POTENTIAL INHIBITORS OF DENGUE AND WEST NILE VIRUS PROTEASES

A Thesis By

Swathi Mohan

Master of Science University of Madras, 2001

Bachelor of Science Osmania University, 1999

Submitted to the Department of Chemistry and the faculty of the Graduate School of Wichita State University in partial fulfillment of the requirement for the degree of Master of Science

July 2006

POTENTIAL INHIBITORS OF
DENGUE AND WEST NILE VIRUS PROTEASES

I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirement for the degree of Master of Science with a major in Chemistry

_____
William C. Groutas, Committee Chair

We have read this thesis
and recommend its acceptance

_____
Erach R. Talaty, Committee Member

_____
Michael Van Stipdonk, Committee Member

_____
Lop-Hing Ho, Committee Member

ACKNOWLEDGEMENTS

I would like to express my deep sense of gratitude to my Research Advisor, Dr. Groutas for giving me an opportunity to work in his research group and for his guidance and support through these years. Dr. Groutas, thank you so much for having taught me all about chemistry and life. You have been a tremendous guiding force to me and you have made my graduate education at Wichita State University a memory of pleasure.

I would all like to thank my committee members, Dr. Talaty, Dr. Stipdonk, and Dr. Ho for their advice and helpful suggestions.

I have had great pleasure in working with my fellow group members for these years and I would like to express my appreciation for all the help they have given me apart from their valuable friendships.

I give special thanks to my family, my Mom and Dad for their love and encouragement. My achievements would never be possible without your support and this degree is as much yours as it is mine. I thank Sumith, not only for his love and encouragement, but also for being my source of strength and support. But most importantly, this accomplishment by me would never be possible without the blessings and grace of God.

ABSTRACT

The 1,2,5-Thiadiazolidin-3-one 1,1-dioxide scaffold was used in the design and synthesis of inhibitors of Dengue Virus and West Nile Virus proteases and human tryptase. The scaffold was successfully used in the synthesis of potential inhibitors of Dengue Virus and West Nile Virus proteases. Inhibitors of Human tryptase synthesized based on the 1,2,5-Thiadiazolidin-3-one 1,1-dioxide scaffold were shown to be effective mechanism-based inhibitors of the enzyme.

TABLE OF CONTENTS

Table of Contents (continued)

Table of Contents (continued)

LIST OF TABLES

TABLE PAGE

LIST OF FIGURES

List of Figures (continued)

LIST OF ABBREVIATIONS AND TERMS

COPD	Chronic Obstructive Pulmonary Disease
HIV	Human Immuno-deficiency Virus
AIDS	Acquired Immune Deficiency Syndrome
E-S complex	Enzyme-Substrate complex
ICE-like	Interleukin-1 beta-converting enzyme-like
RNA	Ribonucleic Acid
NTPase	Nucleotide Triphosphatase
TRIS	Tris (hydroxymethyl) aminomethane
NMR	Nuclear Magnetic Resonance Spectroscopy
TLC	Thin Layer Chromatography
$ClSO_2NCO$	Chlorosulfonyl isocyanate
t-BuOH	tertiary-Butyl Alcohol
TEA	Triethylamine
DCM	Dichloromethane
NaH	Sodium hydride
$ClCH_2SPh$	Chloromethyl phenylsulfide
THF	Tetrahydrofuran
CH_3CN	Acetonitrile
m-CPBA	meta-chloroperbenzoic acid
PPh_3	Triphenylphosphine
DEAD	Diethyl azodicarboxylate
$NaBH_4$	Sodium borohydride

List of Abbreviations and Terms (continued)

$NaBH(OAc)_3$	Sodium triacetoxy borohydride
HOAc	Acetic Acid
$ClSO_2NH_2$	Chlorosulfonylamine
$(CH_3)_3SiI$	Trimethyl silyliodide
NaCl	Sodium Chloride

Chapter 1

INTRODUCTION

1.1 General Introduction to Proteases

Proteins are made up of long chains of amino acids that are bonded together by carbon-nitrogen bonds called amide bonds or peptide bonds. Under normal physiological conditions (pH~7, T=37^{o}C) chemical hydrolysis of amide bond requires activation energy of ~25kcal/mol[1] and hence chemical hydrolysis under physiological conditions would be very slow; however, catalysis of amide bond hydrolysis under physiological conditions is accomplished at a much faster rate by enzymes (proteases) (Fig 1.1). These proteases or proteolytic enzymes not only catalyze protein hydrolysis at a faster rate, but are also known to be specific and selective in order to prevent uncontrolled proteolysis of the organism. Since proteases form one of the largest and important groups of enzymes, they are involved in numerous important physiological processes including protein turnover, digestion, blood coagulation and wound healing, fertilization, cell differentiation and growth, cell signalling, immune response and apoptosis.

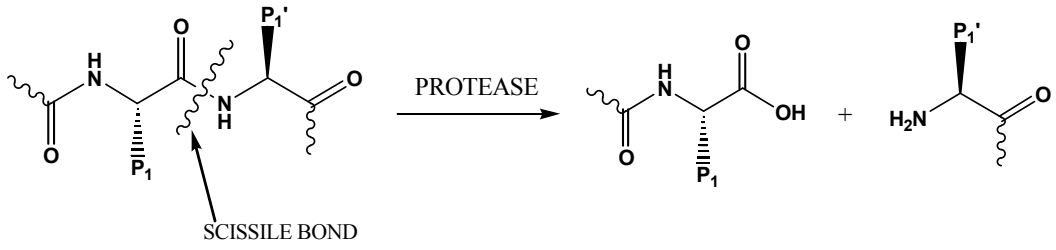


Fig 1.1: Hydrolysis of Peptide Bond Catalyzed by Protease

1.2 Classification of Proteases

According to the NC-IUBMB (Nomenclature Committee of the International Union of Biochemistry and Molecular Biology) the term "peptidase" is used for enzymes that hydrolyze peptide bonds. However, it is still possible to use the term "protease". The term peptidase is the recommended term for protein acting on peptide bonds according to NC-IUBMB. Proteases or peptidases are broadly divided into two groups: the endopeptidases and exopeptidases[2]. Exopeptidases are enzymes, which cleave a protein at either the C terminus or the N-terminus sequentially and endopeptidases cleave the peptide bonds internally in proteins or peptides. Proteases selectively catalyze hydrolysis of peptide bonds and can be divided into five major classes based on their mechanism of action. These classes are serine, cysteine, aspartic, threonine and metallo proteases. Uncontrolled, unregulated and undesired proteolysis can lead to many disease states including emphysema, stroke, viral infections, cancer, inflammatory diseases etc caused by mammalian proteases. There are also some important viral proteases that cause various infectious diseases and protease inhibitors therefore have considerable potential to act as therapeutic agents in a variety of diseases caused by uncontrolled activity of proteases[2]. Some of the examples of mammalian and viral proteases and their corresponding disease states are given in Table 1.1 and Table 1.2

Table 1.1 Examples of Mammalian Proteases and Their Disease States[3]

SERINE PROTEASES	
Human leukocyte elastase (HLE), Proteinase 3 (PR3), Cathepsin G (CatG)	Inflammation, chronic bronchitis, emphysema, COPD
Prostate specific antigen (PSA)	Cancer
Chymase	Cardiovascular disease
Matriptase	Cancer
Urokinase	Cancer, artherosclerosis, arthritis, macular degeneration
Thrombin	Thrombosis
Tryptase	Asthma
CYSTEINE PROTEASES	
Cathepsin K	Bone resorption
Caspases	Bacterial meningitis, heart disease, stroke
ASPARTIC PROTEASES	
Memapsin 2 (β-secretase)	Alzheimer's disease
METALLOPROTEASES	
Metalloproteases	Cancer, cardiovascular disease, pulmonary emphysema and COPD

Table 1.2 Examples of Viral Proteases and Their Disease States[3]

VIRAL PROTEASES	
HIV Virus Protease	AIDS
Rhinovirus 3C Protease	Common Cold
Hepatitis C Virus NS3 Protease	Liver Cirrhosis/liver Cancer
Cytomegalovirus Protease	Retinitis
Herpes Simplex Virus-1 Protease	Oral lesions
Herpes Simplex Virus-2 Protease	Genital Herpes
Varicella Zoster Virus Protease	Chicken Pox, Shingles
Epstein-Barr Virus Protease	Lymphoproliferative diseases
Dengue Virus protease	Dengue fever
West Nile Virus Protease	Encephalitis
BACTERIAL PROTEASES	
Gingipain	Periodontitis
P. aeruginosa Protease IV	Corneal
PARASITIC PROTEASES	
Cruzain	Chagas disease

1.2.1 Nomenclature

Proteases bind to the substrate in a specific manner that is represented by Schechter and Berger system of nomenclature. According to this system, the amino acid residues (or side chains) of the substrate are labeled from the N to C terminus as P_n,……,P_3,P_2,P_1,P_1',P_2',P_3'……..P_n' and the corresponding binding subsites of the enzyme are labeled as S_n,……,S_3,S_2,S_1,S_1',S_2',S_3'……..S_n'[4]. The peptide bond hydrolysis is carried out between P_1 and P_1' and the bond is called the scissile bond. The numbering of the residues is also given from the scissile bond. The P_1 residue of the substrate is called the primary specificity residue. The specificity and selectivity of a protease depends on the nature of the P_1 residue and serine proteases are classified as elastase-like (small hydrophobic residues at P_1), trypsin-like (positively charged residues/basic residues at P_1) or chymotrypsin-like (large hydrophobic residues at P_1).

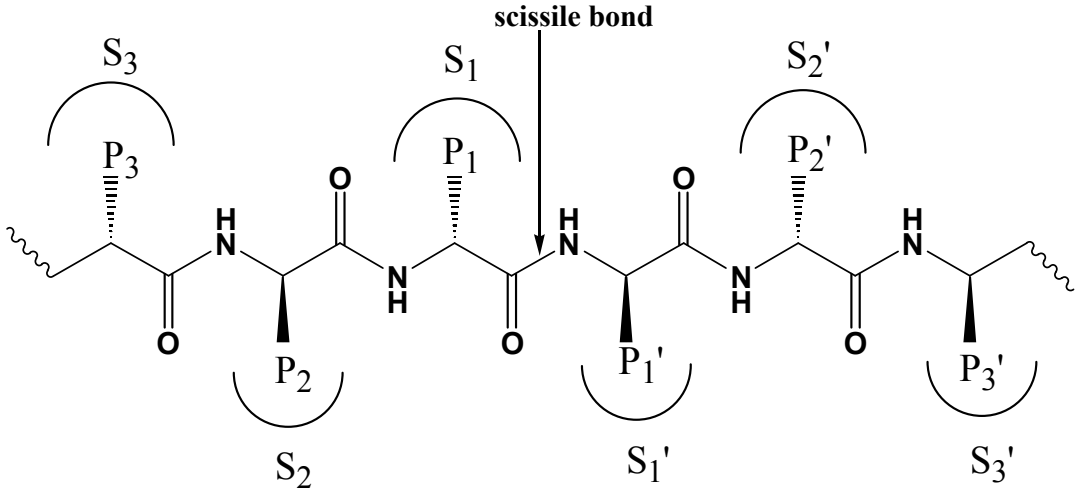


Fig 1.2 Schechter and Berger System of Nomenclature

1.3 Classification of Proteases Based on Catalysis

Based on the mechanism of action, proteases are classified in five groups: namely serine, cysteine, aspartic, metallo and threonine proteases. The catalytic nucleophile in serine and threonine protease is a hydroxyl group of the active site serine and threonine residues respectively. The nucleophile for cysteine proteases is the sulfhydryl group of the active site cysteine. In aspartic proteases, the nucleophile is a water molecule that is directly bound to two aspartic residues present at the active site. Metalloproteases contain two or more metal ions (most cases zinc, but some other metals such as cobalt, nickel or manganese) that are usually bound by three amino acid residues. A water molecule acts as a nucleophile, which is positioned and activated by the active site metal ion[5]. The catalytic mechanism of serine, cysteine, aspartic and metallo proteases will be discussed individually in this thesis.

1.3.1 Mechanism of Action of Serine Proteases

The mechanism of action of serine proteases has been studied extensively over the years using a number of tools such as site-directed mutagenesis, high field NMR and X-ray crystallography[6]. Serine proteases are thought to utilize a combination of mechanisms that are common to enzyme catalysis. First the enzyme binds to the substrate to form an enzyme-substrate Michaelis-Menten complex (E-S complex) utilizing non-covalent bonding interactions such as ionic interactions, dipole-dipole interactions, hydrophobic interactions, hydrogen bonding and Vander Waal's interactions[7]. When the substrate is bound to the active site of the enzyme, the carbonyl group of the scissile amide bond is exposed for catalysis by the enzyme.

The active site of serine proteases consists of three conserved amino acid residues; Ser-195, His-57, Asp-102 (chymotrypsin numbering) that together form the "catalytic triad". The side chain of serine ($-CH_2OH$) is activated by general base catalysis mediated by Asp-102 and His-57 through a hydrogen bonding interaction. The active site serine residue (activated nucleophile) then attacks the amide bond resulting in an oxyanion tetrahedral intermediate. The anion is stabilized in the "oxyanion hole" by means of two hydrogen bonds from amino acid residues Ser-195 and Gly-193 (Fig 1.3 a). The collapse of the oxyanion tetrahedral intermediate occurs by efficient proton transfer from His-57 to form an amine and the acylated enzyme (Fig 1.3 b). During the deacylation step, the covalent acyl-enzyme adduct is attacked by a water molecule activated by His-57, forming a new tetrahedral intermediate (Fig 1.3 c) which subsequently breaks down by acid assisted catalysis to form the C-terminal fragment of the substrate and regenerate the enzyme (Fig 1.3 d).

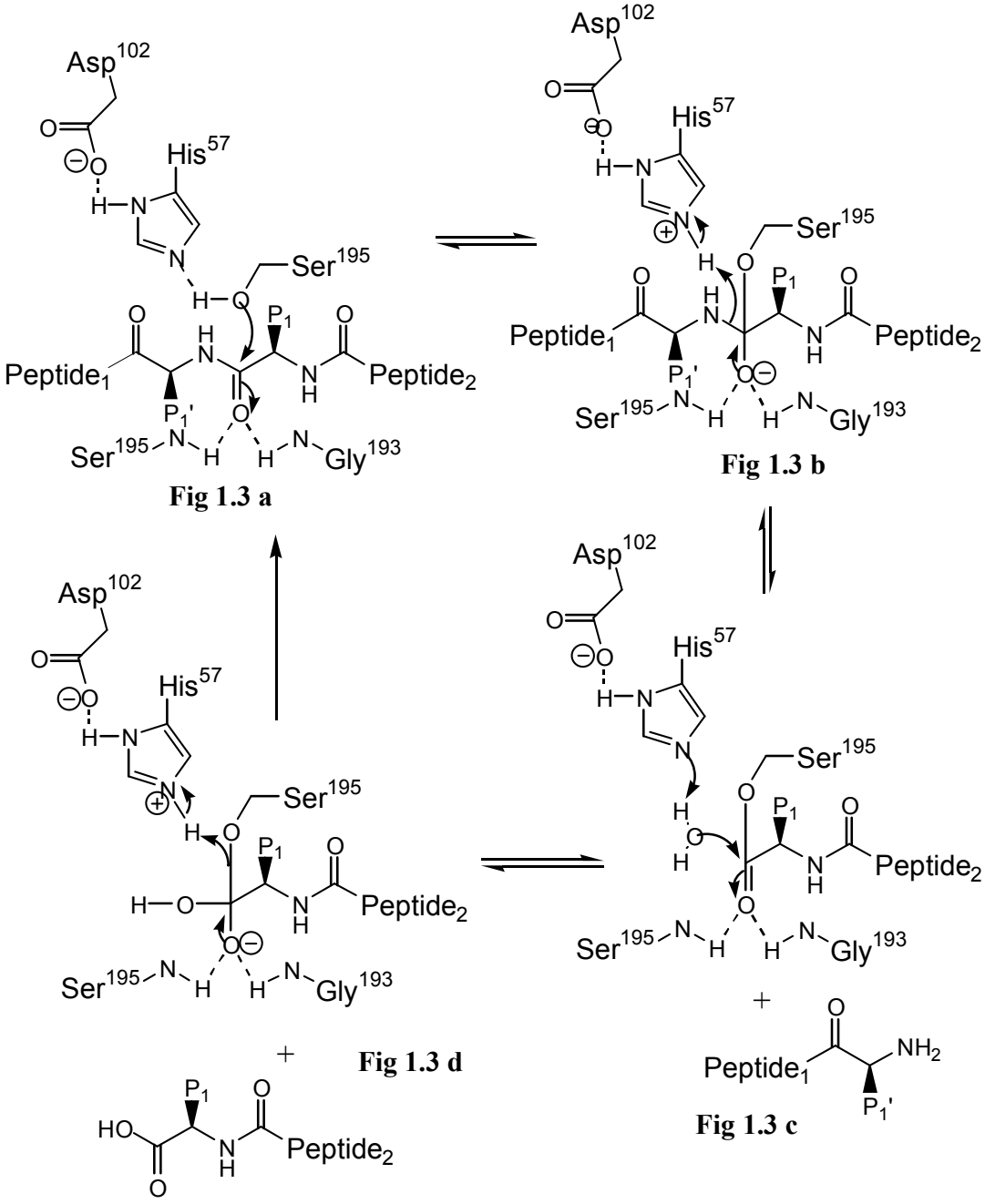


Fig 1.3 Catalytic Mechanism of Serine Proteases[7]

1.3.2 Mechanism of Action of Cysteine Proteases

Cysteine proteases exist in three structurally distinct classes, which are papain-like, ICE-like (caspases) and picorna-viral. The papain group of cysteine proteases has been studied in detail and the catalytic mechanism discussed is based on the active site of papain. The active site of papain contains a catalytic triad of Cys25, His159 and Asn175. However, it is still debatable whether the catalytic dyad of Cys25 and His159 is sufficient for catalytic activity[8]. It has been proposed that the Asn175 residue is able to orient His159, so that the imidazole group is able to activate the thiol group of Cys25 allowing deprotonation at neutral or slightly acidic pH. The resulting thiolate-imidazolium ion pair is then highly nucleophilic. The thiolate ion is able to attack the carbonyl carbon of the scissile amide bond and form the tetrahedral intermediate, which is stabilized by the oxyanion hole. The collapse of the tetrahedral intermediate leads to the formation of the acyl enzyme. The acyl enzyme is subjected to hydrolysis which gives rise to a second tetrahedral intermediate. Following the collapse of the tetrahedral intermediate, the product acid is released and the free enzyme is regenerated (Fig 1.4).

