STUDY OF NATURAL AND SYNTHETIC PROTEASE INHIBITORS AND THEIR BIOLOGICAL ACTIVITIES

A THESIS

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BY

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DEDICATED TO MY

MOTHER

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August 2008 *Mohd Sajid Khan*

CERTIFICATE

Certified that the work incorporated in the thesis entitled "Study of natural and synthetic protease inhibitors and their biological activities" submitted by Mr. Mohd Sajid Khan was carried out under my supervision. Such material as has been obtained from other sources has been duly acknowledged in the thesis.

Dr. M. I. Khan **Date**

Research Guide

DECLARATION OF THE CANDIDATE

I declare that the thesis entitled "Study of natural and synthetic protease inhibitors and their biological activities" submitted by me for the degree of Doctor of Philosophy is the record of work carried out by me during the period from 5th Sep, 2002 to 25th June, 2008 under the guidance of Dr. M. I. Khan and has not formed the basis for the award of any degree, diploma, associateship, fellowship, titles in this or any other University or other institute of Higher learning.

I further declare that the material obtained from other sources has been duly acknowledged in the thesis.

Mohd Sajid Khan **Date**

ABBREVIATIONS

ES-MS	Electrospray mass spectrometer
FTIR	Fourier Transform Infrared Spectroscopy
IC_{50}	50% inhibitory concentration
K_i	Dissociation constant for inhibitor
MIC	Minimum inhibitory concentration
NCIM	National center for industrial microorganisms
RP-HPLC	Reverse phase high performance liquid chromatography
SPI	Serine protease inhibitor
DEPT	Distortionless Enhancement by Polarization Transfer
DMSO	Dimethyl sulphoxide
HMBC	Heteronuclear Multiple Bond Correlation
HMQC	Heteronuclear Multiple Quantum Coherence
HPLC	High Performance Liquid Chromatography
APD	N-acetyl-Lphenylalanyl-L-3, 5-diiodotyrosine
MS	Mass Spectrum
TFA	Trifluoroacetic acid
E_t	Total concentration of enzyme
BAPNA	N-α-benzoyl-L-arginine p-nitroanilide
BAEE	N-α-benzoyl-L-arginine ethyl ester
MBC	Minimum bactericidal concentration
MIC	Minimum Inhibitory Concentration
log P	Logarithm of the partition coefficient
Log S	Aqueous solubility
HBA	Hydrogen Bond acceptor
HBD	Hydrogen Bond donor
RB	Rotational Bonds

ABSTRACT

Proteases play a key role in regulating physiological processes such as digestion, fertilization, growth, differentiation, cell signaling, cell migration, immunological defense, wound healing, apoptosis, protein synthesis, protein turnover and in infection. Proteases are also crucial for disease propagation and inhibitors of such proteases are emerging with promising therapeutic uses in the treatment of diseases such as cancers, parasitic, fungal, and viral infections, inflammatory, immunological, respiratory, cardiovascular and neurodegenerative disorders. Their interaction with inhibitors is the subject of intense investigation with the view of finding lead compounds for drugs that can prevent or treat pathologies dependent on specific protein processing or degradation.

Natural and synthetic protease inhibitors of low molecular weight have great impact on the therapeutics. Marine sources are the richest and unexploited one and full of new kind of compounds whereas analog study of synthetic compounds is the best way to fetch potent inhibitor and *in silico* study proposes them highly accurately as a drug. Investigation was carried out to purify and characterize the natural serine protease inhibitor from marine mollusk and analog study of synthetic cysteine & aspartic protease inhibitors against infectious diseases was also carried out. These analogs were defined as potential drugs on the basis of *in silico* study. The thesis is divided into six chapters.

Chapter 1: General Introduction

This part comprises a literature survey of protease inhibitors with reference to their classification, purification, synthesis, properties and applications.

Chapter 2: Purification and characterization of serine protease inhibitor (SPI) from hemolymph of green mussel, *Perna viridis*

Bioactivity guided fractions of cell free hemolymph of bacterially challenged marine mussel, *Perna viridis* led to the isolation of a novel quaternary alkaloid which was identified by ^{1}H NMR, ^{13}C NMR, DEPT, and 2D NMR, FTIR and LC-MS spectra. The

inhibition constant Ki, determined by the classical double reciprocal plot was 104.68 μM and by Dixon plot was 97.1 μM, which is almost equal to the IC_{50} value (102.5 μM) of the inhibitor. The Line Weaver–Burk reciprocal plot showed that inhibitor was a competitive inhibitor of trypsin and the K_M value for the trypsin with BAPNA was 588 μM. The inhibitor was found to be specific for trypsin and did not show any inhibition against chymotrypsin. The inhibition constants and thermodynamic parameters Free energy, Enthalpy and Entropy for inhibition of trypsin by SPI clearly show that the SPI-Trypsin interaction is driven by positive entropy contribution.

Chapter 3: Synthesized 2,4,5-trisubstituted imidazole derivatives: A candidature towards antibacterials

The 2,4,5-trisubstitued imidazole derivatives were designed and screened against several Gram positive and Gram negative bacteria. The MICs of these compounds, showing inhibition in the preliminary disk diffusion test, were determined by the microtitre plate technique using micro dilution method. The minimum bactericidal concentrations (MBC) were also determined. Study reveals that among C-2 aryl substituted imidazole, a 4-substitution on aryl ring is preferred and more polar substituent makes the molecule more active whereas polar substituents at 2^{nd} position on C-2 aryl ring makes the molecule less active. At C-4 position presence of aromatic ring is required for activity to interact with hydrophobic pocket of enzyme. The candidature of active compounds to be an effective and novel drug was examined based on Lipinski's rule of Five which explained ClogP, LogS, H-bond acceptors, H-Bond donors and Rotational Bonds. Three molecules satisfy Lipinski's rule of Five and could be proposed as potent new antibacterial drugs.

Chapter 4: Antibacterial activities of Cysteine protease inhibitors, 1-substituted pyridylimidazo [1,5-a] pyridine derivatives

The analogs of 1-substituted pyridylimidazo [1,5-a] pyridine were designed and screened to inhibit cysteine proteases using papain as the model enzyme. The

proteolytic activity of the reaction mixtures was determined using Bz-DL-Arg-pNA as the chromogenic substrate. The Lineweaver–Burk plot was used to determine the types of inhibition. The inhibition constants and thermodynamic parameters for inhibition of papain by inhibitors clearly show that the inhibitors-papain interaction is driven by positive entropy contribution and hydrophobic in nature. The MIC_{50} and MIC_{90} of 1-substituted pyridylimidazo [1,5-a] pyridine derivatives, showing inhibition in the preliminary tests, were determined by the microtitre plate technique using micro dilution method. The minimum bactericidal concentrations (MBC) were also determined. Computational study shows all compounds do not show any threat against toxicity risk assessment except compound 1-(2-Pyridyl)-3-(3,5-di-*tert*-butyl-4-hydroxyphenyl) imidazo [1,5-*a*] pyridine which showed threat as tumorogenic and androgenic effect due to the presence of isobutyl group. All molecules have H-bond donors less than 5 and H-bond acceptor less than 10 whereas number of rotational bonds lie in the moderate range i.e. 2-4. Among all tested molecules, molecule 1-(2-Pyridyl)-3-(2-hydroxyphenyl) imidazo [1,5-*a*] pyridine is the most potent molecule whose MIC is the lowest among all molecules and showed maximum drug score and positive values for drug likeness.

Chapter 5: Analog study of synthesized compounds as aspartic protease inhibitors and cysteine protease inhibitors

The analog study of 2,4,5-trisubstituted imidazole derivatives were performed using N-acetyl-L phenylalanyl-L-3,5-diiodotyrosine (APD) as the chromogenic substrate and pepsin as the model enzyme. The inhibition constant Ki determined by the classical double reciprocal plot ranges from 13.75 μM-99.3 μM which is almost equal to the IC_{50} value (15.1 μM-102 μM) of the inhibitors. The K_M value for the Pepsin with APD was 80 μM. For 1,4,5,6-tetrahydropyridine-3-carboxylate derivatives Bz-DL-Arg-pNA as the chromogenic substrate and papain as the model enzyme were chosen. The Lineweaver–Burk plot was used to determine the types of inhibition. The inhibition constant Ki, determined by the classical double reciprocal plot ranges from 0.5 μM-1.1 μM, which is almost equal to the IC_{50} value (0.51 μM-1.2 μM) of the inhibitors. The K_M

value for the Papain with BAPNA was 2.8 mM. The inhibition constants and thermodynamic parameters for inhibition by their respective inhibitors show that the CPI-papain interaction and API-Pepsin interaction is driven by positive entropy contribution and favors hydrophobic interaction.

Chapter 6: General Discussion and Conclusion

This part compares the properties of purified SPI and synthetic protease inhibitors with other natural and synthetic protease inhibitors with respect to their mode of interaction and structure of scaffold.

Chapter 1

General Introduction

Definition

Proteases, also known as proteolytic enzymes, are enzymes that catalyze the breakdown of proteins by hydrolysis of peptide bonds. Using bioinformatics analysis of the mouse and human genomes, at least 500–600 proteases (~2% of the genomes, MEROPS database; http://merops.sanger.ac.uk) have been identified, many of which are orthologous [1]. They catalyze the hydrolysis of peptide bonds, an enzymatic reaction central to many physiological and pathological processes such as cell proliferation, tissue remodeling, embryonic development, blood coagulation, blood pressure control, protein activation and maturation, protein catabolism, protein transport, inflammation, infection, and cancer. The importance of peptide bond cleavage in biological system is reflected by the finding that nature has separately invented the necessary catalytic machinery. Through evolution, proteases have adapted to the wide range of conditions found in complex organisms (variations in pH, reductive environment and so on) and use different catalytic mechanisms for substrate hydrolysis, their mechanism of action classifies them as either serine, cysteine or threonine proteases (amino-terminal nucleophile hydrolases), or as aspartic, metallo and glutamic proteases (with glutamic proteases being the only subtype not found in mammals so far). Primarily proteases were considered as digestive enzymes but now it is well known that they specifically cleave protein substrates either from the N or C termini (aminopeptidases and carboxypeptidases, respectively) and/or in the middle of the molecule (endopeptidases). [2]. However, the pioneering work of Davie and Neurath on trypsinogen activation [3], followed by that of Davie and Ratnoff [4] and MacFarland [5] on the mechanism of blood clotting led to the concept of protein activation by limited proteolysis. It was discovered that precise cleavage of proteins by proteases was the regulatory means of protein activities. The blood coagulation cascade has since remained a prototype for a proteolytic cascade in which a signal is passed through a pathway by the sequential activation of protease zymogens. By cleaving proteins, proteases are involved in the control of a large number of key physiological processes such as cell-cycle progression, cell proliferation and cell death, DNA replication, tissue remodeling, haemostasis (coagulation), wound healing and the immune response. Matrix metalloproteinases (MMPs) are promising targets for the therapy of cancer and other degenerative diseases and their regulation by inhibitors like non-peptidic

hydroxamate-based matrix metalloproteinase inhibitors by inhibiting deep S10 pocket of MMPs enzymes could help to get protected against disease [6]. Proteases involved in protein recycling and degradation are therefore physically separated from the majority of other proteins by being kept in lysosomes or in a proteasome to prevent unwanted proteolysis. Proteases are essential for a number of important metabolic processes. They are essential for

(1) Gain of function (for example, activation by limited proteolysis).
(2) Loss of function (for example, cleavage of inhibitor of caspase-activated DNAse (ICAD) by caspase 3 during apoptosis, thereby releasing caspase-activated DNAse (CAD) and enabling DNA fragmentation [7]).
(3) Switch of function (for example, chemokine processing by matrix metalloproteinases and other proteases [8].

