjee, S.; Josef, K.; Wells, G.; Iqbal, M.; Bihovsky, R.; Mallamo, J. P.; Ator, M.; Bozyczko-Coyne, D.; Mallya, S.; Senadhi, S.; Siman, R. *Bioorganic & Medicinal Chemistry Letters* **1996b**, *6*, 1237-1240.

(24) Huang, Z.; McGowan, E. B.; Detwiler, T. C. *J. Med. Chem.* **1992**, *35*, 2048-2054.

(25) Palmer, J. T.; Rasnik, D.; Klaus, J. L.; Brömme, D. *J. Med. Chem.* **1995**, *38*, 3193-3196.

(26) Parkes, C.; Kembhavi, A. A.; Barrett, A. J. *Biochem. J.* **1985**, *230*, 509-516.

(27) Hoye, T. R.; Crawford, K. B. *J. Org. Chem.* **1994**, *59*, 520-522.

(28) Harris, A. L.; Gregory, J. S.; Maycock, A. L.; Graybill, T. L.; Osifo, I. K.; Schmidt, S. J.; Dolle, R. E. *Bioorg. Medicinal Chem. Letters* **1995**, *5*, 393-398.

(29) Sasaki, T.; Kikuchi, T.; Fukui, I.; Murachi, T. *J. Biochem.* **1986**, *99*, 173-179.

(30) Dolle, R. E.; Singh, J.; Whipple, D.; Osifo, I. K.; Speier, G.; Graybill, T. L.; Gregory, J. S.; Harris, A. L.; Helaszek, C. T.; Miller, R. E.; Ator, M. A. *J. Med. Chem.* **1995**, *38*, 220-222.

(31) Pliura, D. H.; Bonaventura, B. J.; Smith, R. A.; Coles, P. J.; Krantz, A. *Biochem. J.* **1992**, *288*, 759-62.

(32) Prototek, Inc.: US6714484 (**1998**).

(33) Hanada, K.; Tamai, M.; Yamagishi, M.; Ohmura, S.; Sawada, J.; Tanaka, I. *Agric. Biol. Chem.* **1978**, *42*, 523-528.

(34) Inaba, T.; Hirayama, Y.; Fujinaga, N. *Agric. Biol. Chem.* **1979**, *43*, 655.

(35) Hanada, K.; Tamai, M.; Ohmura, S.; Sawada, J.; Seki, T.; Tanaka, I. *Agric. Biol. Chem.* **1978**, *42*, 529-536.

(36) Towatari, T.; Tanaka, K.; Yoshikawa, D.; Katunuma, N. *J. Biochem. (Tokyo)* **1978**, *84*, 659-72.

(37) Suzuki, K. *J. Biochem. (Tokyo)* **1983**, *93*, 1305-12.

(38) Suzuki, K.; Tsuji, S.; Ishiura, S.; Kimura, Y.; Kubota, S.; Imahori, K. *J. Biochem. (Tokyo)* **1981**, *90*, 1787-93.

(39) Sugita, H.; Ishiura, S.; Suzuki, K.; Imahori, K. *J. Biochem. (Tokyo)* **1980**, *87*, 339-41.

(40)   Roush, W. R.; Hernandez, A. A.; McKerrow, J. H.; Selzer, P. M.; Hansell, E.; Engel, J. C. *Tetrahedron* **2000**, *56*, 9747.

(41)   Buttle, D. J.; Saklatvala, J.; Tamai, M.; Barrett, A. J. *Biochem. J.* **1992**, *281 (Pt 1)*, 175-7.

(42)   Kembhavi, A. A.; Buttle, D. J.; Knight, C. G.; Barrett, A. J. *Arch. Biochem. Biophys.* **1993**, *303*, 208-13.

(43)   Chen, Z.; Potempa, J.; Polanowski, A.; Wikstrom, M.; Travis, J. *J. Biol. Chem.* **1992**, *267*, 18896-901.

(44)   Nikawa, T.; Towatari, T.; Katunuma, N. *Eur. J. Biochem.* **1992**, *204*, 381-93.

(45)   Asgian, J. L.; James, K. E.; Li, Z. Z.; Carter, W.; Barrett, A. J.; Mikolajczyk, J.; Salvesen, G. S.; Powers, J. C. *J. Med. Chem.* **2002**, *45*, 4958-60.

(46)   Wadsworth, D. H.; Schupp, O. E. I.; Seus, E. J.; Ford, J. A. J. *Journal of Organic Chemistry* **1965**, *30*, 680-685.

(47)   Wipf, P.; Miller, C. P. *Tetrahedron Letters* **1992**, *33*, 6267-6270.

(48)   Jin, Z.; Kim, S. H.; Fuchs, P. L. *Tetrahedron Letters* **1996**, *37*, 5247-5248.

(49)   Hine, J.; Linden, S.-M.; Wang, A.; Thiagarajan, V. *J. Org. Chem.* **1980**, *45*, 2821-2825.

(50)   Hirata, T.; Sasada, Y.; Ohtani, T.; Asada, T.; Kinoshita, H.; Senda, H.; Inomata, K. *Bulletin of the Chemical Society of Japan* **1992**, *65*, 75-96.

(51)   Inomata, K.; Hirata, T.; Suhara, H.; Kinoshita, H.; Kotake, H.; Senda, H. *Chemistry Letters* **1988**, *12*, 2009-20012.

(52)   Sasaki, T.; Kikuchi, T.; Yumoto, N.; Yoshimura, N.; Murachi, T. *J. Biol. Chem.* **1984**, *259*, 12489-12494.

(53)   Barrett, A. J. *Biochem. J.* **1980**, *187*, 909-12.

(54) Arnon, R. *Immunochemistry* **1965**, *2*, 107-14.

(55) Trost, B. M.; Merlic, C., A. *J. Org. Chem.* **1990**, *55*, 1127-1129.

(56) Chinchilla, R.; Galindo, N.; Najera, C. *Tetrahedron* **1996**, *52*, 1035-1046.

(57) Silverman, R. B. *The Organic Chemistry of Enzyme-Catalyzed Reactions*; Academic Press: San Diego, 2000.

CHAPTER 4

DESIGN, SYNTHESIS, AND EVALUATION OF POTENT AND SELECTIVE

INHIBITORS FOR *S. MANSONI* LEGUMAIN

(ASPARAGINYL ENDOPEPTIDASE)

INTRODUCTION

Schistosomias is after malaria the most prevalent parasitic disease and infects more than 250 million people in tropical regions. There are three distinct parasitic species, that are responsible for schistosomias: *Schistosoma japonicum, Schistosoma haematobium* and *Schistosoma mansoni*. *Schistosoma mansoni*, which is found in 54 countries in Africa, Asia, and South America, is the schistosome species that causes intestinal schistosomias (WHO). The individuals infected with *S. mansoni* suffer from liver fibrosis and portal hypertension. The drug of choice praziquantel has up to date been effective in treating the disease. However recent evidence for an emerging resistance of the parasite to praziquantel has been reported[1-5].

To complete its life-cycle the schistosome parasite requires two separate hosts, a fresh water snail and a mammal or human as the primary host (Figure 4.1). *Schistosoma mansoni* resides in the veins of the mesenteries of its mammalian host, where the female ingests 330 000 red blood cells per hour[6] as a source for amino acids[7]. The red blood cells are lysed by hemolysin in the esophagus of the parasite[8,9]. The parasite employs cysteine and aspartic proteases in a catalytic cascade of hemoglobin degradation[10-12]. The first cysteine proteases to be identified were Sm31 and Sm32[13-15]. Other proteases, which

have so far been identified, include cathepsin L[16], cathepsin D[17], and cathepsin C[18].

Sm31 exhibits clan CA characteristics and has therefore been designated schistosome cathepsin B. It has been studied predominantly over other proteases expressed by adult schistosomes. Sm32 is an asparaginyl endopeptidase homologous to a family of cysteine proteases called legumains, and therefore also known as schistosome legumain[19].
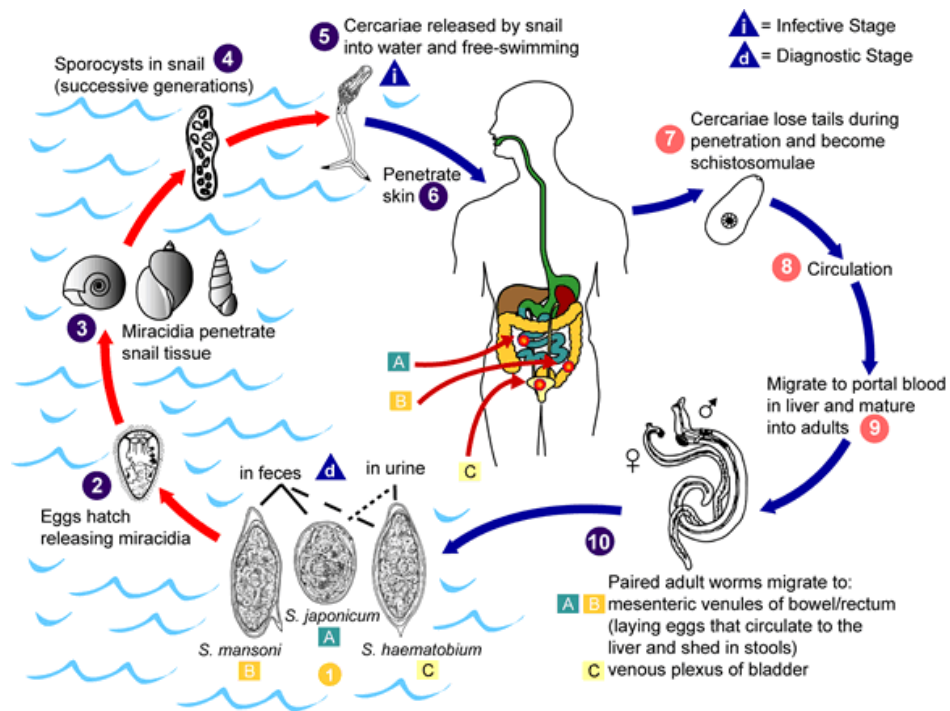
Figure 4.1. Schistosome Life-Cycle[20].

Legumains (EC.3.4.22.34) form a family (C13) of clan CD proteases. Other members of the clan CD proteases include caspases, gingipains, and clostripains. They are related by a shared catalytic-site motif and a common scaffold within their catalytic domains[21]. They were first identified in leguminous plants[22,23], and later in mammalian cells[24]. In mammalian cells legumain has been linked to osteoclast formation and bone

resorption[25], and the processing of bacterial antigens[26] and the potential autoantigen, myelin basic protein, in the major histocompatability (MHC) class II system[27]. In *S. mansoni* the legumain protease is dissimilar to the other proteinases and may process gut-associated clan CA zymogens to their active forms, thus facilitating the digestion of ingested host serum proteins[28,29]. *S. mansoni* legumain is not inhibited by the clan CA cysteine protease inhibitor E-64 or the diazomethane inhibitor Cbz-Phe-Ala-CHN$_2$. Irreversible protease inhibitors would have great potential for the short-term therapeutic administration against parasitic infections[28]. So far there has been no success in the determination of a crystal structure of the asparaginyl endopeptidase. Therefore potent and selective irreversible inhibitors would not only contribute therapeutic properties, but also help determine the structural and enzymatic specificities of the legumain active site. Like all clan CD proteases, the substrate specificity of legumain is controlled by the interactions of the S1 subsite. Legumains selectively hydrolyze substrates with an asparaginyl residue in the P1 position. Mathieu *et al* have mapped the substrate specificity for the P2 and P3 amino acid residues[30], and found that Sm32 is less selective with respect to the P2 and P3 positions, but prefers Ala or Thr. The synthetic substrate Cbz-Ala-Ala-Asn-AMC was effectively cleaved by *S. mansoni* legumain and human legumain with K_M's of 90 and 80 μM respectively. This peptide sequence provides a basis for the design of potential inhibitors.

The synthetic inhibitors reported so far include peptidyl aza-Asn halomethylketones (Cbz-Ala-Ala-AAsn-CH$_2$Cl, k_{obs}/[I] = 139,000 $M^{-1}s^{-1}$, Cbz = benzyloxycarbonyl), peptidyl Michael acceptors derived from Asn (k_{obs}/[I] up to 766 $M^{-1}s^{-1}$)[31], and peptidyl acyloxymethylketones up to 109,000 $M^{-1}s^{-1}$ tested with mammalian

legumain[32] (Figure 4.2). Aspartyl peptidyl fluoromethyl ketones designed specifically for

caspase inhibition have found to be moderate inhibitors of legumain[33].
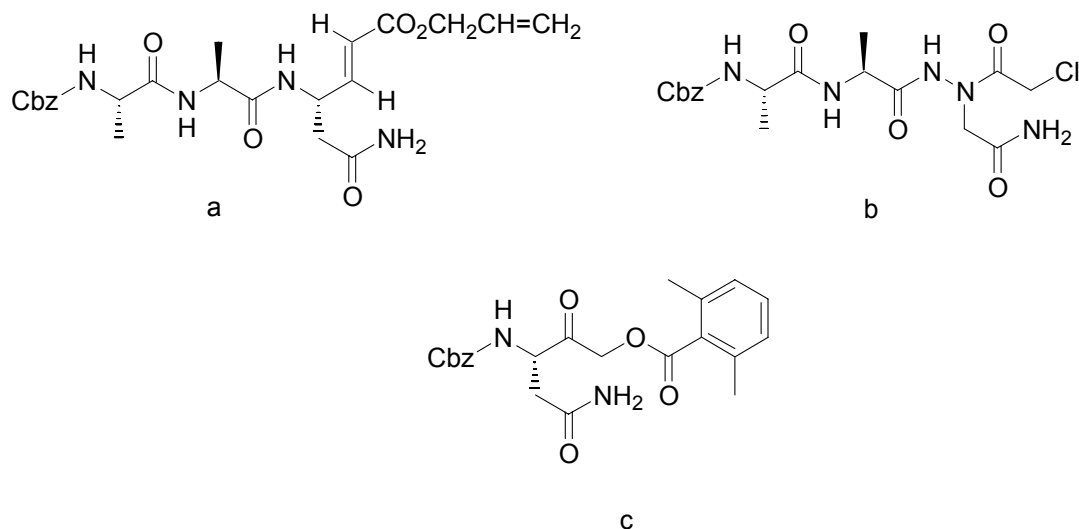
Figure 4.2. Previously Reported Legumain Inhibitors; a) Peptidyl Michael acceptor

inhibitor ($k_{obs}/[I] = 776\ M^{-1}s^{-1}$)[31], b) Aza-peptidyl chloromethyl ketone ($k_{obs}/[I] = 139,088$

$M^{-1}s^{-1}$)[31], c) Acyloxymethylketone inhibitor ($k_{obs}/[I] = 109,000\ M^{-1}s^{-1}$)[32].

In an effort to design and synthesize more specific and selective legumain

inhibitors, we created two new classes of inhibitors. Initially, we based our design on the

previously reported aza-peptide epoxides, a novel class of cysteine protease inhibitors

specific for clan CD proteases developed by our laboratory[34]. We extended this design

towards aza-peptide epoxides, a new class of inhibitors specific for legumain. We have

also replaced the epoxide moiety with the double bond, to create a second new class of

inhibitors, the aza-peptide Michael acceptors, which are even more potent than the

epoxide inhibitors.

4.1. AZA-PEPTIDE EPOXIDES: POTENT AND SELECTIVE

INHIBITORS OF *SCHISTOSOMA MANSONI* LEGUMAIN

INHIBITOR DESIGN

Aza-peptide epoxides (Figure 4.1.1) were designed to closely resemble an extended peptide legumain substrate (**1**) with the placement of the carbonyl group of the epoxide moiety in the inhibitor (**2**) in a location identical to that of the carbonyl of the scissile peptide bond in a legumain substrate. This design allowed the peptide chain of the inhibitor to exactly match that of a good substrate up to the scissile peptide carbonyl group. Conversion of the α-carbon of an amino acid residue into a nitrogen results in the formation of an aza-amino acid residue, which allows the ready synthesis of a variety of epoxide derivatives. In our design of aza-peptide epoxides for legumains, we have used the epoxysuccinyl group from E-64 and attached it to an aza-Asn residue (AAsn) to place the epoxy moiety on the P' site of the inhibitor structure. We abbreviate the epoxide as EP and aza-asparagine as AAsn, thus the above inhibitor is designated Cbz-Ala-Ala-AAsn-EP-R'. In contrast, in E-64 derivatives, the epoxide is usually attached to the N-terminus of a peptide or amino acid[35]. Aza-peptide epoxides (**2**) have the advantage of being easily extended in the P' direction, allowing interactions with the S' subsites of legumain.
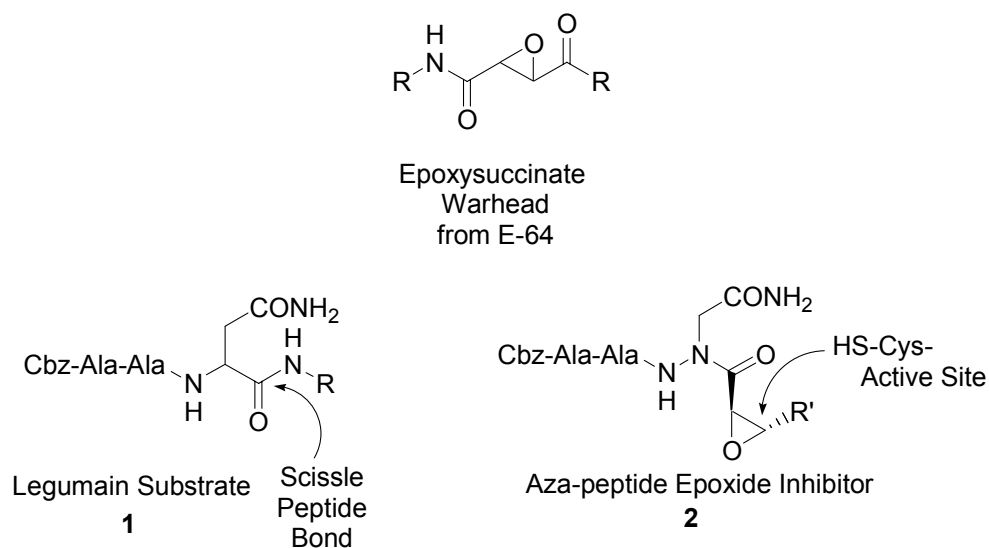
Figure 4.1.1. Design of Aza-Peptide Epoxide Inhibitors.

CHEMISTRY

Methods for synthesizing the various epoxide moities for the new inhibitors are shown in Figure 4.1.3. Enantiomerically pure diethyl epoxysuccinate esters (**3,** 2*S,3S* and 2*R,3R*) were synthesized by Zhao Zhao Li from diethyl D-(-) and L-(+)-tartrate, following the general method developed by Mori and Iwasawa (Figure 4.1.2)[36,37].
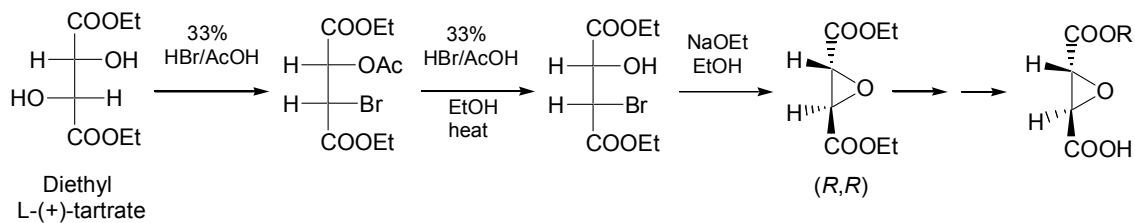
Figure 4.1.2. Synthesis of Enantiomerically Pure Epoxysuccinate Esters.

The *trans*-oxirane-2,3-dicarboxylic acid diethyl ester (**3**, *trans*) was synthesized using a general procedure for the stereocontrolled epoxidation of α,β-unsaturated carbonyl compounds, which was similar to the method developed by Meth-Cohn[38]. The *cis*-oxirane-2,3-dicarboxylic acid diethyl ester was synthesized using the same procedure with diethyl maleate as the starting material. Selective hydrolysis using KOH in ethanol by a procedure similar to that described previously[39,40] yielded the monoethyl epoxysuccinates (**5**). Complete hydrolysis of both esters was accomplished by using 2 eq of NaOH in methanol, to give the oxirane-2,3-dicarboxylic acid (**4,** *trans, 2R,3R*, and *2S,3S*). Additional oxirane-2-carboxylic acid monoester derivatives (**6b, c**) were obtained by adding 1 eq of benzyl alcohol or phenethyl alcohol to **4** using DMAP as a base and EDC as the coupling reagent. Amide derivatives of oxirane-2,3-dicarboxylic acid (**6d-h**) were obtained by coupling the corresponding amine or amino acid (R_2NH where R can be H, CH_3, *n*Bu, CH_2Ph, CH_2CH_2Ph, $CH(CH_3)CONHCH_2Ph$) to **5** using EDC and HOBt, followed by hydrolysis of the ethyl ester using 1.2 eq of KOH in ethanol[41]. The *trans*-3-phenethyloxirane-2-carboxylic acid (**6i**) was synthesized by Özlem Doğan Ekici coupling 3-phenylpropionaldehyde (**7**) with malonic acid to form the α,β-unsaturated acid, which can be further transformed into an ethyl ester (**8**). The double bond was epoxidized using *t*-butyl hydroperoxide and *t*-butyl lithium[38], and then the ethyl ester was hydrolyzed using KOH. Using a similar method, *trans*-3-(4-chlorophenyl)oxirane-2-carboxylic acid (**6j**) was synthesized by epoxidizing 3-(4-chlorophenyl)acrylic acid methyl ester, followed by the deblocking of the methyl ester.

71

We chose the Cbz-Ala-Ala-AAsn sequence for legumain inhibitors since Cbz-Ala-Ala-Asn-NHMec (NHMec = 7-(4-methyl)coumarylamide) is an optimal substrate sequence[42]. We have previously reported a general synthetic method for the preparation of aza-peptide epoxides (Figure 4.1.4)[34]. The peptidyl hydrazide Cbz-Ala-Ala-NHNH$_2$ (**9**) was prepared from Cbz-Ala-Ala-OMe by reaction with excess hydrazine in methanol. The aza-asparagine side chain was introduced by alkylation of Cbz-Ala-Ala-NHNH$_2$ with ethyl bromoacetate and NMM in DMF. The ethyl ester was then converted to the amide (**10**) by ammonolysis with NaCN as a catalyst using the method described by Hogberg *et al*[43]. The substituted hydrazide (**10**) was then coupled to various oxirane derivatives (**6a-j**) using EDC and HOBt to give the desired aza-peptide epoxides (**11a-j**).
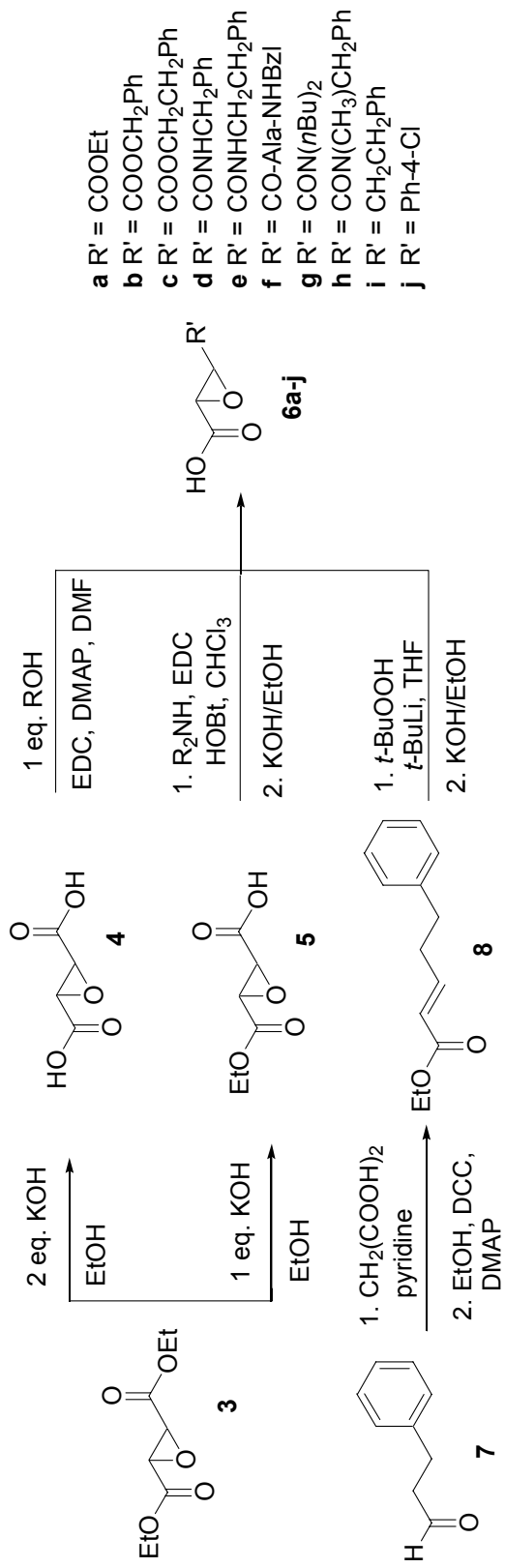
Figure 4.1.3. Synthesis of the Epoxide Moiety.

a R' = COOEt
b R' = COOCH₂Ph
c R' = COOCH₂CH₂Ph
d R' = CONHCH₂Ph
e R' = CONHCH₂CH₂Ph
f R' = CO-Ala-NHBzl
g R' = CON(nBu)₂
h R' = CON(CH₃)CH₂Ph
i R' = CH₂CH₂Ph
j R' = Ph-4-Cl

73
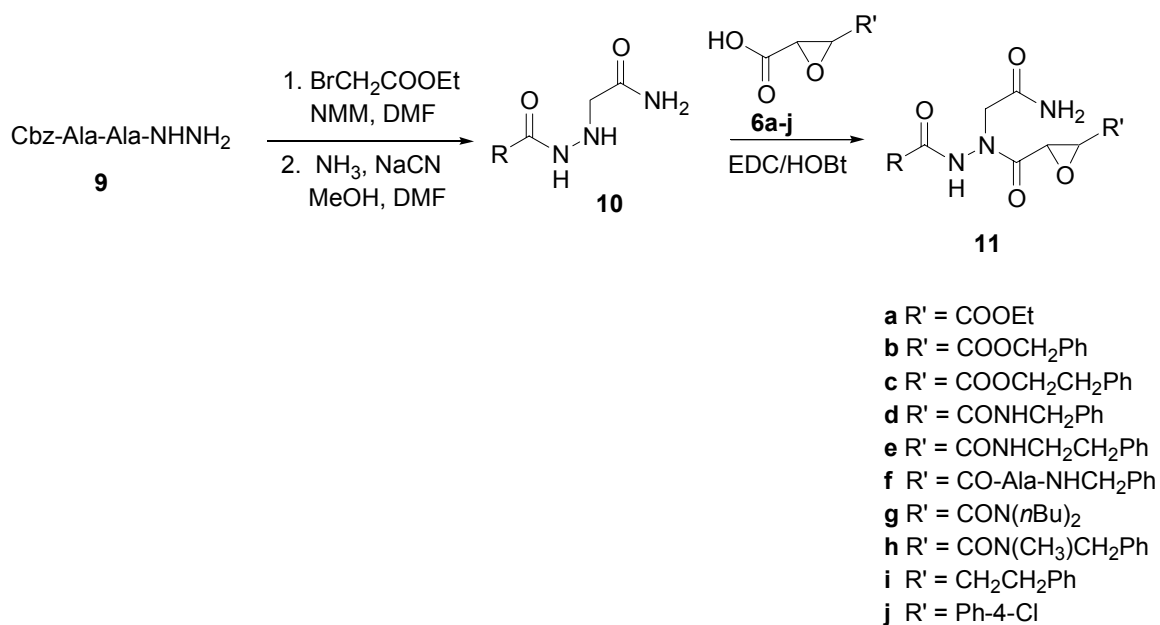
Figure 4.1.4. Synthesis of Aza-Peptide Epoxide Inhibitors.