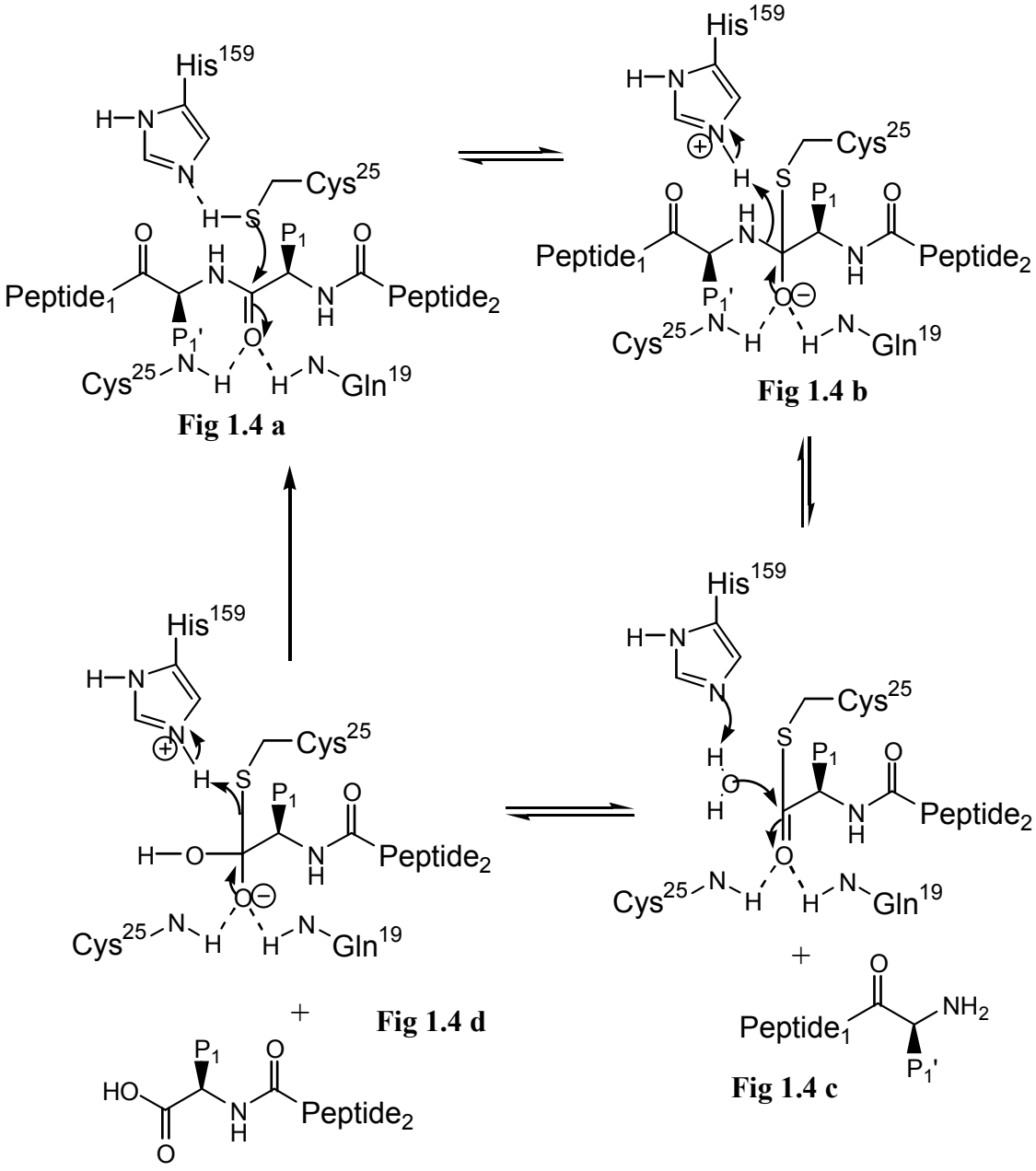


Fig 1.4 Catalytic Mechanism of Cysteine Proteases[8]

1.3.3 Mechanism of Action of Aspartic Proteases

The catalytic mechanism of Aspartic proteases is believed to proceed via general acid-base catalysis. The catalytic mechanism of aspartic proteases is best understood by studying HIV-1 virus protease. In HIV-1 aspartic protease, only one of the two aspartic acid residues is protonated in the enzyme-substrate complex[5]. The scissile amide bond undergoes nucleophilic attack by a water molecule, which is partly activated by one of the aspartic acid residues that acts as a general base. The protonated aspartic acid acts as a general acid and donates a proton to the amide bond nitrogen generating a zwitterionic intermediate, which collapses to release the cleaved products[8]. Thus the catalysis of aspartic proteases is an example of a concerted mechanism[5] (Fig 1.5)

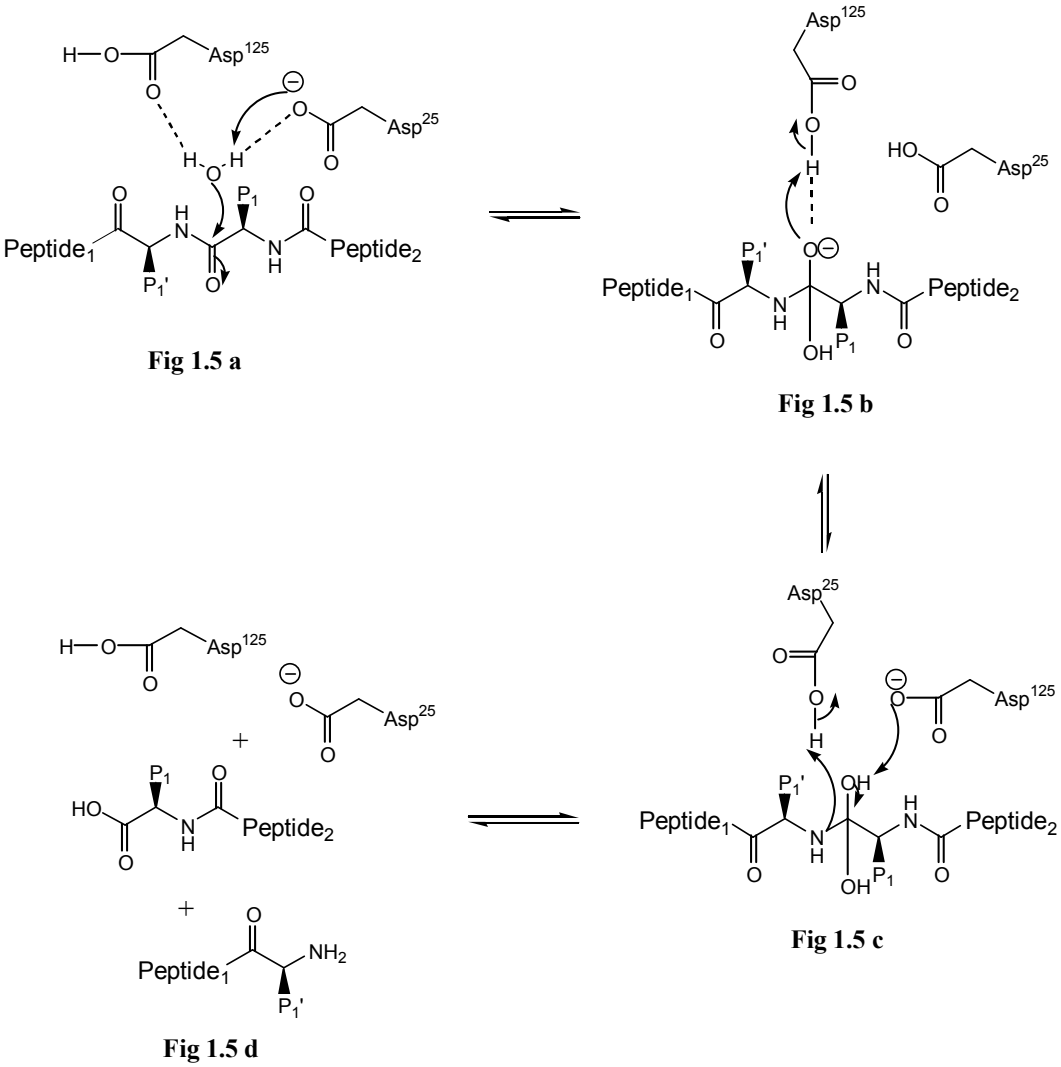


Fig 1.5 a

Fig 1.5 b

Fig 1.5 c

Fig 1.5 d

Fig 1.5 Catalytic Mechanism of Aspartic Proteases[5]

1.3.4 Mechanism of Action of Metalloproteases

Metalloproteases use a zinc atom to effect catalysis of amide bonds. The currently accepted mechanism of matrix metalloproteases (MMPs) is used as an illustration to explain the mechanism of amides bond hydrolysis by metalloproteases. The zinc metal (Zn^{2+}) is tetrahedrally coordinated to three amino acid residues from the backbone of the enzyme and a water molecule. The water molecule is hydrogen-bonded to the side chain

of glutamic acid and activated for nucleophilic attack. Nucleophilic attack by the zinc bound water molecule on to the carbonyl carbon of the scissile bond leads to the formation of a tetrahedral intermediate. The oxyanion formed is stabilized by the zinc atom. Transfer of a proton from glutamic acid to the amide nitrogen leads to the collapse of the tetrahedral intermediate with the generation of a salt bridge between glutamic acid and the free amine of the cleaved substrate (Fig 1.6) [8].

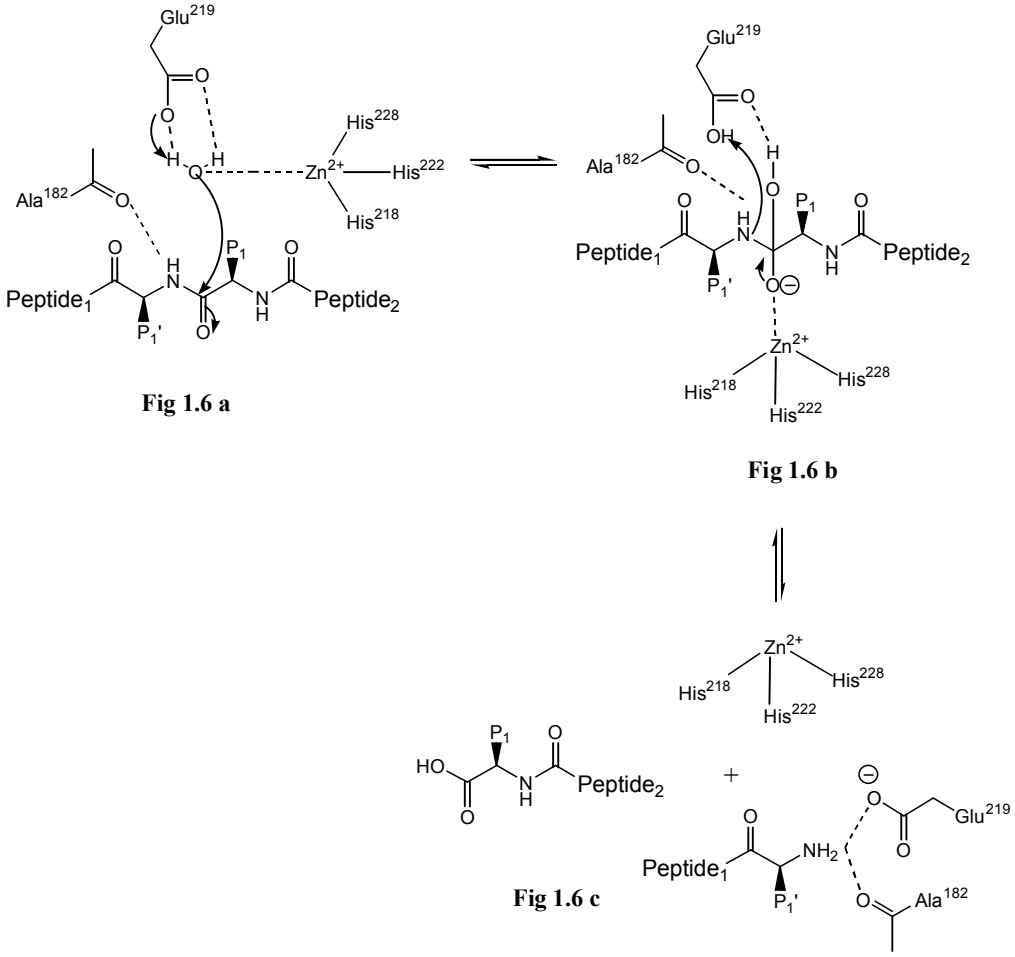


Fig 1.6 Catalytic Mechanism of Metallo-proteases[8]

1.3.5 Threonine Proteases

The only enzymes that utilize a threonine residue as a catalytic nucleophile for amide bond hydrolysis are proteasomes. Proteasomes are barrel-shaped proteins whose main function is the degradation of ubiquitin-labelled proteins, and regulation and turnover of many other proteins that control cellular function. Proteasomes possess three distinct cleavage preferences: trypsin-like (Arg/Lys at P_1), chymotrypsin-like (Phe/Try/Leu at P_1) and caspase-like (Asp/Glu at P_1)[9]. The mechanism of threonine proteases is not yet known in detail.

1.4 Mammalian Serine Proteases

Some of the serine proteases that are known to be involved in diseases such as pulmonary emphysema, chronic bronchitis and cystic fibrosis, are human neutrophil serine proteases. These proteases are stored in the primary (azurophilic) granules of neutrophils and they serve important purposes such as tissue repair and tissue remodeling. The primary granules contain neutral serine proteases such as human leukocyte elastase (HLE), proteinase 3 (PR3) and cathepsin G (CatG) [10]. Human mast cell tryptase is another serine protease that is known to be involved in bronchial asthma and other respiratory infections[11]. Though, the synthesis of inhibitors for these serine proteases is a major part of the research group, the focus of this thesis is on the synthesis of inhibitors for viral proteases. The substrate specificity for mammalian serine proteases is given in Table 1.3.

1.4.1 Human Leukocyte Elastase (HLE)

HLE (EC. 3.4.21.37) is believed to be the most destructive protease released by neutrophils and it is capable of degrading a variety of structural proteins, including elastin, collagen, fibronectin as well as some immunoglobulins and complement proteins[12]. The enzyme is a 218 amino acid glycoprotein, molecular weight 33kDa, possessing four intramolecular disulfide bonds and two sites of glycosylation at Asn-95 and Asn-144. Because of the variations in the carbohydrate side chains, HLE is present in 4-5 isoenzymes that differ in molecular weight, but are identical in catalytic activity and specificity[13, 14]. HLE has a high isoelectric point and is highly basic due to the large number of arginine residues on the outer surface of the glycoprotein.

1.4.2 Cathepsin G (CatG)

Cathepsin G (CatG) is present in approximately equal amounts to that of HLE within the primary granules of neutrophils, but very little amounts are released compared to that of HLE. It is a 30kDa glycoprotein with at least four isomers. It contains three disulfide bonds and is glycosylated once at Asn-51. CatG contains 31 arginine residues and is therefore highly basic in nature than HLE. Cathepsin G is also capable of degrading connective tissue and matrix components such as proteoglycans, collagen, fibronectin and elastin[15]. It also plays a role in activating protease-activated receptor-4 (PAR-4), leading to platelet activation and release of mediators that could contribute to inflammatory responses.