Catalytic mechanisms of proteases

The five major catalytic classes of proteases [9] se two fundamentally different catalytic mechanisms (Fig 1) to stabilize the tetrahedral intermediate. In the serine, cysteine and threonine proteases, the nucleophile of the catalytic

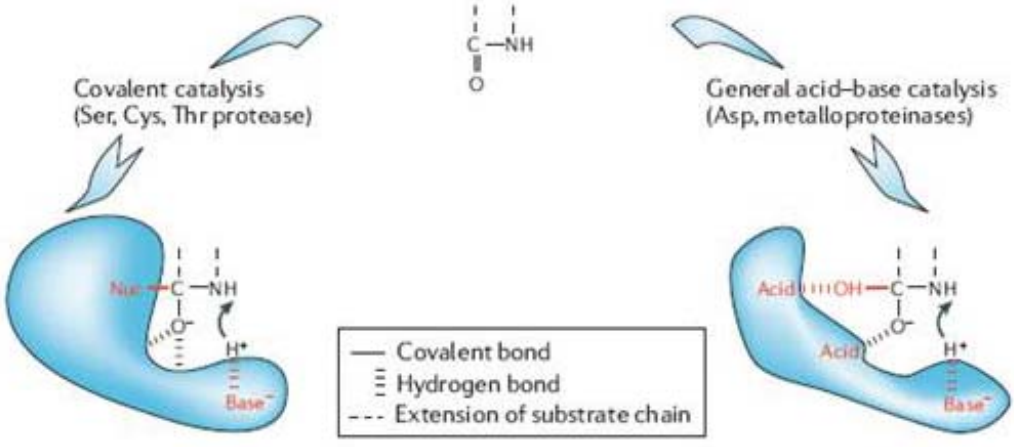


Figure 1

site is part of an amino acid (covalent catalysis), whereas in the metalloproteinases and aspartic proteases the nucleophile is an activated water molecule (non-covalent catalysis). In covalent catalysis, histidines normally function as a base, whereas in non-covalent catalysis Asp or Glu residues and zinc (metalloproteinases) serve as acids and bases. A further difference between the two groups is in the formation of the reaction products from the tetrahedral intermediate, which for cysteine and serine proteases requires an additional intermediate step (acyl-enzyme intermediate)

Modes of substrate cleavage by peptidases:

Endopeptidase (cathepsin L) and exopeptidases (cathepsin H, an aminopeptidase, and cathepsin X, a carboxypeptidase) were taken as examples (Fig 2). Peptide substrate (cyan balls) runs through the entire length of the active site [9] of an endopeptidase framework (blue) and is cleaved in the middle of the molecule (scissile bond-yellow). In exopeptidases, substrate binding is structurally constrained (mini-chain in cathepsin H, orange; mini-loop in cathepsin X, green). In cathepsin exopeptidases these additional structural elements also provide negative charge (cathepsin H) to bind the positively charged amino terminus (blue) of the substrate, or positive charge (cathepsin X) to bind the negatively charged carboxyl terminus (red) of the substrate.

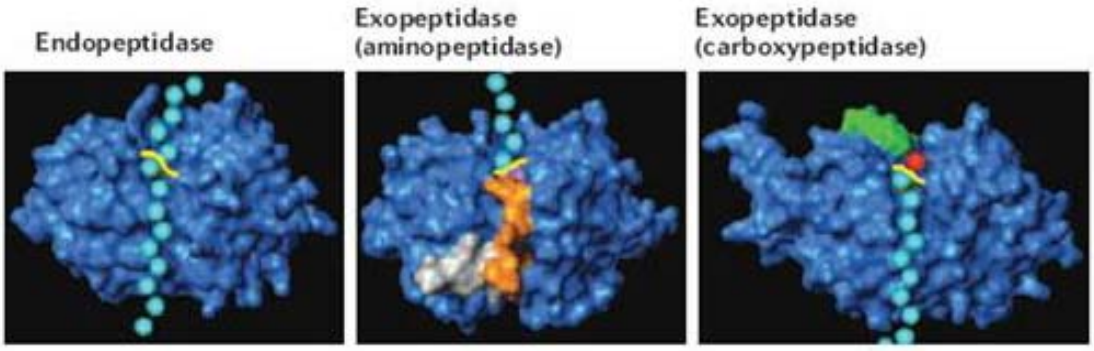


Figure 2

General mechanism of protein substrate binding to a protease - The surface of the protease that is able to accommodate a single side chain of a substrate residue is called the subsite. Subsites are numbered S1–Sn (Fig 3) upwards towards the N terminus of the substrate (non-primed sites), and S1′–Sn′ towards the C terminus (primed sites), beginning from the sites on each side of the scissile bond [9].

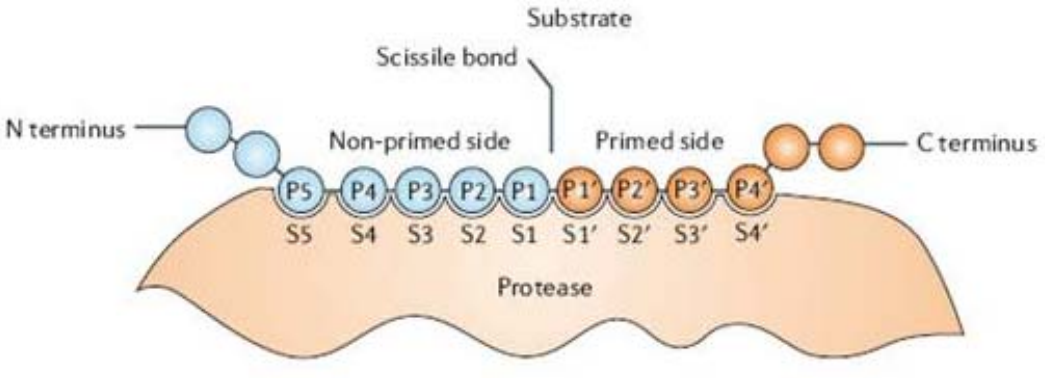


Figure 3

The substrate residues they accommodate are numbered P1–Pn, and P1′–Pn′, respectively. The structure of the active site of the protease therefore determines which substrate residues can bind to specific substrate binding sites of the protease (known as the intrinsic subsite occupancy), thereby determining substrate specificity of a protease.

Protease inhibitors

The action of proteases is tightly controlled to prevent improper cleavage of signalling molecules. Protease activities are regulated at the transcriptional level by differential expression, and at the protein level by activation of inactive zymogens and by the binding of inhibitors and cofactors [9]. During cell and tissue development and organism homeostasis, the protease signalling pathways work normally and are tightly controlled. When the regulation of proteases disturbs then the substrate-cleavage level is either too little or there is too much proteolysis. Diminished proteolysis as a result of insufficient protease activity

Table 1: Protease inhibitors approved for clinical use

Indication	Compound	Company	Target	Protease Class
Hypertension, Myocardial infarction	Captopril	Bristol-Myers Squibb	ACE	Metallo
	Enalapril	Merck		
	Lisinopril	Astra Zeneca		
	Trandolapril	Abbott		
	Ramipril	Aventis		
	Moexipril	Boehringer mannheim		
	Qinapril	Pfizer		
Periodontitis	Periostat	CollaGenex	MMP1, MMP2	
AIDS	Ritonavir	Abbott	HIV Protease	Aspartic
	Fosamprenavir	Glaxo Smithkline		
	Saquinavir	Hoffmann-La-Roche		
Thrombosis	Argatroban	Mitsubishi pharma	Thrombin	Serine
	Lepirudin	Aventis (Hoechst marion Roussell)		
	Desirudin	Novartis		
Thrombosis, Unstable angina	Bivalirudin	The Medicines Company		
Respiratory Disease	Sivelestart	Ono	Human Neutrophil, Elastate	
Pancreatitis	Camostatmesilate	Ono	Trypsin-like	
Pancreatitis, Inflammation	Nafamostat mesilate	Japan Tobacco	Broad-spectrum	
Cancer	Bortezomib	Millennium	Proteasome	Threonine

ACE, angiotensin converting enzyme; MMP,matrix metalloproteinase

Mostly originates from genetic irregularities (endogenous proteases [1]), excessive inhibitory activity or insufficient activation is carried out often by pathogens [10-12]. By contrast, excessive or inappropriate proteolysis is seldom a result of genetic aberrations but most often results from numerous endogenous and/or exogenous factors, which result in unwanted activation of protease signaling pathways, such as the effect of atherosclerotic plaque formation or blood vessel injury in the blood coagulation cascade, which leads to the appearance of intravascular thrombin [13]. Inappropriate proteolysis has been found to have a major role in cancer as well as cardiovascular, inflammatory, neurodegenerative, bacterial, viral and parasitic diseases. Excessive proteolysis can be prevented by blocking the appropriate proteases [14]. Several protease inhibitors (Table 1) have been approved for clinical use [15].

Classification of Enzyme Inhibitors

The initial classification of inhibitors based on the binding of inhibitors with enzymes is given by

(1) **Active site directed** –When inhibitor binds with the active site of enzyme
(2) **Allosteric effector**- When inhibitor binds other than the active site of enzyme

The inhibitors which attack the active site can be divided according to the type of interaction into **covalent/ noncovalent** and **irreversible/reversible** inhibitors.

The **reversible inhibitors** bind to enzymes with non-covalent interactions such as hydrogen bonds, hydrophobic interactions and ionic bonds. Multiple weak bonds between the inhibitor and the active site combine to produce strong and specific binding. In contrast to irreversible inhibitors, reversible inhibitors generally do not undergo chemical reactions when bound to the enzyme and can be easily removed by dilution or dialysis.

There are three kinds of reversible enzyme inhibitors. They are classified according to the effect of varying the concentration of the enzyme's substrate on the inhibitor [16].

In **competitive inhibition**, the substrate and inhibitor compete for access to the enzyme's active site. The substrate and inhibitor cannot bind to the enzyme at the same time. This type of inhibition can be overcome by sufficiently high concentrations of substrate, i.e.,

by out-competing the inhibitor. Competitive inhibitors are often similar in structure to the real substrate.