RESULTS AND DISCUSSION

The synthetic aza-Asn inhibitors (**11a-j**) were tested against *S. mansoni* legumain by Conor Caffrey in the laboratory of Jim McKerrow at the University of California, San Francisco. The IC_{50} values for inhibition of *S. mansoni* legumain varied from 45 to 288 nM and $k_{obs}/[I]$ values ($\sim 10^4$ $M^{-1}s^{-1}$) were obtained for selected inhibitors by the progress curve method (Table 4.1.1).

Table 4.1.1. Inhibition of Legumain by Aza-Peptide Epoxides.

Inhibitor	R'	STEREOCHEMISTRY	S. mansoni legumain	
			IC$_{50}$ (nM)	k$_{obs}$/[I] (M^{-1}s^{-1})
11a	COOEt^a	S,S	53 ± 25	17,400
	COOEt^a	R,R	788 ± 88	
	COOEt^a	cis		
11b	COOCH$_2$Ph^a	S,S	47 ± 33	15,300
	COOCH$_2$Ph^a	trans		
11c	COOCH$_2$CH$_2$Ph	S,S	45	
11d	CONHCH2Ph^a	S,S	NI	
11e	CONHCH$_2$CH$_2$Ph^a	S,S	NI	
11f	CO-Ala-NH-Bzl	R,R	NI	
11g	CON(nBu)$_2$	S,S	68 ± 4	
11h	CON(CH$_3$)CH$_2$Ph	S,S	63 ± 11	
11i	CH$_2$CH$_2$Ph	trans	70 ± 14	
11j	Ph-4-Cl	trans	90 ± 0	

NI = No inhibition after 20 minutes of incubation.
^{a}Compounds were synthesized by Karen James.

Legumains were inhibited by aza-peptide epoxides with a variety of substituents on the prime side of the epoxide such as esters (COOR), alkyl and aryl groups (CH_2CH_2Ph or Ph-4-Cl), monosubstituted or disubstituted amides ($CONR_2$), and disubstituted amides. The epoxide stereochemistry affected the reaction rate, and the order of reactivity is *S,S* > *R,R* > *trans* > *cis*. The more potent form of E-64 also possesses *S,S* stereochemistry at the epoxide moiety, while the *R,R* isomer is less reactive toward cysteine proteases. Interestingly, the placement of esters or disubstituted amides on the epoxide moiety gave more potent inhibitors than the placement of alkyl groups or monosubstituted amides on the epoxide moiety. The ethyl ester on the epoxide (*S,S* isomer of **11a**, $k_{obs}/[I] = 43,000$ $M^{-1}s^{-1}$) was more potent than the benzyl ester (*S,S* isomer of **11b,** $k_{obs}/[I] = 26,700$ $M^{-1}s^{-1}$) with pig kidney legumain (Data obtained for Karen James' compounds by Alan Barrett's lab), but both esters were almost equally potent with *S. mansoni* legumain (**11a** $k_{obs}/[I] = 17,400$ $M^{-1}s^{-1}$, $IC_{50} = 53$ nM and **11b**, $k_{obs}/[I] = 15,300$ $M^{-1}s^{-1}$, $IC_{50} = 47$ nM). The most potent inhibitor of *S. mansoni* legumain was the phenethyl ester Cbz-Ala-Ala-AAsn-(2*S*,3*S*)-EP-COOCH$_2$CH$_2$Ph (**11c**, $IC_{50} = 45$ nM).

Surprisingly, neither the pig kidney nor the *S. mansoni* legumain was inhibited by the monosubstituted amides Cbz-Ala-Ala-AAsn-(2*S*,3*S*)-EP-CONHCH$_2$Ph (**11d**) and Cbz-Ala-Ala-AAsn-(2*S*,3*S*)-EP-CONHCH$_2$CH$_2$Ph (**11e**) nor the amino acid derivative Cbz-Ala-Ala-AAsn-(2*R*,3*R*)-EP-CO-Ala-NHCH$_2$Ph (**11f**). The only difference between the benzyl amide and the benzyl ester is that an NH replaces the oxygen of the carboxylic acid. The lack of inhibition by the benzyl amide could be explained by the formation of different hydrogen bonds within the active site as a result of the NH replacing the oxygen. Interestingly, the disubstituted amides Cbz-Ala-Ala-AAsn-(2*S*,3*S*)-EP-

CON(nBu)$_2$ (**11g**, IC$_{50}$ = 68 nM) and Cbz-Ala-Ala-AAsn-(2S,3S)-EP-CON(CH$_3$)CH$_2$Ph (**11h**, IC$_{50}$ = 63 nM) were quite potent inhibitors of *S. mansoni* legumain. We speculate that this is a result of the lack of a hydrogen bond. Interestingly, aza-peptide epoxides with a P1 aza-Asp and mono- and disubstituted amides or amino acids on the epoxide moiety work extremely well with the caspases (unpublished results). Caspase specific inhibitors with a P1 aza-Asp and an ester functional group on the epoxide moiety have either no reactivity toward legumain or very small rates (15 - 86 M^{-1}s^{-1} with various tetrapeptide inhibitors). No mono- or disubstituted amide with a P1 aza-Asp has been tested with legumain so far. However it would appear that these potent caspase inhibitors should have little or no inhibitory potency toward legumain. The alkyl epoxide moieties **11i** (CH$_2$CH$_2$Ph) and **11j** (Ph-4-Cl) were the poorest *S. mansoni* legumain inhibitors (IC$_{50}$ values > 70 nM). We speculate that for maximum potency, the enzyme needs the carbonyl next to the epoxide moiety (possibly for hydrogen bonding or to activate the epoxide due to its electronegativity).

The aza-peptide epoxides had previously been investigated for their stability (unpublished results). It was found that the caspase-6 specific inhibitor Cbz-Leu-Glu-Thr-AAsp-(S,S)-EP-COOCH$_2$Ph is equally potent with caspase-6 when incubated with buffer containing DTT as when incubated with buffer lacking DTT. The stability of epoxysuccinyl peptide inhibitors under physiological conditions toward simple thiols has been reported in the literature[39]. E-64 is not alkylated by 100 mM cysteine nor does is inactivate lactate dehydrogenase, a non-proteolytic thiol-dependent enzyme[44]. Our laboratory has also synthesized aza-peptide epoxides specific for dipeptidyl peptidase I (DPPI), which is specific for a free N-terminus. The free N-terminus however cyclizes

with the warhead. This was also observed with the dipeptidyl vinyl sulfones (see Chapter

2). The aza-peptide epoxides with a half-life of 350 min at pH 7 and 7.5 are more stable

than the dipeptidyl vinyl sulfones. For example, Nva-AHph-EP-COOEt was 7 and 9-fold

more stable than Nva-Hph-VS-Ph at pH 7 and 7.5, respectively (unpublished results).

Aza-peptide epoxides are specific clan CD cysteine protease inhibitors and do not

inhibit clan CA cysteine proteases, serine proteases, or aspartyl proteases. The aza-Asn

inhibitors are selective for mammalian and *S. mansoni* legumains, since Cbz-Ala-Ala-

AAsn-(2*S*,3*S*)-EP-COOEt **11a** showed essentially no cross reactivity with other enzymes

in clan CD such as caspases ($k_{obs}/[I] = < 1$ $M^{-1}s^{-1}$ with caspases-3, -6 and –8). Aza-

peptide epoxides also displayed little to no inhibition of the clan CA cysteine proteases

papain and cathepsin B and no inhibition of the serine protease α-chymotrypsin[34]. The

inhibitor Cbz-Ala-Ala-AAsn-(2*S*,3*S*)-EP-COOEt (**11a**) did not inhibit the serine protease

granzyme B nor the aspartyl proteases porcine pepsin, human cathepsin D, plasmepsin 2

from *P. falciparum*, HIV-1 protease, and the secreted aspartic proteinase 2 (SAP-2) from

Candida albicans (unpublished results).

SUMMARY

In summary, we have shown that aza-peptide epoxides can be constructed to

selectively and potently inhibit *S. mansoni* legumain. Aza-peptide epoxides are a new

class of cysteine protease inhibitors specific for the clan CD cysteine proteases, as they

show little to no inhibition of clan CA cysteine proteases, serine proteases, or aspartyl

proteases. The aza-peptide epoxides designed for legumains show little to no cross-

reactivity with other clan CD proteases such as capsases. Currently, we are trying to

refine the P' portion of the inhibitors to obtain greater specificity and are testing this class

of inhibitors with other clan CD enzymes.

4.2. AZA-PEPTIDE MICHAEL ACCEPTORS: POTENT AND SELECTIVE INHIBITORS OF *SCHISTOSOMA MANSONI* LEGUMAIN

INHIBITOR DESIGN

In the past Michael acceptors have been employed as warheads against several cysteine proteases[45-47]. Among the most effective inhibitors are vinyl sulfones[48] and α,β-unsaturated carbonyl derivatives against various cysteine proteases[49,50]. The original strategy reported in the literature was to replace the carbonyl group of a good substrate with a moiety, that would trap the enzymatic nucleophile (Ser-OH or Cys-OH) without altering the structural features required for enzyme recognition and binding[45]. The fumarate derivative of the epoxy succinate E-64c, which is one of the first Michael acceptor inhibitors reported, extends the α,β-unsaturated carbonyl by an additional carbonyl in the β-position [44]. This modification allows the preservation of the scissile carbonyl for possible structural recognition and binding requirements within the enzyme active site. Both the fumarate analog of E-64c and the epoxide parent compound do not inhibit clan CD proteases, but are very effective with clan CA proteases. The E-64c epoxide warhead itself, however, can be incorporated into inhibitors specific for clans other than clan CA. This has been described in chapter 4.1 with the design and synthesis of the aza-peptide epoxides specific for the clan CD *Schistosoma mansoni* asparaginyl endopeptidase legumain. The fumarate derivative of E-64c (*trans*-HOOC-CH=CHCO-Leu-NH(CH$_2$)$_2$CH(CH$_3$)$_2$) inhibits cathepsin B ($k_{app} = 625$ M^{-1}s^{-1}), cathepsin H ($k_{app} = 11$ M^{-1}s^{-1}), and cathepsin L ($k_{app} = 2272$ M^{-1}s^{-1}) irreversibly. The α,β-unsaturated carboxyl

moiety undergoes a Michael addition by the active site nucleophile, and thus the inhibitor

irreversibly binds to the enzyme. Since the aza-peptide epoxides described in chapter 4.1

proved to be potent and selective inhibitors for *S. mansoni* legumain, we in parallel

modified this particular design by replacing the epoxide moiety with a double bond to

form a Michael acceptor (Figure 4.2.1). Herein we report the design, synthesis, and

evaluation of aza-peptide Michael acceptors as potent and specific inhibitors for *S.*

mansoni legumain, and provide evidence for the mechanism of inhibition.
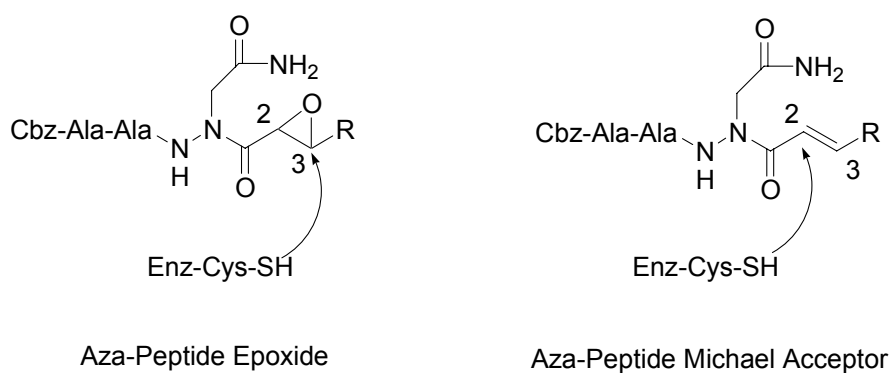
Figure 4.2.1. Aza-Peptidyl Michael Acceptor Design Derived from Aza-Peptidyl

Epoxides.

In addition we synthesized a biotinylated derivative of one of the better Michael

acceptor inhibitors. Biotin has been used in the past as an affinity label for protease

activity. By attaching a biotin label onto a selective inhibitor, it is possible to locate *in*

situ activity of the target enzyme within an organism. A biotinylated Michael acceptor

inhibitor specific for *S. mansoni* legumain would therefore be a useful tool in detecting

legumain activity within the parasite or possible precursors of legumain or other legumain-like proteases.

CHEMISTRY

The synthesis of the aza-peptide fumarate analogues is based on the design of the aza-peptide epoxide inhibitors. The general strategy is to mimic an extended peptide substrate while keeping the synthesis facile. Replacing the α-carbon of the P1 asparaginyl residue with a nitrogen allows practical extension into the P' direction by coupling to the desired fumarate derivatives or commercially available acrylic acid analogues. The fumarate carbonyl is now in the exact place as the scissile peptide carbonyl of the peptidase substrate, and the P' site of the inhibitor can be easily modified to explore the tolerance of the S' subsite interactions between the enzyme and the inhibitor.

A series of amide fumarate derivatives (**13a - x**) were prepared starting with monoethyl fumarate and the corresponding primary or secondary amines by standard mixed anhydride coupling using NMM and iBCF followed by deprotection of the ethyl ester in methanol using aqueous NaOH (Figure 4.2.2). The various disubstituted aromatic amines were synthesized by Tiffany Stark and Amy Campbell by reductive amination starting with aromatic aldehyde precursors and an aromatic primary amine. The benzyl ester fumarate derivative was formed from monoethyl fumarate and benzyl alcohol using NMM and DCC as the coupling reagent. The choice of the *trans* stereochemistry was based on the epoxide inhibitors described in chapter 4.1. The *cis*

epoxysuccinates showed very little potency and therefore the design of the fumarate

analogues did not take the *cis* stereochemistry into consideration.
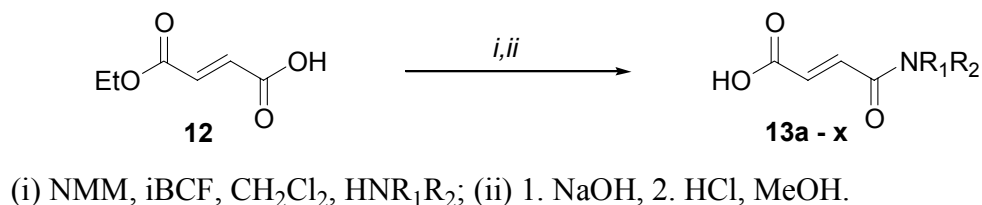
Figure 4.2.2. Preparation of Fumarate Precursors.

(i) NMM, iBCF, CH_2Cl_2, HNR_1R_2; (ii) 1. NaOH, 2. HCl, MeOH.

The peptidyl hydrazides were prepared according to our previously reported

method for the synthesis of aza-peptide epoxides in chapter 4.1. The optimal substrate

sequence Cbz-Ala-Ala-Asn-AMC for legumain determined by Mathieu *et al* provided the

basis for the peptide sequence of the inhibitors[30]. The peptidyl hydrazide **3** was formed

starting with Cbz-Ala-Ala-OMe using excess hydrazine in methanol. The asparaginyl

side chain was synthesized via alkylation of the hydrazide with ethyl bromoacetate and

NMM. The alkylation of the primary nitrogen over the hydrazide nitrogen immediately

next to the carbonyl is based on the more nucleophilic character of the primary nitrogen.

Subsequent conversion of the ethyl ester to the amide (**9**) was accomplished by

ammonolysis with catalytic amounts of NaCN according to the procedure described by

Hogberg *et al*[43]. The peptide precursor was then coupled to the substituted fumarate

amides (**13a – x**), fumarate esters, or commercially available acrylic acids using HOBt

and EDC to complete the synthesis of the various aza-peptide fumarate or acrylic acid

derivatives (**15a – f**) (Figure 4.2.3).

For the design of the biotinylated derivative (Figure 4.2.4), we chose to attach the biotin moiety on the non-prime side of the inhibitor in place of the Cbz protecting group, in order not to interfere with the active site binding interactions. We assumed that the alkyl spacer in the biotin moiety created a sufficient distance between the structural features of the warhead that are essential for inhibition. We chose the ethyl ester **15a** (Cbz-Ala-Ala-AAsn-CH=CHCOOEt) as the most suitable inhibitor to be derivatized, as it is the most potent, and as the monoethyl fumarate precursor is commercially available. For the synthesis of the biotinylated inhibitor we chose to replace the Cbz protecting group with the Boc protecting group, since Cbz deprotection requires catalytic hydrogenation, which could affect the double bond of the fumarate moiety. The Boc group can be removed with TFA using dilute conditions. The Boc-protected aza-peptide Michael acceptor was synthesized in analogous fashion to the Cbz-protected compounds. The coupling of biotin to the free N-terminus of the Michael acceptor inhibitor provided initial difficulties, as the carboxylic acid moiety was not reactive enough for coupling with HOBt/EDC. The mixed anhydride coupling method failed in both methylene chloride as well as THF, as biotin is extremely insoluble in most organic solvents. We initially decided to activate the carboxylic acid moiety of biotin by converting it to the acid chloride. This functionality however turned out to be too reactive. The biotin N-hydroxysuccinimide has previously been successfully coupled to the N-terminus of peptidyl inhibitors[51,52]. Coupling of biotin N-hydroxysuccinimide **17** to the TFA salt of Ala-Ala-AAsn-CH=CHCOOEt (**20**) in the presence of triethylamine gave the biotinylated aza-peptidyl fumarate ethyl ester **21** in good yield.

R3 =

15a -COOEt
15b -COOBzl
15c -COPh
15d -CH=CHCH₃
15e -2-furyl
15f -3-pyridyl

HOBt, EDC, → **15a - f**

HOBt, EDC, **13a - x**
DMF → **14a - x**

9

Cbz-Ala-Ala-N-N-H ... NH₂

NR₁R₂ =

a NEt₂
b N(nBu)₂
c (piperidine)

d NHPh
e NHBzl
f NHBzl-4-F
g NHCH₂CH₂Ph
h N(CH₃)Ph
i N(CH₃)Bzl
j N(CH₃)CH₂-1-Napth
k N(CH₃)CH₂CH₂Ph
l N(Bzl)Ph
m N(Bzl)₂
n N(Bzl-4-OMe)Bzl
o N(Bzl-4-F)Bzl
p N(CH₂-2-furyl)₂
q N(Bzl)-2-CH₂-Napth
r N(Bzl)-1-CH₂-Napth

s **t** **u** **v** **w** **x**
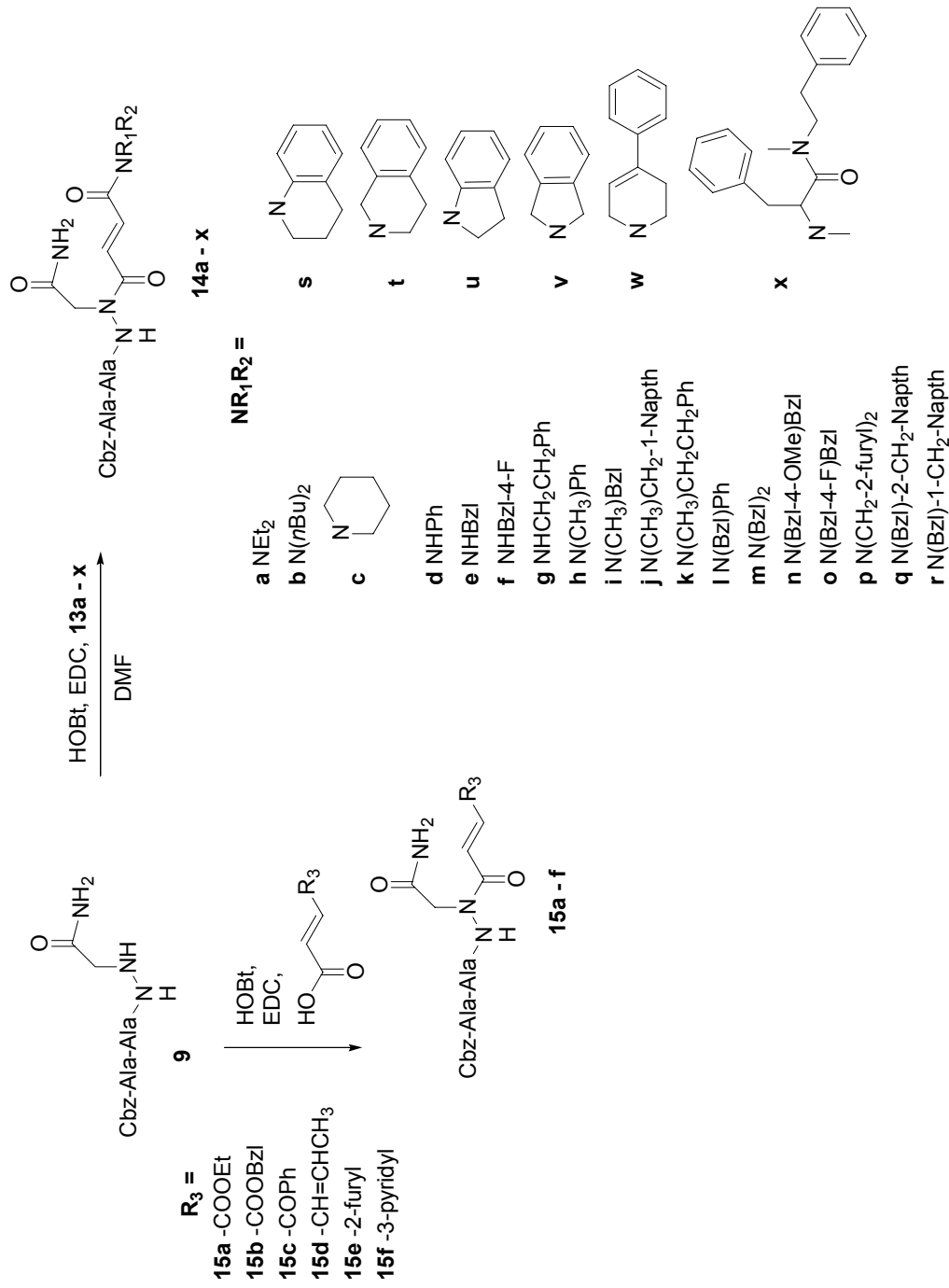
Figure 4.2.3. Coupling of Fumarate and Acryloyl Precursors to Peptidyl Hydrazides.
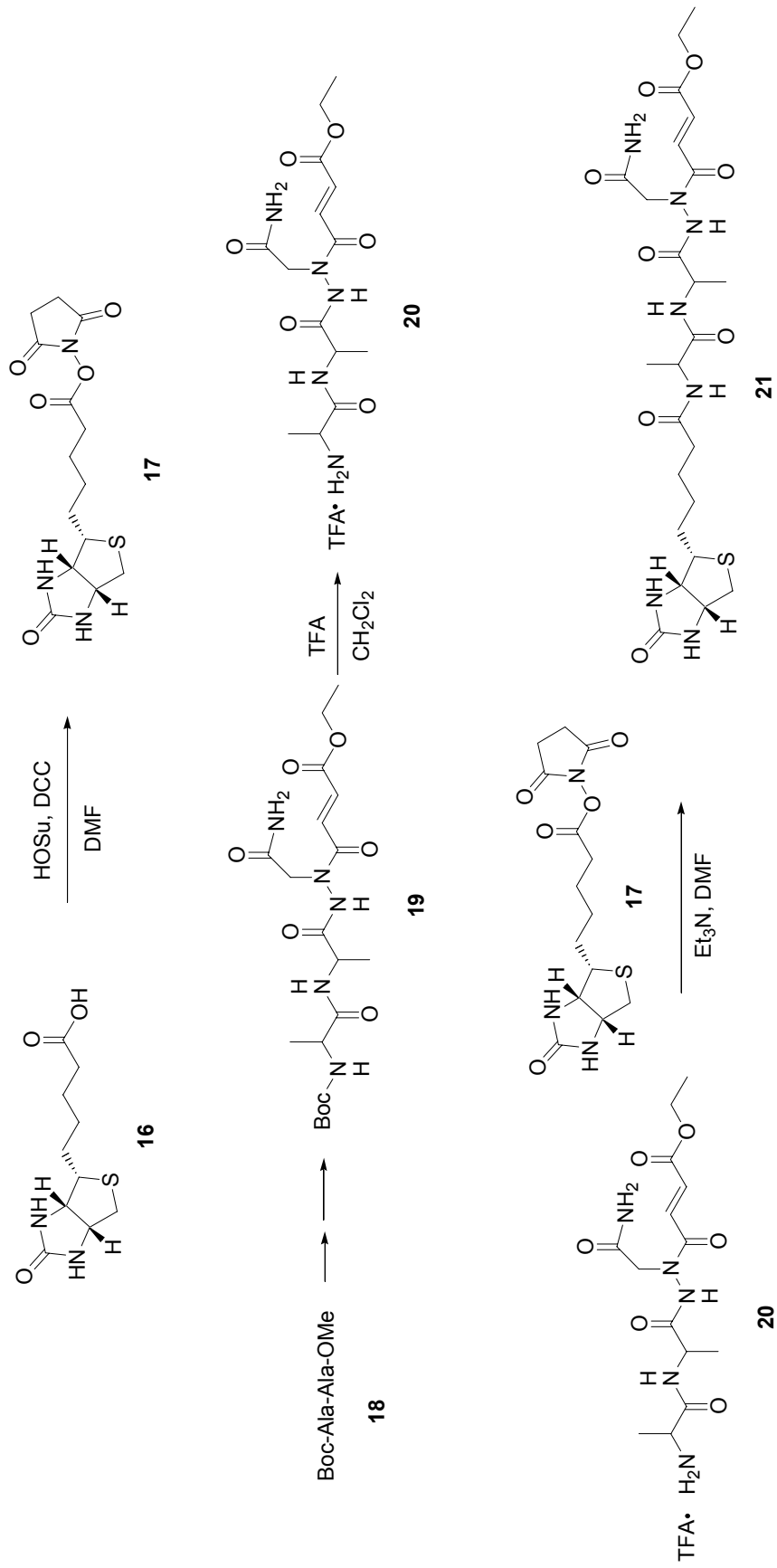
Figure 4.2.4. Preparation of a Biotinylated Michael Acceptor Inhibitor.