1.4.3 Proteinase 3 (PR3)

PR3 is another serine protease present in the primary granules of neutrophils in even greater amounts than HLE. PR3 is a single chain protein containing 228 amino acids with two sites of glycosylation and four intra-molecular disulfide linkages[16]. PR3 displays similarity to HLE and is also capable of connective tissue degradation including elastin.

1.4.4 Tryptase

Human mast cell tryptase (E.C 3.4.21.59) is a trypsin-like serine protease that is secreted by mast cells upon activation in the active form along with numerous proiflammatory mediators such as histamine and arachidonate metabolites[22]. It is a tetramer composed of four identical subunits stabilized by heparin proteoglycans that are stored and secreted with the protease. Each subunit of the enzyme contains an active site that prefers basic amino acids such as lysine and arginine for the S1 pocket [21].

Table 1.3 Subsite Specificity for HLE, PR3, CatG and Tryptase[17, 18, 19,20]

Enzyme	**S_4**	**S_3**	**S_2**	**S_1**	**S_1'**	**S_2'**	**S_3'**	**S_4'**
PR3	Ala	Ala	Pro	Abu	Arg	Gly	Asp	-
HLE	Ala	Val	Pro	Val	-	Phe	-	-
CatG	Ala	Ala	Pro	Phe	-	-	-	-
Tryptase	-	Ile	Leu	Lys	Pro	Val	-	-
Peptide	**P_4**	**P_3**	**P_2**	**P_1**	**P_1'**	**P_2'**	**P_3'**	**P_4'**

1.5 Proteases and Antiproteases

Neutrophils are the most abundant leukocyte that are essential for defense against microbial infection and are responsible for the destruction and detoxification of foreign substances by means of phagocytosis[23]. Neutrophils predominantly produce serine proteases whereas macrophages synthesize a variety of metalloproteases and cysteine proteases[24]. These serine proteases find their way into the extracellular medium by different means and are sometimes released on purpose to aid the migration of neutrophils through dense connective tissue or when phagocytosis of the foreign substance is not possible.

The chronic release of these proteases into the extracellular medium can lead to serious damage to the host. However, the body does possess mechanisms to control the activity of neutrophil protease release and this is accomplished by means of endogenous protein inhibitors called antiproteases. The regulation of neutrophil serine proteases is essential in order for the body to carry out its normal physiological processes. An imbalance in the regulation of these proteases results in various inflammatory and respiratory disorders.

In fact, human plasma contains relatively high concentrations of antiproteases that are highly specific inhibitors of the serine proteases released by neutrophils. The most prominent antiproteases utilized by the human body are α_1-antitrypsin (α_1-AT), α_1-anti-chymotrypsin (α_1-ACT), secretory leukoprotease inhibitor (SLPI), and α_2-macroglobulin (α_2M) [24]. α_1-Antitrypsin (α_1-AT) or α1-proteinase inhibitor (α_1-PI) is a broad-spectrum serine protease inhibitor that inhibits HLE, CatG and PR3. It is thought that the major function of α_1-AT in the lung is to protect it from the degradative action of these serine

proteases. Secretory leukoprotease inhibitor (SLPI) functions as an effective inhibitor of HLE and CatG but not PR3. SLPI is thought to be the most important inhibitor of HLE in the upper respiratory tracts where it is present in nearly double the concentration of α_1-AT[24].

α_1-Antichymotrypsin (α_1-ACT) potently inhibits CatG, but has no effect against HLE and PR3. Its high affinity for CatG suggests that its main physiological function is to control the activity of cathepsin G. α_2- Macroglobulin (α_2-M) is a highly glycosylated protein capable of inhibiting all four classes of proteases (serine, cysteine, aspartic and metallo). Elafin is another neutral serine protease inhibitor produced by airway secretory and epithelial cells that specifically inhibits HLE and PR3[24].

1.6 Viral Serine Proteases

1.6.1 Dengue Virus and West Nile Virus

Dengue Virus (DENV) and West Nile Virus (WNV) are members of the *Flaviviridae* family of viruses. Dengue Virus has four serotypes (DENV I-1V) and is an arthropod-borne human pathogen transmitted by *Aedes aegypti* mosquitoes[25]. The virus causes very mild to severe life threatening form of disease which leads to dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). West Nile Virus is also transmitted to humans by mosquitoes and ticks and it causes severe complications such as encephalitis and neurological sequelae[28].

Flaviviruses are small, enveloped plus-strand RNA viruses. The genome is surrounded by a viral membrane containing embedded envelop proteins and a capsid protein which gives the shape to the virus. The 11-kb positive strand RNA genome

encodes a single polyprotein precursor made up of three structural proteins (Capsid (C), pre-membrane (prM) and Envelop (E) protein) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A and NS4B and NS5)[28]. The polyprotein precursor is co-and post-transationally processed by host and viral proteases to produce the structural and non-structural proteins. Structural proteins are required to form the virus particle, whereas the non-structural proteins are required for genome replication and expression, viral assembly, release and evading the host immune response.

The viral non-structural (NS3) serine protease (Flavivirin, EC 3.4.21.91) is the essential component for the viral replication and maturation of viral polyprotein[25]. Hence it is a promising target for the development of protease inhibitors which can be further developed into antiviral drugs.

1.6.2 Flavivirus Reproductive Cycle

Flaviviruses enter the host cell by receptor mediated endocytosis. The fusion of the viral membrane with the host endosomal vesicle releases the viral genome into the cytosol. Host cell proteases are responsible for cleavage at C/prM, prM/E, E/NS1, NS1/NS2A and NS4A/NS4B junctions. The viral NS2B-NS3 protease complex participates in the cleavage at NS2A/NS2B, NS2B/NS3, NS3/NS4A and NS4B/NS5 junctions and also internal sites within capsid protein, NS3 and NS4A. Viral replication is catalyzed by a replication complex formed by NS5 (RNA-dependent RNA polymerase) and other viral and host factors. The plus strand genome is first transcribed into the minus strand which is then used as a template for making multiple copies of the plus strand RNA genome. After replication, the virus particles are assembled in the endoplasmic

reticulum of the host and then released by exocytosis[29]. The replication cycle is represented in Fig 1.7.

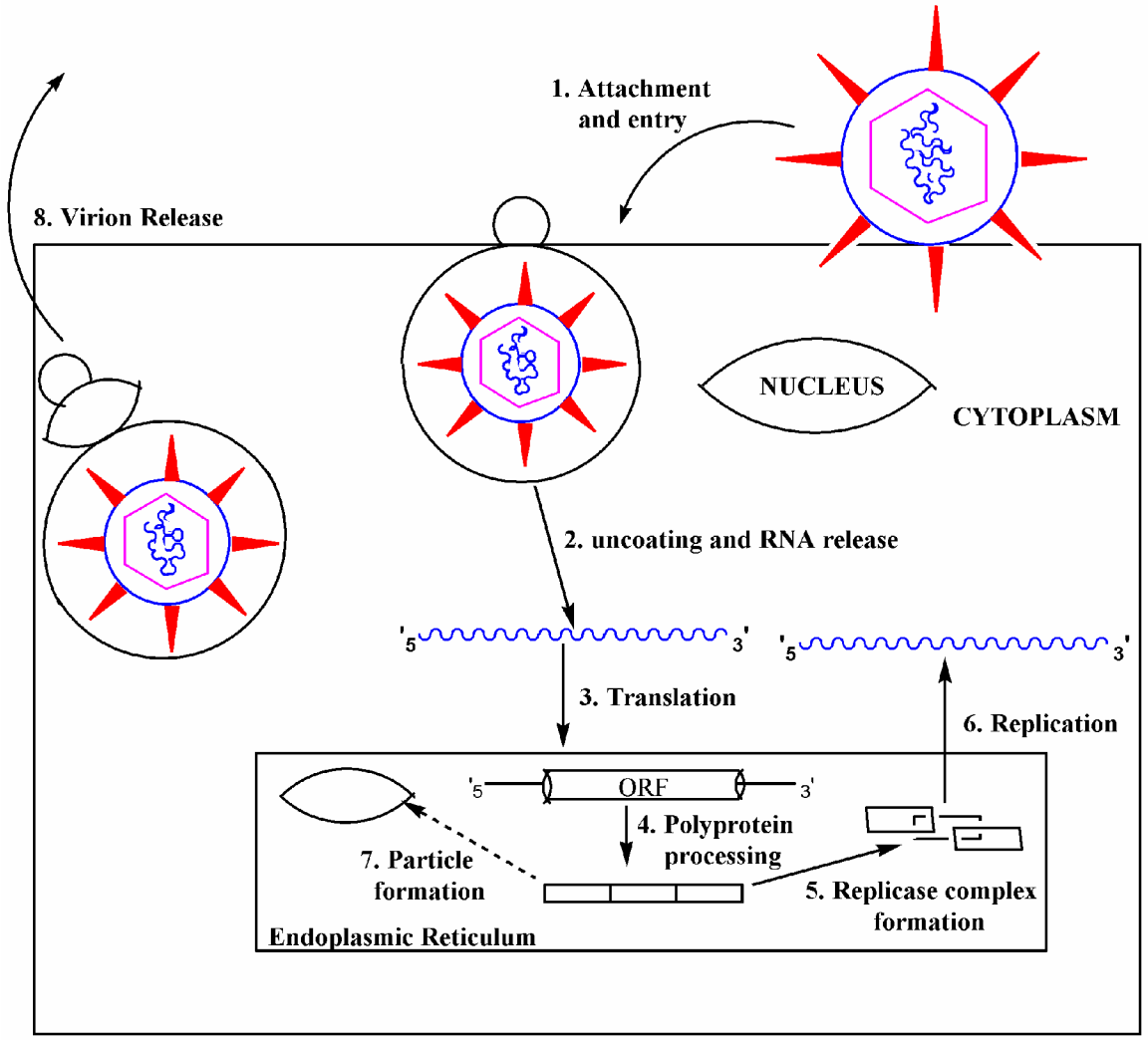


Fig 1.7 Life cycle of Flaviviruses [Figure modified from Ray and Shi][28]

1.6.3 Viral NS3 Protease and Substrate Specificity

The 69-kDa NS3 protein consists of two domains that possess enzymatic activity: a trypsin-like serine protease located in the N-terminal domain of the protein and a C-terminal domain that possesses RNA helicase and NTPase activity. The enzymatic activity of NS3 is enhanced by interactions with NS2B protein that functions as an

essential co-factor[26]. Studies have shown that a 40-residue hydrophilic core fragment of NS2B is sufficient to activate the catalytic activity of NS3 protease[29].

The X-ray crystal structure of Dengue Virus NS3 protease shows that the enzyme exhibits a folding pattern that is characteristic of trypsin- like serine proteases[27]. Substrate specificity studies for Dengue Virus and West Nile Virus NS3 protease show that the enzyme prefers a pair of basic residues (Lys/Arg) at the P_2 and P_1 positions followed by a small branched amino acid residue (Gly/Ala/Ser) at P_1' position. Even though most extensive interactions of the protease are with the P_1 residue, studies show that the P_2 side chain is a very important determinant in substrate binding[26]. Recent studies have shown that NS3 serine protease prefers basic residues extending from P_1-P_4 position and additional basic and/or aliphatic residues at P_5-P_6 position[27]. Though there seems to be interactions in the S' subsites of the protease, recent studies using combinatorial variation of P_2'-P_4' region has lead to several fold increase in potency[26].

1.7 Synthetic Inhibitors of Serine Proteases

Small molecule synthetic inhibitors are an alternative to naturally occurring endogenous inhibitors. Synthetic inhibitors can be designed to provide better selectivity and oral bioavailability than endogenous inhibitors. These inhibitors can be broadly classified into reversible and irreversible inhibitors depending on the mechanism of action.

1.7.1 Reversible Inhibitors

Inhibitors that generally do not form a permanent covalent linkage with the enzyme but have rapid association and dissociation rates are called reversible inhibitors. The most common reversible inhibitors that compete with the substrate to bind to the active site of the enzyme are called reversible competitive inhibitors[30].

The efficiency of inhibition is commonly measured in terms of K_I (k_{off} / k_{on}) which is the equilibrium constant of the chemical reaction shown in Fig 1.8. K_I represents the dissociation constant for the enzyme-inhibitor complex formed. Another method used to determine the potency of the inhibitor is to use a Dixon plot, which is a measure of initial velocity of reaction as a function of inhibitor concentration at two or more substrate concentrations. The data is a plot of 1/v vs [I] for each substrate concentration as shown in Fig 1.9

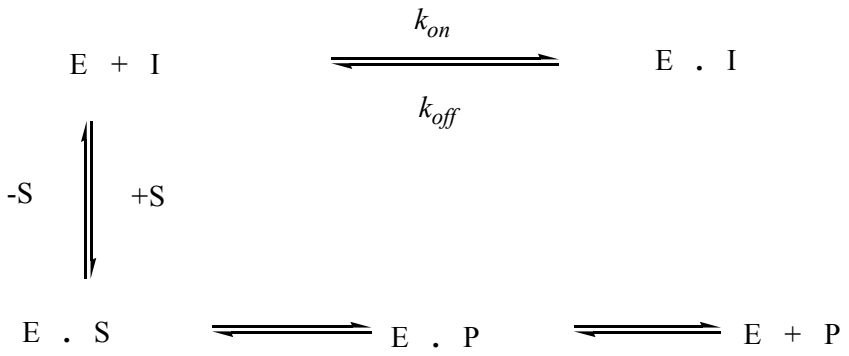


Fig 1.8 Kinetic Scheme for Competitive Reversible Enzyme Inhibition

Another way to measure the potency of a reversible inhibitor is to determine the inhibitor concentration at which the activity of the enzyme is reduced by 50% (IC_{50}). It is common to report the potency of inhibitors in terms of IC_{50} values[30]. The relationship of inhibitor concentration and reaction velocity is described by the following equation:

$$\frac{Vi}{Vo} = \frac{1}{1 + \frac{IC_{50}}{[I]}}$$

where v_0 is the initial velocity in the absence of inhibitor and v_i is the initial velocity in the presence of inhibitor of a certain inhibitor concentration.

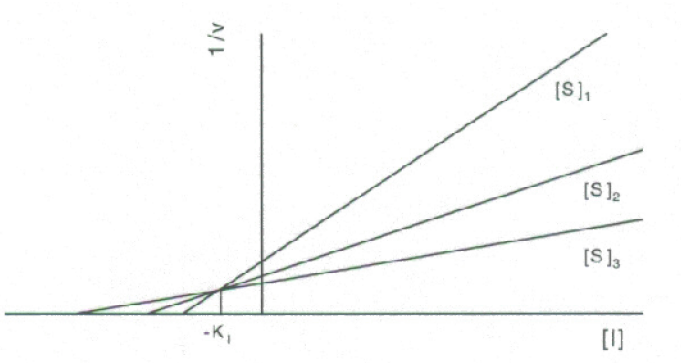


Fig 1.9 Dixon plot

There are four sub-classes of competitive reversible inhibitors which are distinguished based on their enzyme kinetics characteristics: (a) Tight-binding inhibitors that display very high k_{on} values and achieve inhibition at concentrations equal to or slightly higher than that of enzyme; (b) Slow-binding inhibitors display lower k_{on} values and require excess inhibitor to achieve successful inhibition; (c) Slow,Tight-Binding inhibitors are a combination of the first two types and they display low k_{on} values but achieve strong inhibition due to the low k_{off} values; (d) Classical Reversible inhibitors attain rapid equilibrium between inhibitor, enzyme and enzyme-inhibitor complex. The equilibrium is diffusion controlled (k_{on} is very high) and the reaction requires excess of inhibitor to achieve significant inhibition of target enzyme[30].

Since the enzyme catalyzed substrate turnover mainly occurs through stabilization of transition state intermediate, the most significant type of reversible inhibitor are transition state analogs or transition state inhibitors. These inhibitors are designed to mimic the substrate transition state intermediate during the enzyme-catalyzed reaction. Many inhibitors of this type are extremely potent. Peptidyl aldehydes, trifluromethyl ketones (TFMK), α-difluoroketones, α-diketones, α-ketoamides and ketoesters, and boronic acid inhibitors have all been shown to be potent serine protease inhibitors. All these classes of inhibitors have a strong electron-withdrawing group attached to the carbonyl carbon making it more susceptible toward nucleophilic attack. Another feature of these inhibitors is they can orient the various recognition elements in the subsites of the enzyme in order to achieve maximum selectivity and potency[30].