In **mixed inhibition**, the inhibitor binds to a different site on an enzyme. Inhibitor binding to this allosteric site changes the conformation (i.e., tertiary structure or three-dimensional shape) of the enzyme so that the affinity of the substrate for the active site is reduced. This type of inhibition can be reduced, but not overcome by increasing concentrations of substrate. Although it is possible for mixed-type inhibitors to bind in the active site, this type of inhibition generally results from an allosteric effect where the inhibitor binds to a different site on an enzyme.

Non-competitive inhibition is a form of mixed inhibition where the binding of the inhibitor to the enzyme reduces its activity but does not affect the binding of substrate. The extent of inhibition depends only on the concentration of the inhibitor.

Special cases

The mechanism of **partially competitive inhibition** is similar to that of non-competitive, except that the EIS complex has catalytic activity, which may be lower or even higher (partially competitive activation) than that of the enzyme–substrate (ES) complex. This inhibition typically displays a lower V_{max}, but an unaffected K_m value [17].

Uncompetitive inhibition occurs when the inhibitor binds only to the enzyme–substrate complex but not to the free enzyme and the EIS complex is catalytically inactive. This mode of inhibition is rare and causes a decrease in both V_{max} and the K_m value [17].

Substrate and product inhibition is where either the substrate or product of an enzyme reaction inhibit the enzyme's activity. This inhibition may follow the competitive, uncompetitive or mixed patterns. In substrate inhibition there is a progressive decrease in activity at high substrate concentrations. This may indicate the existence of two substrate-binding sites in the enzyme. At low substrate, the high-affinity site is occupied and normal kinetics are followed. However, at higher concentrations, the second inhibitory site becomes occupied, inhibiting the enzyme [18]. Product inhibition is often a regulatory feature in metabolism and can be a form of negative feedback .

Slow-tight inhibition occurs when the initial enzyme–inhibitor complex EI undergoes isomerisation to a second more tightly held complex, EI*, but the overall inhibition

process is reversible. This manifests itself as slowly increasing enzyme inhibition. Under these conditions, traditional Michaelis–Menten kinetics give a false value for K_i, which is time–dependent. The true value of K_i can be obtained through more complex analysis of the on (k_{on}) and off (k_{off}) rate constants for inhibitor association.

Reversible and irreversible inhibitors can be differentiated by lowering the inhibitor concentration, by diluting the reaction preparation, or by gel filtration or dialysis. In the case of a **reversible inhibitor**, the enzyme activity will increase again. Usually reversible inhibition involves a non covalent interaction between enzyme and inhibitor but cases are also known in which covalently bound inhibitors result in reversible inhibition because of hydrolytically labile bonding. Examples are peptidyl aldehydes or nitriles as inhibitors of serine and cysteine proteases.

Irreversible inhibitors (inactivators) always bind to the enzyme covalently. The reaction of irreversible inhibitors with the enzyme occurs via a noncovalent transition state and hence this leads to rapid reduction of enzyme activity. In practice it is often difficult to differentiate between reversible and irreversible inhibitors, for example if a reversible inhibitor binds to the enzyme with such a high affinity that the enzyme-inhibitor complex dissociates very slowly and thus appears irreversible. This type of inhibitor is known as a "tight binding" inhibitor. Normally a rapid equilibrium is observed with reversible inhibitors, whereas reactions which result in modification of the enzyme take place relatively slowly. However, there are also reversible inhibitors which only inhibit enzyme activity very slowly due to conformational changes following enzyme-inhibitor complex formation ("slow binding").

A sub division of the inhibitors (Table 1) which undergo non covalent interactions with the enzyme are

(1) **Simple substrate analogs (reversible competitive inhibitors) -** inhibitors mimick the structure of the substrate e.g. inhibition of succinate dehydrogenase by malonate (a substrate analog) [19].

(2) **Tight binding enzyme complements -** Inhibitors in this group bind tightly to their enzyme target and their potency is critically dependent on the degree of complementarity between enzyme and inhibitor. Extremely tight binding to an enzyme catalyzing a single substrate reaction can be achieved by designing

inhibitors that mimic the substrate in the transition state or high energy intermediates along the reaction pathways [20,21]. Other members in this group include multi-domain inhibitors which are targeted for more than one binding site or domain of the enzyme. These include multi-substrate analogs [22], ground state analogs in the sense proposed by Abeles [23] and essentially any small molecule [24] that has appreciable affinity for more than one binding domain of the enzyme.

Covalent inhibitors can be subdivided according to whether they react with the active site by the normal process of catalysis **("mechanism based"**- the term **"mechanism based"** is often used synonymously for **"enzyme activated"**, **"suicide"**, or better, **"Trojan Horse inhibitors" (k_{cat} inhibitors** [25]**)),** or by another chemical path which does not correspond to the catalytic mechanism of the enzyme **("affinity labeling", affinity marking).**

There are at least four categories of **mechanism-based inhibitors**. Catalytic processing by enzymes may serve to convert inhibitors

(1) to tight binding active-site complements [26]

(2) to reactive intermediates that combine with the enzyme irreversibly in a step that lies outside normal catalysis (mechanism-based inhibitor/reactive intermediate, or suicide inhibitor), as in the classic example of "Bloch" [27], reactions which involve vitamin B_6 and flavin-dependent [28] enzymes.

(3) to compounds that are converted to stable analogs that are unable to proceed to product because they lack the requisite functionality (suburb element) for further processing (dead-end inhibitor) [29,30].

(4) to stable analogs that have the potential for conversion to product, but are trapped in potential energy wells (alternate substrate inhibitors). As opposed to dead-end inhibitors, which simply lack structural features for substrate turnover, alternate substrate inhibitors [31-33] give rise to stable complexes during the normal course of catalysis because the rate of one or more steps in the normal cycle has become extremely slow.

Table 2: Classification of enzyme inhibitors

Type	[a]Binding determinants	[b]Chemically reactive group	Minimal mechanism	Rational	Examples
Substrate analog	**S**	**NO**	**E+S'↔E.S'**	**Structural mimicry**	**Malonate/succinate dehydrogense**
Group I. Tight Binding Enzyme Complements (Non covalent interactions)					
Transition state analog	**t.s**	**NO**	**$E+l_{ts}\leftrightarrow E.l_{ts}$**	**E binds T.S. tightly**	**Phosphonyl peptides/ metallo proteases**
Multisubstrate analog	**S_1+S_2**	**NO**	**$E+S_1\text{-}S_2\leftrightarrow E.S_1\text{-}S_2$**	**Multiple binding effect**	**Phosphono acetyl-L-aspartate/ carbamoyl transferase**
Ground state analog	**S+R**	**NO**	**E+S'-R↔E.S'-R**	**Multiple binding effect**	**---**
Group II. Mechanism-based inhibitors (Covalent interactions)					
Transition state analogs	**t.s**	**NO**	**$E+l_{ts}\leftrightarrow E.l_{ts}$**	**E binds T.S. irreversively**	**Peptidyl aldehydes/cysteine & serine proteases**
Suicide inhibitor	**S**	**latent**	**$E+S'\leftrightarrow E.S'\leftrightarrow E.I_r\rightarrow E.\text{-}l_r$**	**Unmasking of latent reactive group**	**Sulbalactam/β - lactamases**
Dead-end inhibitor	**S**	**NO**	**E+S'↔E.S'→ E'-S'**	**S' lacks structural element for turn over**	**Peptidyl nitriles/cysteine proteases**
Alternate substrate inhibitor	**S**	**NO**	**E+S"↔E.S"↔ E'-S"↔E'-P↔E.P↔E+P**	**Stabilize E'-S", E'-P or E-P**	**Physostigmine/ acetylcholinesterase**
Affinity Labels (Covalent interactions)					
Classical affinity label	**S**	**Yes**	**$E+S_{rx}\leftrightarrow E.S_{rx}\rightarrow E\text{-}S'+X^-$**	**High effective concentration of reactive group at active site**	**Chloromethylketones/ cysteine & serine proteases**
Quiescent affinity label	**S**	**NO**	**$E+S_x\leftrightarrow E.S_x\rightarrow E\text{-}S'+X^-$**	**Different reactivity: Chemical Vs. enzymatic**	**Acyloxymethylketones/ cysteine proteases**

[a] refer to initial reagent; **S**=Substrate; **t.s.** =transition state; **S_1+S_2** = Cosubstrate; R= entity with affinity for non catalytic site. [b] **S'**=Pseudosubstrate; **l_{ts}**=Transition state analog; **l_r**=reactive intermediate; **$E\text{-}l_r.E\text{-}S'$**, **E'-S'**, **E'-S'**, and **E'-P** =enzyme –inhibitor adduct; **S'**=alternate substrate inhibitor; **P**=product; **S_{rx}**=reactive affinity label; **S_x**= quiescent affinity label; **$S_1\text{-}S_2$**= multi-substrate analog; **S'-R**= ground state analog

Affinity labels or active site directed inhibitor is an active-site directed reagent that contains a chemically reactive group which gives rise with its target enzyme to an adduct that is irreversible to gel filtration and to exhaustive dialysis. Compounds are regarded as quiescent affinity labels, if they exploit intrinsic binding to lower the overall free energy of activation of an "aberrant" chemical path and lead to facile enzyme inactivation, with each step being distinct from those which the enzyme has evolved to catalyze. Examples of quiescent affinity labels may include the inhibition of isopentenyl diphosphate isomerase by 3-(fluoromethyl)-3-butenyldiphosphate and the cysteine proteinase inhibitor, E-64. In principle, affinity labels may be of two types, classical or quiescent, which differ in their intrinsic chemical reactivity. The reaction path is nonspecific and does not correspond to the normal catalytic mechanism. If this substance reacts with other molecules that have functional groups corresponding to those of the active site, it is known as a **"chemically reactive affinity label"** or **"Classical affinity label"**. If this is not the case and the reagent has no activity for non enzymatic molecules, it is known as a **"quiescent affinity label"** [34,35].