RESULTS AND DISCUSSION

Synthetic Design. The design of the aza-peptide has been employed with inhibitors of legumain before[31,34]. Replacing the chiral α-carbon with a nitrogen changes the orientation of the scissile peptide bond. The aza-peptide functionality is planar and therefore restricts the conformation and rotational freedom of the original peptide bond. Niestroj *et al* have previously reported the synthesis of aza-asparaginyl chloromethyl ketones[31]. A concern that arose during their synthetic strategy was the intramolecular cyclization of the asparagine's side chain with the warhead. However the rigidity of the aza-peptide backbone prevents the intramolecular cyclization and renders the inhibitor stable after deprotection of the side chain. During the course of our synthetic progress, the asparagine side chain remained unprotected and no cyclization was observed.
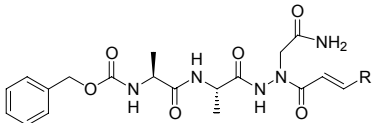
The aza-peptide design is also highly practical for the introduction of a variety of substituents on the P1' position. Fumarates and acrylates can be derivatized prior to coupling to the peptide precursor. This will allow us to perform an extensive SAR study to explore the tolerance of the enzyme's S' subsite.

There are two possible routes to incorporate the aza moiety in the peptide backbone. The hydrazide can be prepared prior to alkylation with the asparagine side chain by conversion of the peptide methyl ester to the peptidyl hydrazide. A second approach is to directly add an amino acid side chain analog bearing the hydrazine moiety. One has to consider the reactivity of the hydrazine moiety. Depending on the hydrazine substituent the reactivity of one hydrazine nitrogen over the other changes. An electron withdrawing substituent, such as esters or amides, places a higher nucleophilic character

on the primary hydrazine carbon. An electron donating substituent deposits the reactivity on the secondary nitrogen[53,54]. For these reasons, we chose to convert the peptide methyl ester into the hydrazide prior to introduction of the side chain, since this would allow us to extend our design onto aza-peptidyl analogs other than the aza-asparagine moiety. We are also currently investigating the use of the aza-peptidyl functionality with ornithine, lysine, and aspartate analogs. The alternate strategy is only limited to a small number of amino acid analogs.

Legumain Inhibition. The compounds were tested for their inhibitory potency towards *S. mansoni* legumain. The IC_{50} values are reported in Table 4.2.1. The results are overall comparable to the general trend of the epoxide inhibitors described in chapter 4.1.

Table 4.2.1. *S. mansoni* Legumain Inhibition with Aza-Peptide Michael Acceptors.

Compound		IC_{50} (nM)
15a	-COOEt[1]	31 ± 25
15b	-COOBzl	38
14a	-CONEt$_2$	N.I.
14b	-CON(nBu)$_2$	550
14c	-CO-Pip	1,000
14d	-CONHPh	700
14e	-CONHBzl	1,000
14f	-COBzl-4-F	800
14g	-CONHCH$_2$CH$_2$Ph	600
14h	-CON(CH$_3$)Ph	60
14i	-CON(CH$_3$)Bzl	55

14j	-CON(CH$_3$)CH$_2$-1-Napth	35
14k	-CON(CH$_3$)CH$_2$CH$_2$Ph	600
14l	-CON(Bzl)Ph	70
14m	-CON(Bzl)$_2$	45
14n	-CON(Bzl-4-OMe)Bzl	90
14o	-CON(Bzl-4-F)Bzl	60
14p	-CON(CH$_2$-2-furyl)$_2$	900
14q	-CON(Bzl)-2-CH$_2$-Napth	70
14r	-CON(Bzl)-1-CH$_2$-Napth	62
14s	-CO-tetrahydroquinoline	70
14t	-CO-tetrahydroisoquinoline	200
14u	-CO-indoline	70
14v	-CO-isoindoline	300
14w	-CO-(4-Ph-pyridine)	750
14x	-CO-MePhe-N(CH$_3$)CH$_2$CH$_2$Ph	750
15c	-COPh	N.I.
15d	-CH=CH-CH$_3$	N.I.
15e	-2-furyl	>2,000
15f	-3-Py	>2,000

N.I. = No inhibition after 20 minutes of incubation.
[1]prepared by Karen James.

The fumarate benzyl and ethyl esters (**15a, 15b**) are one of the most potent

inhibitors. The monosubstituted amides (**14e, f, g**) and the acrylate derivatives (**15d, e, f**)

show little to no inhibition, which leads us to conclude that the hydrogen bonding

network between the inhibitor and the active site is optimal with the carbonyl next to the

double bond and without the H-bond donating monosubstituted amide. The benzoyl

acrylate Cbz-Ala-Ala-AAsn-CH=CHCOPh (**15c**) fulfills both of the aforementioned

requirements, but surprisingly does not inhibit legumain. We found that the aromatic

fumarate extensions give lower IC_{50}'s than the alkyl derivatives (NEt_2, $N(nBu)_2$, Pip).

We speculate that increased Π-stacking of the aromatic residues in one of the enzyme

pockets improves the binding of the inhibitor. The aromatic difuryl derivative **14p**

unexpectedly only inhibits with an IC_{50} of 900 nM.

The positioning and orientation of the phenyl or naphthyl rings plays an important

role. The N-methyl phenyl analog (**14h**) is less potent (IC_{50} = 60 nM) compared to the

N-methyl benzyl analog (**14i**, IC_{50} = 55 nM). However extending the alkyl spacer by one

additional methylene group to the N-methyl phenethyl analog (**14k**) decreases the

inhibition dramatically (IC_{50} = 600 nM).   The extended aromaticity of the N-methyl-1-

methyl naphthyl (**14j**) derivative compared to the N-methyl benzyl amide produced an

even lower IC_{50} (35 nM).   The dibenzyl amide shows the lowest IC_{50} of the aromatic

disubstituted amides (**14m**, IC_{50} = 45 nM). The substitution of one of the benzyl rings

with an electron donating methoxy (**14n**) or an electron withdrawing fluorine (**14o**) only

reduces the potency. The next step was to combine the effects of the naphthyl group with

the benzyl substituent. But neither the N-benzyl-2-methylnaphthyl (**14q**) nor the N-

benzyl-1-methylnaphthyl analog (**14r**) improves the IC_{50} of the dibenzylamide. The

orientation and flexibility of the benzyl group is restricted in the quinoline (**14s**),

isoquinoline (**14t**), indoline (**14u**), and isoindoline (**14v**) analogs. Both quinoline and

indoline exhibit good potency (IC_{50} = 70 nM), but lower in comparison to the benzyl

amide. The aromatic ring is much less flexible in the indoline and quinoline bicyclic

system. The orientation of the aromatic ring in the isoindoline and isoquinoline

compounds is different from their parent compounds due to the different location of the

nitrogen. The altered orientation of the aromatic ring significantly lowers their potency. Both the 4-phenyl tetrahydropyridine (**14w**) and the amino acid derivative (**14x**) could not improve the effectiveness of the warhead. The esters are generally more potent than the amide analogs, however the difficulty of their preparation also limits the variety of possible compounds. The biotinylated inhibitor Biotin-Ala-Ala-AAsn-CH=CHCOOEt (**21**) exhibited the greatest potency with legumain (IC_{50} = 10 nM).

 Stability Studies. The increased reactivity of the ester derivatives, however, is not only limited to enzyme inhibition. In addition we observed that the esters are reactive with thioalkylating agents such as DTT (Table 4.2.2). Upon incubation of various Michael acceptor inhibitors with DTT in legumain assay buffer we observed the disappearance of the Michael acceptor double bond over time. The very potent Cbz-Ala-Ala-AAsn-CH=CHCOOEt (IC_{50} = 31 nM) was found to have a half-life of 3.7 minutes with a first-order rate constant of 3.10×10^{-3} s^{-1}, when reacted with excess DTT in legumain assay buffer at room temperature at pH 6.8. Amide substituted Michael acceptors react with DTT at a slower rate. Half-lives of 20 min and 17 min and a first order rate constant of 5.78×10^{-4} s^{-1} and 6.73×10^{-4} s^{-1} were determined for Cbz-Ala-Ala-AAsn-CH=CHCON(Me)Bzl and Cbz-Ala-Ala-AAsn-CH=CHCON(Bzl)$_2$ respectively.

Table 4.2.2. Degradation of Various Inhibitors in the Presence of DTT.

Inhibitor	pH 5.8		pH 6.8	
	k_{obs} ($M^{-1}s^{-1}$)	$t_{1/2}$ (min)	k_{obs} ($M^{-1}s^{-1}$)	$t_{1/2}$ (min)
R-CH=CHCOOEt	6.06×10^{-4}	19	3.10×10^{-3}	3.7
R-CH=CHCOOBzl			4.60×10^{-3}	2.5
R-CH=CHCON(Me)Bzl	3.07×10^{-5}	376	5.78×10^{-4}	20
R-CH=CHCON(Bzl)$_2$			6.73×10^{-4}	17
R-CH=CHCH=CHCH$_3$	stable		stable	

In general acrylates are more reactive toward nucleophilic attack than acrylamides due to differences in electron withdrawing properties. Freidig *et al* have discovered similar results regarding the reactivity of the non-peptidyl parent compounds, ethyl acrylate and acrylamide, with the thioalkylating glutathione. Their study concludes that ethyl acrylate reacts with glutathione 85 times faster than acrylamide with second order rate constants of 0.66 $M^{-1}s^{-1}$ and 7.8 x 10^{-3} $M^{-1}s^{-1}$ [55]. Our reactivity experiments with peptidyl acrylamides and acrylates and DTT result in a $k_{acrylate}/k_{acrylamide}$ reactivity ratio between 5 and 6. Alkyl substituted acrylates show no reaction with DTT.

We observed that the reactivity of the Michael acceptor with DTT also depends on the pH of the buffer solution. The pk_a of the DTT thiol is 9.2. A higher pH results in a higher concentration of deprotonated DTT thiol in solution, and promotes the nucleophilic attack on the double bond. Lowering the pH in the degradation assay decreases the rate of reactivity dramatically. The ethyl ester, for example, has a half-life of 3.7 minutes in the reaction with DTT at pH 6.8. Lowering the pH to 5.8 increases the half-life to 19 minutes. The effect of pH on the stability of the amide derivatives is much more pronounced. The half-life of Cbz-Ala-Ala-AAsn-CH=CHCON(Me)Bzl increases

92

from 20 minutes to 376 minutes when lowering the pH. In a previous study with caspase specific tetrapeptidyl aza-aspartyl Michael acceptors, we found that the half-life of the caspase specific ethyl ester analog in the reaction with DTT was 10 minutes at pH 7.2. At the same pH the N-methyl benzyl amide had a half-life of 116 minutes. We suspect that the length of the peptide of the inhibitor and the size of the steric bulk of the side chain have an effect on the rate of degradation. Therefore the reactivity of the individual inhibitor with DTT at the pH of the enzyme inhibition assay has to be taken into consideration when evaluating the potency of the inhibitor.

We are certain that the degradation of the legumain Michael acceptor inhibitors upon DTT addition involves thioalkylation of the Michael acceptor double bond by the reactive DTT thiol, since the gradual disappearance of the double bond can be monitored spectroscopically. Initially it was unclear, which carbon of the double bond was alkylated. There are two possible sites of attack, the C2 or C3 carbon of the double bond, to give two alkylated regioisomers (Figure 4.2.5).
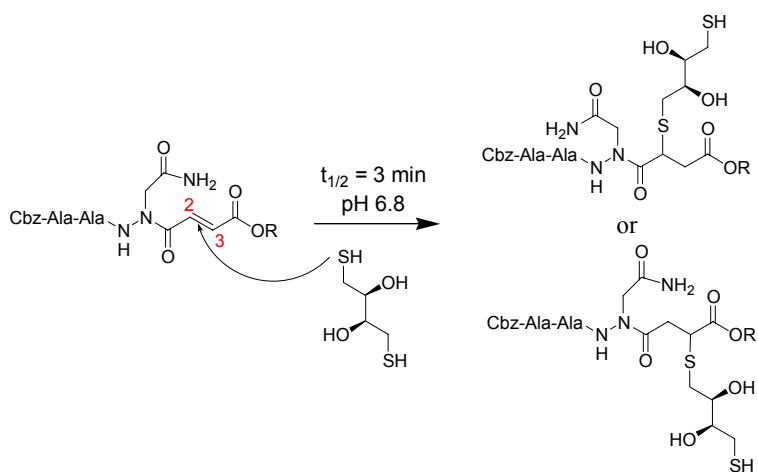
Figure 4.2.5. Mechanism of Thioalkylation of Aza-Peptidyl Michael Acceptor Inhibitors with DTT.

The order of reactivity of the various Michael acceptors with respect to different

P1' substituents, where esters > amides > alkyls, indicates that the thioalkylation occurs at

the C2 position of the double bond. In search of further support for the regioselective

thioalkylation of our inhibitors by DTT, we used ChemDraw to predict the difference of

the two possible products in the ^{1}H NMR spectrum. The estimated chemical shift for a

C2 thioalkylation places the proton of the new thioether linkage at 4.11 ppm. A C3

thioalkylation would result in an estimated chemical shift of 3.59 ppm. Since other

protons in the structure of DTT would interfere with the peaks of interest in our

experimental ^{1}H NMR spectrum, we chose benzyl mercaptan as a thioalkylating agent

instead of DTT. We followed the reaction of Cbz-Ala-Ala-AAsn-CH=CHCOOEt with

benzyl mercaptan in DMSO by ^{1}H NMR for several hours, and observed the expected

gradual disappearance of the vinyl proton at 6.59 ppm together with a simultaneous

increase of a new signal at 4.05 ppm. Unfortunately the new signal coincides with the α-

H of the peptide backbone of the inhibitor, which scrambles the multiplicity of the new

peak. However, the chemical shift of the new signal closely corresponds to the shift

predicted by ChemDraw for a C2 thioalkylation, and thus supports our hypothesis.

Mechanism of Inhibition. Cysteine proteases are generally inhibited by Michael

acceptors in a nucleophilic attack of the side chain thiol of the catalytic cysteine residue

within the active site on the β-carbon of the Michael acceptor double bond. A covalent

bond forms and the inhibitor is irreversibly bound to the enzyme. In our particular

structure, the nucleophilic attack of the active site cysteine thiol is theoretically possible

on either carbon of the double bond. In our recent study with the related aza-peptidyl

epoxide compounds, the X-ray crystal structures of caspase-1 (like legumain, a clan CD

protease) inhibited by PhPr-Val-Ala-AAsp-EP-COOCH$_2$Ph and PhPr-Val-Ala-AAsp-EP-CH$_2$CH$_2$Ph (Ron Rubin, Parke-Davis now Pfizer, unpublished results) revealed that the attack of the cysteine thiol occurred at the C3 carbon of the epoxide moiety. However, one cannot draw similar conclusions for the aza-peptidyl Michael acceptors.

The aforementioned reactivity of the inhibitor with DTT gives insights into the enzymatic mechanism. As the mechanism of legumain inhibition also occurs by nucleophilic attack of the active site cysteine thiol on the Michael acceptor similar to the thiol attack of DTT, we envision mechanistic parallels. We predict that the enzymatic thioalkylation by the active site cysteine attacks the Michael acceptor double bond at the C2 carbon as concluded by the reactivity studies with DTT described above. Furthermore, the ^{1}H NMR gives spectroscopic evidence for a C2 thioalkylation of the inhibitor by the reagent benzyl mercaptan. The decline of inhibitory potency in the series of esters, amides and alkyls provides additional support for our hypothesis, as the reactivity of the enzyme with the inhibitor exhibits the same trend as the reactivity of the inhibitor in the degradation assay with DTT.

Clan CD Specificity. A selection of representative inhibitors was tested with the clan CD proteases caspase 3, 6, and 8, clostripain and gingipain K. Caspases are highly specific for Asp in the P1 position of the substrate and prefer tri- and tetrapeptides over di-peptides. The difference in the amino acid sequence of the substrate further divides the specificity of the individual caspases. Clostripains and gingipains prefer dipeptides with an Orn or Lys P1 amino acid residue. This P1 specificity unique to each member of the clan CD cysteine proteases allows the design of inhibitors that will only target a desired family within the clan CD proteases. It is therefore not surprising that all

95

legumain inhibitors show no inhibition with caspase 3, 6, and 8, clostripain, and only limited inhibition with gingipain (Cbz-Ala-Ala-AAsn-CH=CHCOOEt and Cbz-Ala-Ala-AAsn-CH=CHCON(Me)-2-CH$_2$-Napth, k_{obs}/[I] < 6 M^{-1}s^{-1}).

Specificity with Other Cysteine Proteases. The inhibitors were tested with the clan CA proteases calpain I, papain, and cathepsin B and showed little to no inhibition after 30 minutes of incubation. Papain was only inhibited by Cbz-Ala-Ala-AAsn-CH=CHCONHCH$_2$CH$_2$Ph (11.5 M^{-1}s^{-1}), which surprisingly is not a very potent inhibitor of legumain. The ethyl ester derivative exhibited very limited inhibition with cathepsin B (< 1 M^{-1} s^{-1}). Limited inhibition was also observed with Cbz-Ala-Ala-AAsn-CH=CHCON(Bzl)$_2$ against calpain I (< 1 M^{-1}s^{-1}). The specificity of the clan CA proteases is generally governed by the nature of the P2 amino acid residue of the peptide substrate. We believe that the lack of inhibition of clan CA cysteine proteases arises from the rigid nature of the aza-peptide moiety, which most likely prevents effective binding of the warhead near the enzyme's catalytic residues. For the same rigidity caused by the aza-peptide moiety, the aza-peptide epoxide inhibitors for legumain reported in chapter 4.1 and caspases also show lack of inhibition with clan CA proteases.
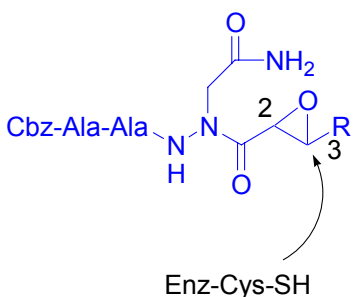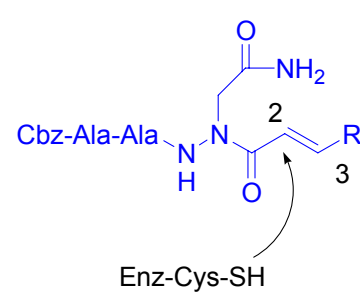
SUMMARY

Aza-peptidyl Michael acceptors are a new class of inhibitors for *Schistosoma mansoni* legumain. The inhibitors are very potent and highly selective within both the clan CD and clan CA cysteine proteases. In our SAR study of the P1' specificity, we discovered that legumain prefers aromatic residues, and that the orientation and extension

of the residue into the S1' subsite plays an important role. Additionally the strength of the electron withdrawing group on the P1' position of the double bond controls the potency of the inhibitor. We have found that the inhibitor's warhead reacts with thioalkylating agents, such as DTT, and that the progress of this reaction is dependent on the pH and the electron withdrawing nature of the P1' substituent. We conclude from the difference in reactivity with thioalkylating agents and from the difference in the IC_{50} values of the inhibition assay with legumain, and our ^{1}H NMR study that the mechanism of inhibition occurs by attack of the active site cysteine on the carbon immediately next to the scissile bond. This insight provides us with new possibilities in the design of more potent inhibitors. Derivatizing this particular carbon with an electron-withdrawing group, as for example a halogen, could promote the effectiveness of our inhibitors. A second task is to increase the stability of the Michael acceptor warhead with regards to its reactivity with thioalkylating agents by strategically introducing a steric bulk on the double bond without affecting the overall potency of the warhead.

Table 4.2.3. Summary of Aza-Peptide Epoxides and Aza-Peptide Michael Acceptors.

Aza-Peptide Epoxides	Aza-Peptide Michael Acceptors
• A new class of inhibitors. • Specific for legumain. • Stereochemistry specificity: $S,S > R,R$ > trans > cis. • P′ specificity: Esters and disubstituted amides.	• A new class of inhibitors. • Specific for legumain. • More reactive than epoxides. • P′ specificity: aromatic residues. • Reactivity with DTT gives insights into the enzyme mechanism.

Comparing aza-peptide epoxide inhibitors to aza-peptide Michael acceptor inhibitors, we found that Michael acceptors are more potent inhibitors of *S. mansoni* legumain. In addition, there is strong evidence for a difference in the site of attack by the active site cysteine thiol. The site of attack on the epoxide inhibitors occurs at the C3 carbon according to the crystal structure of the tetrapeptidyl aza-peptide epoxide bound to caspase, whereas there is strong evidence for a C2 thioalkylation for the Michael acceptors. The increased potency of the Michael acceptors suggests that a transition state oxyanion is not essential for effective binding of the inhibitor. Instead the overall reactivity of the warhead primarily contributes to enzyme inhibition. Therefore, the design of future classes of inhibitors or the modification of the presently known inhibitors should focus on the electronic properties of the warhead. More powerful electron

withdrawing substituents on the prime side of the epoxide or Michael acceptor warhead could improve the current results. A possible future derivative with a halogen at the C2 position of the warhead is shown in figure 4.2.6.
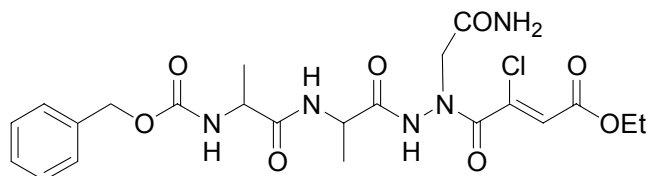
Figure 4.2.6. Proposed Future Modification of the Aza-Peptide Michael Acceptor.

Both the epoxide and the Michael acceptor inhibitors presented in this thesis have great potential to be used as drugs. They are both selective and very potent. Since the medicinal value of peptide inhibitors is generally quite poor due to the high molecular weight and the susceptibility of the amide bonds to enzymatic processing in the due course of metabolism, the peptide backbone of the inhibitor requires some modification to reduce the peptidic character. The cross reactivity of the Michael acceptors with thioalkylating agents, such as DTT or glutathione, presents a second issue that needs to be addressed in the future development of this new class of inhibitors as medicinal agents. However, the groundwork for a promising new class of anti-parasitic agents has been laid to replace the presently used drugs, which are becoming more and more ineffective due to the growing resistance of the schistosome parasite.