1.7.2 Irreversible Inhibitors

Irreversible inhibitors function by forming a covalent bond to an amino acid present in the active site so that the enzyme cannot be reactivated. Irreversible inhibitors include mechanism-based inhibitors, alternate substrate inhibitors (acylating agents) and affinity labels. Like most competitive reversible inhibitors, irreversible inhibitors also compete with the substrate for binding to the active site of the target enzyme[2]. At low concentrations, the rate by which an irreversible inhibitor inactivates the enzyme is related to the inhibitor concentration. Normally very low inhibitor concentrations are required to elicit high inhibition. The potency of the inhibitor is expressed in terms of two types of kinetic constants. The first kinetic constant, $k_{obs}/[I]$ $M^{-1}s^{-1}$ is obtained by incubating the enzyme with excess of inhibitor and monitoring the remaining activity of the enzyme with respect to time by withdrawing aliquots and measuring the initial velocity of the enzyme at incremental time intervals. The second kinetic constant, k_{inact}/K_i $M^{-1}s^{-1}$, is obtained by continuous monitoring of enzyme activity in the presence of excess substrate. The data obtained is expressed in terms of progress curves. In general, the kinetic constants obtained from the two methods are roughly comparable.

1.7.2.1 Affinity Labels

Affinity labels are inhibitors that contain functional groups, which are characteristically reactive towards an active site amino acid residue of the target enzyme. Affinity labeling agents first bind to the enzyme to form a non-covalent complex and then react with an active site amino acid residue to form an irreversible covalent bond. Affinity labels can incorporate recognition elements for a specific enzyme of a particular

class, but show very little selectivity for various enzymes within the class. Since these inhibitors are very reactive, they are capable of producing undesirable side effects resulting from non-specific reactions with untargeted biomolecules. Due to these reasons, affinity labels are rarely used as effective therapeutic drugs[30].

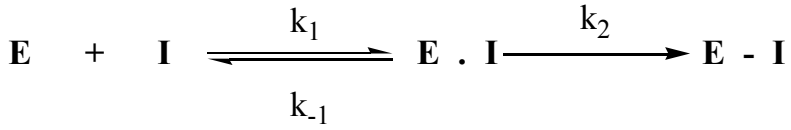


Fig 1.10 Kinetic Scheme for Irreversible Inhibition by an Affinity label

1.7.2.2 Mechanism-Based Inhibitors

A mechanism-based inhibitor is a relatively unreactive compound that, once bound to the active site of the enzyme is designed to react specifically with the catalytic machinery of the enzyme to generate a reactive species, which upon further reaction with an active site residue leads to irreversible inhibition. Since the reactivity of the inhibitor is only attained after the inhibitor is unmasked by the catalytic machinery of the target enzyme, high enzyme specificity can be obtained. In some cases, the inhibitor is activated only after an enzyme-induced rearrangement or fragmentation takes place. However, the effectiveness of a mechanism-based inhibitor is determined by the partition ratio, which is defined as ratio of k_3 to k_4 (Fig 1.11). Since k_3 is the rate constant for the pathway leading to enzyme regeneration, lower partition ratios lead to more effective mechanism-based inhibitors with fewer side effects[30].

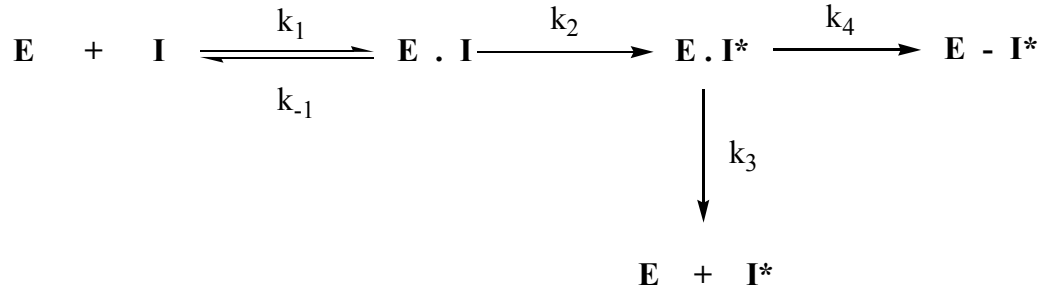


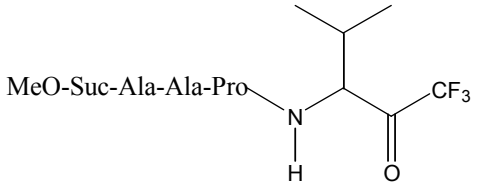
Fig 1.11 Kinetic Scheme for Irreversible Mechanism-based Enzyme Inactivation

1.7.2.3 Acylating Agents

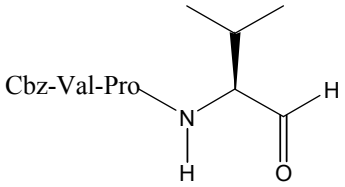
Acylating agents are also called alternate substrate inhibitors that inhibit enzymes by acylating the active site nucleophile to form a stable acyl enzyme intermediate. The acyl enzyme is very stable and the enzyme is essentially irreversibly inactivated. However, in some cases, the acyl enzyme will hydrolyze to regenerate the active enzyme by a slow deacylation process. The potency of the inhibitor is compared by the magnitude of the deacylation rate (k_{off}) and the acylation rate $(k_{on})^2$.

Fig 1.12 Kinetic Scheme for Irreversible Inhibition by Acylating agents[2]

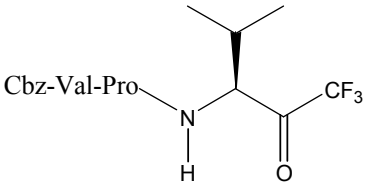
Table 1.4 Examples of Reversible Inhibitors of Serine Proteases[12]



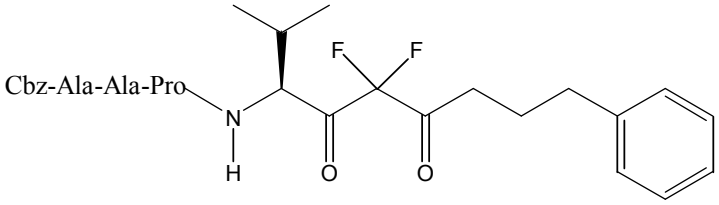
Ki = 0.014 uM
(HLE)



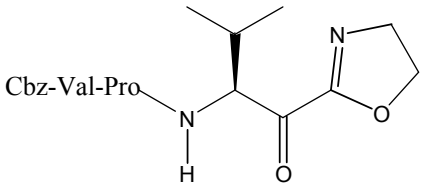
Ki = 0.041 uM
(HLE)



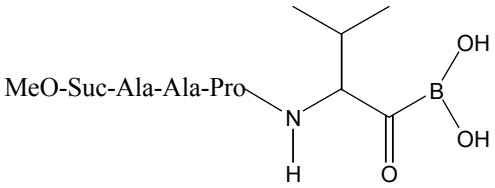
Ki = 0.8 nM
(HLE)



Ki = 0.23 nM
(HLE)

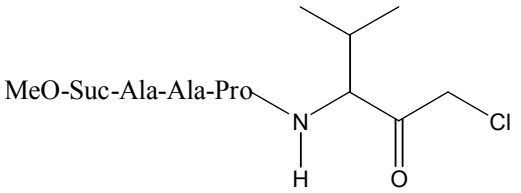


Ki = 0.6 nM
(HLE)

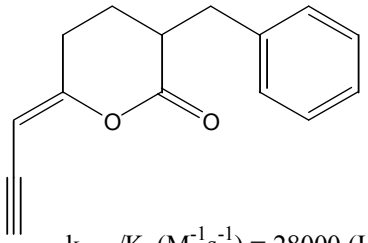


Ki = 0.57 nM
(HLE)

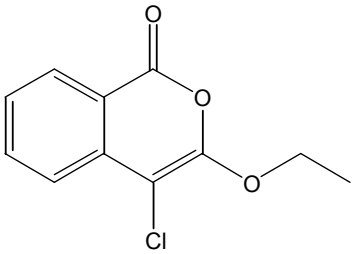
Table 1.5 Examples of Irreversible Inhibitors of Serine Proteases[12]



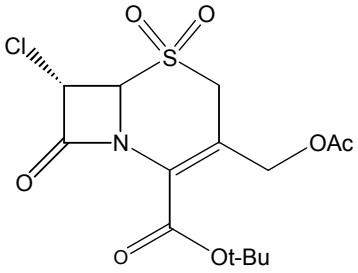
$k_{obs}/[I](M^{-1}s^{-1})$ = 1560 (HLE)
Affinity label



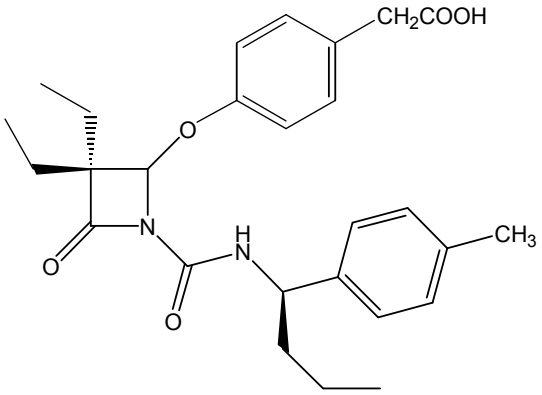
k_{inact}/K_i ($M^{-1}s^{-1}$) = 28000 (HLE)
Mechanism-based



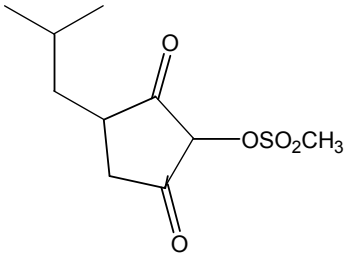
$k_{obs}/[I](M^{-1}s^{-1})$ = 43000 (HLE)
Acylating Agent



$k_{obs}/[I](M^{-1}s^{-1})$ = 161000 (HLE)
Mechanism-based



k_{inact}/K_i ($M^{-1}s^{-1}$) = 622000 (HLE)
Acylating Agent



$k_{obs}/[I](M^{-1}s^{-1})$ = 49300 (HLE)
Mechanism-based

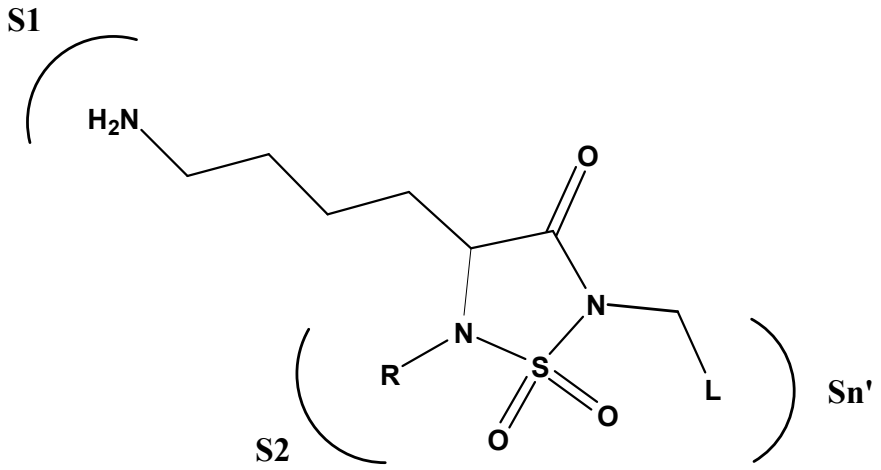
Chapter 2

RESEARCH GOALS

Based on the forgoing discussion, the objectives of the research described in this thesis were the following:

a) Exploratory studies focusing on the utilization of the 1,2,5-thiadiazolidin-3-one 1,1 dioxide scaffold (I) in the design and synthesis of potential inhibitors of West Nile Virus (WNV) and Dengue Virus (DENV) proteases.

b) Utilization of the heterocyclic scaffold (1,2,5-thiadiazolidin-3-one 1,1 dioxide) cited above in the design, synthesis and *in vitro* biochemical evaluation of mechanism-based inhibitors (II) of human tryptase.



When R = X-NH_2 (I), where X= Spacer, R = Benzyl (II) and L = Leaving group

Fig 2.1 1, 2, 5-Thiadiazolidin-3-one 1, 1 dioxide based Inhibitors (I-II)

Chapter 3

EXPERIMENTAL

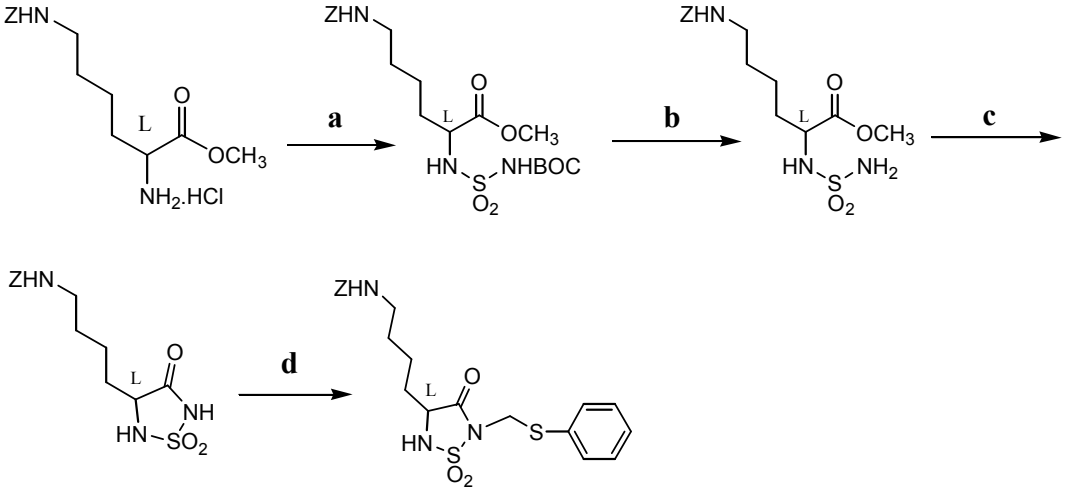
All reported melting points were recorded on a Mel-Temp apparatus and are uncorrected. All NMR spectra were recorded on a Varian XL-300 or XL-400 NMR spectrometer. Reagents used for synthesis were purchased from a number of chemical suppliers including ALDRICH Chemical Co, TCI America, SIGMA Chemical Co, ACROS, Bachem and Alfa Aesar.

Purification of compounds by flash chromatography was accomplished by using Silica Gel (230-450 mesh, 60 Å) purchased from Sorbent Technologies (Atlanta, GA). Solvents used for flash chromatography were purchased from Fisher Scientific Co. Thin Layer Chromatography was performed using Analtech Silica Gel Plates. The TLC plates were visualized using iodine vapor and/or UV-light.

N-p-Tosyl-Gly-Pro-Lys-p-nitroanilide acetate salt, papain and bovine trypsin were purchased from SIGMA Chemicals Co, St Louis, MO. Human tryptase was obtained from Dr. Norman M. Schechter (University of Pennsylvania). A Hewlett-Packard diode array UV/VIS spectrophotometer was used in the enzyme assays and inhibition studies.

3.1 Synthesis of Template I

The synthesis of the 1,2,5-thiadiazolidin-3-one 1,1 dioxide scaffold was accomplished by following the scheme shown in Fig 3.1. Detailed procedures for the synthesis of each compound follows.



[a]$ClSO_2NCO$/ t-BuOH/ TEA/ DCM [b]4.0M HCl in dioxane [c]NaH/ THF [d]$ClCH_2SPh$/ TEA/ CH_3CN

Fig 3.1 General Scheme for Synthesis of Scaffold

Synthesis of Compound I-1

To a chilled solution of dry methanol (400 mL), thionyl chloride (24.60 g; 206.8 mmol) was added slowly and stirred for few minutes. To this solution mixture, L-Lysine (Z)-OH (50 g; 178.4 mmol) was added slowly and the reaction mixture was allowed to warm to RT and stirred for 2-3 hours in a water bath maintained at 40^{o}C. The solvent was completely evaporated and the residue was washed with ether (1500 mL). The compound was collected by vacuum filtration and pumped to dryness to yield L-Lysine (Z)-methyl ester hydrochloride salt as a pure white solid (59 g; 100% yield).

Synthesis of Compound I-2

A solution of t-butyl alcohol (11.20 g; 151.14 mmol) in dry methylene chloride (100 mL) was added dropwise to a solution of N-chlorosulfonyl isocyanate (21.4 g; 151.14 mmol) in dry methylene chloride (200 mL) kept in an ice bath with continuous stirring. After addition is completed, the solution was stirred for an additional 15 minutes. After 15 minutes, the resulting solution was added dropwise to a solution of I-1 (50.0 g; 151.14 mmol) and triethylamine (30.6 g; 303.3 mmol) in dry methylene chloride (450 mL) kept in an ice bath. The reaction mixture was stirred at room temperature for 4 h. The reaction mixture was then washed with 5% aqueous HCl (2 X 300 mL). The organic layer was collected and washed with brine (2 X 300 mL). The organic layer was separated and dried over anhydrous sodium sulfate. The solvent was removed on the rotovac to give the product as a viscous sticky oil (56.89 g; 79.5% yield).