Serine Protease

Serine proteases [36-40] are classified by their substrate specificity, particularly by the type of residue found at P1, as either trypsin-like (positively charged residues Lys/Arg preferred at P1), elastase-like (small hydrophobic residues Ala/Val at P1), or chymotrypsin-like (large hydrophobic residues Phe/Tyr/Leu at P1). The active site of these enzymes consists of a catalytic triad of Ser195, His57, and Asp102 residues (chymotrypsin numbering system) and an oxyanion hole. The substrate binds in the active site forming a Michaelis complex, exposing the carbonyl group of the scissile amide bond to nucleophilic attack by the active site serine hydroxyl, under base catalysis by the imidazole side chain of His57 (Fig 4). The resulting tetrahedral intermediate is stabilized by hydrogen bonding to the backbone NH of Ser195 and Gly193, which form the oxyanion hole. Proton transfer from His57 to the amine of the tetrahedral intermediate facilitates expulsion of the amine fragment as leaving group. The covalent acyl-enzyme

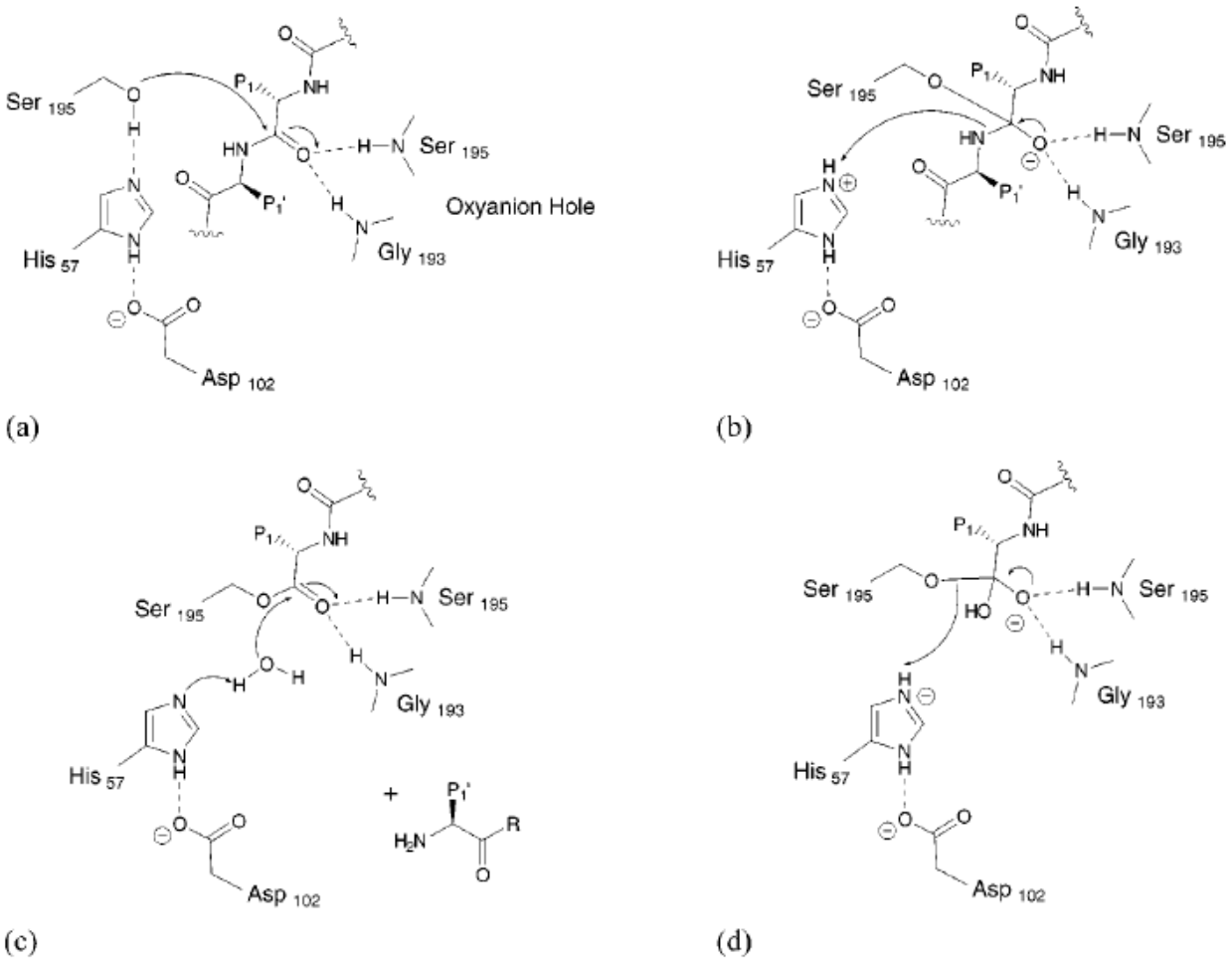


Figure 4: General catalytic mechanism for serine proteases. (a) Hydrogen-bonding interactions with histidine and aspartate residues of the catalytic triad activate the serine hydroxyl for nucleophilic attack of the scissile amide bond, forming the first hemiacetal tetrahedral intermediate. **(b)** Proton transfer from His57 to the amine of the tetrahedral intermediate facilitates expulsion of the C-terminal fragment of the substrate to give the covalent acyl complex. **(c)** Water attacks the complex to form the second tetrahedral intermediate **(d)** which collapses via acid-assisted catalysis by His57 to regenerate Ser195 and the N-terminal fragment of the cleaved substrate.

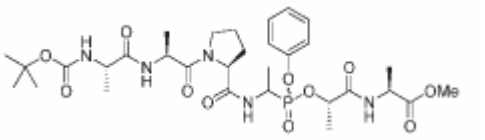
complex is attacked by water, with formation of a new tetrahedral intermediate which subsequently breaks down via acid-assisted catalysis by His57 to form the carboxyl fragment of the cleaved substrate and regenerates Ser195.

Serine Protease Inhibitors

Important classes of serine protease inhibitors are given by

Phosphorus-based inhibitors

Organophosphorus-based synthetic serine protease inhibitors represent an excellent model for the evolution of inhibitors with enhanced chemical and biological properties for the study of serine proteases in a variety of biological systems and as potential lead compounds for therapeutic use. These agents were identified as one of the first inhibitors exhibiting selectivity of action for the serine proteases. Diisopropylphosphofluoridate (DFP) is still one of the most widely used broad – spectrum inhibitors of these enzymes [41]. Its mode of action involves the formation of a single covalent bond between the phosphorus atom of the inhibitor and the Oγ atom of the active-site serine hydroxyl [42, 43]. Lambden and Bartlett prepared N-protected analogues of phenylalanine with a phosphonyl fluoride group [44]. These were shown to be excellent, selective inhibitors of chymotrypsin, but the presence of the highly labile P–F bond renders the inhibitor unstable, being rapidly hydrolysed in physiological buffers. Oleksyzyn *et al*. [45] have described peptidyl-(α-aminoalkyl) phosphonate diphenyl esters containing P1 4-amidinophenyl groups. Bartlett's group have reported inhibitors of α-lytic protease based on mixed phosphonate esters containing C-terminal groups which mimic extended peptide sequences [46, 47]. Analogues such as



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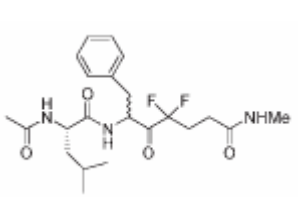
(1) were prepared with the aim of fulfilling additional subsite interactions in the S' subsites. As with the diphenyl phosphonates, these inhibitors were found to form a formal covalent bond between Ser 195 and the phosphorus atom.

Fluorine-containing serine protease inhibitors

In order to prepare metabolically stable analogues of inhibitors containing electrophilic carbonyl groups, trifluoromethyl ketones were prepared (TFMKs) as potential serine protease inhibitors [48]. It is proposed that the trifluoromethyl group facilitates nucleophilic attack by the serine residue, on the carbonyl group of the inhibitor [49]. ^{13}C NMR studies suggest that the TFMKs are hydrated in the presence of water, due to their high susceptibility to nucleophilic attack. This does not inactivate the inhibitor, although the nonhydrated form is the more effective inhibitor species [50]. Systematic modification of the difluoro ketone, Ac-Leu-Phe-CF_2H, involving replacement of the hydrogen group with moieties increasingly peptidic in nature led to the discovery of inhibitors **2** and **3** with



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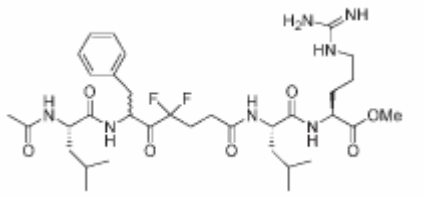

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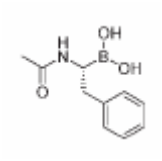
enhanced potency, thus illustrating the substantial contribution made by S*'* subsite interactions in this series of compounds. Abeles' group have described *β, β* -difluoro-*α*-keto esters and acids as serine protease inhibitors which are more potent than analogous amino acid-based TFMKs and *α*-keto ester analogues [51]. Addition of Ala-Leu-Arg-OMe, replacing the ethyl ester, results in a slow, tight-binding inhibitor of chymotrypsin. Pentafluoroethyl ketones have also been shown to be excellent inhibitors of serine proteases.

Peptidyl boronic acids

Chymotrypsin and subtilisin were the first serine proteases shown to be inhibited by aromatic boronic acid derivatives [52, 53].The crystal structure of subtilisin, complexed with phenylethane boronic acid [54], clearly shows that binding and interaction of the trigonal boronic acid moiety, with the active site serine residue leads to the formation of a tetrahedral adduct in complex with the enzyme, mimicking the putative transition state. In

this structure, one –OH group interacts with the oxyanion binding site, whereas the other occupies a position analogous to the –NH– leaving group found during normal peptide bond hydrolysis. (*R*)-acetamidophenylethane boronic acid **(4)**, a phenylalanine analogue, was shown to be several times more effective as a chymotrypsin inhibitor than aromatic boronates, being active in the micromolar range [55]. Shevni [56] prepared the first *α*-amino boronate containing as inhibitors of chymotrypsin and elastase like



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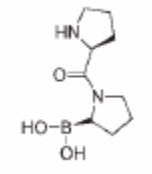

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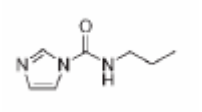
enzymes. Flentke *et al* [71] reported the first boroPro-containing dipeptides as inhibitors of CD 26. H-Ala-bPro-OH (*K*i = 15 nM) and H-Pro-bPro-OH **(5)** (*K*i = 16 pM) were found to be potent inactivators of the enzyme, acting in a slow, tight-binding manner [57].

Serine protease inhibitors based on heterocyclic structures

Some peptide-based compounds have had considerable utility as agents for the study of the role of particular serine proteases in biological systems and a few have progressed to late-stage clinical trials. The utilization of any compounds based on peptides has a number of well-documented pharmacological drawbacks, including poor oral bioavailability, solubility, stability and effects. Dramatic advances in the development of new drug delivery and formulation strategies may eventually circumvent many of these drawbacks, however, strategically, medicinal chemists continue to pursue heterocyclic and other nonpeptide scaffolds as the basis for the design of new protease inhibitors.