EXPERIMENTAL

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

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

The incubation method method was also used for papain. The inhibition

incubation buffer for papain was 50 mM Hepes buffer at pH 7.5, containing 2.5 mM DTT

and 2.5 mM EDTA. The assay used the substrate Cbz-Phe-Arg-pNA (53.7 μM) in the

same buffer. The approximate concentration of papain added to the incubation buffer

was 0.29 mg/mL. The release of p-nitroanilide was monitored at 405 nm with a

Molecular Devices Thermomax microplate reader.

Caspase 3, -6, and –8 Assays. All caspase assays were performed by Özlem

Doğan Ekici in our laboratory. Assays using the fluorogenic substrate Ac-DEVD-AMC

(λ_{ex} = 360 nm, λ_{em} = 465 nm) were carried out on a Tecan Spectra Fluor microplate

reader at 37 ^{o}C. Inhibition rates were determined by the progress curve method. The

concentration of the caspase-3 stock solution was 2 nM in the assay buffer. Assay buffer

consists of a 1:1 mixture of 40 mM Pipes, 200 mM NaCl, 0.2% (w/v) CHAPS, and

sucrose 20% (w/v) to 20 mM DTT solution in H_2O at pH 7.2. The concentration of the

substrate stock solution was 2 mM in DMSO. The enzyme was pre-activated for 10 min

at 37 ^{o}C in the assay buffer. The standard 100 μL reaction was started by adding 40 μL of

assay buffer, 5 μL of various amounts of inhibitor (stock solution concentrations varied

from 5 x 10^{-3} M to 4.84 x 10^{-7} M in DMSO), and 5 μL of substrate in DMSO (100 μM

final concentration) at 37 ^{o}C. Enzyme stock solution (50 μL of 2 nM, final concentration:

1 nM) was added to the mixture after 1 min and reading started immediately for 20 min at

37 ^{o}C. Inhibition experiments were repeated in duplicate and standard deviations were

determined.

Caspase-6 kinetic assays were performed using the same conditions and the same

substrate (Ac-DEVD-AMC, 2 mM stock solution in DMSO). The enzyme stock solution

was 10 nM (final concentration in the well: 5 nM) in the assay buffer. The inhibitor stock solution concentrations varied from 5 x 10^{-3} M to 2.42 x 10^{-6} M in DMSO.

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

G. Salvesen determined the K_M values for Ac-DEVD-AMC with caspase-3 (K_M = 9.7 μM), caspase-6 (K_M = 236.35 μM), and caspase-8 (K_M = 6.79 μM). The k_2 values are 11.309-fold higher than the apparent rate for caspase-3 because of the 100 mM [S] and K_M = 9.7 μM. The k_2 values are 1.4231-fold higher than the apparent rate for caspase-6 because of the 100 mM [S] and K_M = 236.35 μM. The k_2 values are 15.7275-fold higher than the apparent rate for caspase-8 because of the 100 mM [S] and K_M = 6.79 μM.

Gingipain K and Clostripain Assays. The clostripain and gingipain assays were performed by Brian Rukamp in our laboratory. Clostripain was purchased from Sigma Chemical Co. (St. Louis, MO) as a solid which was dissolved in an activation solution of 8 mM DTT at a concentration of 5.962 μM and stored at -20 °C prior to use. The inhibition of clostripain began with the addition of 25 μl of stock inhibitor solution (concentration varies by inhibitor) in DMSO to a solution of 250 μl of 20 mM Tris/HCl, 10 mM $CaCl_2$, 0.005% Brij 35, 2 mM DTT buffer at pH 7.6 (clostripain buffer) and 5 μl of the stock enzyme solution. Aliquots (25 μl) of this incubation mixture were taken at various time points and added to a solution containing 100 μl of the clostripain buffer and 5 μl of Z-Phe-Arg-AMC substrate solution (0.139 mM) in DMSO. The enzymatic

activity was monitored by following the change in fluorescence at 465 nm. The all data obtained was processed by pseudo-first order kinetics.

Gingipain K 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 $CaCl_2$, 0.02% NaN_3, 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 $CaCl_2$, 2 mM DTT at pH 8.0 (gingipain K buffer) and kept at 0 °C. This solution was used only for one day, as freezing the enzyme at this concentration destroyed all activity. The inhibition of gingipain K began with the addition of 25 μl of stock inhibitor solution (concentration varies by inhibitor) in DMSO to 244 μl of the diluted enzyme solution (4.61 nM) in gingipain K buffer warmed to rt. Aliquots (20 μl) of this were taken at various time points and added to a solution containing 100 μl of the gingipain K buffer and 5 μl of Suc-Ala-Phe-Lys-AMC · TFA as the substrate (0.910 mM stock) in DMSO. The enzymatic activity was monitored by following the change in fluorescence at 465 nm. The data for gingipain K was processed by pseudo-first order kinetics.

Legumain Assays. The legumain assays were performed by Conor Caffrey and Elizabeth Hansell at Jim McKerrow's laboratory at UCSF. The zymogen form of schistosome legumain SmAE was expressed in *Pichia*[56]. The lyophilized enzyme (50-100 mg) was reconstituted in 1.5 mL 0.5 M sodium acetate, pH 4.5 containing 4 mM DTT, and left to stand at 37 °C for 3-4 hours to allow for auto-activation of the zymogen. In a black 96-well microtiter plate, 50 μL of activated enzyme was added to an equal volume of 0.1 M citrate-phosphate buffer pH 6.8 containing 4 mM DTT. The inhibitors,

added as 1 µL aliquots (serial water dilutions of DMSO stock solutions, 2 to 0.00002 µM (final)), were preincubated with the protease at room temperature for 20 min. After incubation, 100 uL of the same buffer containing 20 µM substrate (Cbz-Ala-Ala-Asn-AMC) was added to the wells and the reaction monitored for 20 minutes. A plot of the RFU/min versus the inhibitor concentration (µM) permitted calculation of an IC_{50} value.

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

Stability Studies of Aza-peptidyl Michael Acceptors. An aliquot (15 µL) of 10 mM aza-peptidyl Michael acceptors in DMSO was added to legumain buffer (385 µL, pH 5.8 or 6.8) containing DTT (3.4 mM) and the reaction of the double bond of the inhibitor with DTT was monitored spectrophotometrically at 250 nm over time. The k_{obs} was obtained from pseudo first-order rate plots of ln (A_t/A_0) versus time and the half-lives were derived from $t_{1/2} = $ ln $2/k_{obs}$.

General Procedure for Mixed Anhydride Coupling.

N-Benzyloxycarbonylalanylalanyl Methyl Ester (Cbz-Ala-Ala-OMe). N-Methylmorpholine (2 mmol) was added to Cbz-Ala-OH in CH_2Cl_2 at -15 ºC followed by isobutyl chloroformate. N-Methylmorpholine (2 mmol) was added to a cooled solution (-15 ºC) of HCl·H-Ala-OMe (2 mmol) in CH_2Cl_2. This solution was added to the Cbz-Ala-OH mixture, which had been stirring at -15 ºC. The mixture was continued to stir at -15 ºC for 30 minutes, then warmed to room temperature and continued to stir over night. The amount of solvent was doubled, then washed with citric acid (10 %, 3 x 50 ml), saturated $NaHCO_3$ (3 x 50 ml), and brine (3 x 50 ml), and finally dried ($MgSO_4$). The solvent was evaporated to give Cbz-Ala-Ala-OMe as a pure white solid in good yield (90 %). ^1H NMR ($CDCl_3$): 1.18 (t, 9H, CH_3), 3.5 (s, 3H, OCH_3), 4.1 (m, 1H, α-H), 4.2 (m, 1H, α-H), 5.03 (m, 2H, Cbz), 5.18 (m, 1H, NH), 7.22-7.40 (m, 5H, Ph), 7.9 (m, 1H, NH).

N-Benzyloxycarbonylalanylalanyl Hydrazide (Cbz-Ala-Ala-NHNH₂) (3) was synthesized from Cbz-Ala-Ala-OMe by hydrazinolysis. Anhydrous hydrazine (10 eq) was added to a solution of Cbz-Ala-Ala-OMe (1 eq) in MeOH at room temperature, and the resulting mixture was then stirred at room temperature for 16 hours. Excess hydrazine and solvent were removed by evaporation. The resulting residue was washed with ethanol and ether to give Cbz-Ala-Ala-NHNH₂ as a white solid (57% yield). ^1H NMR (DMSO-d_6): 1.1-1.3 (d, CH_3), 4.0-4.1 (m, 1H, α-H), 4.1-4.3 (m, 2H, α-H and NH), 5.05 (s, 2H, Cbz), 7.3-7.4 (m, 5H, Ph), 7.5 (d, 1H, NH), 7.9 (d, 1H, NH), 9.05 (s, 1H, NH).

N^1-(N-Benzyloxycarbonylalanylalanyl)-N^2-ethoxycarbonylmethylhydrazine (Cbz-Ala-Ala-NHNHCH₂COOEt). Ethyl bromoacetate (1.1 eq) was added dropwise to

a stirred solution of Cbz-Ala-Ala-NHNH$_2$ (1 eq) and NMM (1.1 eq) in DMF that was cooled to -10 °C. The resulting solution was stirred for 30 min at -10 °C, after which the mixture was allowed to react at room temperature for 36 hours. The DMF was evaporated, and the residue was purified on a silica gel column using 1:9 MeOH:CH$_2$Cl$_2$ as the eluting solvent system to give the ethyl ester as a white solid (yield 36%). ^{1}H NMR (DMSO-d$_6$): 1.18 (t, 9H, CH$_3$), 3.5 (d, 2H, NCH$_2$COOEt), 4.0-4.15 (m, 3H, α-H and OCH$_2$CH$_3$), 4.2 (m, 1H, α-H), 5.03 (m, 2H, Cbz), 5.18 (m, 1H, NH), 7.22-7.40 (m, 5H, Ph), 7.4-7.5 (d, 1H, NH), 7.9 (m, 1H, NH), 9.35 (m, 1H, NH). MS (FAB) *m/z* 395 [(M + 1)$^+$].

N^1-(N-Benzyloxycarbonylalanylalanyl)-N^2-carbamoylmethylhydrazine (Cbz-Ala-Ala-NHNHCH$_2$CONH$_2$) (4). The ethyl ester Cbz-Ala-Ala-NHNHCH$_2$COOEt (1 eq) was dissolved in a 9 M solution (100 eq) of NH$_3$ in methanol and a small amount of DMF, and allowed to stir on an ice bath. To this solution was added catalytic NaCN (0.1 eq). The flask was closed with a rubber septum and allowed to stir at 0 °C for three days. The solvent was evaporated and the product was precipitated with 1:9 MeOH:CH$_2$Cl$_2$ and methanol to yield a white solid (68% yield). ^{1}H NMR (DMSO-d$_6$): 1.18 (d, 6H, CH$_3$), 3.2 (d, 2H, NCH$_2$CONH$_2$), 4.0-4.12 (m, 1H, α-H), 4.2 (m, 1H, α-H), 5.03 (m, 2H, Cbz), 5.22 (m, 1H, NH), 7.18 (d, 1H, NH), 7.3-7.5 (m, 6H, Ph and NH), 8.0 (m, 1H, NH), 9.38 (m, 1H, NH). MS (FAB) *m/z* 366 [(M + 1)$^+$]. HRMS (FAB) Calcd. for C$_{16}$H$_{24}$N$_5$O$_5$: 366.17774. Observed *m/z* 366.17665.

General Procedure: HOBt/EDC Coupling Method. To a stirred solution of the peptidyl hydrazide precursor (1 eq) (**9**) and the epoxysuccinate, fumarate and acrylic acid precursor (2 eq) (**13a – x** and **15a – f**), HOBt (2 eq) and EDC (2 eq) was added. The

mixture was allowed to react for 16 h at room temperature. The DMF was evaporated, and the residue was redissolved in EtOAc. The organic layer was washed with 2% citric acid, saturated NaHCO$_3$, saturated NaCl, dried over MgSO$_4$, and concentrated. Chromatography on a silica gel column using 10% MeOH/CH$_2$Cl$_2$ as the eluent afforded the aza-peptidyl fumarate, epoxysuccinates or acrylate derivatives. MS and ^{1}H NMR (CDCl$_3$ or DMSO-d$_6$) were consistent with the proposed structures.

(2S,3S)-3-(N^2-(N-Benzyloxycarbonylalanylalanyl)-N^1-carbamoylmethylhydrazinocarbonyl)oxirane-2-carboxylic Acid Phenethyl Ester (11c, Cbz-Ala-Ala-AAsn-(2S,3S)-EP-COOCH$_2$CH$_2$Ph). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography on silica gel using 10% MeOH/CH$_2$Cl$_2$ as the eluent and then recrystallized from EtOAc/ hexane to give a white powder (9% yield). ^{1}H-NMR ((CD$_3$)$_2$CO): 1.34 (m, 6H, 2 x Ala-CH$_3$), 2.87 (s, 2H, NCH$_2$CO), 2.98 (t, 2H, CH$_2$-Ph), 3.48 (s, 1H, EP), 4.04 (m, 1H, α-H), 4.17 (m, 1H, α-H), 4.28-4.43 (m, 3H, *CH$_2$*-CH$_2$-Ph and EP), 5.10 (m, 2H, Cbz), 6.57 (s, 1H, NH), 6.65 (s, 1H, NH), 7.19-7.38 (m, 10H, 2 x Ph), 7.77 (s, 1H, NH), 9.89 (s, 1H, NH). MS (ESI) m/z 584 [(M + 1)$^+$]. HRMS (ESI) calculated for C$_{28}$H$_{34}$N$_5$O$_9$: 584.233. Observed m/z 584.235653. Anal. Calcd. for C$_{28}$H$_{33}$N$_5$O$_9$·0.1H$_2$O·0.5EtOAc: C, 57.18; H, 6.06; N, 11.11. Found: C, 56.96; H, 5.88; N, 11.08.

(2S,3S)-3-(N^2-(N-Benzyloxycarbonylalanylalanyl)-N^1-carbamoylmethylhydrazinocarbonyl)oxirane-2-carboxylic Acid N,N-Dibutyl-amide (11g, Cbz-Ala-Ala-AAsn-(2S,3S)-EP-CON(nBu)$_2$). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography on silica gel using 1:9 MeOH/CH$_2$Cl$_2$ as the eluent and then recrystallized from EtOAc/ hexane to

give a white powder (26% yield). ^{1}H-NMR (DMSO-d_6): 0.84-0.90 (m, 6H, 2 x N-CH$_2$-CH$_2$-CH$_2$-*CH$_3$*), 1.17-1.27 (m, 10H, 2 x Ala-CH$_3$ and 2 x N-CH$_2$-CH$_2$-*CH$_2$*-CH$_3$), 1.41 (t, 2H, N-CH$_2$-*CH$_2$*-CH$_2$-CH$_3$), 1.51 (t, 2H, N-CH$_2$-*CH$_2$*-CH$_2$-CH$_3$), 3.20-3.32 (m, 7H, NCH$_2$CO and CH$_2$-N-CH$_2$ and EP), 3.63 (s, 1H, EP), 4.04 (m, 1H, α-H), 4.26 (m, 1H, α-H), 4.99 (m, 2H, Cbz), 7.21 (s, 1H, NH), 7.33 (m, 5H, Ph), 7.49 (s, 1H, NH), 8.15 (d, 1H, NH), 10.62 (s, 1H, NH). MS (ESI) m/z 591 [(M + 1)$^+$]. HRMS (ESI) calculated for C$_{28}$H$_{43}$N$_6$O$_8$: 591.3059. Observed m/z 591.314238. Anal. Calcd. for C$_{28}$H$_{42}$N$_6$O$_8$·1H$_2$O: C, 55.25; H, 7.29; N, 13.81. Found: C, 55.32; H, 7.10; N, 13.80.

(2*S*,3*S*)-3-(N^2-(N-Benzyloxycarbonylalanylalanyl)-N^1-carbamoylmethylhydrazinocarbonyl)oxirane-2-carboxylic Acid N,N-Methylbenzylamide (11h, Cbz-Ala-Ala-AAsn-(2*S*,3*S*)-EP-CON(Me)-Bzl). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography on silica gel using 10% MeOH/CH$_2$Cl$_2$ as the eluent and then recrystallized from EtOAc/hexane to give a white powder (10% yield). ^{1}H-NMR (DMSO-d_6): 1.11-1.27 (m, 6H, 2 x Ala-CH$_3$), 2.95 (d, 3H, N-CH$_3$), 3.20-3.32 (d, 3H, NCH$_2$CO and EP), 3.34 (s, 1H, EP), 4.04-4.61 (m, 4H, 2 x α-H, CH$_2$-Ph), 5.02 (m, 2H, Cbz), 7.21 (s, 1H, NH), 7.33 (m, 5H, Ph), 7.42 (d, 1H, NH), 8.20 (d, 1H, NH), 10.73 (s, 1H, NH). MS (ESI) m/z 583 [(M + 1)$^+$]. HRMS (ESI) calculated for C$_{28}$H$_{35}$N$_6$O$_8$: 583.2567. Observed m/z 583.251638. Anal. Calcd. for C$_{28}$H$_{34}$N$_6$O$_8$·1.3H$_2$O: C, 55.49; H, 6.09; N, 13.87. Found: C, 55.45; H, 5.89; N, 13.85.

trans-3-(N^2-(N-Benzyloxycarbonylalanylalanyl)-N^1-carbamoylmethylhydrazinocarbonyl)oxirane-2-phenethyl (11i, Cbz-Ala-Ala-AAsn-*trans*-EP-CH$_2$-CH$_2$-Ph). This compound was obtained using the HOBt/EDC coupling

method and purified by column chromatography on silica gel using 1:9 MeOH/CH$_2$Cl$_2$ as the eluent and then recrystallized from EtOAc/ hexane to give a white powder (40% yield). ^{1}H-NMR (DMSO-d$_6$): 1.15-1.17 (d, 3H, CH$_3$), 1.23-1.25 (d, 3H, CH$_3$), 1.67 (m, 2H, *CH$_2$*-CH$_2$-Ph), 2.65 (m, 2H, CH$_2$-*CH$_2$*-Ph), 2.86 (m, 1H, EP), 3.28-3.31 (d, 2H, NCH$_2$CO), 3.63 (s, 1H, EP), 4.01 (m, 1H, α-H), 4.24 (m, 1H, α-H), 4.99 (m, 2H, Cbz), 7.13 (s, 1H, NH), 7.17-7.41 (m, 10H, 2 x Ph), 7.48 (s, 1H, NH), 8.17 (d, 1H, NH), 10.67 (s, 1H, NH). MS (ESI) m/z 540 [(M + 1)$^+$]. HRMS (ESI) calculated for C$_{27}$H$_{34}$N$_5$O$_7$: 540.2392. Observed m/z 540.2458. Anal. Calcd. for C$_{27}$H$_{33}$N$_5$O$_7$·0.2H$_2$O: C, 59.70; H, 6.20; N, 12.89. Found: C, 59.46; H, 6.15; N, 12.76.

4-Chlorophenyl-*trans*-oxirane Carboxylic Acid (HOOC-EP-Ph-Cl). The starting 4-chloro-trans-cinnamic acid (1 eq) was cooled to –20 °C in dry methanol. Thionyl chloride (3 eq) was added dropwise to the cooled solution over one hour. The mixture was stirred at –15 °C for an additional 30 minutes and subsequently stirred at room temperature over night. Evaporation of the volatiles yielded the methyl ester MeOOC-CH=CH-Ph-Cl as a white solid (89% yield). Without further purification the methyl ester was subjected to epoxidation. An anhydrous solution of t-butyl hydroperoxide in toluene (3.3 M, 1.5 eq) was added to freshly distilled THF at -78 °C under argon. A solution of butyllithium in pentane (1.7 M, 1.1 eq) was added drop wise. The mixture was stirred at -78 °C for 5 minutes when a solution of MeOOC-CH=CH-Ph-Cl (1 eq) in tetrahydrofuran was added drop wise. The reaction was stirred at -78 °C for 30 minutes then at room temperature for 16 hours. Solid sodium sulfite was added and the mixture was continued to stir for 20 minutes, then diluted with ether and filtered over celite. The solvent was evaporated, and the crude residue was subjected to column

chromatography on silica gel using 3:1 hexane/EtOAc as the eluent. ^{1}H-NMR (CDCl$_3$): 3.47 (d, 1H, EP), 3.83 (s, 3H, CH$_3$), 4.08 (d, 1H, EP), 7.21 (d, 2H, Ph), 7.33 (d, 2H, Ph). Standard deblocking of the methyl ester with sodium hydroxide (1 N) followed by acidic workup (1 N HCl) produced the 3-(4-chlorophenyl) glycidic acid. ^{1}H-NMR (DMSO-d$_6$): 3.65 (s, 1H, EP), 4.14 (s, 1H, EP), 7.37 (d, 2H, Ph), 7.43 (d, 2H, Ph).

trans-3-(N^2-(N-Benzyloxycarbonylalanylalanyl)-N^1-carbamoylmethylhydrazinocarbonyl)oxirane-2-(4-chlorophenyl) (11j, Cbz-Ala-Ala-AAsn-*trans*-EP-Ph-Cl). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography on silica gel using 1:9 MeOH/CH$_2$Cl$_2$ as the eluent and then recrystallized from EtOAc/CH$_2$Cl$_2$ to give a white powder (50% yield). ^{1}H-NMR (DMSO-d$_6$): 1.02-1.03 (d, 3H, CH$_3$), 1.14-1.18 (d, 3H, CH$_3$), 3.29-3.31 (d, 2H, NCH$_2$CO), 3.94-4.16 (m, 4H, epoxy and 2 x α-H), 4.99 (m, 2H, Cbz), 7.20 (s, 1H, NH), 7.32 (m, 9H, Ph and Ph-Cl), 8.07-8.09 (d, 1H, NH), 10.64 (s, 1H, NH). HRMS (ESI) calculated for C$_{25}$H$_{29}$N$_5$O$_7$Cl : 546.1758. Observed m/z 546.175551. Anal. Calcd. for C$_{25}$H$_{28}$N$_5$O$_7$Cl·0.2H$_2$O: C, 54.63; H, 5.21; N, 12.74. Found: C, 54.65; H, 5.25; N, 12.47.

General Procedure: Mixed Anhydride Coupling Method. Coupling of the amine precursors to the fumarates was accomplished using the mixed anhydride coupling method. To a solution of the fumarate (1 eq) in CH$_2$Cl$_2$ at -20 °C was added N-methylmorpholine (NMM, 1 eq) followed by isobutyl chloroformate (IBCF, 1 eq). After the reaction mixture was allowed to stir for 30 min, the amine (1 eq) was added to the mixture. Hydrochloride salts of the amine were pretreated with NMM (1 eq) at −20 °C in CH$_2$Cl$_2$ prior to addition. After 30 min the reaction was continued to stir for 4 hours at

room temperature. The DMF was evaporated and the residue was washed and purified

using the same procedure as described above for the EDC/HOBt coupling method. MS

and ^{1}H NMR (DMSO-d$_6$ or CDCl$_3$) were consistent with the proposed structures.

trans-3-Benzyloxycarbonylacrylic Acid or Monobenzyl Fumarate

(HOOCCH=CHCOOBzl). Equimolar amounts of fumaric acid and benzyl alcohol were

dissolved in anhydrous DMF. NMM (1 eq) was added at 0 ºC followed by EDC after 15

minutes. The reaction was stirred over night at room temperature. DMF was evaporated

and the crude residue was redissolved in EtOAc. The product was extracted with

saturated aqueous NaHCO$_3$. The aqueous layer was then acidified with 1N HCl to pH 2.

The product was extracted with EtOAc, and the organic layer was washed with water and

dried (MgSO$_4$). The solvent was evaporated and the crude residue was subjected to

column chromatography (MeOH/CH$_2$Cl$_2$) to give a white powder (51% yield). ^{1}H-NMR

(DMSO-d$_6$): 5.21 (s, 2H, CH=CH-COO*CH$_2$*Ph), 6.73 (s, 2H, *CH=CH*-COOCH$_2$Ph),

7.29-7.43 (m, 5H, Ph). MS (ESI) *m/z* 207 [(M + 1)$^+$].

N^2-(N-Benzyloxycarbonylalanylalanyl)-N^1-_trans_-(3-

benzyloxycarbonylacryloyl)-N^1-carbamoylmethylhydrazine (15b, Z-Ala-Ala-AAsn-

CH=CHCOOBzl). This compound was obtained using the HOBt/EDC coupling method

and purified by column chromatography on silica gel using 10% MeOH/CH$_2$Cl$_2$ as the

eluent and then recrystallized from EtOAc/hexane to give a white powder (20% yield).

^{1}H-NMR ((CD$_3$)$_2$CO): 1.37 (m, 6H, 2 x Ala-CH$_3$), 2.88 (s, 2H, NCH$_2$CO), 4.21-4.24 (m,

1H, α-H), 4.50 (m, 1H, α-H), 5.10 (m, 2H, Cbz), 5.24 (s, 2H, O-*CH$_2$*-Ph), 6.58-6.33 (m,

2H, NH and CH=CH), 6.74-6.88 (d, 1H, CH=CH), 7.31-7.44 (m, 11H, 2 x Ph and NH),

7.76 (s, 1H, NH), 8.16 (d, 1H, NH), 9.89 (s, 1H, NH). MS (ESI) *m/z* 554 [(M + 1)$^+$].

HRMS (ESI) calculated for $C_{27}H_{32}N_5O_8$: 554.2219. Observed *m/z* 554.225088. Anal.