Synthesis of Compound I-3

To a solution of I-2 (56.89 g; 120.14 mmol) in 200 mL of 1, 4-dioxane, 4.0 M HCl in 1, 4-dioxane (350 mL) was added dropwise. The reaction mixture was stirred at room temperature until most of the starting material disappears (6 h). The solvent was removed and the residue was washed with ether (600 mL). The ether was removed and the compound was purified by flash chromatography (hexane/ ethyl acetate gradient) to remove any unreacted starting material to give pure compound as a white solid (33.85 g; 75% yield).

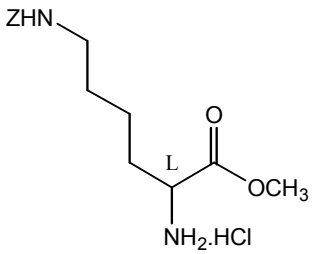
Synthesis of Compound I-4

A solution of I-3 (33.80 g; 90.5 mmol) in dry tetrahydrofuran (300 mL) was kept in an ice bath and treated in portions with 60 % sodium hydride (5.80 g; 145 mmol). The reaction mixture was allowed to stir overnight at room temperature under nitrogen atmosphere. The solvent was removed to near dryness and the residue was dissolved in water (100 mL) and acidified to pH=7 using 6N HCl solution. The aqueous solution was extracted using ethyl acetate (500 mL) to remove starting material. The aqueous layer was collected and kept in an ice bath and acidified to pH<2 using 6N HCl solution. The aqueous layer was extracted using ethyl acetate (3 X 500 mL). The organic layer was collected and dried over anhydrous sodium sulfate. The solvent was removed to give the product as a solid (29.79 g; 96% yield).

Synthesis of Compound I-5

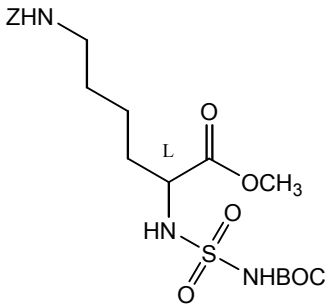
To a solution of I-4 (24.88 g; 73 mmol) and chloromethyl phenylsulfide (12.70 g; 80 mmol) in dry acetonitrile (250 mL) was added triethylamine (7.4 g; 73 mmol) and the resulting reaction mixture was refluxed for 12-15 h. The solvent was evaporated and the residue was dissolved in ethyl acetate (300 mL) and washed with 5% aqueous HCl (2 X 50 mL) followed by 5% aqueous sodium bicarbonate (2 X 50 mL) and brine (100 mL). The organic layer was collected and dried over anhydrous sodium sulfate. The solvent was removed to give a crude product which was purified by flash chromatography (hexane/ethyl acetate gradient) to give a pure product (18.02 g; 53 % yield) as a white solid.

Fig 3.2 Structures of Compounds I-1 to I-5



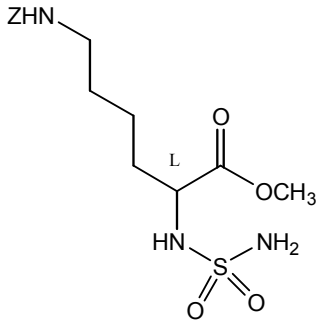
Compound I-1

$C_{15}H_{23}ClN_2O_4$
Mol. Wt.: 330.81



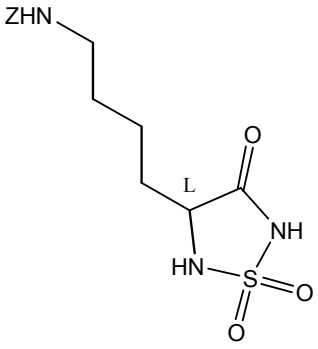
Compound I-2

$C_{20}H_{31}N_3O_8S$
Mol. Wt.: 473.54



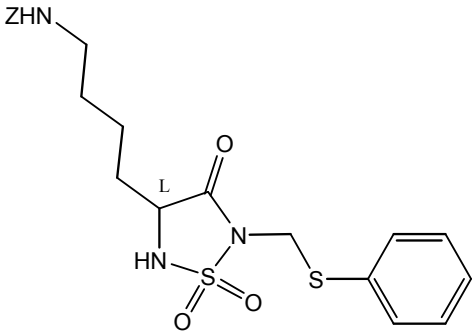
Compound I-3

$C_{15}H_{23}N_3O_6S$
Mol. Wt.: 373.43



Compound I-4

$C_{14}H_{19}N_3O_5S$
Mol. Wt.: 341.38



Compound I-5

$C_{21}H_{25}N_3O_5S_2$
Mol. Wt.: 463.57

3.2 Synthesis of Inhibitors Based on Template (I)

Synthesis of the inhibitor was accomplished by using two schemes. In Scheme A, different amino alcohols were first protected by using Boc-group. The protected amino alcohols were then coupled to compound I-5 using the Mitsunobu reaction. The resulting sulfides were oxidized to the corresponding sulfones using m-CPBA. In Scheme B, different nitro-benzyl bromides were coupled to compound I-5 by alkylation reaction. The resulting sulfides were oxidized to corresponding sulfones using m-CPBA. The nitro group was then reduced to the amine by using 10% Palladium-Carbon and sodium borohydride[33]. The Cbz-group was also removed during the reduction reaction. The general schemes showing the reaction sequence is given below.

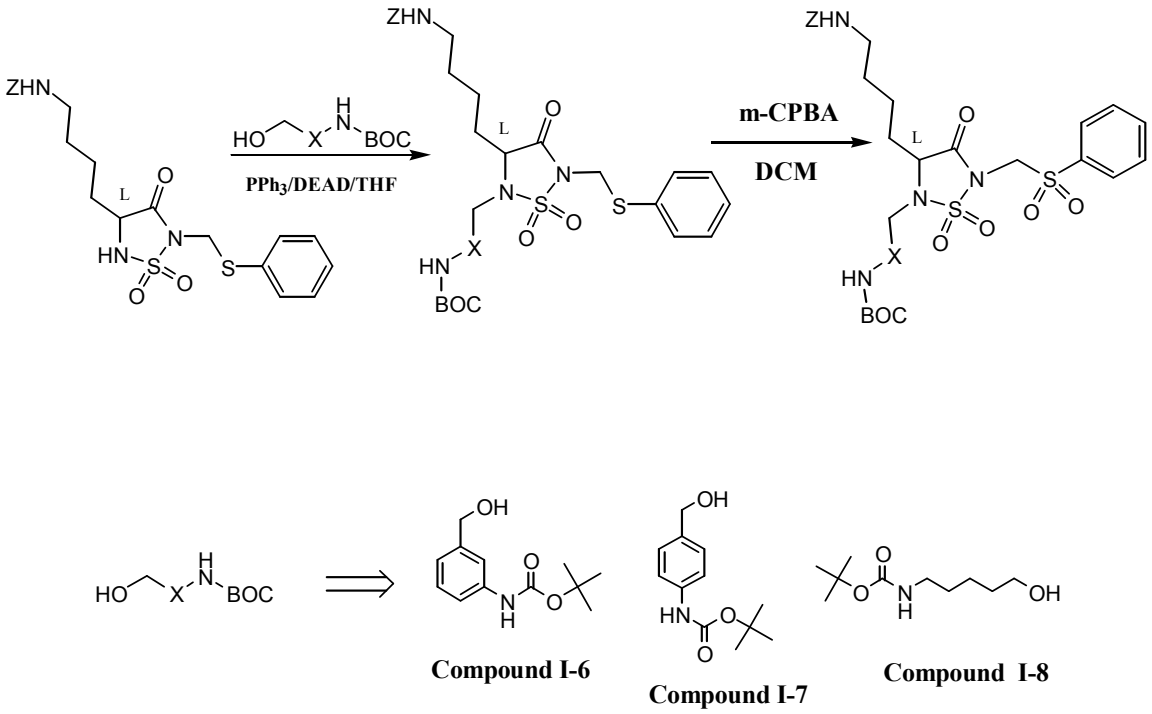


Fig 3.3 Synthesis of Inhibitor-Scheme A

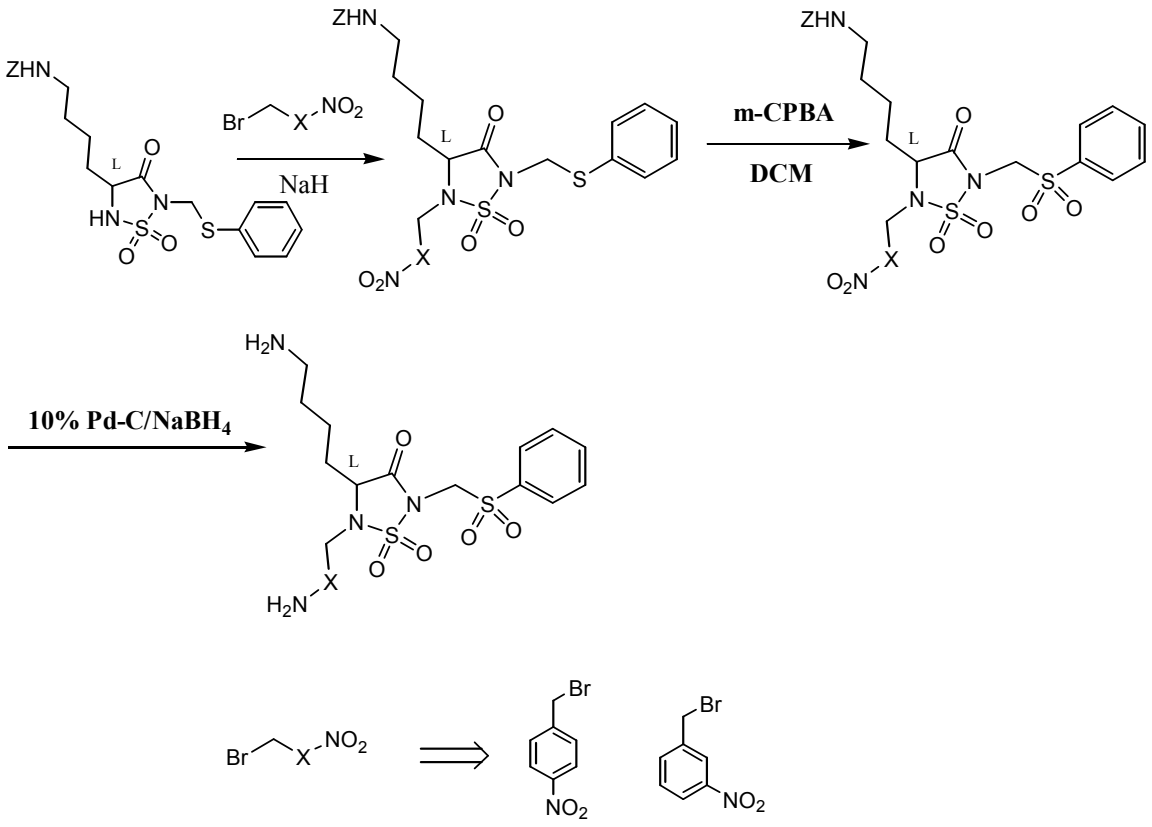


Fig 3.4 Synthesis of Inhibitor-Scheme B

Synthesis of Compound I-6

A mixture of di(t-butyl-dicarbonate (10.7 g; 49 mmol) in 1, 4-dioxane (40 mL) was added dropwise to a solution of 3-aminobenzyl alcohol (5 g; 40.6 mmol) and triethylamine (6.17 g; 61 mmol) in dioxane (50 mL) at 0^{o}C. The solution was allowed to stir in an ice bath for 0.5 h. The ice bath was removed and the reaction mixture was stirred overnight at room temperature. The solvent was evaporated and the residue was dissolved in water (15 mL), acidified with dilute HCl (pH 3-4), and extracted with ethyl acetate (3 x 100 mL). The combined organic extracts were dried over anhydrous sodium sulfate. The solvent was evaporated to give a crude product which was purified by flash chromatography (hexane/ethyl acetate gradient) to give pure product (5.91 g; 65 % yield).

Synthesis of Compound I-7

A mixture of di(t-butyl-dicarbonate (10.7 g; 49 mmol) in 1, 4-dioxane (40 mL) was added dropwise to a solution of 4-aminobenzyl alcohol (5 g; 40.6 mmol) and triethylamine (6.17 g; 61 mmol) in dioxane (50 mL) at 0^{o}C. The solution was allowed to stir in an ice bath for 0.5 h. The ice bath was removed and the reaction mixture was stirred overnight at room temperature. The solvent was evaporated and the residue was dissolved in water (15 mL), acidified with dilute HCl (pH 3-4) and, extracted with ethyl acetate (3 x 100 mL). The combined organic extracts were dried over anhydrous sodium sulfate. The solvent was evaporated to give a crude product which was purified by flash chromatography (hexane/ethyl acetate gradient) to give pure product (6.9 g; 76 % yield).

Synthesis of Compound I-8

A mixture of di(t-butyl-dicarbonate (12.65 g; 58 mmol) in 1, 4-dioxane (50 mL) was added dropwise to a solution of 5-aminopentanol (5 g; 48.4 mmol) and triethylamine (9.7 g; 96 mmol) in dioxane (50 mL) at 0^{o}C. The solution was allowed to stir in an ice bath for 0.5 h. The ice bath was removed and the reaction mixture was stirred overnight at room temperature. The solvent was evaporated and the residue was dissolved in water (15 mL), acidified with dilute HCl (pH 3-4) and, extracted with ethyl acetate (3 x 100 mL). The combined organic extracts were dried over anhydrous sodium sulfate. The solvent was evaporated to give a crude product which was purified by flash chromatography (hexane/ethyl acetate gradient) to give pure product (4.75 g; 48 % yield).

Synthesis of Compound I-9

To a solution of I-5 (3.0 g; 6.48 mmol) and I-6 (1.44 g; 6.48 mmol) dissolved in 50 mL of dry tetrahydrofuran was added triphenylphosphine (2.4 g; 9.0 mmol) and stirred for 10 minutes. Diethyl azodicarboxylate (1.56 g; 9.0 mmol) dissolved in 20 mL of dry tetrahydrofuran was added dropwise to the reaction mixture and the solution was stirred overnight at room temperature. The solvent was removed and the crude product was purified by flash chromatography (hexane/ ethyl acetate gradient) to give pure product (1.64 g; 38 % yield).

Synthesis of Compound I-10

To a solution of I-5 (3.0 g; 6.48 mmol) and I-7 (1.44 g; 6.48 mmol) dissolved in 50 mL of dry tetrahydrofuran was added triphenylphosphine (2.4 g; 9.0 mmol) and stirred for 10 minutes. Diethyl azodicarboxylate (1.56 g; 9.0 mmol) dissolved in 20 mL of dry tetrahydrofuran was added dropwise to the reaction mixture and the solution was stirred overnight at room temperature. The solvent was removed and the crude product was purified by flash chromatography (hexane/ ethyl acetate gradient) to give pure product (2.86 g; 66 % yield).

Synthesis of Compound I-11

To a solution of I-5 (3.0 g; 6.48 mmol) and I-8 (1.32 g; 6.48 mmol) dissolved in 50 mL of dry tetrahydrofuran was added triphenylphosphine (2.4 g; 9.0 mmol) and stirred for 10 minutes. Diethyl azodicarboxylate (1.56 g; 9.0 mmol) dissolved in 20 mL of dry tetrahydrofuran was added dropwise to the reaction mixture and the solution was stirred

overnight at room temperature. The solvent was removed and the crude product was purified by flash chromatography (hexane/ ethyl acetate gradient) to give pure product (3.89 g; 88 % yield).