(a) ***N*-hydroxysuccinimide heterocycles and related compounds**

William Groutas who designed and synthesize irreversible heterocyclic serine protease inhibitors has reported a number of mechanism-based inactivators containing an *N*-hydroxysuccinimide moiety or closely related analogue (such as saccharins). He [58] designed the mechanism-based inhibitors of PPE of general structure **(6)**. It was proposed that abstraction of a proton from the R– NHCO–



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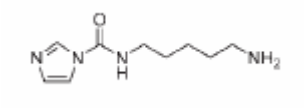

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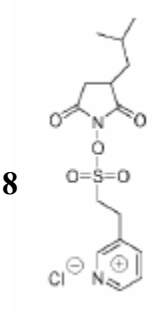

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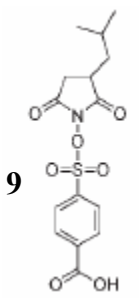

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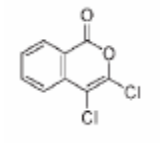
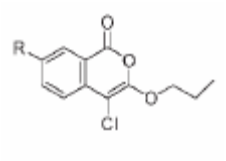
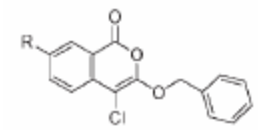
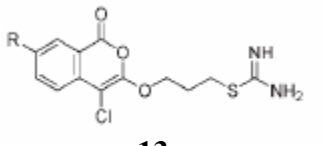
portion of the inhibitor by the imidazole group of His 57 causes decomposition, yielding an alkyl isocyanate (R–N=C=O) which covalently modifies the active site serine. This hypothesis was expanded to prepare a series of inhibitors of trypsin-like serine proteases by replacing the alkyl R group by NH_2–$(CH_2)_6$– to fulfill primary specificity requirements at the S1 subsite **(7)** [59]. A further series of 3-alkyl-*N*-hydroxysuccinimides were prepared containing pyridinium **(8)** and phenyl carboxylate **(9)** ionic groups attached to the sulphone, substitutions known to improve solubility and bioavailability. These were found to retain their capacity to be inhibitors of HNE and cathepsin G [60]. Oxidation of the sulphur atom in 3-(alkylthio)-*N*-hydroxysuccinimides does not adversely affect the biological activity of these compounds. An exciting expansion of this concept has been the development of compounds with protease inhibitor and antioxidant functionality.

(b) Isocoumarins

Compounds based on the isocoumarin scaffold have been developed by Powers' group at Georgia Tech. The original derivative, 3,4-dichloroisocoumarin **(10)** has been shown to act as a broad-spectrum inhibitor of a number of serine proteases, including chymotrypsin, trypsin and elastase like enzymes [61]. This compound does not generally inhibit other cysteine, or indeed metallo- or aspartyl proteases, and as such has often been utilized to classify the action of serine proteases in biological systems. In attempts to enhance selectivity of isocoumarins for specific subclasses of serine protease, a large number of analogues have been prepared, particularly based on 3- alkoxy-4-chloro-7-substituted analogues. Generally, isocoumarins with 3-methoxy- or -ethoxy **(11)**

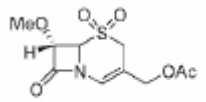
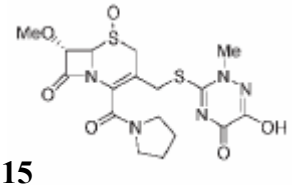
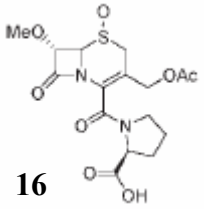





10 **11** **12** **13**

substitution confers selectivity for elastases, 3-benzyloxy- **(12)** or 3- phenylethoxy substituents inhibit chymotrypsin-like enzymes and incorporation of a basic group **(13)** enhances activity against trypsin-like enzymes. The isocoumaryl carbon is attacked by the active site serine, forming an acyl-enyzme intermediate. This intermediate is then believed to be converted to a highly reactive quinonimine methide intermediate, which may undergo further reactions.

(c) ***β*-lactam-based inhibitors**

Intensive investigations into the SAR of *β*-lactam based ring system, against HNE led to the conclusion that cephalosporin-based compounds with 7*α* substitution, such as **(14)**, are more potent than their 7*β*-substituted counterparts, with the enzyme preferring small 7*α* substituents, consistent with the known subsite specificity of HNE for small aliphatic side chains at P1. Numerous attempts have been made to elucidate the mechanism of the reaction of different inhibitors with HNE and PPE. Knight *et al* [62] provided evidence for the formation of a noncovalent (Michaelis) complex between enzyme and inhibitor, since k_{app} values obtained for the inhibition of HNE by two cephalosporin derivatives, L-658 758 **(15)** and L-659 286 **(16)**, were

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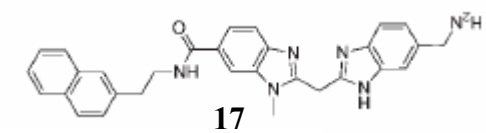
saturable. Monocyclic *β*-lactams have been reported as inhibitors of prostate-specific antigen (PSA). Han's group has developed monocyclic *β*-lactams as inhibitors of other serine proteases, describing the synthesis and biological evaluation of *β*-lactam analogues designed to act against the trypsin-like serine proteases, in particular, thrombin [63, 64].

Metal-potentiated compounds

Katz *et al* have recently reported an entirely new concept in the development of highly selective trypsin-like serine protease inhibitors. Based on the use of compounds with the ability to form a ternary complex with enzyme and a 'bridging' Zn(II) atom, the mediation of Zn(II) in the enzyme- inhibitor interaction enhances potency by anything from 1000–100,000-fold [65]. Originally based on the nonspecific trypsin-like serine protease inhibitor, bis (5-amidino-2- benzimidazolyl) methane, additional functionalities incorporated into this molecule facilitate binding to the specificity sites of individual enzymes, thus enhancing selectivity **(17–19)**.



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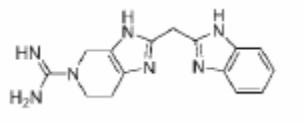

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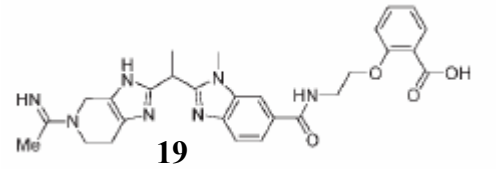

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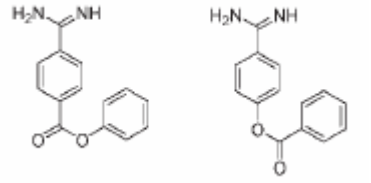
More recently, kinetic studies aimed at elucidating the mechanism of inhibition of tryptase by metal-potentiated inhibitors illustrate a slow, tight-binding mechanism with extremely slow dissociation rates, with half-lives in the order of hours [66].

Inverse substrates

In an extension of early work investigating the use of esters of *p*-amidinobenzoic acid such as **20** as potential substrates for the trypsin-like serine proteases [67, 68], Tanizawa's group prepared a series of closely analogous 'inverse substrates', in which the scissile ester bond is effectively reversed **(21)**. After mixing of a *p*-amidinophenyl based inverse substrate with trypsin, rapid acylation of the enzyme occurs, followed by slow deacylation. This steady-state hydrolysis reaction results in accumulation of an acyl-enzyme intermediate. The unusual feature of such compounds is that the amidinium ion is both the targeting and leaving group of these compounds, and thus the exact opposite of traditional substrates. Unfortunately, the major drawback in the use of inverse substrates as therapeutic inhibitors *in vivo* with these early agents is their comparative lack of selectivity for enzymes within the same serine protease subclass.



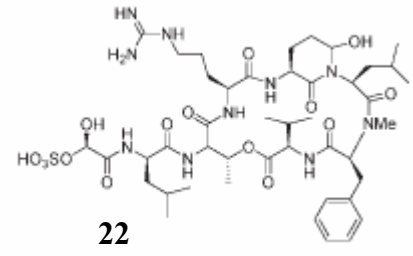
20 **21**

The *p* amidinophenyl esters will readily inactivate trypsin-like serine proteases such as thrombin, plasmin, kallikrein and uPA [69]. Whereas the deacylation rates for these respective acyl protease complexes have been shown to vary considerably, *p*-amidinophenyl inverse esters will inactivate all these species with similar binding and acylation rate constants.

Natural product inhibitors of serine proteases

Natural products have been the focus of attention for protease researchers for many years. Since the discovery of the peptide aldehydes such as leupeptin and antipain in the late sixties and early seventies, there has been an enormous interest in the development of modified and synthetic analogues of these compounds as inhibitors of serine proteases. It was shown that judicious use of peptide-targeting sequences can achieve selectivity for individual enzymes. The most significant discovery in this field over the last number of years has been that of the cyclotheonamides, derived from the Japanese marine sponge *Theonella* sp. These are a series of macrocyclic pentapeptides analogues that inhibit various trypsin-like serine proteases [70–72]. Five cyclotheonamides have been discovered till now. All are orthologous to the same molecular template, in particular an α-keto amide bond, with variations in composition of the amino acids or the substitution of the side-chain groupings [73]. All analogues have been tested and found to have excellent inhibitory properties. The total chemical synthesis of these compounds has been achieved, and significant structural data has been obtained from nuclear magnetic resonance (NMR) studies [74, 75], which has enabled SAR studies to be carried out, particularly against thrombin. Studies screening of natural products from blue-green algae have also led to the discovery of a number of interesting serine protease inhibitors. Bonjoulikan et al. [76] have reported the isolation and structural elucidation of a

nonspecific serine protease inhibitor, designated A90720A **(22)**, from *Microchaete lokatensis*. This compound, structurally related to a number of other blue-green algal derived agents such as aeruginopeptins, cyanopeptolins and micropeptins, was active against thrombin (IC_{50} = 270 ng/ml), trypsin (IC_{50} = 10 ng/ml) and plasmin (IC_{50} = 30 ng/ml).



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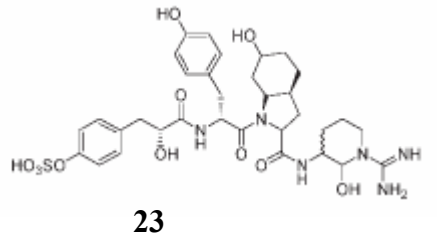

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Microcystin species, such as *M. aeruginosa* and *M. viridis* have been shown to contain a number of interesting biological compounds, not least the aeruginosins, which have been investigated as inhibitors of trypsin-like serine proteases. Matsuda *et al* [77] described the aeruginosins 102-A [(L-Arg)] and 102-B [(D-Arg)] **(23)** as inhibitors of trypsin, thrombin and plasmin. A number of other aeruginosins with inhibitory activity have subsequently been discovered and recently their crystal structures in complex with target proteases have been reported. Aeruginosin 298-A, which contains a C-terminal alcohol analogue, was shown to inhibit thrombin and trypsin (IC_{50}s 0.5 mM and 1.7 mM, respectively), but it has little or no activity against chymotrypsin, elastase or plasmin. It has been shown that amino and peptide alcohols are generally poor inhibitors of the serine proteases [78, 79], Steiner *et al* made an important contribution that agrees with a previous observation that thrombin is generally very tolerant of imprecision in substrate/inhibitor recognition, compared with other serine proteases. This may explain the unusual mode of inhibition observed in the aeruginosin 298-A-thrombin interaction. Aeruginosin 98-B which contains a C-terminal agmatine moiety, inhibits trypsin (IC_{50} = 0.9 mM), plasmin (IC_{50} = 10 mM) and thrombin (IC_{50} = 15 mM). Interestingly, this occurs without the inhibitor making any formal contact with the catalytic triad residues. Inhibition is primarily mediated through interactions with specificity elements within the substrate recognition/binding region [80]. Although it was stated that this mode of inhibition is unique to a natural product, this interaction closely resembles that previously observed

with some synthetic compounds, including (D) Phe-Pro-agmatine [81], argatroban and napsagratran.