Calcd. for $C_{27}H_{31}N_5O_8 \cdot 0.2H_2O$: C, 58.20; H, 5.68; N, 12.57. Found: C, 58.16; H, 5.60;

N, 12.47.

trans-3-Diethylcarbamoylacrylic Acid Ethyl Ester (EtOOCCH=CHCONEt₂)

was obtained by mixed anhydride coupling of equimolar amounts of monoethyl fumarate

and diethylamine. The crude product was recrystallized from hexane/EtOAc to give a

white powder (95% yield). ^{1}H-NMR (CDCl₃): 1.17-1.21 (t, 3H, N-CH₂*CH₃*), 1.22-1.24

(t, 3H, N-CH₂*CH₃*), 1.32 (t, 3H, *CH₃*CH₂O), 3.40-3.47 (m, 4H, 2 x N-CH₂), 4.24-4.26 (q,

2H, CH₃*CH₂*O), 6.78-6.82 (d, 1H, *J* = 14.8 Hz, *CH*=CHCON), 7.31-7.35 (d, 1H, *J* = 14.8

Hz, CH=*CH*CON).

trans-3-Diethylcarbamoylacrylic Acid (13a, HOOCCH=CHCONEt₂).

EtOOCCH=CHCONEt₂ was hydrolyzed in MeOH using NaOH (1M aqueous, 1.1 eq)

under standard deblocking conditions to give a white crystalline solid (53% yield).

N²-(N-Benzyloxycarbonylalanylalanyl)-N¹-carbamoylmethyl-N¹-*trans*-(3-

diethylcarbamoylacryloyl)hydrazine (14a, Z-Ala-Ala-AAsn-CH=CHCONEt₂). This

compound was obtained using the HOBt/EDC coupling method and purified by column

chromatography on silica gel using 10% MeOH/CH₂Cl₂ as the eluent and then

recrystallized from EtOAc/hexane to give a white powder (11% yield). ^{1}H-NMR

(DMSO-d₆): 1.02-1.05 (t, 3H, NCH₂*CH₃*), 1.08-1.11 (t, 3H, NCH₂*CH₃*), 1.17-1.19 (d, 2H,

Ala-CH₃), 1.24-1.26 (d, 2H, Ala-CH₃), 3.20-3.32 (d, 6H, NCH₂CO and 2 x N-CH₂), 4.02-

4.07 (m, 1H, α-H), 4.27-4.31 (m, 1H, α-H), 4.99 (m, 2H, Cbz), 7.00-7.04 (d, 1H,

CH=CHCON), 7.18 (s, 1H, NH), 7.33 (m, 6H, Ph and CH=*CH*CON), 7.39 (d, 1H, NH),

8.20 (d, 1H, NH), 10.68 (s, 1H, NH). MS (ESI) *m/z* 519 [(M + 1)⁺]. HRMS (ESI)

calculated for $C_{24}H_{35}N_6O_7$: 519.2608. Observed *m/z* 519.2657. Anal. Calcd. for

$C_{24}H_{34}N_6O_7 \cdot 0.7H_2O$: C, 54.27; H, 6.72; N, 15.82. Found: C, 54.25; H, 6.69; N, 15.87.

trans-3-Dibutylcarbamoylacrylic Acid Ethyl Ester

(EtOOCCH=CHCON(*n*Bu)$_2$) was obtained by mixed anhydride coupling of equimolar

amounts of monoethyl fumarate and dibutylamine. The crude product was recrystallized

from hexane/EtOAc to give a white powder (95% yield). ^{1}H-NMR (CDCl$_3$): 0.95 (m,

6H, 2 x *n*Bu-CH$_3$), 1.32 (m, 7H, 2 x CH$_2$CH$_2$*CH$_2$*CH$_3$ and *CH$_3$*CH$_2$O), 1.51-1.59 (m, 4H,

2 x CH$_2$*CH$_2$*CH$_2$CH$_3$), 3.33 (t, 2H, N-CH$_2$), 3.39 (t, 2H, N-CH$_2$), 4.24 (q, 2H, CH$_3$*CH$_2$*O),

6.76-6.81 (d, 1H, J = 15.6 Hz, *CH*=CHCON), 7.33-7.36 (d, 1H, J = 15.2 Hz,

CH=*CH*CON).

trans-3-Dibutylcarbamoylacrylic Acid (13b, HOOCCH=CHCON(*n*Bu)$_2$).

EtOOCCH=CHCON(*n*Bu)$_2$ was hydrolyzed in MeOH using NaOH (1M aqueous, 1.1 eq)

under standard deblocking conditions to give a clear, colorless syrup (80% yield). ^{1}H-

NMR (CDCl$_3$): 0.95 (m, 6H, 2 x *n*Bu-CH$_3$), 1.30-1.37 (m, 4H, 2 x CH$_2$CH$_2$*CH$_2$*CH$_3$),

1.53-1.59 (m, 4H, 2 x CH$_2$*CH$_2$*CH$_2$CH$_3$), 3.33 (t, 2H, N-CH$_2$), 3.39 (t, 2H, N-CH$_2$), 6.79-

6.83 (d, 1H, J = 15.6 Hz, *CH*=CHCON), 7.33-7.36 (d, 1H, J = 15.2 Hz, CH=*CH*CON).

N^2-(N-Benzyloxycarbonylalanylalanyl)- N^1-carbamoylmethyl-N^1-*trans*-(3-

dibutylcarbamoylacryloyl)hydrazine (14b, Z-Ala-Ala-AAsn-CH=CHCON(*n*Bu)$_2$).

This compound was obtained using the HOBt/EDC coupling method and purified by

column chromatography on silica gel using 10% MeOH/CH$_2$Cl$_2$ as the eluent and then

recrystallized from EtOAc/hexane to give a white powder (15% yield). ^{1}H-NMR

(DMSO-d$_6$): 0.95 (m, 6H, 2 x *n*Bu-CH$_3$), 1.11-1.27 (m, 6H, 2 x Ala-CH$_3$), 1.30-1.37 (m,

4H, 2 x CH$_2$CH$_2$*CH$_2$*CH$_3$), 1.53-1.59 (m, 4H, 2 x CH$_2$*CH$_2$*CH$_2$CH$_3$), 3.20-3.32 (d, 6H,

NCH$_2$CO and 2 x N-CH$_2$), 4.00-4.01 (m, 1H, α-H), 4.25-4.30 (m, 1H, α-H), 4.99 (m, 2H,

Cbz), 7.00-7.04 (d, 1H, *CH*=CHCON), 7.21 (s, 1H, NH), 7.33 (m, 6H, Ph and

CH=*CH*CON), 7.42 (d, 1H, NH), 8.20 (d, 1H, NH), 10.73 (s, 1H, NH). MS (FAB) *m/z*

575 [(M + 1)$^+$]. HRMS (FAB) calculated for C$_{28}$H$_{43}$N$_6$O$_7$: 575.32052. Observed *m/z*

575.31932. Anal. Calcd. for C$_{28}$H$_{42}$N$_6$O$_7$·0.8H$_2$O: C, 57.16; H, 7.35; N, 14.28. Found:

C, 57.18; H, 7.35; N, 14.28.

trans-3-(1-Piperidyloxo)acrylic Acid Ethyl Ester (EtOOCCH=CHCO-Pip)

was obtained by mixed anhydride coupling of equimolar amounts of monoethyl fumarate

and piperidine to give a clear syrup (99% yield).

trans-3-(1-Piperidyloxo)acrylic Acid (13c, HOOCCH=CHCO-Pip).

EtOOCCH=CHCO-Pip was hydrolyzed in MeOH using NaOH (1M aqueous, 1.1 eq)

under standard deblocking conditions to give a clear, colorless syrup which was

recrystallized over night from hexane/EtOAc to give a white powder (27% yield). ^{1}H-

NMR (DMSO-d$_6$): 1.46-1.60 (m, 6H, 3 x piperidine CH$_2$), 3.45-3.49 (m, 4H, CH$_2$-N-

CH$_2$), 6.41-6.44 (d, 1H, *J* = 15.2 Hz, *CH*=CHCON), 7.33-7.37 (d, 1H, *J* = 15.2 Hz,

CH=*CH*CON).

N^2-(N-Benzyloxycarbonylalanylalanyl)-N^1-carbamoylmethyl-N^1-trans-(3-(1-

piperidyloxo)acryloyl)hydrazine (14c, Z-Ala-Ala-AAsn-CH=CHCO-Pip). This

compound was obtained using the HOBt/EDC coupling method and purified by column

chromatography on silica gel using 10% MeOH/CH$_2$Cl$_2$ as the eluent and recrystallized

from hexane/EtOAc to give a white powder (10% yield). ^{1}H-NMR ((CD$_3$)CO): 1.28 (m,

2H, piperidine-CH$_2$), 1.37 (d, 3H, Ala-CH$_3$), 1.41 (d, 3H, Ala-CH$_3$), 1.52-1.67 (m, 4H, 2

x piperidine-CH$_2$), 2.80 (s, 2H, NCH$_2$CO), 3.55 (m, 4H, CH$_2$-N-CH$_2$), 4.24 (m, 1H, α-H),

4.50 (m, 1H, α-H), 5.10 (m, 2H, Cbz), 7.11-7.15 (d, 1H, J = 14.8 Hz, CH=CHCON),

7.30-7.44 (m, 7H, CH=CHCON and Ph and NH), 7.77 (s, 1H, NH), 9.82 (s, 1H, NH).

MS (ESI) m/z 531 [(M + 1)$^+$]. HRMS (ESI) calculated for $C_{25}H_{35}N_6O_7$: 531.2532.

Observed m/z 531.256723. Anal. Calcd. for $C_{25}H_{34}N_6O_7 \cdot 0.7H_2O$: C, 55.32; H, 6.50; N,

15.48. Found: C, 55.26; H, 6.43; N, 15.28.

trans-3-Phenylcarbamoylacrylic Acid Ethyl Ester (EtOOCCH=CHCONHPh)

was obtained by mixed anhydride coupling of equimolar amounts of monoethyl fumarate

and aniline to give a white solid (59% yield). ^{1}H-NMR (CDCl$_3$): 1.34 (t, 3H, CH_3CH$_2$),

4.29 (q, 2H, CH$_3$$CH_2$), 6.93-6.97 (d, 1H, J = 15.2 Hz, CH=CHCON), 7.10-7.14 (d, 1H, J

= 15.2 Hz, CH=CHCON), 7.15 (t, 1H, Ph), 7.34 (t, 2H, Ph), 7.61 (d, 2H, Ph), 7.85 (s, 1H,

NH).

trans-3-Phenylcarbamoylacrylic Acid (13d, HOOCCH=CHCONHPh).

EtOOCCH=CHCONHPh was hydrolyzed in MeOH using NaOH (1M aqueous, 1.1 eq)

under standard deblocking conditions to give a white solid (81% yield). ^{1}H-NMR

(DMSO-d$_6$): 6.61-6.65 (d, 1H, J = 15.2 Hz, CH=CHCON), 7.02-7.14 (m, 1H,

CH=CHCON), 7.14-7.31 (m, 2H, Ph), 7.32 (t, 2H, Ph), 7.66 (d, 2H, Ph), 10.47 (s, 1H,

NH).

N^2-(N-Benzyloxycarbonylalanylalanyl)-N^1-carbamoylmethyl-N^1-_trans_-(3-

phenylcarbamoylacryloyl)hydrazine (14d, Z-Ala-Ala-AAsn-CH=CHCONHPh).

This compound was obtained using the HOBt/EDC coupling method with minimal

NaHCO$_3$ washing during the workup. The product was isolated as a yellow solid without

chromatography by recrystallization from 10% MeOH/CH$_2$Cl$_2$ (21% yield). ^{1}H-NMR

(DMSO-d$_6$): 1.18-1.27 (m, 6H, 2 x Ala-CH$_3$), 3.20-3.32 (d, 2H, NCH$_2$CO), 4.04-4.08 (m,

1H, α-H), 4.28-4.31 (m, 1H, α-H), 4.99 (m, 2H, Cbz), 7.05-7.42 (m, 11H, 2 x Ph, NH

CH=*CH*CON and *CH*=CHCON), 7.63-7.65 (d, 2H, Ph), 7.52 (s, 1H, NH), 8.15 (d, 1H,

NH), 10.42 (s, 1H, NH), 10.72 (s, 1H, NH). MS (ESI) *m/z* 539 [(M + 1)$^+$]. HRMS (ESI)

calculated for $C_{26}H_{31}N_6O_7$: 539.2208. Observed *m/z* 539.2254. Anal. Calcd. for

$C_{26}H_{30}N_6O_7$: C, 57.98; H, 5.61; N, 15.60. Found: C, 57.73; H, 5.58; N, 15.72.

trans-3-Benzylcarbamoylacrylic Acid Ethyl Ester

(EtOOCCH=CHCONHBzl) was obtained by mixed anhydride coupling of equimolar

amounts of monoethyl fumarate and benzylamine to give a white powder (72% yield).

trans-3-Benzylcarbamoylacrylic Acid (13e, HOOCCH=CHCONHBzl).

EtOOCCH=CHCONHBzl was hydrolyzed in MeOH using NaOH (1M aqueous, 1.1 eq)

under standard deblocking conditions to give a white solid (81% yield). ^{1}H-NMR

(DMSO-d_6): 4.37 (d, 2H, N-*CH$_2$*-Ph), 6.52-6.56 (d, 1H, *J* = 15.2 Hz, *CH*=CHCON),

6.94-6.98 (d, 1H, *J* = 15.6 Hz, CH=*CH*CON), 7.14-7.31 (m, 5H, Ph), 8.97 (t, 1H, NH).

N^2-(N-Benzyloxycarbonylalanylalanyl)-N^1-carbamoylmethyl-N^1-*trans*-(3-benzylcarbamoylacryloyl)hydrazine (14e, Z-Ala-Ala-AAsn-CH=CHCONHBzl).

This compound was obtained using the HOBt/EDC coupling method with minimal

washing during the workup and purified by recrystallization from ice cold EtOAc without

column chromatography (18% yield). ^{1}H-NMR (DMSO-d_6): 1.17-1.26 (m, 6H, 2 x Ala-

CH$_3$), 3.30-3.32 (d, 2H, NCH$_2$CO), 4.02-4.07 (m, 1H, α-H), 4.27-4.32 (m, 1H, α-H),

4.35-4.36 (d, 2H, CH$_2$Ph), 4.99 (m, 2H, Cbz), 6.89-6.92 (d, 1H, *J* = 14.2 Hz,

CH=CHCON), 7.06-7.10 (d, 1H, *J* = 14.8 Hz, CH=*CH*CON), 7.16-7.41 (m, 11H, 2 x Ph

and NH), 7.42 (d, 1H, NH), 8.16 (d, 1H, NH), 8.94 (t, 1H, NH), 10.72 (s, 1H, NH). MS

(ESI) *m/z* 553 [(M + 1)$^+$]. HRMS (ESI) calculated for $C_{27}H_{33}N_6O_7$: 553.2397. Observed

m/z 553.2411.   Anal. Calcd. for $C_{27}H_{32}N_6O_7 \cdot 0.3H_2O$: C, 58.12; H, 5.89; N, 15.06.

Found: C, 58.07; H, 5.81; N, 15.03.

trans-3-(4-Fluorobenzylcarbamoyl)acrylic Acid Ethyl Ester

(EtOOCCH=CHCONH-Bzl-4-F) was obtained by mixed anhydride coupling of

equimolar amounts of monoethyl fumarate and 4-fluoro benzylamine to give a pink solid

(66% yield). ^{1}H-NMR (CDCl$_3$): 1.32 (t, 3H, CH$_2$*CH$_3$*), 4.21 (q, 2H, *CH$_2$*CH$_3$), 4.47 (d,

2H, N-*CH$_2$*-Ph), 6.52-6.56 (d, 1H, *J* = 15.2 Hz, *CH*=CHCON), 6.94-6.98 (d, 1H, *J* = 15.6

Hz, CH=*CH*CON), 6.99-7.04 (m, 2H, Ph), 7.20-7.27 (m, 2H, Ph).

trans-3-(4-Fluorobenzylcarbamoyl)acrylic Acid (13f, HOOCCH=CHCONH-

Bzl-4-F). EtOOCCH=CHCONH-Bzl-4-F was hydrolyzed in MeOH using NaOH (1M

aqueous, 1.1 eq) under standard deblocking conditions to give a clear, colorless syrup

(45% yield). ^{1}H-NMR (DMSO-d$_6$): 4.34-4.35 (d, 2H, N-*CH$_2$*-Ph), 6.52-6.55 (d, 1H, *J* =

15.2 Hz, *CH*=CHCON), 6.93-6.94 (d, 1H, *J* = 15.6 Hz, CH=*CH*CON), 7.14-7.16 (t, 2H,

Ph), 7.27-7.31 (t, 2H, Ph), 8.99 (t, 1H, NH).

N^2-(N-Benzyloxycarbonylalanylalanyl)-N^1-carbamoylmethyl-N^1-trans-(3-(4-

fluorobenzyl)carbamoylacryloyl)hydrazine (14f, Z-Ala-Ala-AAsn-CH=CHCONH-

Bzl-4-F). This compound was obtained using the HOBt/EDC coupling method and

purified by column chromatography using 10% MeOH/CH$_2$Cl$_2$ as the eluent to give a

pink powder (8% yield). ^{1}H-NMR (DMSO-d$_6$): 1.17-1.26 (m, 6H, 2 x Ala-CH$_3$), 3.30-

3.32 (d, 2H, NCH$_2$CO), 4.02-4.07 (m, 1H, α-H), 4.27-4.35 (m, 3H, α-H and CH$_2$PhF),

4.99 (m, 2H, Cbz), 6.88-6.92 (d, 1H, *J* = 16 Hz, *CH*=CHCON), 7.06-7.32 (m, 11H,

CH=*CH*CON and NH and 2 x Ph), 7.43 (d, 1H, NH), 7.53 (s, 1H, NH), 8.19 (d, 1H, NH),

8.97 (t, 1H, NH), 10.72 (s, 1H, NH). MS (ESI) *m/z* 571 [(M + 1)$^+$]. HRMS (ESI)

calculated for $C_{27}H_{32}N_6O_7F$: 571.2323. Observed m/z 571.231651. Anal. Calcd. for

$C_{27}H_{31}N_6O_7F$: C, 56.84; H, 5.48; N, 14.73. Found: C, 56.73; H, 5.58; N, 14.68.

trans-3-Phenethylcarbamoylacrylic Acid Ethyl Ester

(EtOOCCH=CHCONHCH$_2$CH$_2$Ph) was obtained by mixed anhydride coupling of

equimolar amounts of monoethyl fumarate and phenethylamine to give a clear colorless

syrup (78% yield).

trans-3-Phenethylcarbamoylacrylic Acid (13g,

HOOCCH=CHCONHCH$_2$CH$_2$Ph). EtOOCCH=CHCONHCH$_2$CH$_2$Ph was hydrolyzed

in MeOH using NaOH (1M aqueous, 1.1 eq) under standard deblocking conditions to

give a clear, colorless syrup (81% yield). ^{1}H-NMR (DMSO-d$_6$): 3.54 (t, 2H, N-CH$_2$-

CH$_2$-Ph), 3.61 (t, 2H, N-*CH$_2$*-CH$_2$-Ph) 6.43-6.47 (d, 1H, J = 15.2 Hz, *CH*=CHCON),

6.98-7.02 (d, 1H, J = 15.6 Hz, CH=*CH*CON), 7.14-7.31 (m, 5H, Ph).

N^2-(N-Benzyloxycarbonylalanylalanyl)-N^1-carbamoylmethyl-N^1-*trans*-(3-

phenethylcarbamoylacryloyl)hydrazine (14g, Z-Ala-Ala-AAsn-

CH=CHCONHCH$_2$CH$_2$Ph). This compound was obtained using the HOBt/EDC

coupling method with minimal washing during the workup and purified by column

chromatography on silica gel using 10% MeOH/CH$_2$Cl$_2$ as the eluent and then washed

with EtOAc to give a white powder (12% yield). ^{1}H-NMR (DMSO-d$_6$): 1.18-1.27 (m,

6H, 2 x Ala-CH$_3$), 2.74 (m, 2H, N-CH$_2$*CH$_2$*Ph), 3.20-3.32 (d, 2H, NCH$_2$CO), 3.52-3.58

(m, 2H, N-*CH$_2$*CH$_2$Ph), 4.04-4.08 (m, 1H, α-H), 4.28-4.31 (m, 1H, α-H), 4.99 (m, 2H,

Cbz), 6.86-6.89 (d, 1H, J = 14.4 Hz, *CH*=CHCON), 7.04-7.08 (d, 1H, J = 14.8 Hz,

CH=*CH*CON), 7.16-7.41 (m, 11H, 2 x Ph and NH), 7.50 (d, 1H, NH), 8.16 (d, 1H, NH),

8.53 (s, 1H, NH), 10.72 (s, 1H, NH). MS (ESI) m/z 567 [(M + 1)$^+$]. HRMS (ESI)

calculated for $C_{28}H_{35}N_6O_7$: 567.2596. Observed *m/z* 567.256723. Anal. Calcd. for

$C_{28}H_{34}N_6O_7 \cdot 0.3H_2O \cdot 0.2$hexane: C, 59.52; H, 6.40; N, 14.26. Found: C, 59.71; H, 6.18;

N, 13.99.

trans-N-Methylphenylcarbamoylacrylic Acid Ethyl Ester

(EtOOCCH=CHCON(CH3)Ph) was obtained by mixed anhydride coupling of

equimolar amounts of monoethyl fumarate and N-methyl aniline to give a white solid

(99% yield). ^{1}H-NMR (CDCl$_3$): 1.25 (t, 3H, *CH$_3$*CH$_2$), 3.39 (s, 3H, N-CH$_3$), 4.17 (q, 2H,

CH$_3$*CH$_2$*), 6.93-6.97 (d, 1H, *J* = 15.2 Hz, *CH*=CHCON), 7.10-7.14 (d, 1H, *J* = 15.2 Hz,

CH=*CH*CON), 7.15 (t, 1H, Ph), 7.34 (t, 2H, Ph), 7.61 (d, 2H, Ph).

trans-N-Methylphenylcarbamoylacrylic Acid (13h,

HOOCCH=CHCON(CH3)Ph). EtOOCCH=CHCON(CH$_3$)Ph was hydrolyzed in

MeOH using NaOH (1M aqueous, 1.1 eq) under standard deblocking conditions to give a

white solid (29% yield). ^{1}H-NMR (DMSO-d$_6$): 3.14 (s, 3H, N-CH$_3$), 6.50-6.54 (d, 1H, *J*

= 15.2 Hz, *CH*=CHCON), 6.60-6.64 (d, 1H, *J* = 15.2 Hz, CH=*CH*CON), 7.32 (t, 2H,

Ph), 7.40 (d, 1H, Ph), 7.47 (d, 1H, Ph).

N^2-(N-Benzyloxycarbonylalanylalanyl)-N^1-carbamoylmethyl-N^1-trans-(3-

(methylphenylcarbamoyl)acryloyl)hydrazine (14h, Z-Ala-Ala-AAsn-

CH=CHCON(CH3)Ph). This compound was obtained using the HOBt/EDC coupling

method. The product was isolated by chromatography with 10% MeOH/CH$_2$Cl$_2$ as the

eluent and recrystallized from hexane/EtOAc to give a white powder (34% yield). ^{1}H-

NMR (DMSO-d$_6$): 1.18-1.27 (m, 6H, 2 x Ala-CH$_3$), 3.20-3.32 (m, 5H, NCH$_2$CO and N-

CH$_3$), 4.02-4.07 (m, 1H, α-H), 4.30 (m, 1H, α-H), 4.99 (m, 2H, Cbz), 6.56 (d, 1H,

CH=CHCON), 7.06-7.10 (d, 1H, CH=*CH*CON), 7.14 (s, 1H, NH), 7.29-7.46 (m, 11H, 2

x Ph, NH), 8.14 (d, 1H, NH), 10.65 (s, 1H, NH). MS (ESI) m/z 553 [(M + 1)$^+$]. HRMS

(ESI) calculated for $C_{27}H_{33}N_6O_7$: 553.2356. Observed m/z 553.2411. Anal. Calcd. for

$C_{27}H_{32}N_6O_7$: C, 58.69; H, 5.84; N, 15.21. Found: C, 58.43; H, 5.90; N, 15.20.

trans-3-Benzylmethylcarbamoylacrylic Acid Ethyl Ester

(EtOOCCH=CHCON(CH₃)Bzl) was obtained by mixed anhydride coupling of

equimolar amounts of monoethyl fumarate and N-methyl benzylamine to give a clear,

pink syrup (76% yield).

trans-3-Benzylmethylcarbamoylacrylic Acid (13i,

HOOCCH=CHCON(CH₃)Bzl). EtOOCCH=CHCON(CH₃)Bzl was hydrolyzed in

MeOH using NaOH (1M aqueous, 1.1 eq) under standard deblocking conditions to give a

clear, colorless syrup (77% yield). ^{1}H-NMR (CDCl$_3$): 3.03 (s, 3H, N-CH$_3$), 4.62-4.67 (d,

2H, N-*CH$_2$*-Ph), 6.83-6.87 (d, 1H, J = 16 Hz, *CH*=CHCON), 7.15-7.17 (d, 1H, J = 8 Hz,

CH=*CH*CON), 7.25-7.50 (m, 5H, Ph).