Synthesis of Compound I-12

A solution of I-9 (0.52 g; 0.77 mmol) in dry methylene chloride (10 mL) was treated with m-chloroperbenzoic acid (0.6 g; 2.3 mmol) under nitrogen and the reaction mixture was stirred overnight at room temperature. The solvent was removed and the residue was dissolved in ethyl acetate (250 mL) and washed with saturated sodium bicarbonate (50 mL) and brine (50 mL). The organic layer was collected and dried over anhydrous sodium sulfate and the solvent was removed on the rotovac to give the crude product. The crude product was purified by flash chromatography (hexane/ethyl acetate gradient) to give pure product (0.42 g; 77 % yield)

Synthesis of Compound I-13

A solution of I-10 (0.52 g; 0.77 mmol) in dry methylene chloride (10 mL) was treated with m-chloroperbenzoic acid (0.6 g; 2.3 mmol) under nitrogen and the reaction mixture was stirred overnight at room temperature. The solvent was removed and the residue was dissolved in ethyl acetate (250 mL) and washed with saturated sodium bicarbonate (50 mL) and Brine (50 mL). The organic layer was collected and dried over anhydrous sodium sulfate and the solvent was removed on the rotovac to give the crude product. The crude product was purified by flash chromatography (hexane/ethyl acetate gradient) to give pure product (0.39 g; 72 % yield)

Synthesis of Compound I-14

A solution of I-11 (0.50 g; 0.77 mmol) in dry methylene chloride (10 mL) was treated with m-chloroperbenzoic acid (0.6 g; 2.3 mmol) under nitrogen and the reaction mixture was stirred overnight at room temperature. The solvent was removed and the residue was dissolved in ethyl acetate (250 mL) and washed with saturated sodium bicarbonate (50 mL) and Brine (50 mL). The organic layer was collected and dried over anhydrous sodium sulfate and the solvent was removed on the rotovac to give the crude product. The crude product was purified by flash chromatography (hexane/ethyl acetate gradient) to give pure product (0.25 g; 48 % yield)

Synthesis of Compound I-15

To a solution of I-5 (5.0 g; 10.8 mmol) and 4-nitrobenzyl bromide (4.7 g; 21.6 mmol) dissolved in 75 mL of dry acetonitrile and kept in an ice bath, 60 % sodium hydride (0.64 g; 16 mmol) was added slowly and the reaction mixture was stirred overnight at room temperature. The solvent was removed and the residue was dissolved in ethyl acetate (250 mL) and washed with water (50 mL) followed by 5 % aqueous HCl (50 mL) and finally washed with brine (50 mL). The organic layer was collected and dried over anhydrous sodium sulfate. The solvent was removed on the rotovac to give the crude product. The crude product was purified by flash chromatography (hexane/ethyl acetate gradient) to give pure product (6.08 g; 94 % yield)

Synthesis of Compound I-16

To a solution of I-5 (2.5 g; 5.4 mmol) and 3-nitrobenzyl bromide (2.33 g; 10.8 mmol) dissolved in 40 mL of dry acetonitrile and kept in an ice bath, 60 % sodium hydride (0.32 g; 8 mmol) was added slowly and the reaction mixture was stirred overnight at room temperature. The solvent was removed and the residue was dissolved in ethyl acetate (250 mL) and washed with water (50 mL) followed by 5 % aqueous HCl (50 mL) and finally washed with brine (50 mL). The organic layer was collected and dried over anhydrous sodium sulfate. The solvent was removed on the rotovac to give the crude product. The crude product was purified by flash chromatography (hexane/ethyl acetate gradient) to give pure product (2.49 g; 77 % yield)

Synthesis of Compound I-17

A solution of I-15 (6.08 g; 10.15 mmol) in dry methylene chloride (60 mL) was treated with m-chloroperbenzoic acid (7.52 g; 30.5 mmol) under nitrogen and the reaction mixture was stirred overnight at room temperature. The solvent was removed and the residue was dissolved in ethyl acetate (500 mL) and washed with saturated sodium bicarbonate (2 x 100 mL) and brine (2 x 100 mL). The organic layer was collected and dried over anhydrous sodium sulfate and the solvent was removed on the rotovac to give the crude product. The crude product was purified by flash chromatography (hexane/ethyl acetate gradient) to give pure product (5.25 g; 82 % yield)

Synthesis of Compound I-18

A solution of I-16 (2.49 g; 4.2 mmol) in dry methylene chloride (30 mL) was treated with m-chloroperbenzoic acid (3.1 g; 12.5 mmol) under nitrogen and the reaction mixture was stirred overnight at room temperature. The solvent was removed and the residue was dissolved in ethyl acetate (400 mL) and washed with saturated sodium bicarbonate (2 x 100 mL) and brine (2 x 100 mL). The organic layer was collected and dried over anhydrous sodium sulfate. The solvent was removed on the rotovac to give the crude product. The crude product was purified by flash chromatography (hexane/ethyl acetate gradient) to give pure product (2.18 g; 82 % yield)

Synthesis of Compound I-19

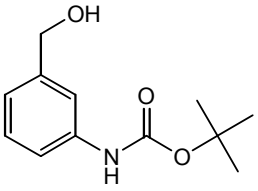
A solution of I-17 (1.89 g; 3 mmol) in dry tetrahydrofuran (15 mL) was kept in an ice bath and 10 % Palladium-Carbon (0.15 g) was added slowly under nitrogen atmosphere. Sodium borohydride (0.28 g, 7.5 mmol) was added slowly in 3 portions over a period of 10 minutes. The ice bath was removed and the reaction mixture was stirred at room temperature for a period of 0.5 h and the progress of the reaction was monitored by TLC. The reaction was stopped when the starting material disappeared. The residue was removed by filtration through a Celite pad. The filtrate was collected and kept in an ice bath and acidified with 6 N HCl until pH=3. The filtrate was then treated with saturated sodium bicarbonate solution until pH=7. The filtrate was then extracted with ethyl acetate (2 x 200 mL). The combined organic extracts were collected and dried over anhydrous sodium sulfate. The solvent was removed on the rotovac to give the crude product. The

crude product was washed with ether to give pure product as a yellow hygroscopic solid (1.38 g; 73 % yield)

Synthesis of Compound I-20

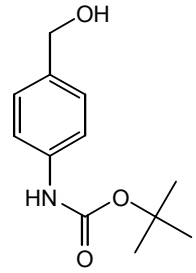
A solution of I-18 (1.89 g; 3 mmol) in dry tetrahrofuran (15 mL) was kept in an ice bath and 10 % Palladium-Carbon (0.15 g) was added slowly under nitrogen atmosphere. Sodium borohydride (0.28 g, 7.5 mmol) was added slowly in 3 portions over a period of 10 minutes. The ice bath was removed and the reaction mixture was stirred at room temperature for a period of 0.5 h and the progress of the reaction was monitored by TLC. The reaction was stopped when the starting material disappeared. The residue was removed by filtration through a Celite pad. The filtrate was collected and kept in an ice bath and acidified with 6 N HCl until pH=3. The filtrate was then treated with saturated sodium bicarbonate solution until pH=7. The filtrate was then extracted with ethyl acetate (2 x 200 mL). The combined organic extracts were dried over anhydrous sodium sulfate. The solvent was removed on the rotovac to give the crude product. The crude product was washed with ether to give pure product as a white hygroscopic solid (1.34 g; 71 % yield)

Fig 3.5 Structures of Compounds I-6 to I-14



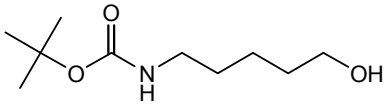
Compound I-6

$C_{12}H_{17}NO_3$
Mol. Wt.: 223.27



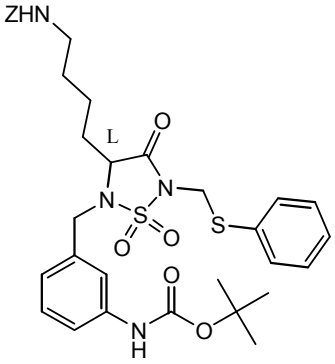
Compound I-7

$C_{12}H_{17}NO_3$
Mol. Wt.: 223.27



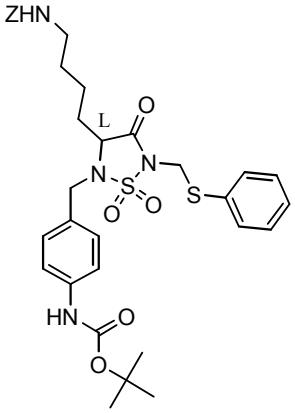
Compound I-8

$C_{10}H_{21}NO_3$
Mol. Wt.: 203.28



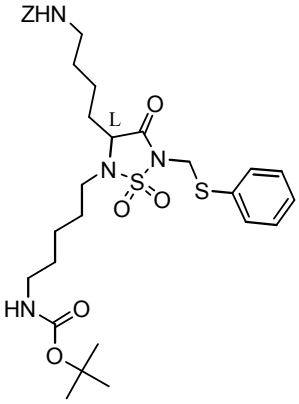
Compound I-9

$C_{33}H_{40}N_4O_7S_2$
Mol. Wt.: 668.83



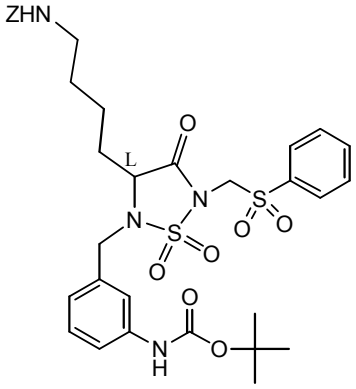
Compound I-10

$C_{33}H_{40}N_4O_7S_2$
Mol. Wt.: 668.83



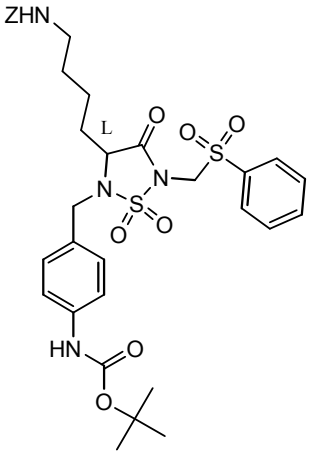
Compound I-11

$C_{31}H_{44}N_4O_7S_2$
Mol. Wt.: 648.84



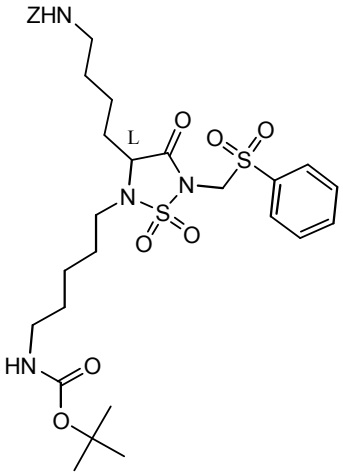
Compound I-12

$C_{33}H_{40}N_4O_9S_2$
Mol. Wt.: 700.82



Compound I-13

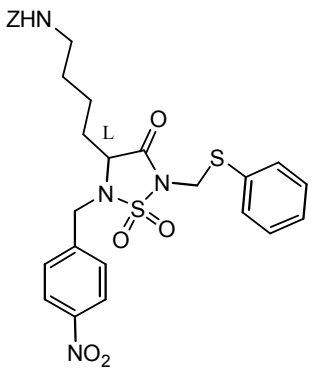
$C_{33}H_{40}N_4O_9S_2$
Mol. Wt.: 700.82



Compound I-14

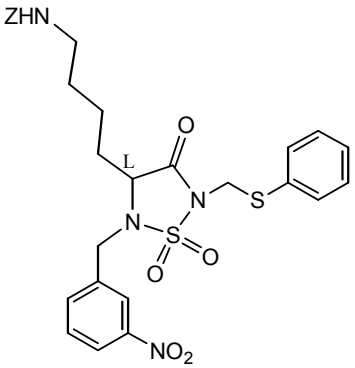
$C_{31}H_{44}N_4O_9S_2$
Mol. Wt.: 680.83

Fig 3.6 Structures of Compounds I-15 to I-20



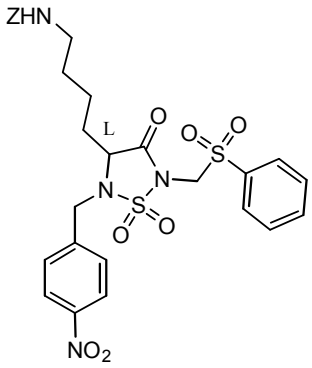
Compound I-15

$C_{28}H_{30}N_4O_7S_2$
Mol. Wt.: 598.69



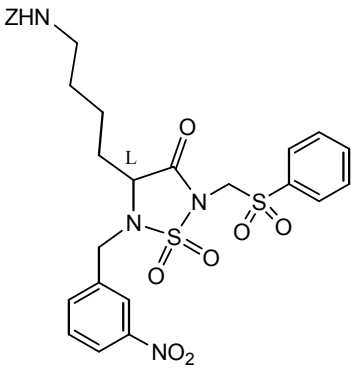
Compound I-16

$C_{28}H_{30}N_4O_7S_2$
Mol. Wt.: 598.69



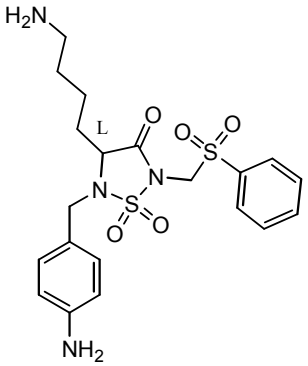
Compound I-17

$C_{28}H_{30}N_4O_9S_2$
Mol. Wt.: 630.69



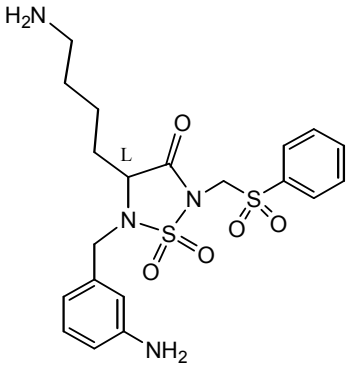
Compound I-18

$C_{28}H_{30}N_4O_9S_2$
Mol. Wt.: 630.69



Compound I-19

$C_{20}H_{26}N_4O_5S_2$
Mol. Wt.: 466.58



Compound I-20

$C_{20}H_{26}N_4O_5S_2$
Mol. Wt.: 466.58

Table 3.1 Physical and Spectral Data of Compounds

Compound	Formula	M.W g/mol	M.P (^{0}C)	1H-NMR Data (δ ppm)
I-1	$C_{15}H_{23}ClN_2O_4$	330.81	110-112	[$CDCl_3$]: 1.52 (bs,4H); 2.03 (bs,2H); 3.16 (bs,2H); 3.74 (s,3H); 4.13 (bs,1H); 5.05 (s,2H); 5.53 (bs,1H); 7.38 (s,5H)
I-2	$C_{20}H_{31}N_3O_8S$	473.54	Oil	[$CDCl_3$]: 1.41-1.55 (m,4H); 1.48 (s,9H); 1.7-1.8 (m,2H); 3.18 (m,2H); 3.75 (s,3H); 4.17 (m,1H); 4.85 (t,1H); 5.1 (s,2H); 7.35 (s,5H)
I-3	$C_{15}H_{23}N_3O_6S$	373.43	83-84	[$CDCl_3$]: 1.41-1.55 (m,4H); 1.73 (m,1H); 1.81 (m,1H); 3.19 (m,2H); 3.75 (s,3H); 4.17 (m,1H); 4.9 (d,2H); 5.15 (s,2H); 5.75 (d,1H); 7.35 (s,5H)
I-4	$C_{14}H_{19}N_3O_5S$	341.38	77-81	[$CDCl_3$]: 1.41-1.55 (m,4H); 1.73 (m,1H); 1.81 (m,1H); 3.19 (m,2H); 4.18 (m,1H); 5.1 (d,2H); 5.2 (t,1H); 6.36 (bs,1H); 7.35 (s,5H)
I-5	$C_{21}H_{25}N_3O_5S_2$	463.57	83-84	[$CDCl_3$]: 1.25-1.32 (m,2H); 1.34-1.49 (m,2H); 1.76 (m,1H); 1.92 (m,1H); 3.2 (m,2H); 4.13 (m,1H); 5.06 (dd,2H); 5.12 (dd,2H); 6.2 (bs,1H); 7.29-7.36 (m,8H); 7.56 (m,2H)
I-6	$C_{12}H_{17}NO_3$	223.27	82-84	[$CDCl_3$]: 1.49 (s,9H); 2.96 (bs,1H); 4.56 (s,2H); 6.81 (s,1H); 6.97 (t,1H); 7.21 (d,2H); 7.35 (s,1H)
I-7	$C_{12}H_{17}NO_3$	223.27	83-85	[$CDCl_3$]: 1.5 (s,9H); 2.82 (bs,1H); 4.56 (s,2H); 6.92 (s,1H); 7.2 (d,2H); 7.3 (d,2H)
I-8	$C_{10}H_{21}NO_3$	203.28	Oil	[$CDCl_3$]: 1.36-1.61 (m,6H); 1.44 (s,9H); 1.85 (bs,1H); 3.13 (m,2H); 3.64 (t,2H); 4.6 (bs,1H)