Proteinaceous inhibitors

Serine proteases and their natural protein inhibitors are among the most intensively studied protein complexes. About 20 structurally [82] diverse inhibitor families have been identified, comprising α-helical, β sheet, and α/β proteins, and different folds of small disulfide rich proteins. Three different types of inhibitors can be distinguished based on their mechanism of action:

(1) **Canonical (standard mechanism):** The canonical inhibitors bind to the enzyme through an exposed convex binding loop, which is complementary to the active site of the enzyme. The mechanism of inhibition in this group is always very similar and resembles that of an ideal substrate.

(2) **Non-canonical inhibitors**: The non-canonical inhibitors interact through their N-terminal segment. There are also extensive secondary interactions outside the active site, contributing significantly to the strength, speed, and specificity of recognition.

(3) **Serpins:** Like canonical inhibitors, they interact with their target proteases in a substrate-like manner; however, cleavage of a single peptide [82] bond in the binding loop leads to dramatic structural changes.

Cysteine Protease

Cysteine (thiol) proteases [83-86] exist in three structurally distinct classes which are either papain-like (e.g.cathepsins), ICE-like (caspases), or picorna-viral (similar to serine proteases with cysteine replacing serine). The papain group of cysteine proteases has been the most studied until recently. The active site of Papain contains a catalytic triad of Cys25, His159, and Asn175. Asn175 has been proposed to orientate His159, so that the

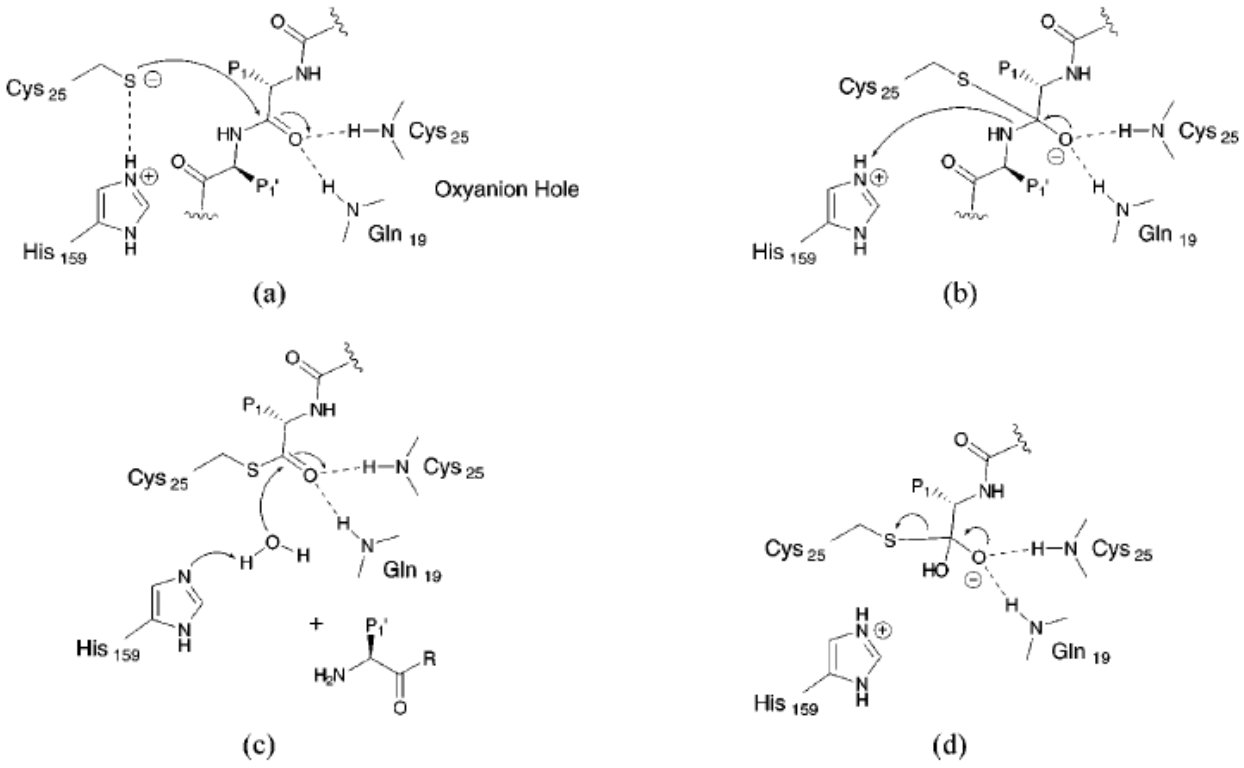


Figure 5. Proposed catalytic mechanism for cysteine proteases. (a) The thiol group of the active site cysteine and the imidazole ring of histidine are believed to exist as a thiolate/imidazolium ion pair. The thiolate anion is therefore highly nucleophilic and readily attacks the scissile amide bond. **(b)** The tetrahedral intermediate produced is stabilized by the oxyanion hole. This intermediate collapses, via acid-assisted catalysis, to the thioester intermediate **(c)** with release of the C-terminal substrate fragment. **(d)** Water hydrolysis gives the regenerated active site and the N-terminal substrate fragment.

imidazole group of His159 polarizes the thiol group of Cys25, allowing deprotonation even at neutral to weakly acidic pH; the resulting thiolate/imidazolium ion pair is highly nucleophilic. Cysteine proteases hydrolyze amide bonds in much the same manner as serine proteases. A noncovalent Michaelis complex is formed upon substrate binding. The thiolate anion then attacks the carbonyl carbon of the scissile amide bond (Figure 5a). A tetrahedral intermediate is produced which is stabilized by the oxyanion hole (Figure 5b). This is followed by the acylation of the enzyme and the liberation of the first product. Hydrolysis of the acyl-enzyme leads to the formation of the second tetrahedral intermediate (Figure 5c). Following the collapse of the second intermediate, the product acid is released and the free enzyme is regenerated (Figure 5d).

Cysteine Protease Inhibitors

Early developments of cysteine protease inhibitors provided useful tools to study the protease activity, only recently significant progress has been accomplished to develop cysteine protease inhibitors into drugs. This development was mostly driven by the dramatic increase in understanding of Papain like cysteine proteases as pharmaceutically valid targets. Cysteine protease inhibitors have been used to develop new drugs. The design and synthesis of cysteine protease inhibitors has a long history and has been extensively reviewed in recent years [87-91]. Compounds synthesized include a wide range of peptide aldehydes, methyl ketones and nitriles as reversibly acting inhibitors and diazomethanes, halomethyl ketones, acyloxymethyl ketones, *O*-acylhydroxamates, and epoxysuccinyl derivatives as irreversible inhibitors.

Aldehydes

Aldehydes and its analogues continue to be attractive moieties despite their well-established chemical reactivity. “Slow binding” inhibition is observed by aldehydes, i.e. the slow attainment of steady state during inhibition with a lag phase having a half life of several minutes, is not due to induction of conformational changes in the enzyme (“slow binding” in the usual sense) but is rather a result of the low concentration of free aldehyde in solution [92]. Recent efforts in designing aldehyde-based inhibitors are focused on achieving high selectivity toward cathepsins. Since cysteine and metalloproteinases have been implicated in tumor invasion and metastasis, double headed inhibitors would be desirable. Development of peptidyl aldehydes as inhibitors of cysteine and serine proteases is based on the assumption that a tetrahedral intermediate is involved in enzymatic hydrolysis. This has led to an investigation of the effect of carbonyl compounds on these proteases, with the intention of developing analogs of this transition state (e.g. Ac-Phe-Glyal) [93, 94]. These are the leupeptines [95], chymostatins [95], antipain [95], elastinal [95], and *β*- MAPI [95]. Peptidyl aldehydes are not selective inhibitors *per se*. They inhibit both cysteine and serine proteases. Peptidyl aldehydes are reversible inhibitors despite binding covalently to the enzyme and peptidyl aldehydes are transition-state analogs [96, 97].

Acyclic and Cyclic Ketones

Investigations of inhibition of the serine protease elastase by peptidyl aldehydes *in vivo* have shown that rapid loss of activity occurs if the aldehyde is oxidized to a carboxylic acid. For this reason, the aldehyde group was replaced by the metabolically more stable trifluoromethyl ketone (TFMK). This produced significantly more potent inhibitors of serine proteases, also *in vitro*. It has also been shown that a tetrahedral hemiketal is formed as a covalent enzyme-inhibitor adduct. Many derivatives have been developed which inhibit different serine proteases [98-101], metalloproteases and aspartate proteases such as pepsin [102], rennin [103], carboxypeptidase A [102] and ACE [102], and esterases such as acetylcholinesterase [104]. In contrast, TFMK and difluoromethyl ketone peptides, and also the corresponding methyl ketones, are much weaker inhibitors of cysteine proteases. The design of mercaptomethyl ketones as cruzipain inhibitors was based on specificity information from diazomethyl ketones and vinyl sulfone inhibitors. Several modifications of the structures such as the incorporation of pyridine, homophenylalanine, and leucine to improve solubility and bioavailability while maintaining selectivity and potency, proved to be successful. Conformationally constrained cyclic ketones were aimed at locking the bioactive conformation of the compounds and to improve bioavailability. The cyclic conformers did not lose potency toward cathepsin K and thus provided a useful scaffold for further modifications. The inhibitors covering extended binding sites of cathepsin K were significantly more potent than the single site analogue . The introduction of a sulfonamide moiety in combination with a six membered ring oxy ketone resulted in a very potent and selective cathepsin K inhibitor [105, 106]. The introduction of an azepanone moiety to prevent epimerization of the chiral center in the cyclic ketone analogues resulted in a further improvement of the potency into the subnanomolar range for human cathepsin K and in increased bioavailabilities of the compounds [107].

Epoxysuccinyl Derivatives

In 1978, Hanada *et al.* succeeded in isolating a highly active, irreversible inhibitor of papain from culture extract of *Aspergillus japonicas* [108]. The substance was identified as 1-[[*N*-(L-3-*trans*-carboxyoxiran- 2-carbonyl)-L-leucyl]amino]-4-guanidinobutane [109], E-64 . Systematic studies were carried out to investigate the role of the different structural components of the inhibitor in enzyme inhibition and the *trans*-L-(*S*,*S*)-epoxysuccinic acid was discovered to be the reactive group essential for inhibition [110]. A change in configuration of the epoxide residue or the neighboring amino acids reduces the activity by a factor of 10-100 [111]. Structural information about the reverse mode of binding of the propeptide to active cathepsin B was utilized in designing novel, extended active site spanning E-64 peptidyl analogues. Selective cathepsin B or cathepsin L inhibitors were synthesized with the most active compounds spanning over six subsites (P4 to P2' residues). The most potent cathepsin B inhibitor derivative contained a Gly-Gly-Leu peptide in the P4-P2 position and a Leu-Pro-OH in the P1'-P2' position [112]. A similar selective and potent cathepsin L inhibitor was obtained by introducing a Phe-dimethylamide moiety into the P2 residue. This compound was effective in reducing tumor-induced hypercalcemia in mice [113,114]. Peptidyl epoxides with tyrosine and biotin moieties allowing iodination and streptavidin-based detection proved very valuable as functional proteomics tools [115,116]. Some analogues like ACB41, ACD15, and ACD16, having 2-hydroxybenzoic acid moiety, were designed and reported to inhibit the trypanosome alternative oxidase (TAO) which ultimately arrest the parasite growth *in vitro* [117].