N^2-(N-Benzyloxycarbonylalanylalanyl)-N^1-carbamoylmethyl-N^1-trans-(3-

benzylmethylcarbamoylacryloyl)hydrazine (14i, Z-Ala-Ala-AAsn-

CH=CHCON(CH₃)Bzl). This compound was synthesized using the HOBt/EDC

coupling method and purified by column chromatography on silica gel using 10%

MeOH/CH$_2$Cl$_2$ as the eluent and then recrystallized from EtOAc/hexane to give a white

powder (54% yield). ^{1}H-NMR (DMSO-d$_6$): 1.18-1.27 (m, 6H, 2 x Ala-CH$_3$), 2.89 (s, 3H,

N-CH$_3$), 3.20-3.32 (d, 2H, NCH$_2$CO), 4.04-4.08 (m, 1H, α-H), 4.28-4.31 (m, 1H, α-H),

4.57 (m, 2H, N-*CH$_2$*-Ph), 4.99 (m, 2H, Cbz), 7.05-7.41 (m, 13H, *CH*=CHCON and

CH=*CH*CON and 2 x Ph and NH), 7.49 (d, 1H, NH), 8.16 (d, 1H, NH), 10.72 (s, 1H,

NH). MS (ESI) m/z 567 [(M + 1)$^+$]. HRMS (ESI) calculated for $C_{28}H_{35}N_6O_7$: 567.2604.

Observed *m/z* 567.256723. Anal. Calcd. for $C_{28}H_{34}N_6O_7 \cdot 0.9H_2O$: C, 57.70; H, 6.19; N, 14.42. Found: C, 57.91; H, 6.25; N, 14.27.

trans-3-(Methyl-1-naphthylmethylcarbamoyl)acrylic Acid Ethyl Ester
(EtOOCCH=CHCON(CH₃)CH₂-1-Napth) was obtained by mixed anhydride coupling
of equimolar amounts of monoethyl fumarate and N-methyl-1-naphthyl methylamine
hydrochloride to give a clear, colorless syrup (99% yield). ^{1}H-NMR (CDCl₃): 1.31 (t,
3H, *CH₃*CH₂), 3.03 (s, 3H, N-CH₃) 4.29 (q, 2H, CH₃*CH₂*), 5.30 (s, 2H, N-CH₂-naphthyl),
6.92 (d, 1H, *J* = 15.2 Hz, *CH*=CHCON), 7.10-7.52 (m, 5H, naphthyl and CH=*CH*CON),
7.81-7.91 (m, 2H, naphthyl), 7.85 (d, 1H, naphthyl).

trans-3-(Methyl-1-naphthylmethylcarbamoyl)acrylic Acid (13j,
HOOCCH=CHCON(CH₃)CH₂-1-Napth). EtOOCCH=CHCON(CH₃)CH₂-1-Napth
was hydrolyzed in MeOH using NaOH (1M aqueous, 1.1 eq) under standard deblocking
conditions to give a white solid after recrystallization from cold EtOAc (13% yield). ^{1}H-
NMR (DMSO-d₆): 3.01 (s, 3H, CH₃), 5.01 (s, 2H, CH₂), 6.61-6.65 (d, 1H, *J* = 15.2 Hz,
CH=CHCON), 7.17-7.21 (d, 1H, CH=*CH*CON), 7.37-7.60 (m, 4H, naphthyl), 7.85-8.01
(m, 3H, naphthyl).

N^2-(N-Benzyloxycarbonylalanylalanyl)-N^1-carbamoylmethyl-N^1-*trans*-(3-
(methyl-1-naphthylmethylcarbamoyl)acryloyl)hydrazine (14j, Z-Ala-Ala-AAsn-
CH=CHCON(CH₃)CH₂-1-Napth). This compound was obtained using the HOBt/EDC
coupling method and purified by column chromatography on silica gel using 10%
MeOH/CH₂Cl₂ as the eluent and then recrystallized from EtOAc/hexane to give a yellow
powder (31% yield). ^{1}H-NMR (DMSO-d₆): 1.18-1.27 (m, 6H, 2 x Ala-CH₃), 3.10 (s, 3H,
N-CH₃), 3.20-3.32 (d, 2H, NCH₂CO), 4.04-4.08 (m, 1H, α-H), 4.28-4.31 (m, 1H, α-H),

4.99 (m, 4H, Cbz and N-CH$_2$-naphthyl), 7.07-7.61 (m, 12H, naphthyl and Ph

CH=*CH*CON and *CH*=CHCON and NH), 7.85-8.10 (m, 3H, naphthyl), 8.15 (d, 1H, NH),

10.42 (s, 1H, NH), 10.72 (s, 1H, NH). MS (ESI) *m/z* 617 [(M + 1)$^+$]. HRMS (ESI)

calculated for C$_{32}$H$_{37}$N$_6$O$_7$: 617.265. Observed *m/z* 617.2724. Anal. Calcd. for

C$_{32}$H$_{36}$N$_6$O$_7$·0.5H$_2$O: C, 61.43; H, 5.96; N, 13.43. Found: C, 61.48; H, 6.03; N, 13.25.

trans-3-(Methylphenethylcarbamoyl)acrylic Acid Ethyl Ester

(EtOOCCH=CHCON(CH$_3$)CH$_2$CH$_2$Ph) was obtained by mixed anhydride coupling of

equimolar amounts of monoethyl fumarate and N-methyl phenethylamine to give a clear

colorless syrup (64% yield).

trans-3-(Methylphenethylcarbamoyl)acrylic Acid (13k,

HOOCCH=CHCON(CH$_3$)CH$_2$CH$_2$Ph). EtOOCCH=CHCON(CH$_3$)CH$_2$CH$_2$Ph was

hydrolyzed in MeOH using NaOH (1M aqueous, 1.1 eq) under standard deblocking

conditions to give a clear, colorless syrup (81% yield). ^{1}H-NMR (DMSO-d$_6$): 2.82 (s,

3H, N-CH$_3$), 3.54 (t, 2H, N-CH$_2$-*CH$_2$*-Ph), 3.61 (t, 2H, N-*CH$_2$*-CH$_2$-Ph), 6.43-6.47 (d, 1H,

J = 15.2 Hz, *CH*=CHCON), 6.98-6.7.02 (d, 1H, *J* = 15.6 Hz, CH=*CH*CON), 7.14-7.31

(m, 5H, Ph).

N^2-(N-Benzyloxycarbonylalanylalanyl)-N^1-carbamoylmethyl-N^1-trans-(3-

(Methylphenethylcarbamoyl)acryloyl)hydrazine (14k, Z-Ala-Ala-AAsn-

CH=CHCON(CH$_3$)CH$_2$CH$_2$Ph). This compound was obtained using the HOBt/EDC

coupling method and purified by column chromatography on silica gel using 10%

MeOH/CH$_2$Cl$_2$ as the eluent and then recrystallized from EtOAc/hexane to give a white

powder (28% yield). ^{1}H-NMR (DMSO-d$_6$): 1.18-1.27 (m, 6H, 2 x Ala-CH$_3$), 2.74-2.82

(m, 2H, N-CH$_2$*CH$_2$*Ph), 2.86 (s, 3H, N-CH$_3$), 3.20-3.32 (d, 2H, NCH$_2$CO), 3.52-3.58 (m,

2H, N-*CH₂*CH₂Ph), 4.04-4.08 (m, 1H, α-H), 4.28-4.31 (m, 1H, α-H), 4.99 (m, 2H, Cbz),

6.86-6.89 (d, 1H, J = 14.4 Hz, *CH*=CHCON), 7.04-7.08 (d, 1H, J = 14.8 Hz,

CH=*CH*CON), 7.16-7.41 (m, 11H, 2 x Ph and NH), 7.50 (d, 1H, NH), 8.16 (d, 1H, NH),

10.72 (s, 1H, NH). MS (ESI) *m/z* 581 [(M + 1)$^+$]. HRMS (ESI) calculated for

$C_{29}H_{37}N_6O_7$: 581.2717. Observed *m/z* 581.272373. Anal. Calcd. for

$C_{29}H_{36}N_6O_7 \cdot 0.6H_2O \cdot 0.1$hexane: C, 59.25; H, 6.48; N, 14.01. Found: C, 59.27; H, 6.50;

N, 13.82.

        trans-3-Phenylbenzylcarbamoylacrylic Acid Ethyl Ester

(EtOOCCH=CHCON(Bzl)Ph) was obtained by mixed anhydride coupling of equimolar

amounts of monoethyl fumarate and phenyl benzylamine to give an orange oil (87%

yield).

        trans-3-Phenylbenzylcarbamoylacrylic Acid (13l,

HOOCCH=CHCON(Bzl)Ph). EtOOCCH=CHCON(Bzl)Ph was hydrolyzed in MeOH

using NaOH (1M aqueous, 1.1 eq) under standard deblocking conditions to give a clear,

colorless syrup (74% yield). ^{1}H-NMR (DMSO-d$_6$): 4.97 (s, 2H, N-*CH₂*-Ph), 6.61-6.62

(d, 1H, *CH*=CHCON), 7.15-7.43 (m, 11H, CH=*CH*CON and 2 x Ph).

        N^2-(N-Benzyloxycarbonylalanylalanyl)-N^1-carbamoylmethyl-N^1-trans-(3-

phenylbenzylcarbamoylacryloyl)hydrazine (14l, Z-Ala-Ala-AAsn-

CH=CHCON(Bzl)Ph). This compound was obtained using the HOBt/EDC coupling

method and purified by column chromatography on silica gel using 10% MeOH/CH$_2$Cl$_2$

as the eluent to give a white powder (14% yield). ^{1}H-NMR (DMSO-d$_6$): 1.20-1.21 (d,

3H, Ala-CH$_3$), 1.26 (d, 3H, Ala-CH$_3$), 3.31 (s, 2H, NCH$_2$CO), 4.04-4.08 (m, 1H, α-H),

4.28-4.31 (m, 1H, α-H), 4.96-4.99 (d, 4H, N-*CH₂*-Ph and Cbz), 6.57-6.61 (d, 1H, J =

15.2 Hz, CH=CHCON), 7.14-7.45 (m, 18H, CH=CHCON and 3 x Ph and 2 x NH), 8.16

(d, 1H, NH), 10.71 (s, 1H, NH). MS (ESI) m/z 629 [(M + 1)$^+$]. HRMS (ESI) calculated

for $C_{33}H_{37}N_6O_7$: 629.2691. Observed m/z 629.272373. Anal. Calcd. for

$C_{33}H_{36}N_6O_7 \cdot 0.09H_2O \cdot 0.17$hexane: C, 62.15; H, 6.05; N, 12.83. Found: C, 62.15; H, 5.96;

N, 12.66.

trans-3-Dibenzylcarbamoylacrylic Acid Ethyl Ester

(EtOOCCH=CHCON(Bzl)$_2$) was obtained by mixed anhydride coupling of equimolar

amounts of monoethyl fumarate and dibenzylamine to give a clear, pink syrup (87%

yield).

trans-3-Dibenzylcarbamoylacrylic Acid (13m, HOOCCH=CHCON(Bzl)$_2$).

EtOOCCH=CHCON(Bzl)$_2$ was hydrolyzed in MeOH using NaOH (1M aqueous, 1.1 eq)

under standard deblocking conditions to give a clear, colorless syrup which was

recrystallized over night from hexane/EtOAc (30% yield). ^{1}H-NMR (DMSO-d$_6$): 4.57 (s,

2H, N-CH_2-Ph), 4.65 (s, 2H, N-CH_2-Ph), 6.59-6.63 (d, 1H, J = 15.2 Hz, CH=CHCON),

7.15-7.17 (d, 1H, J = 8 Hz, CH=CHCON), 7.25-7.50 (m, 10H, 2 x Ph).

N^2-(N-Benzyloxycarbonylalanylalanyl)- N^1-carbamoylmethyl-N^1-trans-(3-

dibenzylcarbamoylacryloyl)hydrazine (14m, Z-Ala-Ala-AAsn-CH=CHCON(Bzl)$_2$).

This compound was obtained using the HOBt/EDC coupling method and purified by

column chromatography on silica gel using 10% MeOH/CH$_2$Cl$_2$ as the eluent to give a

white powder (34% yield). ^{1}H-NMR (DMSO-d$_6$): 1.19 (d, 3H, Ala-CH$_3$), 1.26 (d, 3H,

Ala-CH$_3$), 3.31 (s, 2H, NCH$_2$CO), 4.04-4.08 (m, 1H, α-H), 4.28-4.31 (m, 1H, α-H), 4.56

(s, 2H, N-CH_2-Ph), 4.63 (s, 2H, N-CH_2-Ph), 5.00 (m, 2H, Cbz), 7.13-7.41 (m, 17H,

CH=CHCON and CH=CHCON and 3 x Ph and NH), 7.48 (s, 1H, NH), 8.14 (d, 1H, NH),

10.71 (s, 1H, NH). MS (ESI) *m/z* 643 [(M + 1)$^+$]. HRMS (ESI) calculated for

C$_{34}$H$_{39}$N$_6$O$_7$: 643.2843. Observed *m/z* 643.288023. Anal. Calcd. for C$_{34}$H$_{38}$N$_6$O$_7$: C,

63.54; H, 5.96; N, 13.08. Found: C, 63.75; H, 6.02; N, 12.81.

trans-3-(Benzyl-4-methoxybenzylcarbamoyl)acrylic Acid Ethyl Ester

(EtOOCCH=CHCON(Bzl-4-OMe)Bzl) was obtained by mixed anhydride coupling of

equimolar amounts of monoethyl fumarate and 4-methoxy benzyl benzylamine to give a

clear colorless syrup (91% yield).

trans-3-(Benzyl-4-methoxybenzylcarbamoyl)acrylic Acid (13n,

HOOCCH=CHCON(Bzl-4-OMe)Bzl). EtOOCCH=CHCON(Bzl-4-OMe)Bzl was

hydrolyzed in MeOH using NaOH (1M aqueous, 1.1 eq) under standard deblocking

conditions to give a clear, colorless syrup (44% yield). ^{1}H-NMR (DMSO-d$_6$): 3.73 (s,

3H, OCH$_3$), 4.50-4.54 (d, 2H, N-*CH$_2$*-Ph), 4.56-4.61 (d, 2H, N-*CH$_2$*-Ph), 6.59-6.63 (d,

1H, *J* = 15.2 Hz, *CH*=CHCON), 6.86-6.92 (2 x d, 2H, Ph), 7.07-7.09 (d, 1H, *J* = 8 Hz,

CH=*CH*CON), 7.14-7.39 (m, 7H, 2 x Ph).

N^2-(N-Benzyloxycarbonylalanylalanyl)-N^1-trans-(3-benzyl-(4-

methoxybenzyl)carbamoyl)acryloyl-N^1-carbamoylmethylhydrazine (14n, Z-Ala-

Ala-AAsn-CH=CHCON(Bzl-4-OMe)Bzl). This compound was obtained using the

HOBt/EDC coupling method and purified by recrystallization from 10% MeOH/CH$_2$Cl$_2$

to give a yellow powder (9% yield). ^{1}H-NMR (DMSO-d$_6$): 1.19-1.20 (d, 3H, Ala-CH$_3$),

1.26-1.28 (d, 3H, Ala-CH$_3$), 3.32 (s, 2H, NCH$_2$CO), 3.72 (s, 3H, OCH$_3$), 4.04-4.09 (m,

1H, α-H), 4.28-4.32 (m, 1H, α-H), 4.48-4.54 (d, 2H, N-*CH$_2$*-Ph), 4.54-4.59 (d, 2H, N-

CH$_2$-Ph), 5.00 (m, 2H, Cbz), 6.84-6.91 (2 x d, 2H, Ph), 7.06-7.08 (d, 1H, *J* = 8.8 Hz,

CH=CHCON), 7.12-7.42 (m, 14H, CH=*CH*CON and 3 x Ph and NH), 7.50 (s, 1H, NH),

8.16-8.17 (d, 1H, NH), 10.71 (s, 1H, NH). MS (ESI) *m/z* 673 [(M + 1)$^+$]. HRMS (ESI)

calculated for $C_{35}H_{41}N_6O_8$: 673.3001. Observed *m/z* 673.298588. Anal. Calcd. for

$C_{35}H_{40}N_6O_8 \cdot 0.5CH_2Cl_2$: C, 59.61; H, 5.78; N, 11.75. Found: C, 59.76; H, 5.71; N, 11.54.

<ins>trans</ins>-3-Benzyl(4-fluorobenzyl)carbamoylacrylic Acid Ethyl Ester

(EtOOCCH=CHCON(Bzl-4-F)Bzl) was obtained by mixed anhydride coupling of

equimolar amounts of monoethyl fumarate and 4-fluorobenzyl benzylamine to give a

clear, colorless syrup (95% yield).

<ins>trans</ins>-3-Benzyl(4-fluorobenzyl)carbamoylacrylic Acid (13o,

HOOCCH=CHCON(Bzl-4-F)Bzl). EtOOCCH=CHCON(Bzl-4-F)Bzl was hydrolyzed

in MeOH using NaOH (1M aqueous, 1.1 eq) under standard deblocking conditions to

give a clear, colorless syrup (44% yield). ^{1}H-NMR (DMSO-d$_6$): 4.50-4.54 (d, 2H, N-

CH$_2$-Ph), 4.56-4.61 (d, 2H, N-*CH$_2$*-Ph), 6.59-6.63 (d, 1H, *J* = 15.2 Hz, *CH*=CHCON),

6.99-7.44 (m, 11H, CH=*CH*CON and *CH*=CHCON and 2 x Ph).

N^2-(N-Benzyloxycarbonylalanylalanyl)-N^1-<ins>trans</ins>-(3-benzyl(4-

fluorobenzyl)carbamoylacryloyl)-N^1-carbamoylmethylhydrazine (14o, Z-Ala-Ala-

AAsn-CH=CH-CON(Bzl-4-F)Bzl). This compound was obtained using the HOBt/EDC

coupling method and purified by column chromatography using 10% MeOH/CH$_2$Cl$_2$ as

the eluent. Recrystallization with hexane/EtOAc gave a white powder (11% yield). ^{1}H-

NMR (DMSO-d$_6$): 1.19-1.20 (d, 3H, Ala-CH$_3$), 1.26-1.28 (d, 3H, Ala-CH$_3$), 3.32 (s, 2H,

NCH$_2$CO), 4.05-4.09 (m, 1H, α-H), 4.28-4.32 (m, 1H, α-H), 4.35 (s, 2H, *CH$_2$*Ph), 4.62-

4.64 (d, 2H, N-*CH$_2$*-Ph), 5.00 (m, 2H, Cbz), 7.09-7.34 (m, 17H, *CH*=CHCON and

CH=*CH*CON and 3 x Ph and NH), 7.40-7.42 (d, 1H, NH), 7.50 (s, 1H, NH), 8.15-8.16 (d,

1H, NH), 10.71 (s, 1H, NH). MS (ESI) *m/z* 661 [(M + 1)$^+$]. HRMS (ESI) calculated for

$C_{34}H_{38}N_6O_7F$: 661.2781. Observed *m/z* 661.278601. Anal. Calcd. for

$C_{34}H_{37}N_6O_7F \cdot 0.35H_2O$: C, 61.22; H, 5.70; N, 12.60. Found: C, 61.16; H, 5.71; N, 12.60.

trans-3-(Bis-(2-furylmethyl)carbamoyl)acrylic Acid Ethyl Ester

(EtOOCCH=CHCON(CH$_2$-2-furyl)$_2$) was obtained by mixed anhydride coupling of

equimolar amounts of monoethyl fumarate and bis-2-furanmethyl amine to give a brown

syrup (83% yield). ^{1}H-NMR (CDCl$_3$): 1.26 (t, 3H, *CH$_3$*CH$_2$), 2.94 (d, 1H, *J* = 3.2 Hz,

furyl), 3.47 (t, 1H, furyl), 3.64 (d, 1H, N-CH$_2$), 3.97 (d, 1H, N-CH$_2$), 4.12 (q, 2H,

CH$_3$*CH$_2$*), 4.36 (d, 1H, N-CH$_2$), 4.71 (d, 1H, N-CH$_2$), 5.25 (d, 1H, furyl), 6.26-6.33 (m,

3H, furyl), 6.51-6.53 (d, 1H, *J* = 15.2 Hz, *CH*=CHCON), 7.37 (s, 1H, CH=*CH*CON).

trans-3-(Bis-(2-furylmethyl)carbamoyl)acrylic Acid (13p,

HOOCCH=CHCON(CH$_2$-2-furyl)$_2$). EtOOCCH=CHCON(CH$_2$-2-furyl)$_2$ was

hydrolyzed in MeOH using NaOH (1M aqueous, 1.1 eq) under standard deblocking

conditions to give a brown solid (91% yield). ^{1}H-NMR (DMSO-d$_6$): 2.73 (d, 1H, *J* = 3.6

Hz, furyl), 3.13 (t, 1H, furyl), 3.49 (d, 1H, N-CH$_2$), 4.03 (d, 1H, N-CH$_2$), 4.35-4.49 (dd,

2H, N-CH$_2$), 5.17 (d, 1H, furyl), 6.30-6.40 (m, 3H, furyl), 6.63-6.64 (d, 1H, *J* = 15.2 Hz,

CH=CHCON), 7.60 (s, 1H, CH=*CH*CON).

N^2-(N-Benzyloxycarbonylalanylalanyl)-N^1-carbamoylmethyl-N^1-_trans_-(3-bis-

(2-furylmethyl)carbamoylacryloyl)hydrazine (14p, Z-Ala-Ala-AAsn-

CH=CHCON(CH$_2$-2-furyl)$_2$). This compound was obtained using the HOBt/EDC

coupling method. The product was isolated by chromatography with 10% MeOH/CH$_2$Cl$_2$

as the eluent and recrystallized from hexane/EtOAc to give a white powder (34% yield).

^{1}H-NMR (DMSO-d$_6$): 1.18-1.27 (m, 6H, 2 x Ala-CH$_3$), 2.92 (d, 1H, furyl), 3.28-3.38 (m,

4H, N-CH$_2$ and NCH$_2$CO and furyl), 3.47-3.50 (d, 1H, N-CH$_2$), 3.99-4.07 (m, 2H, α-H

and N-CH$_2$), 4.30-4.49 (m, 2H, α-H and N-CH$_2$), 4.99 (m, 2H, Cbz), 6.02 (s, 1H, NH), 6.11 (s, 1H, NH), 6.31(s, 1H, *CH*=CHCON), 6.39 (s, 1H, CH=*CH*CON), 6.55 (t, 1H, furyl), 7.12 (s, 1H, NH), 7.32 (m, 5H, Ph), 7.41-7.59 (m, 3H, furyl), 8.21 (d, 1H, NH), 10.58 (s, 1H, NH). MS (ESI) *m/z* 623 [(M + 1)$^+$]. HRMS (ESI) calculated for C$_{30}$H$_{35}$N$_6$O$_9$: 623.2486. Observed *m/z* 623.2466. Anal. Calcd. for C$_{30}$H$_{34}$N$_6$O$_9$·1H$_2$O: C, 56.24; H, 5.66; N, 13.12. Found: C, 56.38; H, 5.58; N, 13.15.

trans-3-(Benzyl-2-naphthylmethylcarbamoyl)acrylic Acid Ethyl Ester (EtOOCCH=CHCON(Bzl)-2-CH$_2$-Napth) was obtained by mixed anhydride coupling of equimolar amounts of monoethyl fumarate and benzyl-2-naphthylmethylamine to give a clear colorless syrup (87% yield). ^{1}H-NMR (CDCl$_3$): 1.31 (t, 3H, *CH$_3$*CH$_2$), 4.23 (q, 2H, CH$_3$*CH$_2$*), 4.55-4.69 (d, 2H, N-CH$_2$), 4.71-4.81 (d, 2H, N-CH$_2$), 6.95-6.91 (d, 1H, *J* = 14.4 Hz, *CH*=CHCON), 7.16-7.18 (d, 1H, CH=*CH*CON), 7.23-7.57 (m, 10H, naphthyl and Ph), 7.85 (m, 2H, naphthyl).

trans-3-(Benzyl-2-naphthylmethylcarbamoyl)acrylic Acid (13q, HOOCCH=CHCON(Bzl)-2-CH$_2$-Napth). EtOOCCH=CHCON(Bzl)-2-CH$_2$-Napth was hydrolyzed in MeOH using NaOH (1M aqueous, 1.1 eq) under standard deblocking conditions to give a white solid after recrystallization from cold EtOAc (13% yield). ^{1}H-NMR (DMSO-d$_6$): 4.82 (d, 2H, N-CH$_2$), 5.01 (d, 2H, N-CH$_2$), 6.61-6.65 (d, 1H, *J* = 15.2 Hz, *CH*=CHCON), 7.17-7.21 (d, 1H, CH=*CH*CON), 7.37-7.60 (m, 9H, naphthyl and Ph), 7.85-8.01 (m, 3H, naphthyl).