Table 3.1 Physical and Spectral Data of Compounds continued

Compound	Formula	M.W g/mol	M.P (^{0}C)	1H-NMR Data (δ ppm)
I-9	$C_{33}H_{40}N_4O_7S_2$	668.63	Oil	[$CDCl_3$]: 1.25-1.32 (m,2H); 1.35-1.49 (m,2H); 1.5 (s,9H); 1.83 (m,2H); 3.1 (m,2H); 3.8 (t,1H); 4.2-4.4 (dd,2H); 4.9 (d,2H); 5.16 (s,2H); 6.9 (s,1H); 7.1 (d,2H); 7.2-7.4 (m,10H); 7.6 (m,2H)
I-10	$C_{33}H_{40}N_4O_7S_2$	668.63	Oil	[$CDCl_3$]: 1.2-1.3 (m,4H); 1.5 (s,9H); 1.62 (m,1H); 1.82 (m,1H); 3.15 (m,2H); 3.9 (t,1H); 4.3-4.5 (dd,2H); 4.9 (t,1H); 5.05 (s,2H); 5.15 (s,2H); 7.3-7.4 (m,10H); 7.6 (d,2H); 8.2 (d,2H)
I-11	$C_{31}H_{44}N_4O_7S_2$	648.84	Oil	[$CDCl_3$]: 1.25-1.45 (m,8H); 1.4 (s,9H); 1.62- 1.82 (m,4H); 3.15 (m,2H); 3.9 (t,1H); 4.98 (s,2H); 5.05 (s,2H); 7.3-7.4 (m,8H); 7.58 (m,2H)
I-12	$C_{33}H_{40}N_4O_9S_2$	700.82	Oil	[$CDCl_3$]: 1.25-1.49 (m,4H); 1.5 (s,9H); 1.83 (m,2H); 2.9 (m,1H); 3.15-3.2 (m,4H); 3.22(m,1H); 3.81 (t,1H); 4.9 (d,2H); 5.16 (s,2H); 6.9 (s,1H); 7.1 (d,2H); 7.2-7.4 (m,10H); 7.6 (m,2H)
I-13	$C_{33}H_{40}N_4O_9S_2$	700.82	Oil	[$CDCl_3$]: 1.2-1.3 (m,4H); 1.5 (s,9H); 1.62 (m,1H); 1.82 (m,1H); 3.15 (m,2H); 3.9 (t,1H); 4.3-4.5 (dd,2H); 4.9 (s,2H); 5.15 (s,2H); 7.3-7.4 (m,10H); 7.6 (d,2H); 8.2 (d,2H)
I-14	$C_{31}H_{44}N_4O_9S_2$	680.83	Oil	[$CDCl_3$]: 1.3-1.5 (m,6H); 1.42 (s,9H); 1.62 -1.98 (m,6H); 3.1-3.2 (m,4H); 3.33 (p,2H); 3.92 (t,1H); 4.87 (d,2H); 5.08 (s,2H); 7.34 (m,5H); 7.6 (m,3H); 7.97 (m,2H)

Compound	Formula	M.W g/mol	M.P (^{0}C)	1H-NMR Data (δ ppm)
I-15	$C_{28}H_{30}N_4O_7S_2$	598.69	Oil	[$CDCl_3$]: 1.15 (m,2H); 1.31 (m,2H); 1.62 (m,1H); 1.82 (m,1H); 3.15 (m,2H); 3.81 (t,1H); 4.45 (dd,2H); 4.77 (t, 1H); 4.91 (d,2H); 5.1 (s,2H); 7.35 (s,8H); 7.5 (m,4H); 8.2 (d,2H)
I-16	$C_{28}H_{30}N_4O_7S_2$	598.69	Oil	[$CDCl_3$]: 1.15 (m,2H); 1.3 (m,2H); 1.7 (m,1H); 1.82 (m,1H); 3.15 (m,2H); 3.82 (t,1H); 4.4 (dd,2H); 4.99 (d,2H); 5.1 (s,2H); 7.34 (s,7H); 7.56 (m,4H); 7.74 (d,1H); 8.17 (d,1H); 8.23 (s,1H)
I-17	$C_{28}H_{30}N_4O_9S_2$	630.69	94-95	[$CDCl_3$]: 1.2-1.4 (m,4H); 1.72 (m,1H); 1.83 (m,1H); 3.12 (m,2H); 3.93 (t,1H); 4.45 (dd,2H); 4.79 (t,1H); 4.9 (s,2H); 5.07 (s,2H); 7.33 (s,5H); 7.58 (t,3H); 7.71 (t,2H); 7.97 (d,2H); 8.24 (d,2H)
I-18	$C_{28}H_{30}N_4O_9S_2$	630.69	84-87	[$CDCl_3$]: 1.2-1.39 (m,4H); 1.71 (m,1H); 1.83 (m,1H); 3.11 (m,2H); 3.94 (t,1H); 4.44 (dd,2H); 4.78 (s,1H); 4.9 (s,2H); 5.07 (s,2H); 7.55 (s,6H); 7.59 (t,3H); 7.68 (dd,2H); 7.97 (d,2H); 8.22 (d,2H)
I-19	$C_{20}H_{26}N_4O_5S_2$	466.58	Hygroscopic solid	[$CDCl_3$]: 1.24-1.42 (m,6H); 3.08 (m,2H); 3.82 (t,1H); 4.52 (dd,2H); 5.06 (s,2H); 7.32 (s,5H); 7.62 (d,2H); 8.16 (d,2H)
I-20	$C_{20}H_{26}N_4O_5S_2$	466.58	Hygroscopic solid	[$CDCl_3$]: 1.24-1.42 (m,6H); 3.1 (m,2H); 3.85 (t,1H); 4.5 (dd,2H); 5.07 (s,2H); 7.26-7.34 (s,5H); 7.51 (t,1H); 7.84 (d,1H); 8.11(d,1H); 8.28 (s,1H)

Chapter 4

RESULTS AND DISCUSSION

4.1 Inhibitor Design Rationale

The substrate specificity studies of the NS3 protease show that the enzyme prefers a pair of basic residues (Lys/Arg) at P_1 and P_2 positions. Recently a tetrapeptide aldehyde (Bz-Nle-Lys-Arg-Arg-CHO) was reported to inhibit Dengue Virus NS3 protease with K_i=5.8 uM[26]. The study shows that presence of the two basic residues is required in order to have effective inhibition. Based on the substrate specificity studies we were able to synthesize potential inhibitors of Dengue Virus and West Nile Virus protease by having a pair of basic resiudes (Lys) at P_1 and P_2 positions. At pH=7 the free amine groups of the inhibitor (I) side chains are protonated and can therefore strongly interact with the acidic groups present in the S1 and S2 pocket (Fig 4.1). The 1,2,5-thiadiazolidin-3-one 1,1 dioxide scaffold is known to dock in the active site of the target protease like a substrate and orient the recognition groups towards the subsites of the enzyme and was therefore used to synthesize the inhibitors.

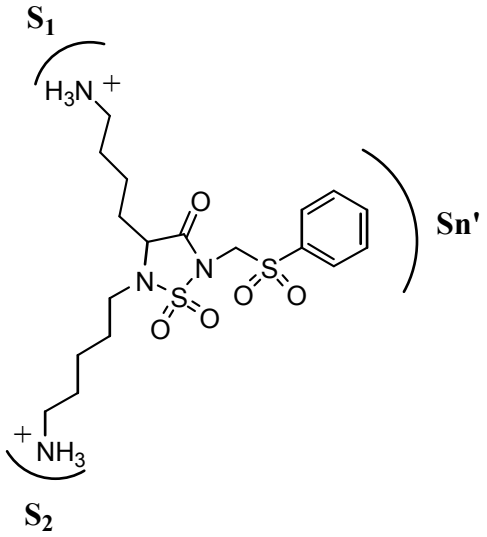


Fig 4.1 Interaction of the Inhibitor (I) with NS3 Protease

4.2 Synthesis of Inhibitors

Since the enzyme has a preference for basic side chain residues, L-Lysine (Z)-OH was used as starting material. L-Lysine (Z)-methyl ester hydrochloride was treated with the solution of N-chlorosulfonyl isocyanate and t-butyl alcohol in the presence of triethylamine. The removal of Boc protective group was followed by cyclization using sodium hydride generated the desired 1,2,5-thiadiazolidin-3-one 1,1 dioxide scaffold (compound I-5). In order to synthesize the inhibitors, initially compound I-5 was reacted with different Boc-protected amino alcohols using the Mitsunobu reaction. The resulting sulfides were then oxidized to the sulfones using m-CPBA and the inhibitors would be obtained by removal of the amine protecting groups (Fig 3.3 Scheme A). The Mitsunobu reaction gave very low yields and hence an alternative scheme was used to synthesize the potential inhibitors. In Scheme B (Fig 3.4), compound I-5 was alkylated with 4-nitro benzyl bromide and 3-nitobenzyl bromide. The resulting sulfides were oxidized to the corresponding sulfones using m-CPBA and the nitro group was reduced to the amine in

the presence of sodium borohydride and 10% Palladium-carbon[33]. The reduction reaction was very effective in reducing the nitro group and at the same time was able to remove the Cbz-protecting group. The alternate method gave high yields in each step and the reduction reaction was an effective method not only to reduce the nitro group but also to remove the amine protective group. The three inhibitors described in this thesis were synthesized using Scheme A and Scheme B.

Chapter 5

INHIBITORS OF HUMAN TRYPTASE BASED ON TEMPLATE (II)

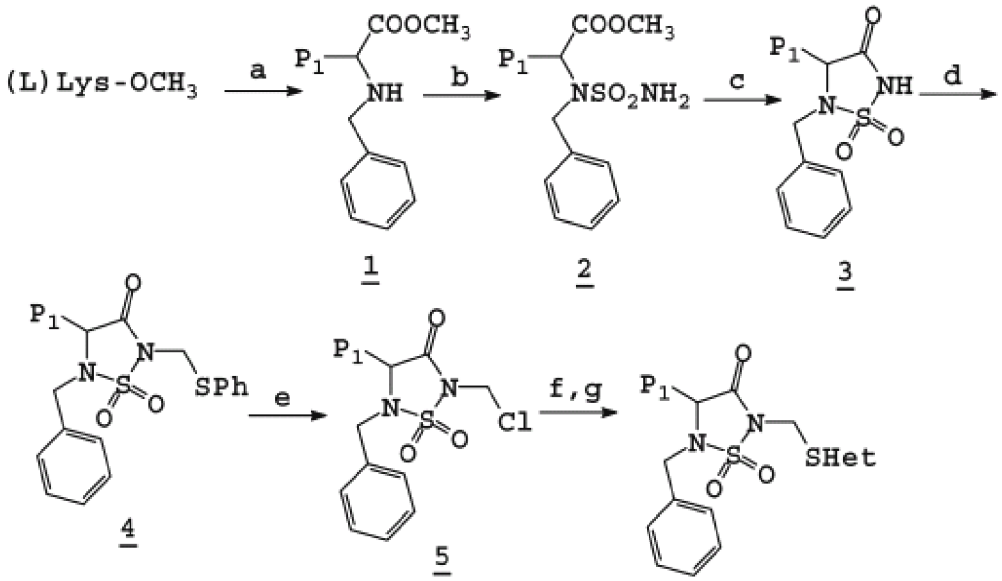
5.1 Inhibitor Design

Human tryptase is a trypsin-like serine protease that is stored in the cytoplasmic granules of mast cells and is released upon activation along with other pro-inflammatory substances. The uncontrolled release of human tryptase has been associated with a number of allergic diseases such as asthma, psoriasis, chronic pancreatitis and other inflammatory diseases. Hence, tryptase is a validated target for the development of therapeutic agents for such diseases.

Previous studies have shown that mechanism-based inhibitors based on the 1,2,5-thiadiazolidin-3-one 1,1 dioxide template are highly effective in inactivating (chymo) trypsin-like serine proteases[31]. Since the preferred P_1 residue for tryptase is Lys, we reasoned that compound (II) (Fig 5.1) where P_1= $NH_2(CH_2)_4$ may serve as inhibitors of tryptase. The inhibitor was designed in a way that it binds to the target enzyme and orients the appropriate recognition elements towards the enzyme S and S' subsites in order to achieve effective binding. The enzyme selectivity can be optimized by exploiting differences in the S' subsites of closely related enzymes

5.2 Synthesis of Inhibitors of Human Tryptase

The synthesis of inhibitors was accomplished by former members of the group using L-lysine methyl ester as starting material and different commercially available heterocyclic thiols as leaving groups[32], using the reaction sequence shown below (Fig 5.1).



Template (II)

[a]Benzaldehyde/$NaBH(OAc)_3$/HOAc; [b]$ClSO_2NH_2$/TEA; [c]NaH/THF [d]$ClCH_2SPh$/TEA; [e]SO_2Cl_2; [f]HetSH/TEA; [g]$(CH_3)_3SiI$

P1= $ZNH(CH_2)_4$ and CH_2SHet =L (Template II where L= leaving group)

Fig 5.1 Synthesis of 1, 2, 5-Thiadiazolidin-3-one 1, 1 Dioxide-based Heterocyclic Sulfides[32]

Table 5.1 List of Inhibitors with Different Heterocyclic Thiols as Leaving Groups

P1	SHet
$ZNH(CH_2)_4$	2-mercaptobenzoxazolyl (6)
$ZNH(CH_2)_4$	5-phenyl-1,3,4-oxadiazole-2-mercapto(7)
$ZNH(CH_2)_4$	3-phenyl-1,2,4-oxadiazole-5-mercapto(8)
$NH_2(CH_2)_4$	2-mercaptobenzoxazolyl (9)
$NH_2(CH_2)_4$	5-phenyl-1,3,4-oxadiazole-2-mercapto(10)
$NH_2(CH_2)_4$	3-phenyl-1,2,4-oxadiazole-5-mercapto(11)

5.3 Biochemical Studies

5.3.1 *In vitro* Biochemical Studies Using Human Tryptase

5.3.1.1 Time-dependent Loss of Enzyme Activity

Human lung tryptase was assayed by mixing 10 µL of 1.04 µM enzyme solution (in 0.05 M TRIS-HCl buffer, pH 7.62 containing 0.12 M NaCl and 0.1 mg/mL heparin) ($[E]_f$ = 10.4 nM), 100 µL dimethyl sulfoxide and 890 µL 0.05 M TRIS-HCl buffer, pH 7.62, in a thermostat test tube. A 100 µL aliquot was transferred to a thermostat cuvette containing 890 µL of 0.05 M TRIS-HCl buffer, pH 7.62 and 10 µL of 10 mM solution of N-p-Tosyl-Gly-Pro-Lys-pNA ($[S]_f$ = 0.1 mM), and the change in absorbance was monitored at 410 nm for one minute. In a typical inhibition run, 10 µL of a 20.8 µM inhibitor solution in dimethyl sulfoxide was mixed with 90 µL dimethyl sulfoxide, 10 µL of 1.04 µM enzyme solution and 890 µL of 0.05 M TRIS-HCl buffer, pH 7.62 and placed in a constant temperature bath. Aliquots (100 µL) were withdrawn at different time intervals and transferred to a cuvette containing 10 µL of 10 mM solution of N-p-Tosyl-Gly-Pro-Lys-pNA and 890 µL of 0.05 M TRIS-HCl buffer, pH 7.62. The absorbance was monitored at 410 nm for one minute. The remaining activity of tryptase was expressed as % remaining activity = (v/v_o) X 100 and is the average of duplicate or triplicate determinations. The pseudo first-order deacylation rate constant (k_{deacyl}) was obtained by determining the slope of the semi logarithmic plot of enzymatic activity remaining versus time, using equation (1) where $[E_t]/[E_o]$ is the amount of enzyme remaining at time t. These are the average of two or three determinations.

$$\ln([E_t]/[E_o]) = k_{deacyl} \text{ x } t \qquad (1)$$

5.3.1.2 Progress Curve Method

The apparent second-order rate constants k_{inact}/K_I M^{-1} s^{-1} for inhibitors 9-11 listed in Table 5.2 were determined using progress curve method. Thus, in a typical run 40 µL of a 0.104 µM human lung tryptase solution ($[E]_f$ =4.16 nm) was added to a solution containing 10 µL of inhibitor (20.8 µM solution in dimethyl sulfoxide), 50 µL substrate (10 mM solution of N-p-Tosyl-Gly-Pro-Lys-pNA) ($[S]_f$ = 0.5 mM) and 900 µL of 0.05 M TRIS-HCl buffer, pH 7.62, containing 0.12 M NaCl and 0.1 mg/mL heparin, and the absorbance was continuously monitored at 410 nm for 1000s. Control progress curves in the absence of inhibitor are linear. The pseudo first-order rate constant k_{obs}, for the inhibition of tryptase as a function of time were determined according to equation (2) shown below, where A = absorbance at 410 nm, v_o= reaction velocity when t = 0, v_s = final steady-state velocity, k_{obs} = observed first-order rate constant, and A_o= absorbance at t = 0. The k_{obs} was determined by fitting the A~t data into equation (2) and performing non-linear regression analysis using Sigma Plot, Jander Scientific. The second order rate constants k_{inact}/K_I were determined in duplicate or triplicate by calculating $k_{obs}/[I]$ and then correcting for the substrate concentration and Michealis-Menten constant using equation (3) given below.

$$A = v_s t + \{(v_o - v_s)(1 - e^{-k_{obs} t})/k_{obs}\} + A_o \qquad (2)$$

$$k_{obs}/[I] = k_{inact}/K_I \{1 + [S]/K_m\} \qquad (3)$$

5.3.2 *In vitro* Biochemical Studies Using Bovine Trypsin

5.3.2.1 Inhibition Experiment

In a typical inhibition experiment, 10 μL of 8.4 μM bovine trypsin was incubated with 10 μL of a 2.1 mM solution of inhibitor in dimethyl sulfoxide at an [I]/[E] ratio of 250, and 970 μL of 0.025 M phosphate buffer, pH 7.51, containing 0.1 M NaCl at 25^{o}C. After the solution was incubated for 0.5 hr, 10 μL of 50 mM of N-p-Tosyl-Gly-Pro-Lys-pNA was added and the amount of active enzyme was determined by monitoring the release of p-nitoaniline at 410 nm for four minutes. A control experiment in the absence of inhibitor, containing 10 μL of 8.4 μM bovine trypsin, 10 μL of dimethyl sulfoxide and 970 μL of 0.025 M phosphate buffer containing 0.1 M NaCl , pH 7.51, was run under identical conditions. The remaining activity of enzyme was expressed as % remaining activity = (v/v_o) X 100 and is an average of duplicate or triplicate determinations.