Vinyl Sulfones

Peptidyl vinyl sulfone inhibitors are remarkably potent irreversible inhibitors of cathepsins [118]. They have been shown to be effective in mice arthritis models by significantly reducing inflammation as well as bone and cartilage erosion [119]. However, due to the irreversible mode of action, vinyl sulfone inhibitors are unlikely to be developed as therapeutic drugs for chronic diseases such as osteoporosis. High potency of vinyl sulfones against various parasitic cysteine proteases makes them ideal

drug candidates for the treatment of acute and chronic infections. Structural analysis of the cruzipain inhibitor complex revealed a covalent Michael adduct with the active site cysteine residue and strong hydrogen bonding interactions in the S1' subsite [120]. The same compound was also orally effective in a mouse model of malaria [121,122].

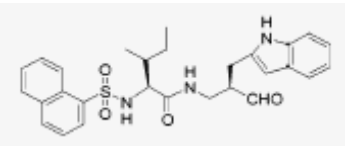
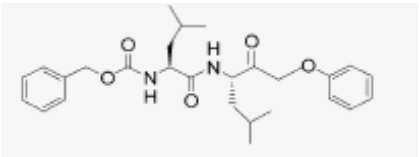
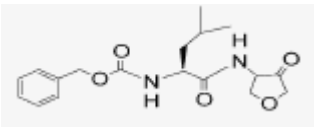
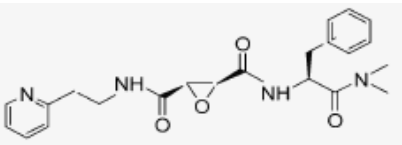
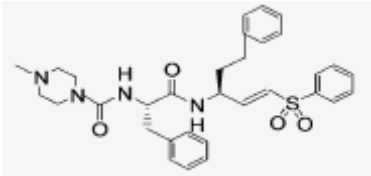
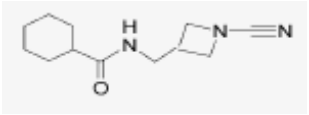
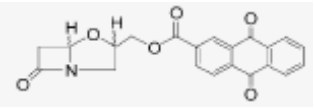
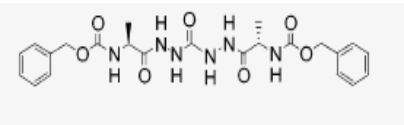
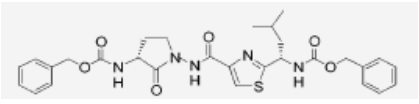
Nitriles

Nitriles are weaker inhibitors than the corresponding aldehydes. Nonpeptidyl nitriles act as reversible, but time dependent inhibitors by forming a cleavable isothiourea ester link with the enzyme [123]. Nonpeptidyl derivatives of nitriles employing pyrrolidine or azetidine rings have been demonstrated to be potent cysteine protease inhibitors. Interestingly, a four-membered ring derivative was approximately 10-fold more potent than the five-membered ring analogue which was possibly due to the increased chemical reactivity of the azetidine ring. Peptidyl nitriles are reversible inhibitors of cysteine proteases. New dipeptidyl cyanamides targeting cathepsin B revealed IC_{50} values in the low nanomolar range whereas cathepsins L and S required approximately 100-fold higher inhibitor concentrations for a comparable inhibition. These compounds inhibited cathepsin B with a reversible mode of inhibition by forming an unstable thioimidazole ester linkage with the active site cysteine residue of the protease [124]. The isothioamides, which are produced by nucleophilic attack of the thiolate on the nitrile carbon, are thought to be the "dead-end" adducts, on the basis of NMR spectroscopic investigations [125,126]. Hydrolysis of the thioamide does not take place [127, 125] and the thioamides are themselves weak inhibitors [128, 129].

***β*-Lactams**

The development of *β*-lactams as cysteine protease inhibitors is, however, very recent. Single ring as well as bicyclic ring β-lactam moieties have been evaluated as inhibitors of cysteine proteases. The *β*-lactam moiety serves as thiol reactive species and is linked

Table 3: Novel Inhibitors of Papain-Like Cysteine Proteases [138]

Chemical Class	Structure	Activity	Mode of inhibition
Aldehydes		Cat L IC_{50}=1.9 nm	Reversible
acycli ketone		Cat K K_{iapp}= 8nm	Irreversible
Cyclic ketone		Cat K K_{iapp}= 140 nm	Reversible competitive
epoxysuccinyl analog		Cat L 63% inhibition at 100nm	Irreversible
Vinyl sulfones		Potent cruzipain inhibitor	Irreversible
Nitrile		CatK IC50=5nm	Reversible time dependent
β-lactam		Cat K IC50=60 nm	Irreversible
Diacyl-bis hydrazide		K_{iapp} >1000 nm	Irreversible
Diamino pyrrolidinone		Cat K K_i= 330 nm	Unknown

with nonpeptidyl or amino acid or peptide portions targeting binding site pockets of relevant cathepsins. 2-Substituted oxapenams employing a nonpeptidyl aromatic or alkyl moiety as subsite motif displayed surprisingly potent inhibitory efficacy in the mid-nanomolar range. On the other hand, the incorporation of amino acid or peptidyl moieties as targeting sequence for individual proteases at the other side of the ring resulted in a significant enhancement of the potency and specificity of the compounds. IC_{50} values in the nanomolar range were observed and the selectivity of the compounds was improved [130-132]. The replacement of the oxygen in the oxapenam ring by sulfur further improved the potency. Mono β-lactams revealed a reversible mode of inhibition. Specificity knowledge based modifications of P and P' positions in peptidyl Mono β-lactams led to the development of more selective cathepsin inhibitors. Selective cathepsin K inhibitors contained heterocyclic and aromatic rings targeting the S' binding sites, while a hydrophobic cyclohexane moiety targeting the S2 pocket produced cathepsin S specific inhibitors [133, 134].

Diacyl Bis Hydrazides

Diacyl bis hydrazides derived from diaminopropanones have been developed as potent and selective cathepsin K inhibitors spanning both S and S' subsites of the substrate binding cleft [135]. The incorporation of bis aza analogues increased the potency while maintaining the selective profile for cathepsin K. The most potent compounds possess a leucine residue either in the P2 or P2' position or in both. Cyclic reversible cysteine protease inhibitor which contains a peptidomimetic thiazole ring in place of an amide bond may form an acyl adduct with the enzyme. In contrast, the acyclic inhibitors are irreversible inhibitors of cathepsin K. The importance of a proper S2P2 and S2'P2' binding interaction was demonstrated by the replacement of the leucine residues by alanine residues. The second-order rate constant of the alanine- containing derivative dropped by more than 2-orders of magnitude with most of the loss due to a dramatically reduced affinity of the compound. In contrast, replacements of the P2' leucine residue with other hydrophobic residues such as norvaline or with a non-amino acid aromatic moiety retained the potency of the compounds [136].

Diamino Pyrrolidinone

Diacyl hydrazides and cyclic thiazole ring analogues were susceptible to acylation which may lead to the proteolytic cleavage of the inhibitors. Therefore, the bis hydrazide moiety was modified by the incorporation of five-membered rings under maintaining the P2 and P2' isobutyl structures. This compound retained the nanomolar affinity toward cathepsin K. However, the removal of the P2 isobutyl chain increased the K_i value 10-fold iterating the importance of P2S2 hydrophobic interactions [137].

Proteinaceous cysteine protease inhibitors

The recent decade has witnessed tremendous development in the field of proteinaceous cysteine protease inhibitors. Though the prototype cystatins discovered in the 1960s remain the best-characterized group, several new large and a few smaller families are now recognized. Currently 10 are described, and the accompanying growth in a number of known inhibitors and processes involving these molecules is even higher. Many natural protein inhibitors of cysteine proteases, called cystatins, have been isolated and characterized. They act both intra- and extracellularly, forming complexes with their target enzymes. In this role, cystatins are general regulators of harmful cysteine protease activities. The roles of cystatins in health and disease have been reviewed by Henskens *et al.* and Grubb. The human superfamily of cystatins is divided into three families. Family I, called stefins, comprises intracellular cystatins A and B. Family II includes extracellular and/or transcellular cystatins (cystatins: C, D, E, F, S, SA, and SN). Kininogens, the intravascular cystatins, form family III of cystatins. Proteinaceous inhibitors of cysteine proteases outside the papain family are scarce.

Aspartic protease

Aspartic proteases [139] generally bind 6-10 amino acid regions of their polypeptide substrates which are typically processed with the aid of two catalytic aspartic acid residues in the active site. Thus there is usually considerable scope for building inhibitor

specificity for a particular aspartic protease by taking advantage of the collective interactions between a putative inhibitor, on both sides

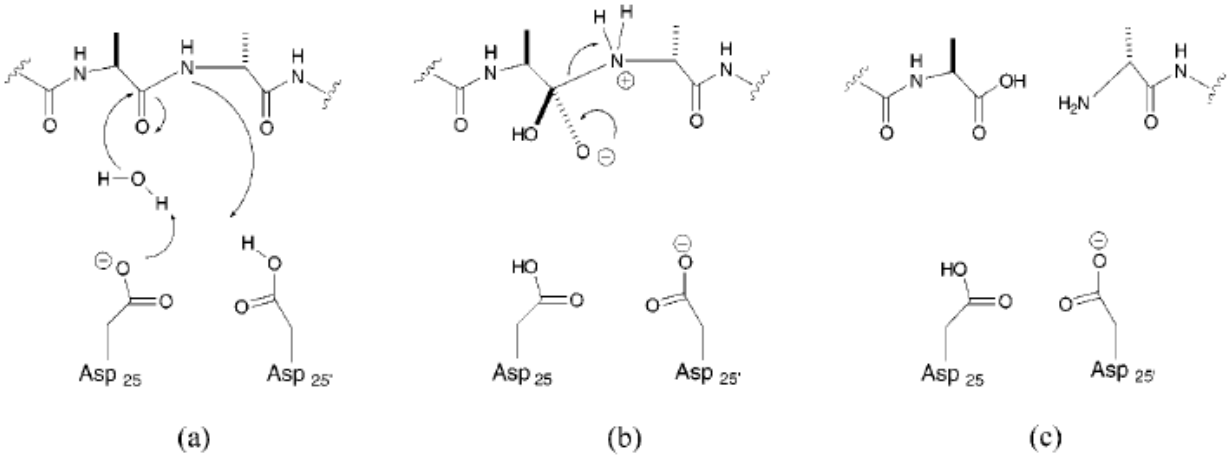


Figure 6: Catalytic mechanism for substrate hydrolysis by aspartic proteases: Nucleophilic attack of an activated water molecule on the scissile amide bond **(a)** and protonation of the amide nitrogen **(b)** give the zwitterionic intermediate **(c)** which collapses to the cleaved products.

of its scissile amide bond, and a substantial portion of the substrate-binding groove of the enzyme. Some aspartic proteases also have one or more flaps that close down on top of the inhibitor further adding to inhibitor-protease interactions and increasing the basis for selectivity. The general acid-base mechanism that is considered most likely for polypeptide hydrolysis catalyzed by aspartic proteases is depicted in Figure 6. The scissile amide bond undergoes nucleophilic attack by a water molecule which itself is partially activated by a deprotonated catalytic aspartic acid residue (Figure 6a). The protonated aspartic acid donates a proton to the amide bond nitrogen, generating a zwitterionic intermediate (Figure 6b) which collapses to the cleaved products (Figure 6c). The water molecule that binds between the enzyme (Ile50 and Ile150) and inhibitor is thought to position a peptide substrate, stretching the peptide bond out of planarity toward a tetrahedral transition state that is stabilized by a second water molecule [140]. Aspartic protease-inhibitor crystal structures are currently available on the PDB database [141] for viral proteases (HIV-1, HIV-2, SIV, FIV), cathepsin D, renin, renin/chymosin,

penicillopepsin, secreted aspartic protease, pepsin, mucoropepsin, retropepsin, saccharopepsin,rhizopuspepsin, and plasmepsin II.