N^2-(N-Benzyloxycarbonylalanylalanyl)-N^1-_trans_-(3-benzyl-2-naphthylmethylcarbamoylacryloyl)-N^1-carbamoylmethylhydrazine (14q, Z-Ala-Ala-AAsn-CH=CHCON(Bzl)-2-CH$_2$-Napth). This compound was obtained using the

HOBt/EDC coupling method and purified by column chromatography on silica gel using 10% MeOH/CH_2Cl_2 as the eluent and then recrystallized from EtOAc/hexane to give a yellow powder (11% yield). ^{1}H-NMR (DMSO-d_6): 1.18-1.28 (m, 6H, 2 x Ala-CH_3), 3.29-3.32 (d, 2H, NCH_2CO), 4.04-4.08 (m, 1H, α-H), 4.28-4.31 (m, 1H, α-H), 4.63-4.69 (d, 2H, N-CH_2), 4.73-4.80 (d, 2H, N-CH_2), 4.99 (m, 2H, Cbz), 7.14-7.48 (m, 18H, naphthyl and 2 x Ph and CH=CHCON and CH=CHCON and 2 x NH), 7.84-7.90 (m, 3H, naphthyl), 8.15 (d, 1H, NH), 10.72 (s, 1H, NH). MS (ESI) m/z 693 [(M + 1)$^+$]. HRMS (ESI) calculated for $C_{38}H_{41}N_6O_7$: 693.2998. Observed m/z 693.303673. Anal. Calcd. for $C_{38}H_{40}N_6O_7 \cdot 0.63H_2O$: C, 64.82; H, 5.91; N, 11.94. Found: C, 64.83; H, 6.02; N, 11.83.

trans-3-(Benzyl-1-naphthylmethylcarbamoyl)acrylic Acid Ethyl Ester (EtOOCCH=CHCON(Bzl)-1-CH$_2$-Napth) was obtained by mixed anhydride coupling of equimolar amounts of monoethyl fumarate and benzyl-1-naphthylmethylamine to give a yellow oil (79% yield). ^{1}H-NMR (CDCl$_3$): 1.31 (t, 3H, CH_3CH$_2$), 4.23 (q, 2H, CH$_3$$CH_2$), 4.55-4.69 (d, 2H, N-$CH_2$), 4.71-4.81 (d, 2H, N-$CH_2$), 6.95-6.91 (d, 1H, J = 14.4 Hz, CH=CHCON), 7.16-7.18 (d, 1H, CH=CHCON), 7.23-7.57 (m, 10H, naphthyl and Ph), 7.85 (m, 2H, naphthyl).

trans-3-(Benzyl-1-naphthylmethylcarbamoyl)acrylic Acid (13r, HOOCCH=CHCON(Bzl)-1-CH$_2$-Napth). EtOOCCH=CHCON(Bzl)-1-CH$_2$-Napth was hydrolyzed in MeOH using NaOH (1M aqueous, 1.1 eq) under standard deblocking conditions to give a white solid after recrystallization from cold EtOAc (62% yield). ^{1}H-NMR (DMSO-d_6): 4.82 (d, 2H, N-CH_2), 5.01 (d, 2H, N-CH_2), 6.61-6.65 (d, 1H, J = 15.2 Hz, CH=CHCON), 7.17-7.21 (d, 1H, CH=CHCON), 7.37-7.60 (m, 9H, naphthyl and Ph), 7.85-8.01 (m, 3H, naphthyl).

N²-(N-Benzyloxycarbonylalanylalanyl)-N¹-*trans*-(3-benzyl-1-naphthylmethylcarbamoylacryloyl)-N¹-carbamoylmethylhydrazine (14r, Z-Ala-Ala-AAsn-CH=CHCON(Bzl)-1-CH₂-Napth). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography on silica gel using 10% MeOH/CH₂Cl₂ as the eluent and then recrystallized from EtOAc/hexane to give a white powder (17% yield). ^{1}H-NMR (DMSO-d₆): 1.19-1.29 (m, 6H, 2 x Ala-CH₃), 3.30-3.32 (d, 2H, NCH₂CO), 4.04-4.08 (m, 1H, α-H), 4.29-4.33 (m, 1H, α-H), 4.64-4.67 (d, 2H, N-CH₂), 4.99 (m, 2H, Cbz), 5.06-5.17 (d, 2H, N-CH₂), 7.14-7.48 (m, 18H, naphthyl and 2 x Ph and CH=*CH*CON and *CH*=CHCON and 2 x NH), 7.84-7.90 (m, 3H, naphthyl), 8.15 (d, 1H, NH), 10.72 (s, 1H, NH). MS (ESI) m/z 693 [(M + 1)⁺]. Anal. Calcd. for C₃₈H₄₀N₆O₇·0.2H₂O: C, 65.54; H, 5.85; N, 12.07. Found: C, 65.54; H, 5.93; N, 11.81.

***trans*-3-(3,4-Dihydro-2H-quinolin-1-yloxo)acrylic Acid Ethyl Ester (EtOOCCH=CHCO-tetrahydroquinoline)** was obtained by mixed anhydride coupling of equimolar amounts of monoethyl fumarate and 1,2,3,4-tetrahydroquinoline to give a brown syrup (83% yield). ^{1}H-NMR (CDCl₃): 1.29 (t, 3H, *CH₃*CH₂OC), 1.99-2.02 (m, 2H, N-CH₂-*CH₂*-CH₂), 2.73-2.76 (t, 1H, N-CH₂-CH₂-*CH₂*), 3.86-3.98 (t, 1H, N-*CH₂*-CH₂-CH₂), 4.24-4.30 (q, 2H, CH₃*CH₂*OOC), 6.78-6.82 (dd, 1H, $J = 14.8$ Hz, *CH*=CHCON), 7.18-7.22 (m, 4H, quinoline), 7.44-7.48 (d, 1H, $J = 14.8$ Hz, CH=*CH*CON).

***trans*-3-(3,4-Dihydro-2H-quinolin-1-yloxo)acrylic Acid (13s, HOOCCH=CHCO-tetrahydroquinoline).** EtOOCCH=CHCO-tetrahydroquinoline was hydrolyzed in MeOH using NaOH (1M aqueous, 1.1 eq) under standard deblocking

conditions to give a clear syrup, which was recrystallized using cold EtOAc to give a yellow powder (68% yield).

N^2-(N-Benzyloxycarbonylalanylalanyl)-N^1-carbamoylmethyl-N^1-*trans*-(3-(3,4-dihydro-2H-quinolin-1-yloxo)acryloyl)hydrazine (14s, Z-Ala-Ala-AAsn-CH=CHCO-tetrahydroquinoline). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography on silica gel using 10% MeOH/CH_2Cl_2 as the eluent and then recrystallized from EtOAc/hexane to give a yellow powder (28% yield). ^{1}H-NMR (DMSO-d_6): 1.18-1.27 (m, 6H, 2 x Ala-CH_3), 1.87 (m, 2H, N-CH_2-CH_2-CH_2), 2.70 (t, 2H, N-CH_2-CH_2-CH_2), 3.29-3.32 (d, 2H, NCH_2CO), 3.73 (m, 2H, N-CH_2-CH_2), 4.02-4.06 (m, 1H, α-H), 4.31 (m, 1H, α-H), 4.99 (m, 2H, Cbz), 7.02-7.07 (dd, 2H, J = 14.8 Hz, CH=CHCON), 7.15-7.41 (m, 11H, quinoline and Ph and NH and CH=CHCON), 7.49 (s, 1H, NH), 8.16 (d, 1H, NH), 10.73 (s, 1H, NH). MS (ESI) *m/z* 579 [(M + 1)$^+$]. HRMS (ESI) calculated for $C_{29}H_{35}N_6O_7$: 579.2525. Observed *m/z* 579.2567. Anal. Calcd. for $C_{29}H_{34}N_6O_7·0.7H_2O$: C, 58.91; H, 6.03; N, 14.21. Found: C, 58.87; H, 6.00; N, 14.24.

trans-3-(3,4-Dihydro-2H-quinolin-1-ylcarbonyl)acrylic Acid Ethyl Ester (EtOOCCH=CHCO-tetrahydroisoquinoline) was obtained by mixed anhydride coupling of equimolar amounts of monoethyl fumarate and 1,2,3,4-tetrahydroquinoline to give a brown syrup (83% yield). ^{1}H-NMR (CDCl$_3$): 1.29 (t, 3H, CH_3CH$_2$OC), 1.99-2.02 (m, 2H, N-CH$_2$-CH_2-CH$_2$), 2.73-2.76 (t, 1H, N-CH$_2$-CH$_2$-CH_2), 3.86-3.98 (t, 1H, N-CH_2-CH$_2$-CH$_2$), 4.24-4.30 (q, 2H, CH$_3$$CH_2$OOC), 6.78-6.82 (dd, 1H, J = 14.8 Hz, CH=CHCON), 7.18-7.22 (m, 4H, quinoline), 7.44-7.48 (d, 1H, J = 14.8 Hz, CH=CHCON).

trans-3-(3,4-Dihydro-2H-quinolin-1-ylcarbonyl)acrylic Acid (13t,

HOOCCH=CHCO-tetrahydroisoquinoline). EtOOCCH=CHCO-tetrahydroquinoline

was hydrolyzed in MeOH using NaOH (1M aqueous, 1.1 eq) under standard deblocking

conditions to give a clear syrup, which was recrystallized using cold EtOAc to give a

yellow powder (68% yield).

N^2-(N-Benzyloxycarbonylalanylalanyl)-N^1-carbamoylmethyl-N^1-trans-(3-(3,4-

dihydro-2H-quinolin-1-yloxo)acryloyl)hydrazine (14t, Z-Ala-Ala-AAsn-CH=CHCO-

tetrahydroisoquinoline). This compound was obtained using the HOBt/EDC coupling

method and purified by column chromatography on silica gel using 10% MeOH/CH$_2$Cl$_2$

as the eluent and then recrystallized from EtOAc/hexane to give a yellow powder (28%

yield). ^{1}H-NMR (DMSO-d$_6$): 1.18-1.27 (m, 6H, 2 x Ala-CH$_3$), 1.87 (m, 2H, N-CH$_2$-*CH$_2$*-

CH$_2$), 2.70 (t, 2H, N-CH$_2$-CH$_2$-*CH$_2$*), 3.29-3.32 (d, 2H, NCH$_2$CO), 3.73 (m, 2H, N-*CH$_2$*-

CH$_2$), 4.02-4.06 (m, 1H, α-H), 4.31 (m, 1H, α-H), 4.99 (m, 2H, Cbz), 7.02-7.07 (dd, 2H,

J = 14.8 Hz, *CH*=CHCON), 7.15-7.41 (m, 11H, quinoline and Ph and NH and

CH=*CH*CON), 7.49 (s, 1H, NH), 8.16 (d, 1H, NH), 10.73 (s, 1H, NH). MS (ESI) *m/z*

579 [(M + 1)$^+$]. HRMS (ESI) calculated for C$_{29}$H$_{35}$N$_6$O$_7$: 579.2525. Observed *m/z*

579.2567. Anal. Calcd. for C$_{29}$H$_{34}$N$_6$O$_7$·0.9H$_2$O·0.1hexane: C, 58.92; H, 6.21; N, 13.92.

Found: C, 58.81; H, 6.04; N, 13.79.

trans-3-(2,3-Dihydroindol-1-ylcarbonyl)acrylic Acid Ethyl Ester (EtOOC-

CH=CHCO-indoline) was obtained by mixed anhydride coupling of equimolar amounts

of monoethyl fumarate and indoline to give a bright yellow solid (84% yield).

trans-3-(2,3-Dihydroindol-1-yloxo)acrylic Acid (13u, HOOC-CH=CHCO-

indoline). EtOOC-CH=CHCO-indoline was hydrolyzed in MeOH using NaOH (1M

aqueous, 1.1 eq) under standard deblocking conditions to give a clear syrup, which was washed several times with CH_2Cl_2 to give a bright yellow solid (40% yield). ^{1}H-NMR (DMSO-d_6): 3.16 (t, 2H, N-CH_2-CH_2), 4.27 (t, 1H, N-CH_2-CH_2), 6.64-6.67 (dd, 1H, J = 15.2 Hz CH=CHCON,), 7.03 (t, 1H, indoline-H), 7.17 (t, 1H, indoline-H), 7.24-7.26 (d, 1H, J = 7.2 Hz, indoline-H), 7.28-7.32 (d, 1H, J = 15.2 Hz, CH=CHCON), 8.11-8.13 (d, 1H, J = 8 Hz, indoline-H).

N^2-(N-Benzyloxycarbonylalanylalanyl)-N^1-carbamoylmethyl-N^1-*trans*-(3-(2,3-dihydroindol-1-ylcarbonyl)acryloyl)hydrazine (14u, Z-Ala-Ala-AAsn-CH=CHCO-indoline). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography on silica gel using 10% MeOH/CH_2Cl_2 as the eluent and then recrystallized from EtOAc/hexane to give a bright yellow, flaky powder (15% yield). ^{1}H-NMR (DMSO-d_6): 1.18-1.19 (d, 3H, Ala-CH_3), 1.27-1.28 (d, 3H, Ala-CH_3), 3.15 (t, 2H, N-$CH_2$$CH_2$), 3.20-3.31 (d, 2H, NCH_2CO), 4.02-4.06 (m, 1H, α-H), 4.06 (t, 2H, N-CH_2-CH_2), 4.31 (m, 1H, α-H), 4.99 (m, 2H, Cbz), 7.02 (t, 1H, indoline-H), 7.13-7.41 (m, 10H, indoline and Ph and NH and CH=CHCON and CH=CHCON), 7.52 (s, 1H, NH), 8.13 (d, 1H, NH), 8.15-8.16 (d, 1H, indoline-H), 10.76 (s, 1H, NH). MS (ESI) *m/z* 565 [$(M + 1)^+$]. HRMS (ESI) calculated for $C_{28}H_{33}N_6O_7$: 565.2416. Observed *m/z* 565.241073. Anal. Calcd. for $C_{28}H_{32}N_6O_7 \cdot 0.4H_2O$: C, 58.82; H, 5.78; N, 14.70. Found: C, 58.87; H, 5.82; N, 14.68.

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

***trans*-3-(1,3-Dihydroisoindol-2-ylcarbonyl)acrylic Acid (13v,**

HOOCCH=CHCO-isoindoline). EtOOCCH=CHCO-isoindoline was then hydrolyzed

in MeOH using NaOH (1M aqueous, 1.1 eq) under standard deblocking conditions to

give a white solid after recrystallization with cold EtOAc (37% yield). ^{1}H-NMR

(DMSO-d_6): 4.73 (s, 2H, NCH$_2$), 5.02 (s, 2H, NCH$_2$), 6.58-6.62 (d, 1H, *J* = 15.2 Hz,

CH=CHCON), 7.25 (m, 5H, isoindoline and CH=*CH*CON).

N^2-(N-Benzyloxycarbonylalanylalanyl)-N^1-carbamoylmethyl-N^1-*trans*-(3-(1,3-

dihydroisoindol-2-ylcarbonyl)acryloyl)hydrazine (14v, Z-Ala-Ala-AAsn-

CH=CHCO-isoindoline). This compound was obtained using the HOBt/EDC coupling

method and purified by column chromatography on silica gel using 10% MeOH/CH$_2$Cl$_2$

as the eluent and then recrystallized from EtOAc/hexane to give a bright yellow, flaky

powder (15% yield). ^{1}H-NMR (DMSO-d_6): 1.18-1.19 (d, 3H, Ala-CH$_3$), 1.27-1.28 (d,

3H, Ala-CH$_3$), 3.20-3.31 (d, 2H, NCH$_2$CO), 4.04-4.07 (m, 1H, α-H), 4.29-4.33 (m, 1H,

α-H), 4.72 (s, 2H, NCH$_2$), 4.99 (m, 2H, Cbz and NCH$_2$), 7.11-7.32 (m, 11H, indoline and

Ph and NH and *CH*=CHCON and CH=*CH*CON), 7.39 (d, 1H, NH), 7.52 (s, 1H, NH),

8.14 (d, 1H, NH), 10.76 (s, 1H, NH). MS (ESI) *m/z* 565 [(M + 1)$^+$]. HRMS (ESI)

calculated for C$_{28}$H$_{33}$N$_6$O$_7$: 565.2414. Observed *m/z* 565.241073. Anal. Calcd. for

C$_{28}$H$_{32}$N$_6$O$_7$·0.3H$_2$O: C, 59.00; H, 5.76; N, 14.74. Found: C, 59.04; H, 5.54; N, 14.56.

***trans*-3-(4-Phenyl-5,6-dihydro-2H-pyridin-1-ylcarbonyl)acrylic Acid**

Ethyl Ester (EtOOCCH=CHCO-(4-Ph-Py)) was obtained by mixed anhydride

coupling of equimolar amounts of monoethyl fumarate and 4-phenyl-1,2,3,6-

tetrahydropyridine hydrochloride to give a pink solid (80% yield). ^{1}H-NMR (CDCl$_3$):

1.32 (t, 3H, CH$_2$*CH$_3$*), 2.64 (s, 2H, pyridyl-CH$_2$), 3.78-3.81 (t, 1H, pyridyl-CH$_2$), 3.90-

3.93 (t, 1H, pyridyl-CH$_2$), 4.24-4.33 (m, 4H, *CH$_2$*CH$_3$ and pyridyl-CH$_2$), 6.02-6.09 (d, 1H, pyridyl-CH=), 6.78-6.82 (d, 1H, *J* = 15.6 Hz, *CH*=CHCON), 6.99-7.49 (m, 6H, Ph and CH=*CH*CON).

trans-3-(4-Phenyl-5,6-dihydro-2H-pyridin-1-ylcarbonyl)acrylic Acid (13w, HOOCCH=CHCO-(4-Ph-Py)). EtOOCCH=CHCO-(4-Ph-Py) was hydrolyzed in MeOH using NaOH (1M aqueous, 1.1 eq) under standard deblocking conditions to give a white solid (59% yield). ^{1}H-NMR (DMSO-d$_6$): 2.57 (s, 2H, pyridyl-CH$_2$), 3.73-3.76 (t, 2H, pyridyl-CH$_2$), 4.17-4.26 (d, 2H, pyridyl-CH$_2$), 6.14-6.17 (d, 1H, pyridyl CH=), 7.23-7.27 (t, 1H, Ph), 7.31-7.35 (t, 2H, Ph), 7.39-7.48 (m, 3H, *CH*=CHCON and Ph).

N^2-(N-Benzyloxycarbonylalanylalanyl)-N^1-carbamoylmethyl -N^1-trans-(3-(4-phenyl-5,6-dihydro-2H-pyridin-1-ylcarbonyl)acryloyl)hydrazine (14w, Z-Ala-Ala-AAsn-CH=CHCO-(4-Ph-Py)). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH$_2$Cl$_2$ as the eluent to give a white solid (11% yield). ^{1}H-NMR (DMSO-d$_6$): 1.15-1.26 (m, 6H, 2 x Ala-CH$_3$), 2.56 (s, 2H, pyridyl-CH$_2$), 3.30-3.32 (d, 2H, NCH$_2$CO), 3.74 (t, 2H, pyridyl-CH$_2$), 4.02-4.07 (m, 1H, α-H), 4.17 (s, 2H, pyridyl-CH$_2$), 4.27-4.35 (m, 1H, α-H), 4.99 (m, 2H, Cbz), 6.16 (s, 1H, pyridyl-CH=), 7.02-7.06 (d, 1H, *J* = 15.2 Hz, *CH*=CHCON), 7.19 (s, 1H, NH), 7.23-7.42 (m, 11H, CH=*CH*CON and 2 x Ph), 7.51 (d, 1H, NH), 8.14-8.15 (d, 1H, NH), 10.72 (s, 1H, NH). MS (ESI) *m/z* 605 [(M + 1)$^+$]. HRMS (ESI) calculated for C$_{31}$H$_{37}$N$_6$O$_7$: 605.2757. Observed *m/z* 605.272373. Anal. Calcd. for C$_{31}$H$_{36}$N$_6$O$_7$·1H$_2$0: C, 59.80; H, 6.15; N, 13.50. Found: C, 59.71; H, 6.02; N, 13.27.

Methyl-(1-methylphenethylcarbamoyl)phenylethylcarbamic Acid *tert*-Butyl Ester (Boc-Phe(Me)-N(Me)(CH$_2$)$_2$Ph) was obtained by mixed anhydride coupling of

equimolar amounts of Boc-Phe(Me)-OH and N-methyl phenethylamine. The product

was purified by column chromatography using hexane/EtOAc (1:1) as the eluent to give a

colorless oil (89% yield). ^{1}H-NMR (CDCl$_3$): 1.14-1.35 (m, 9H, tBu), 2.73-2.98 (m, 10H,

Phe-CH_2 and CH_2Ph and 2 x Me), 3.40-3.51 (m, 2H, N-CH$_2$), 5.20 (m, 1H, α-H), 7.10-

7.33 (m, 10H, 2 x Ph).

N-Methyl-2-methylamino-N-phenethyl-3-phenylpropionamide

Hydrochloride (Phe(Me)-N(Me)(CH$_2$)$_2$Ph). Boc-Phe(Me)-N(Me)(CH$_2$)$_2$Ph was

hydrolyzed under standard deblocking conditions using 12 eq. of HCl in EtOAc (4 N) to

give a white powder (100% yield).

trans-**3-(Methyl-(1-methylphenethylcarbamoyl)phenylethylcarbamoyl)acrylic**

Acid Ethyl Ester (EtOOCCH=CH-Phe(Me)-N(Me)(CH$_2$)$_2$Ph) was obtained by mixed

anhydride coupling of equimolar amounts of monoethyl fumarate and Phe(Me)-

N(Me)(CH$_2$)$_2$Ph to give a clear colorless syrup (52% yield). ^{1}H-NMR (CDCl$_3$): 1.20-

1.25 (t, 3H, CH_3CH$_2$O), 2.73-2.98 (m, 10H, Phe-CH_2 and CH_2Ph and 2 x Me), 3.40-3.51

(m, 2H, N-CH$_2$), 4.19-4.34 (q, 2H, CH$_3$$CH_2$O), 5.20 (m, 1H, α-H), 6.63-5.78 (2 x t, 1H,

CH=CHCON), 6.80-6.99 (m, 1H, CH=CHCON), 7.10-7.33 (m, 10H, 2 x Ph).

trans-**3-(Methyl-(1-methylphenethylcarbamoyl)phenylethylcarbamoyl)acrylic**

Acid (13x, HOOCCH=CH-Phe(Me)-N(Me)(CH$_2$)$_2$Ph). (HOOCCH=CH-Phe(Me)-

N(Me)(CH$_2$)$_2$Ph) was hydrolyzed in MeOH using NaOH (1M aqueous, 1.1 eq) under

standard deblocking conditions to give a clear, colorless syrup (75% yield).

N^2-(N-Benzyloxycarbonylalanylalanyl)-N^1-carbamoylmethyl-N^1-*trans*-(3-

methyl-(1-methylphenethylcarbamoyl)phenylethylcarbamoylacryloyl)hydrazine

(14x, Z-Ala-Ala-AAsn-CH=CHCO-Phe(Me)-N(Me)(CH$_2$)$_2$Ph). This compound was

obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH$_2$Cl$_2$ as the eluent. Recrystallization with hexane/EtOAc gave a yellow powder (11% yield). ^{1}H-NMR (DMSO-d$_6$): 1.19-1.20 (d, 3H, Ala-CH$_3$), 1.26-1.28 (d, 3H, Ala-CH$_3$), 2.61-2.95 (m, 10H, Phe-*CH$_2$* and CH$_2$Ph and 2 x N-Me), 3.32 (s, 2H, NCH$_2$CO), 3.62 (m, 2H, N-CH$_2$), 4.05-4.09 (m, 1H, α-H), 4.28-4.32 (m, 1H, α-H), 4.99 (m, 2H, Cbz), 5.52 (m, 1H, α-H), 6.93-7.32 (m, 18H, *CH*=CHCON and CH=*CH*CON and 3 x Ph and NH), 7.40-7.42 (d, 1H, NH), 7.50 (s, 1H, NH), 8.15-8.16 (d, 1H, NH), 10.71 (s, 1H, NH). MS (ESI) *m/z* 607 [(M – HN(CH$_3$)CH$_2$CH$_2$Ph + 1, 100 %)$^+$]. HRMS (ESI) calculated for C$_{39}$H$_{48}$N$_7$O$_8$: 742.3575. Observed *m/z* 742.3564. Anal. Calcd. for C$_{39}$H$_{47}$N$_7$O$_8$·1.23H$_2$0·0.3hexane: C, 62.09; H, 6.78; N, 12.42. Found: C, 61.99; H, 6.62; N, 12.49.

trans-N^2-(N-Benzyloxycarbonylalanylalanyl)-N^1-carbamoylmethyl-N^1-trans-(3-benzoylacryloyl)hydrazine (15c, Z-Ala-Ala-AAsn-CH=CHCOPh). This compound was obtained using the HOBt/EDC coupling method starting from the peptide precursor Z-Ala-Ala-NHNHCH$_2$CONH$_2$ and commercially available *trans*-3-benzoylacrylic acid. The workup omitted the NaHCO$_3$ washings. The crude product was purified by column chromatography on silica gel using 10% MeOH/CH$_2$Cl$_2$ as the eluent and then washed with EtOAc to give a white powder (39% yield). ^{1}H-NMR (DMSO-d$_6$): 1.18-1.27 (m, 6H, 2 x Ala-CH$_3$), 3.20-3.32 (d, 2H, NCH$_2$CO), 3.98-4.05 (m, 1H, α-H), 4.26-4.29 (m, 1H, α-H), 4.99 (m, 2H, Cbz), 7.14-7.21 (m, 2H, *CH*=CHCOPh and NH), 7.32-7.35 (m, 5H, Ph), 7.54 (t, 2H, Ph), 7.69 (t, 1H, Ph), 7.76-7.80 (d, 1H, *J* = 15.6 Hz, CH=*CH*COPh), 7.97-7.99 (d, 2H, Ph), 8.14 (d, 1H, NH), 10.77 (s, 1H, NH). MS (ESI) *m/z* 524 [(M +

1)$^+$]. HRMS (ESI) calculated for $C_{26}H_{30}N_5O_7$: 524.2056. Observed *m/z* 524.2145. Anal.

Calcd. for $C_{26}H_{29}N_5O_7$: C, 59.65; H, 5.58; N, 13.38. Found: C, 59.42; H, 5.50; N, 13.16.