5.3.2.2 Progress Curve Method

The apparent second-order rate constants k_{inact}/K_I M^{-1} s^{-1} for inhibitors 9-11 listed in Table 5.2 were determined using progress curve method. Thus, in a typical run 5 μL of an 8.4 μM bovine trypsin solution was added to a solution containing 10 μL of inhibitor (0.21 μM solution in dimethyl sulfoxide), 10 μL substrate (50 mM solution of N-p-Tosyl-Gly-Pro-Lys-pNA) and 975 μL of 0.025 M phosphate buffer, pH 7.51, containing 0.10 M NaCl, and the absorbance was continuously monitored at 410 nm for 600s. Control progress curves in the absence of inhibitor are linear. The k_{inact}/K_I values were extracted from the data in the same manner as described above for human tryptase.

5.4 Results and Discussion

We have described that 1,2,5-thiadiazolidin-3-one 1,1 dioxide-based heterocyclic sulfides can be used as potent mechanism-based inhibitors of human tryptase. The biochemical studies show that compounds **9-11** are potent inhibitors of human tryptase (Table 5.2). The progress curves for the inhibition of tryptase by compound **9** are given in Fig 5.2. These compounds were also found to inhibit bovine trypsin to a certain extent (Table 5.2). Though the enzyme selectivity is low, the differences in the S' subsites of the two enzymes can be exploited to optimize selectivity. As expected, these inhibitors are devoid of any inhibitory activity toward the neutral serine protease elastase and also with papain, a prototypical cysteine protease.

Table 5.2 Inhibitory Activity of Compounds 9-11[32]

Compound	k_{inact}/K_I M^{-1} s^{-1}	
	Tryptase	**Trypsin**
9	49,600	4,180
10	69,200	10,600
11	112,000	36,500

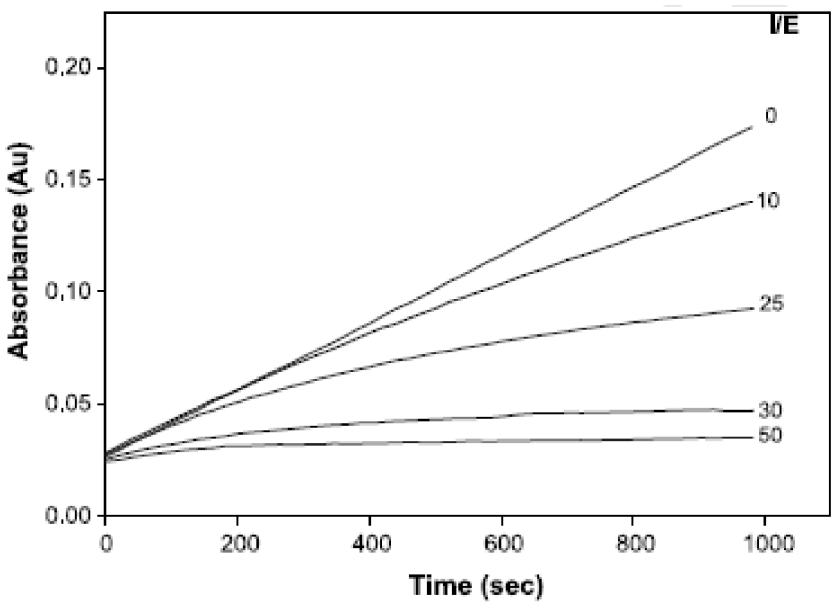


Fig 5.2 Progress Curves for Inhibition of Human Tryptase by Compound 9[32]

The incubation of compound **9** with tryptase, leads to rapid, time-dependent loss of enzyme activity (Fig 5.3). The enzyme regained activity slowly but fully after 24 hours with a half-life of reactivation of about 5 hours. Since tryptase regains activity after 24 hours, it suggests that the mechanism of action of these inhibitors involves the formation of an acyl enzyme that undergoes slow deacylation. From previous studies[31], we can propose that acyl enzyme arises from a nucleophilic attack of the active site serine-195 on the carbonyl carbon of the inhibitor to form a tetrahedral intermediate which collapses to form a sulfonyl imine and subsequent hydrolysis of the imine leads to the acyl enzyme shown in Fig 5.4.

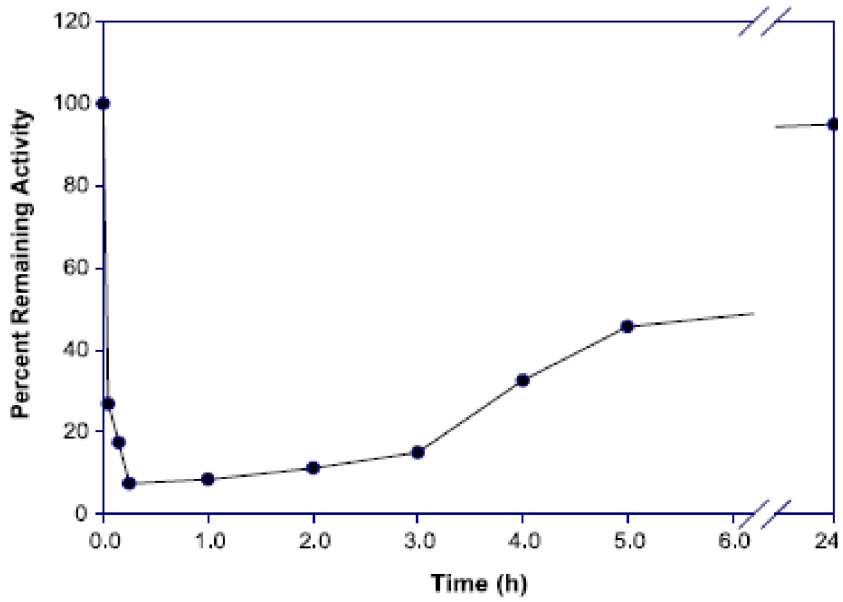


Fig 5.3 Time-dependent Loss of Enzyme Activity of Tryptase when Incubated with Excess of Compound 9[32]

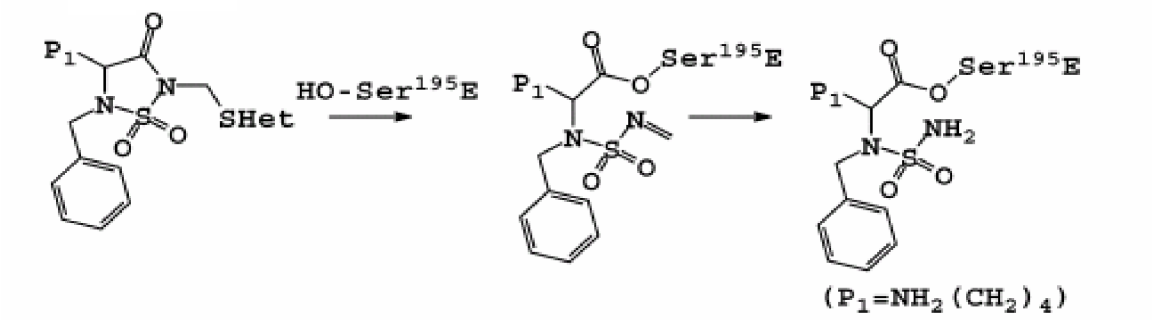


Fig 5.4 Proposed Mechanism of Action of Inhibitor II[32]

Chapter 6

CONCLUSIONS

In this thesis, the design and synthesis of potential inhibitors of Dengue Virus and West Nile Virus is described. The 1,2,5-thiadiazolidin-3-one 1,1 dioxide-based scaffold was used to synthesize inhibitors based on Template (I) and demonstrate that inhibitors based on Template (II) are potent inhibitors of Human tryptase. Inhibitors based on Template (II) are mechanism-based, time-dependent inhibitors of Human tryptase. The thesis also describes how the 1,2,5-thiadiazolidin-3-one 1,1 dioxide scaffold can be used to generate potential and potent inhibitors of (chymo)trypsin-like serine proteases based on the differences in the S and S' subsites.

REFERENCES

REFERENCES

1. Dunn B.M **(1992)** *Reaction Mechanisms of the Proteolytic Enzymes* in **Advances in Detailed Reaction Mechanisms, Vol. 2:213-241,** JAI Press Inc.

2. Powers C. J, Asgian L. J, Ekici D. O and James E. K **(2002)** *Irreversible Inhibitors of Serine, Cysteine and Threonine Proteases.* **Chem Rev. 102: 4639-4750**

3. Zhong J and Groutas W.C. **(2004)** *Recent Developments in the Design of Mechanism-based and Alternate Substrate Inhibitors of Serine Proteases.* **Current Topics in Medicinal Chemistry, 4: 1203-1216**

4. Schechter I and Berger A **(1967)** *On the Size of the Active Site in Proteases. I. Papain.* **Biochem Biophys Res Commun: 27(2) 157-162**

5. Proteinase and Peptidase Inhibition-Recent Potential Targets for Drug Development. **Edited by H. J. Smith and Claire Simons**

6. Silverman R.B **(1992)** *The Organic Chemistry of Drug Design and Drug Action* **Academic press: NY**

7. Silverman R.B. **(2000)** *The Organic Chemistry of Enzyme-Catalyzed Reactions* **Academic press: San Diego**

8. Leung Donmienne , Abbenante Giovanni and Fairlie P. David **(2000)** *Protease Inhibitors: Current Status and Future Prospects.* **J Med Chem. 43(3): 305-341**

9. Giovanni Abbenante and David P. Fairlie **(2005)** *Protease Inhibtiors in the Clinic.* **Medicinal Chemistry, 1: 71-104**

10. Abrahamson S.L, Malech H.L and Gallin J.I **(1991)** *Neutrophils in* **The Lung: Scientific Foundations, Vol.1 553-563**, Raven Press, New York

11. *Mast Cells and Basophils* **(2000)** Edited by Gianni Marone, Lawrence M. Lichenstein and Stephen J. Galli- Academic Press

12. Edwards D.P and Bernstein R.P **(1994)** *Synthetic Inhibitors of Elastase* **Medical Research Reviews, Vol.14, No.2, 127-194**

13. Sina S, Watorek W, Karr S, Giles J, Bode W, and Travis J **(1987)** *Primary Structure of Human Neutrophil Elastase.* **Proc. Natl. Acad. Sci. 84: 2228-2232**

14. Green B.G, Weston H, Ashe B.M, Doherty J, Finke P, Hagmann W, Lark M, Mao J, Maycock A, Moore V, Mumford R, Shah S, Walakovits L and Knight W.B **(1991)** *Polymorphonuclear Elastases: A Comparison of the Specificity of Human Isozymes and the Enzyme from Other Species Towards Substrates and Inhibitors.* **Arch. Biochem. Biophys 286(1): 284-292**

15. Bruce E. Maryanoff **(2004)** *Inhibitors of Serine Proteases as Potential Therapeutic Agents: The Road from Thrombin to Tryptase to Cathepsin G.* **J. Med. Chem Vol. 47(4): 769-787**

16. Campanelli D, Melchior M, Fu Y, Nakata M, Shuman H, Nathan C and Gabay J.E **(1990) J.Exp. Med 172: 1709-1715**

17. Hof P, Mayr I, Huber R, Korzus E, Potempa J, Travis J, Powers J.C, Bode W **(1996)** *The 1.8 A^o Crystal Structure of Human Cathepsin G in Complex with Succ-Val-Pro-Phe^P-$(OPh)_2$: a Janus-faced Proteinase with Two Opposite Specificities.* **EMBO J. 15: 5481-5491**

18. Navia et al **(1989)** *Structure of Human Leukocyte Elastase in Complex with a Peptide Chloromethyl Ketone at 1.84 A^o Resolution.* **PNAS-USA. 86:7-11**

19. Alter S.C, Kramps J.A, Janoff A, Schwartz L.B **(1990)** *Interactions of Human Mast Tryptase with Biological Protease Inhibitors.* **Arch. Biochem.Biophys. 276:26-31**

20. Brubaker M.J, Groutas W.C, Hoidal J.R and Rao N.V **(1992)** *Human Neutrophil Proteinase-3: Mapping of the Substrate Binding Site Using Peptidyl Thiobenzyl Esters.* **Biochem. Biophys. Res. Commun. 188(3): 1318-1324**

21. Combrink D. Keith, Gulgeze B.H, Meanwell A.N, Pearce C. B, Zulan P, Bisacchi S.G, Roberts G.M.D, Stanley P and Seiler M.S **(1998)** *1,2-Benzisothiazol-3-one 1,1-Dioxide Inhibitors of Human Mast Cell Tryptase.* **J. Med. Chem, 41:4854-4860**

22. Costanzo J.M, Yabut C.S, Almond Jr., R.H, Andrade-Gordon P, Corcoran W.T, de Garavilla L, Kauffman A.J, Abraham M.W, Recacha R, Chattopadhyay D and Maryanoff E.B **(2003)** *Potent, Small-Molecule Inhibitors of Human Mast Cell Tryptase. Antiasthmatic Action of a Dipeptide-Based Transition State Analogue Containing a Benzothiazole Ketone.* **J. Med. Chem, 46: 3865-3876**

23. Hubbard R.C, Brantly M.L and Crystal R.G **(1991)** *Proteases in* **The Lung: Scientific Foundations, Vol. 2, 1763-1773**, Raven Press, New York

24. Hubbard R.C and Crystal R.G **(1991)** *Antiproteases in* **The Lung: Scientific Foundations, Vol. 2, 1775-1787**, Raven Press, New York

25. Chanprapaph S, Saparpakorn P, Sangma C, Niyomrattanakit P, Hannongbua S, Angsuthanasombat C and Katzenmeier G **(2005)** *Competitive Inhibition of Dengue Virus NS3 Serine Protease by Synthetic Peptides Representing Polyprotein Cleavage Sites.* **Biochem. Biophys. Res. Commun, 330: 1237-1246**

26. Yin Z, Patel J.S, Wang Wei-Ling, Wang G, Chan Wai-Ling, Ranga Rao R.K, Alam J, Jeyaraj A.D, Ngew X, Patel V, Beer D, Lim P.S, Vasudevan G.S and Keller H.T **(2006)** *Peptide Inhibitors of Dengue Virus NS3 Protease. Part 1: Warhead* **Bioorg. Med. Chem. Lett, 16: 36-39**

27. Niyomrattanakit P, Yahorava S, Mutule I, Mutulis F, Petrovska R, Prusis P, Katzenmeier G and Wikberg E.S.J **(2006)** *Probing the Substrate Specificity of the Dengue Virus Type 2 NS3 Serine Protease by Using Internally Quenched Fluorescent Peptides* **Biochem. J Immediate Publication**. doi : 10.1042/BJ20051767

28. Debashish Ray and Pei-Yong Shi **(2006)** *Recent Advances in Flavivirus Antiviral Drug Discovery and Vaccine Development.* **Recent Patents on Anti-Infective Drug Discovery, 1: 45-55**

29. Shirayaev S. A, Ratnikov B. I, Chekanov A. V, Sikora S, Rozanov D. V, Godzik A, Wang J, Smith J. W, Lindberg I, Samuel M. A, Diamond M. S and Strongin A. Y **(2006)** *Cleavage Targets and the D-Arginine-based Inhibitors of the West Nile Virus NS3 Processing Proteinase***. Biochem. J, 393: 503-511**

30. Siegel H.I **(1993)** *Enzyme Kinetics-Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems*. **Wiley Interscience Publication**

31. Groutas W.C, Kuang R, Venkatraman R, Epp J. B, Prakash O **(1997)** *Structure-Based Design of a General Class of Mechanism-Based Inhibitors of the Serine Proteinases Employing a Novel Amino Acid-Derived Heterocyclic* **Biochemistry, 36: 4739-4750**

32. Wong T, Groutas C. S, Mohan S, Lai Z, Alliston K.R, Vu N, Schechter N. M and Groutas W.C **(2005)** *1,2,5-Thiadiazolidin-3-one 1,1-dioxide-based Heterocyclic Sulfides are Potent Inhibitors of Human Tryptase.* **Arch. Biochem. Biophys 436(1): 1-7**

33. Petrini Marino, Ballini Roberto and Rosini Goffredo **(1987)** *Reduction of Aliphatic and Aromatic Nitro Compounds with Sodium Borohydride in Tetrahydrofuran Using 10% Palladium-on-Carbon as Catalyst.* **Synthetic Communications: 713-714**