N^2-(N-Benzyloxycarbonylalanylalanyl)-N^1-carbamoylmethyl-N^1-*trans-trans*-hexa-2,4-dienoylhydrazine (15d, Z-Ala-Ala-AAsn-CH=CHCH=CHCH$_3$). This

compound was synthesized by coupling the peptide precursor Z-Ala-Ala-

NHNHCH$_2$CONH$_2$ and commercially available 2,4-hexadienoic acid using HOBt/EDC

and was purified by column chromatography on silica gel using 10% MeOH/CH$_2$Cl$_2$ as

the eluent and then recrystallized from CH$_2$Cl$_2$/ hexane to give a white powder (14%

yield). ^{1}H-NMR (DMSO-d$_6$): 1.18-1.24 (m, 6H, 2 x Ala-CH$_3$), 1.79 (d, 3H, *CH$_3$*-

CH=CH-), 3.30-3.32 (d, 2H, NCH$_2$CO), 4.06 (m, 1H, α-H), 4.25 (m, 1H, α-H), 4.98 (m,

2H, Cbz), 6.19 (m, 3H, CH$_3$-*CH=CH-CH*), 7.21 (m, 2H, NH and CH=*CH*-CO), 7.33 (m,

5H, Ph), 7.5 (d, 1H, NH), 8.19 (d, 1H, NH), 10.53 (s, 1H, NH). MS (ESI) m/z 460 [(M +

1)$^+$]. HRMS (ESI) calculated for $C_{22}H_{30}N_5O_6$: 460.2157. Observed m/z 460.219609.

Anal. Calcd. for $C_{22}H_{29}N_5O_6$·0.3EtOAc: C, 57.39; H, 6.44; N, 14.42. Found: C, 57.14;

H, 6.72; N, 14.44.

N^2-(N-Benzyloxycarbonylalanylalanyl)-N^1-carbamoylmethyl-N^1-*trans*-(3-(2-furyl)acryloyl)hydrazine (15e, Z-Ala-Ala-AAsn-CH=CH-2-furyl). This compound

was obtained using the HOBt/EDC coupling method starting with the peptide precursor

Cbz-Ala-Ala-NHNHCH$_2$CONH$_2$ and commercially available 2-furylacrylic acid and

purified by column chromatography on silica gel using 10% MeOH/CH$_2$Cl$_2$ as the eluent.

Recrystallization from hexane/EtOAc gave a white powder (23% yield). ^{1}H-NMR

((CD$_3$)CO): 1.37 (d, 3H, Ala-CH$_3$), 1.44 (d, 3H, Ala-CH$_3$), 2.88 (s, 2H, NCH$_2$CO), 4.24

(m, 1H, α-H), 4.52 (m, 1H, α-H), 5.10 (m, 2H, Cbz), 6.41 (m, 1H, furyl-H), 6.55 (m, 1H,

furyl-H), 6.68 (m, 1H, *CH*=CHCON), 6.88 (s, 2H, NH$_2$), 7.31-7.36 (m, 5H, Ph), 7.39-

7.43 (d, 1H, *J* = 15.6 Hz, CH=*CH*CON), 7.48 (s, 1H, NH), 7.65 (s, 1H, furyl-H), 7.81 (s,

1H, NH), 9.83 (s, 1H, NH). MS (ESI) *m/z* 486 [(M + 1)$^+$]. HRMS (ESI) calculated for

C$_{23}$H$_{28}$N$_5$O$_7$: 486.1957. Observed *m/z* 486.198874. Anal. Calcd. for

C$_{23}$H$_{27}$N$_5$O$_7$·0.3H$_2$O·0.1hexane: C, 56.14; H, 5.91; N, 13.87. Found: C, 56.09; H, 5.85;

N, 13.78.

N^2-(N-Benzyloxycarbonylalanylalanyl)-N^1-carbamoylmethyl-N^1-*trans*-(3-(3-pyridyl)acryloyl)hydrazine (15f, Z-Ala-Ala-AAsn-CH=CH-3-Py). This compound

was obtained using the HOBt/EDC coupling method starting from the peptide precursor

Z-Ala-Ala-NHNHCH$_2$CONH$_2$ and commercially available 3-pyridyl acrylic acid. Upon

completion of the reaction sat. NaHCO$_3$ was added, and the volatiles were evaporated.

Without further workup, the crude product was chromatographed on silica gel using 10%

to 20% MeOH/CH$_2$Cl$_2$ as the eluent and recrystallized from hexane/EtOAc to give a

white powder (7% yield). ^{1}H-NMR ((CD$_3$)CO): 1.37 (d, 3H, Ala-CH$_3$), 1.44 (d, 3H, Ala-

CH$_3$), 2.91 (s, 2H, NCH$_2$CO), 4.24 (m, 1H, α-H), 4.42 (m, 1H, α-H), 5.10 (m, 2H, Cbz),

6.53 (m, 1H, pyridine-H), 6.68 (d, 1H, *CH*=CHCON), 7.31-7.36 (m, 5H, Ph), 7.59-7.64

(m, 1H, NH and CH=*CH*CON), 7.98 (s, 1H, pyridine-H), 8.32 (s, 1H, NH), 7.53 (s, 1H,

pyridine-H), 8.90 (s, 1H, pyridine-H), 10.08 (s, 1H, NH). MS (ESI) *m/z* 497 [(M + 1)$^+$].

HRMS (ESI) calculated for C$_{24}$H$_{29}$N$_6$O$_6$: 497.2078. Observed *m/z* 497.2149. Anal.

Calcd. for C$_{24}$H$_{28}$N$_6$O$_6$·1H$_2$O: C, 56.02; H, 5.88; N, 16.33. Found: C, 56.21; H, 5.77; N,

16.93.

N-*tert*-Butyloxycarbonylalanylalanyl Hydrazide (Boc-Ala-Ala-NHNH$_2$) was

synthesized from Boc-Ala-Ala-OMe by hydrazinolysis. Anhydrous hydrazine (10 eq)

was added to a solution of Boc-Ala-Ala-OMe (1 eq) in MeOH at room temperature, and

the resulting mixture was then stirred at room temperature for 16 hours. Excess

hydrazine and solvent were removed by evaporation. The resulting residue was washed

with ethanol and ether to give Boc-Ala-Ala-NHNH$_2$ as a white solid (95% yield). ^{1}H

NMR (DMSO-d$_6$): 1.1-1.3 (d, 6H, CH$_3$), 1.36 (s, 9H, Boc), 4.0-4.1 (m, 1H, α-H), 4.1-4.3

(m, 2H, α-H and NH), 7.5 (d, 1H, NH), 7.9 (d, 1H, NH), 9.05 (s, 1H, NH).

N^1-(N-*tert*-Butyloxycarbonylalanylalanyl)-N^2-

ethoxycarbonylmethylhydrazine (Boc-Ala-Ala-NHNHCH$_2$COOEt). Ethyl

bromoacetate (1.1 eq) was added dropwise to a stirred solution of Boc-Ala-Ala-NHNH$_2$

(1 eq) and NMM (1.1 eq) in DMF that was cooled to -10 °C. The resulting solution was

stirred for 30 min at -10 °C, after which the mixture was allowed to react at room

temperature for 36 hours. The DMF was evaporated, and the residue was purified on a

silica gel column using 1:9 to 2:8 MeOH:CH$_2$Cl$_2$ as the eluting solvent system to give the

ethyl ester as a white solid (yield 34%). ^{1}H NMR (DMSO-d$_6$): 1.18 (t, 9H, CH$_3$), 1.41 (s,

9H, Boc), 3.5 (d, 2H, N*CH$_2$*COOEt), 4.0-4.15 (m, 3H, α-H and O*CH$_2$*CH$_3$), 4.2 (m, 1H,

α-H), 5.18 (m, 1H, NH), 7.22-7.40 (m, 5H, Ph), 7.4-7.5 (d, 1H, NH), 7.9 (m, 1H, NH),

9.35 (m, 1H, NH).

N^1-(N-*tert*-Butyloxycarbonylalanylalanyl)-N^2-carbamoylmethylhydrazine

(Boc-Ala-Ala-NHNHCH$_2$CONH$_2$). The ethyl ester Boc-Ala-Ala-NHNHCH$_2$COOEt (1

eq) was dissolved in a 9 M solution (100 eq) of NH$_3$ in methanol and a small amount of

DMF, and allowed to stir on an ice bath. To this solution was added catalytic NaCN (0.1

eq). The flask was closed with a rubber septum and allowed to stir at 0 °C for 5 days.

The solvent was evaporated and, the crude product was purified by column

chromatography (1:9 MeOH:CH$_2$Cl$_2$) to yield a white solid (53% yield). ^{1}H NMR

(DMSO-d$_6$): 1.15 (2d, 6H, CH$_3$), 1.36 (s, 9H, Boc), 3.3 (d, 2H, NCH$_2$CONH$_2$), 3.9-4.0

(m, 1H, α-H), 4.1-4.2 (m, 1H, α-H), 5.22 (m, 1H, NH), 6.93 (d, 1H, NH), 7.1 (s, 1H,

NH), 7.4 (s, 1H, NH), 7.85 (d, 1H, NH), 9.3 (s, 1H, NH).

N^2-(N-*tert*-Butyloxycarbonylalanylalanyl)-N^1-carbamoylmethyl-N^1-*trans*-(3-
ethylesteracryloyl)hydrazine (19, Boc-Ala-Ala-AAsn-CH=CHCOOEt) was

synthesized using the EDC/HOBt coupling method, purified by chromatography on a

silica gel column using 1:9 MeOH:CH$_2$Cl$_2$ as the eluent, and then recrystallized from

EtOAc/hexane to give a white solid, yield 17%. ^{1}H NMR (DMSO-d$_6$): 1.14-1.16 (d, 3H,

Ala-CH$_3$), 1.21-1.24 (t, 3H, OCH$_2$CH$_3$), 1.24-1.26 (d, 3H, Ala-CH$_3$), 1.36 (s, 9H, Boc),

3.33 (m, 2H, NCH$_2$CO), 3.96 (m, 1H, α-H), 4.07-4.11 (q, 2H, OCH$_2$CH$_3$), 4.26-4.29 (m,

1H, α-H), 6.56-6.60 (d, 1H, J = 15.6 Hz, CH=CH,), 7.16-7.20 (m, 2H, NH and CH=CH),

7.51 (s, 1H, NH), 8.03-8.04 (d, 1H, NH), 10.78 (s, 1H, NH).

N^2-(Alanylalanyl)-N^1-carbamoylmethyl-N^1-*trans*-(3-
ethylesteracryloyl)hydrazine (20, TFA·Ala-Ala-AAsn-CH=CHCOOEt). Boc-Ala-

Ala-AAsn-CH=CHCOOEt was deblocked with trifluoroacetic acid/methylene chloride

(1:5) for 3 hours at room temperature. The volatiles were evaporated, and the crude TFA

salt was washed several times with to give a white solid, yield 99%. ^{1}H NMR (DMSO-

d$_6$): 1.14-1.16 (d, 3H, Ala-CH$_3$), 1.21-1.24 (t, 3H, OCH$_2$CH$_3$), 1.24-1.26 (d, 3H, Ala-

CH$_3$), 3.41 (m, 2H, NCH$_2$CO), 3.83 (m, 1H, α-H), 4.14-4.19 (q, 2H, OCH$_2$CH$_3$), 4.38-

4.39 (m, 1H, α-H), 6.63-6.67 (d, 1H, J = 15.6 Hz, CH=CH,), 7.16-7.23 (m, 2H, NH and

CH=CH), 7.54 (s, 1H, NH), 8.07 (s, 2H, 2 x NH), 10.97 (s, 1H, NH).

N^2-(N-Biotinylalanylalanyl)-N^1-carbamoylmethyl-N^1-*trans*-(3-ethylesteracryloyl)hydrazine (21, Biotin-Ala-Ala-AAsn-CH=CHCOOEt). To a solution of TFA·Ala-Ala-AAsn-CH=CHCOOEt (1 eq) in DMF triethylamine (1 eq) and biotin N-hydroxysuccinimide ester (1.5 eq) in DMF were added. The reaction was stirred over night at room temperature. The solvent was evaporated, and the crude residue was washed several times with cold methanol to give biotin-Ala-Ala-AAsn-CH=CHCOOEt as a white solid, yield 31%. ^{1}H NMR (DMSO-d$_6$): 1.14-1.16 (d, 3H, Ala-CH$_3$), 1.19-1.23 (t, 3H, OCH$_2$*CH$_3$*), 1.23-1.24 (d, 3H, Ala-CH$_3$), 1.27-1.28 (m, 2H, biotin-CH$_2$), 1.45-1.64 (m, 4H, biotin-CH$_2$CH$_2$), 2.06-2.10 (t, 2H, biotin-CH$_2$CO), 2.54-2.57 (d, 1H, CHS), 2.78-2.82 (dd, 1H, CH$_2$S, J = 5.2 and 12.4 Hz), 3.05-3.10 (m, 1H, CH$_2$S), 3.31 (s, 2H, N*CH$_2$*CO), 4.08-4.13 (m, 2H, biotin-CH and α-H), 4.13-4.19 (q, 2H, O*CH$_2$*CH$_3$), 4.23-4.30 (m, 1H, biotin-CH and α-H), 6.35 (s, 1H, biotin-NH), 6.41 (s, 1H, biotin-NH), 6.57-6.61 (d, 1H, *J* = 15.2 Hz, CH=CH,), 7.16-7.23 (m, 2H, NH and CH=CH), 7.51 (s, 1H, NH), 7.92 (d, 1H, NH), 8.15-8.16 (d, 1H, NH), 10.76 (s, 1H, NH). MS (ESI) *m/z* 584 [(M + 1)$^+$]. HRMS (ESI) calculated for C$_{24}$H$_{38}$N$_7$O$_8$S : 584.2447. Observed *m/z* 584.24971. Anal. Calcd. for C$_{24}$H$_{37}$N$_7$O$_8$S·1H$_2$O: C, 47.91; H, 6.53; N, 16.30. Found: C, 47.77; H, 6.36; N, 16.16.

Biotinyl-N-hydroxy Succinimide Ester (17, Biotin-OSu) was prepared by dissolving biotin (1 eq) in DMF at 80 ºC. Upon cooling to room temperature N-hydroxysuccinimde (1.3 eq) was added together with DCC (1 eq). The reaction was stirred for 12 hours at room temperature. Dicyclohexylurea was filtered off, and the solution was evaporated to dryness. The residue was taken up in boiling isopropanol, and the resulting suspension was cooled to room temperature. The solid was filtered and

characterized; yield 72 %. ^{1}H NMR (DMSO-d$_6$): 1.31-1.55 (m, 4H, biotin-CH$_2$CH$_2$), 1.55-1.69 (m, 2H, biotin-CH$_2$), 2.54-2.57 (d, 1H, CHS), 2.63-2.70 (t, 2H, biotin-CH$_2$CO), 2.78-2.90 (m, 7H, CH$_2$S and Su-CH$_2$CH$_2$, and CHS), 4.1-4.2(m, 1H, biotin-CH), 4.25-4.31 (m, 1H, biotin-CH), 6.35 (s, 1H, biotin-NH), 6.41 (s, 1H, biotin-NH).

REFERENCES

(1)   King, C. H.; Muchiri, E. M.; Ouma, J. H. *Emerg. Infect. Dis.* **2000**, *6*, 585-94.

(2)   Ismail, M.; Botros, S.; Metwally, A.; William, S.; Farghally, A.; Tao, L. F.; Day, T. A.; Bennett, J. L. *Am. J. Trop. Med. Hyg.* **1999**, *60*, 932-5.

(3)   Ismail, M. M.; Farghaly, A. M.; Dyab, A. K.; Afify, H. A.; el-Shafei, M. A. *J. Egypt. Soc. Parasitol.* **2002**, *32*, 589-600.

(4)   Ismail, M. M.; Taha, S. A.; Farghaly, A. M.; el-Azony, A. S. *J. Egypt. Soc. Parasitol.* **1994**, *24*, 685-95.

(5)   Liang, Y. S.; Coles, G. C.; Doenhoff, M. J. *Trop. Med. Int. Health.* **2000**, *5*, 72.

(6)   Lawrence, J. D. *J. Parasitol.* **1973**, *59*, 60-3.

(7)   Timms, A. R.; Bueding, E. *Br. J. Pharmacol.* **1959**, *14*, 68-73.

(8)   Bogitsh, B. J. *Exp. Parasitol.* **1978**, *45*, 247-54.

(9)   Kasschau, M. R.; Robinson, D. C.; Dreden, M. H. *Exp. Parasitol.* **1986**, *62*, 442-9.

(10)   Dalton, J. P.; Hola-Jamriska, L.; Brindley, P. J. *Parasitology* **1995**, *111 (Pt 5)*, 575-80.

(11)   Klinkert, M. Q.; Ruppel, A.; Beck, E. *Mol. Biochem. Parasitol.* **1987**, *25*, 247-55.

(12)   Takeda, O.; Miura, Y.; Mitta, M.; Matsushita, H.; Kato, I.; Abe, Y.; Yokosawa, H.; Ishii, S. *J. Biochem. (Tokyo)* **1994**, *116*, 541-6.

(13)   Ruppel, A.; Diesfeld, H. J.; Rother, U. *Clin. Exp. Immunol.* **1985**, *62*, 499-506.

(14)   Ruppel, A.; Shi, Y. E.; Wei, D. X.; Diesfeld, H. J. *Clin. Exp. Immunol.* **1987**, *69*, 291-8.

(15)   Klinkert, M. Q.; Felleisen, R.; Link, G.; Ruppel, A.; Beck, E. *Mol. Biochem. Parasitol.* **1989**, *33*, 113-22.

(16)   Smith, A. M.; Dalton, J. P.; Clough, K. A.; Kilbane, C. L.; Harrop, S. A.; Hole, N.; Brindley, P. J. *Mol. Biochem. Parasitol.* **1994**, *67*, 11-9.

(17)   Wong, J. Y.; Harrop, S. A.; Day, S. R.; Brindley, P. J. *Biochim. Biophys. Acta* **1997**, *1338*, 156-60.

(18)   Butler, R.; Michel, A.; Kunz, W.; Klinkert, M. Q. *Prot. Pept. Letts.* **1995**, *2*, 313-320.

(19)   Dalton, J. P.; Brindley, P. J. In *Handbook of Proteolytic Enzymes*; Barrett, A. J., Rawlings, N. D., Woessner, J. F., Eds.; Academic Press: London, 1998, p 749 754.

(20)   http://www.dpd.cdc.gov/dpdx/HTML/Schistosomiasis.htm.

(21)   Chen, J. M.; Rawlings, N. D.; Stevens, R. A.; Barrett, A. J. *FEBS Lett.* **1998**, *441*, 361-5.

(22)   Kembhavi, A. A.; Buttle, D. J.; Knight, C. G.; Barrett, A. J. *Arch. Biochem. Biophys.* **1993**, *303*, 208-13.

(23)   Hara-Nishimura In *Handbook of Proteolytic Enzymes*; Barrett, A. J., Rawlings, N. D., Woessner, J. F., Eds.; Academic Press: London, 1998, p 746-749.

(24)   Chen, J. M.; Dando, P. M.; Stevens, R. A.; Fortunato, M.; Barrett, A. J. *Biochem. J.* **1998**, *335 (Pt 1)*, 111-7.

(25)   Choi, S. J.; Reddy, S. V.; Devlin, R. D.; Menaa, C.; Chung, H.; Boyce, B. F.; Roodman, G. D. *J. Biol. Chem.* **1999**, *274*, 27747-53.

(26)   Manoury, B.; Hewitt, E. W.; Morrice, N.; Dando, P. M.; Barrett, A. J.; Watts, C. *Nature* **1998**, *396*, 695-9.

(27)   Beck, H.; Schwarz, G.; Schroter, C. J.; Deeg, M.; Baier, D.; Stevanovic, S.; Weber, E.; Driessen, C.; Kalbacher, H. *Eur. J. Immunol.* **2001**, *31*, 3726-36.

(28)   Sajid, M.; McKerrow, J. H. *Mol. Biochem. Parasitol.* **2002**, *120*, 1-21.

(29)   Dalton, J. P.; Brindley, P. J. *Parasitology Today* **1996**, *12*, 125.

(30)   Mathieu, M. A.; Bogyo, M.; Caffrey, C. R.; Choe, Y.; Lee, J.; Chapman, H.; Sajid, M.; Craik, C. S.; McKerrow, J. H. *Mol. Biochem. Parasitol.* **2002**, *121*, 99-105.

(31)   Niestroj, A. J.; Feussner, K.; Heiser, U.; Dando, P. M.; Barrett, A.; Gerhartz, B.; Demuth, H. U. *Biol. Chem.* **2002**, *383*, 1205-14.

(32)   Loak, K.; Li, D. N.; Manoury, B.; Billson, J.; Morton, F.; Hewitt, E.; Watts, C. *Biol. Chem.* **2003**, *384*, 1239-46.

(33) Rozman-Pungercar, J.; Kopitar-Jerala, N.; Bogyo, M.; Turk, D.; Vasiljeva, O.; Stefe, I.; Vandenabeele, P.; Bromme, D.; Puizdar, V.; Fonovic, M.; Trstenjak-Prebanda, M.; Dolenc, I.; Turk, V.; Turk, B. *Cell Death Differ.* **2003**, *10*, 881-8.

(34) Asgian, J. L.; James, K. E.; Li, Z. Z.; Carter, W.; Barrett, A. J.; Mikolajczyk, J.; Salvesen, G. S.; Powers, J. C. *J. Med. Chem.* **2002**, *45*, 4958-60.

(35) Powers, J. C.; Asgian, J. L.; Ekici, O. D.; James, K. E. *Chem. Rev.* **2002**, *102*, 4639-750.

(36) Korn, A.; Rudolph-Böhner, S.; Moroder, L. *Tetrahedron* **1994**, *50*, 8381-8392.

(37) Mori, K.; Iwasawa, H. *Tetrahedron* **1980**, *36*, 87-90.

(38) Meth-Cohn, O.; Moore, C.; Taljaard, H. C. *J. Chem. Soc. Perkin Trans. I* **1998**, 2663.

(39) Meara, J. P.; Rich, D. H. *J. Med. Chem.* **1996**, *39*, 3357-66.

(40) Schaschke, N.; Assfalg-Machleidt, I.; Machleidt, W.; Turk, D.; Moroder, L. *Bioorg. & Med. Chem. Lett.* **1997**, *5*, 1789-1797.

(41) Therrien, C.; Lachance, P.; Sulea, T.; Purisima, E. O.; Qi, H.; Ziomek, E.; Alvarez-Hernandez, A.; Roush, W. R.; Menard, R. *Biochemistry* **2001**, *40*, 2702-11.

(42) Chen, J. M.; Dando, P. M.; Rawlings, N. D.; Brown, M. A.; Young, N. E.; Stevens, R. A.; Hewitt, E.; Watts, C.; Barrett, A. J. *J. Biol. Chem.* **1997**, *272*, 8090-8.

(43) Hogberg, T.; Strom, P.; Ebner, M.; Ramsby, S. *Journal of Organic Chemistry* **1987**, 2033-2036.

(44) Barrett, A. J.; Kembhavi, A. A.; Brown, M. A.; Kirschke, H.; Knight, C. G.; Tama, M.; Hanada, K. *Biochem. J.* **1982**, *201*, 189-198.

(45) Hanzlik, R. P.; Thompson, S. A. *J. Med. Chem.* **1984**, *27*, 711-2.

(46) Thompson, S. A.; Andrews, P. R.; Hanzlik, R. P. *J. Med. Chem.* **1986**, *29*, 104-11.

(47) Liu, S.; Hanzlik, R. P. *J. Med. Chem.* **1992**, *35*, 1067-75.

(48) Palmer, J. T.; Rasnik, D.; Klaus, J. L.; Brömme, D. *J. Med. Chem.* **1995**, *38*, 3193-3196.

(49)   Johnson, T. O.; Hua, Y.; Luu, H. T.; Brown, E. L.; Chan, F.; Chu, S. S.; Dragovich, P. S.; Eastman, B. W.; Ferre, R. A.; Fuhrman, S. A.; Hendrickson, T. F.; Maldonado, F. C.; Matthews, D. A.; Meador, J. W., 3rd; Patick, A. K.; Reich, S. H.; Skalitzky, D. J.; Worland, S. T.; Yang, M.; Zalman, L. S. *J. Med. Chem.* **2002**, *45*, 2016-23.

(50)   Dragovich, P. S.; Zhou, R.; Skalitzky, D. J.; Fuhrman, S. A.; Patick, A. K.; Ford, C. E.; Meador, J. W., 3rd; Worland, S. T. *Bioorg. Med. Chem.* **1999**, *7*, 589-98.

(51)   Schaschke, N.; Assfalg-Machleidt, I.; Lassleben, T.; Sommerhoff, C. P.; Moroder, L.; Machleidt, W. *FEBS Lett.* **2000**, *482*, 91-6.

(52)   Schaschke, N.; Dominik, A.; Matschiner, G.; Sommerhoff, C. P. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 985-8.

(53)   Knobloch, W.; Niedrich, H. *Journal für Praktische Chemie* **1962**, *4*, 263-281.

(54)   Niedrich, H. *Chem. Ber.* **1969**, *102*, 1557-69.

(55)   Freidig, A. P.; Verhaar, H. J. M.; Hermens, J. L. M. *Environ. Sci. Technol.* **1999**, *33*, 3038-3043.

(56)   Caffrey, C. R.; Mathieu, M. A.; Gaffney, A. M.; Salter, J. P.; Sajid, M.; Lucas, K. D.; Franklin, C.; Bogyo, M.; McKerrow, J. H. *FEBS Lett.* **2000**, *466*, 244-8.

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