Aspartic protease inhibitors

As an enzyme family, aspartic proteases are a relatively small group. Nevertheless, they have received enormous interest because of their significant roles in human diseases. The best-known examples are the involvement of renin in hypertension, cathepsin D in metastasis of breast cancer, and the protease of human immunodeficiency virus (HIV) in acquired immune deficiency syndrome (AIDS). Therefore, the new understanding of the structure and function relationships of these enzymes has a direct impact on the design of inhibitor drugs.

Aspartic protease inhibitors can be grouped under two categories:

1) Proteinaecous Inhibitors

In a sharp contrast to the ubiquitous presence of multiple forms of proteinaceous inhibitors of other classes of proteases from different sources of plants, animals and microorganisms, there is a paucity of proteinaceous inhibitors of aspartic proteases. With the exception of macroglobulins, which inhibit proteases of all classes, individual protein inhibitors inhibit only proteases belonging to a single mechanistic class. Protein inhibitors of aspartic proteases are relatively uncommon and are found in only a few specialized locations [142]. Few of the examples include renin-binding protein in mammalian kidney [143], a 17-kDa inhibitor of pepsin and cathepsin E from the parasite Ascaris lumbicoides, proteins from plants such as potato, tomato, and squash [144], and a pluoripotent inhibitor from sea anemone of cysteine protease as well as cathepsin D [145]. There is a report of an 8-kDa polypeptide inhibitor from yeast of the vacuolar aspartic protease [146] (protease A or saccharopepsin).

2) Low-Molecular Weight Inhibitors

In contrast to the proteinaceous nature of the protease inhibitors from plants and animals, the inhibitors produced by microorganisms are of smaller molecular nature. Presence of protease inhibitors in microorganisms came into existence from the studies on antibiotics as they act as inhibitors of enzymes which are involved in growth and multiplication. Extracellular proteolytic enzymes hydrolyze organic nitrogen compounds in the medium and are thought to be harmful to cells. The production of inhibitors of the proteolytic enzymes by microorganisms has probably evolved as a mechanism to provide cell protection. Specific inhibitors of microbial origin have been used as useful tools in biochemical analysis of biological functions and diseases. Polysaccharide sulfates have been reported to be pepsin inhibitors, however, their antipepsin activity is weak, and the effect of such polyanionic compounds is not specific. Pepstatin, a low molecular weight aspartic protease inhibitor, isolated from various species of Streptomyces, is a specific inhibitor of pepsin [147]. Streptomyces testacus was reported to produce various pepstatins that differed from one another in the fatty acid moiety (C2-C10) [148]. A pepstatin containing an isovaleryl group has been most widely used for biological and biochemical studies. Moreover, as minor components, pepstanone [149], containing (S)-3-amino-5-methylhexane-2-one instead of the C-terminal (3S, 4S)-4-amino-3-hydroxy-6-methylheptanoic acid (AHMHA), and hydroxypepstatin [150], containing L-serine instead of L-alanine, have also been isolated. Pepstatin containing an acetyl group and propanoyl or isobutyryl groups were isolated from Streptomyces naniwaensis [151]. Pepstatins, pepstanones, and hydroxypepstatins have almost identical activity against pepsin and cathepsin D. However, Pepstatin is more effective against renin than are pepstanone or hydroxypepstatin [152] and its potency against renin increases with the increasing numbers of carbon atoms in the fatty acid moiety [153]. Esters of pepstatin, pepstatinal and pepstatinol, possess anti-pepsin activity similar to pepstatins. Several pepstatin analogs have also been synthesized to date. The acetyl-valyl-AHMHA-L-alanine is the smallest molecular structure that exhibits inhibition against pepsin and cathepsin D similar to pepstatin [152]. Acetyl-L-valyl-L-valyl-[(3S, 4R)-4-amino-3-hydroxy-6-methyl] heptanoic acid prepared by chemical synthesis shows absence of activity [154]. This suggests that the 4S-configuration of AHMHA is essential for

activity. The bacterial enzyme that hydrolyzes the isovaleryl bond in pepstatin has been identified, and from the residual peptide, benzoyl-L-valyl-AHMHA-L-alanyl-AHMHA and L-lactyl-L-valyl-AHMHA-L-alanyl-AHMHA have been synthesized. These analogs are more water-soluble than pepstatin and have almost identical activity against pepsin and cathepsin D, as that of pepstatin. However, these water-soluble analogs have much weaker activity against renin as compared to pepstatin. The addition of aspartic acid or arginine to the C-terminus of pepstatin increases its water solubility. Such water-soluble analogs have same activity against renin as does pepstatin and also have a hypotensive action. Pepstatin has been reported to be effective against experimental muscle dystrophy and enhances the effect of leupeptin [155]. Pepstatin also inhibits leukokinin formation and ascites accumulation in ascites carcinoma of mice [156]. Pepstatin inhibits the growth of Plasmodium beghei [157], and also inhibits focus formation in murine sarcoma virus [158].

HIV-1 Protease Inhibitors

The protease of the human immunodeficiency virus (HIV-1 PR) has proved to be an attractive drug target due to its essential role in the replicative cycle of HIV. A sensitive luminometric assay for high-throughput screening of retroviral protease inhibitors have been developed to produce potent inhibitors [159]. Several low molecular weight inhibitors of HIV-1 PR are now used in humans, including saquinavir, ritonavir, indinavir, nelfinavir, and amprenavir. These are the first successful examples of receptors/structure-based designer drugs and were developed using structures of compounds bound in the active site of HIV-1 PR and with the knowledge of inhibitors of other aspartic proteases (e.g. renin) [160]. All HIV-1 PR inhibitors developed so far target the active site substrate binding groove of the homodimeric enzyme, a long cylindrical cavity that binds 6-7 amino acids via ionic, van der Waals, or hydrogen bonding interactions [161]. Two catalytic aspartates in the center of this cavity promote amide bond hydrolysis. Saquinavir became the first protease inhibitor designed from a three dimensional structure of a protease (structure-based design) to be approved for human use in 1996 [162], despite its low oral bioavailability due to poor absorption and extensive firstpass degradation by cytochrome P450 [163]. Ritonavir is itself a potent

inhibitor of HIV-1 PR with high oral bioavailability [164]. Indinavir or Crixivan is another potent inhibitor of HIV-1 and HIV-2 protease, which halts the spread of HIV infection in MT4 lymphoids cells and is orally bioavailable. The mesylate salt of nelfinavir, approved for human use in 1997, is a lipophilic protease inhibitor with good oral bioavailability in rats and monkeys [165]. Computational modifications in Nelfinavir, optimizing its affinity to the most conserved amino acids in protease, in order to increase the efficiency of new inhibitor also play important role to combat the pathogens [166]. Ampenavir, is a water-soluble, orally bioavailable inhibitor with long half life, allows less frequent administration of drug thereby having the potential for less side effects with respect to other marketed HIV protease inhibitors described above. Viral resistance to "monotherapy" with any of these drugs is a significant problem [167]. Serial passages of HIV-1 in vitro in the presence of increasing concentrations of a protease inhibitor cause rapid emergence of drug-resistant viral strains of HIV-1. Thus, new HIV protease inhibitors with different resistance profiles are still being actively pursued. A number of second-generation inhibitors have been developed. ABT -378 was designed to inhibit mutant proteases produced in response to ritonavir. It is 10 fold more potent against ritonavir-resistant strains and displays lower binding to serum proteins. CGP-73547 inhibits indinavir resistant and saquinavir resistant strains of HIV-1, is orally bioavailable [168]. One of the most promising preclinical candidates for HIV protease inhibition is palinavir. This compound is a very potent, orally active inhibitor of HIV-1 and HIV-2 proteases with high antiviral activity.

Renin Inhibitors

The aspartic protease, renin, is involved in the rate-limiting step of the renin-angiotensin (RAS) system, by hydrolyzing the α 2-globulin angiotensinogen to release the 10-residue peptide angiotensin I. Because of its specificity, renin inhibitors are antihypertensive agents similar in action to ACE inhibitors, and AII antagonists, but are free of some side effects associated with ACE inhibitor administration. For example Zankiren (A-72517), a potent inhibitor of human plasma renin, is the peptidic inhibitor with significant oral absorption [169]. Renin inhibitors have mainly been developed by modifying substrate

fragments from the angiotensinogen cleavage site, but their clinical progress has been hampered by their peptidic character, which confers low stability and poor oral bioavailability in humans. Renin inhibitors generally need to interact with five subsites (S4-S1') of the enzyme to bind tightly and selectively compared with only three for ACE inhibitors. Low molecular weight and less peptidic inhibitor like CP-108 671 was designed from the cleavage site of angiotensinogen and the structure of the general aspartic protease inhibitor pepstatin [170]. It uses a cyclohexylnorstatine transition-state analogue, a (R)-benzylsuccinate, at P3 for chymotrypsin stability and is a potent inhibitor of human plasma renin. It is highly selective over most aspartic proteases but does weakly inhibit cathepsin D. BILA 2157 BS is another potent renin inhibitor with some selectivity towards cathepsin D and oral activity. A combination of the X-ray crystal structure of CGP38560 bound rennin, and previous information [171] that the S3 subsite can be accessed by extending the P1 residue has helped in developing several other nonpeptidic inhibitors with good activity and specificity. These nonpeptidic, low molecular weight compounds represent excellent progress towards the necessary features (oral bioavailability and economic production) for renin-binding drugs but may require improved selectivity.

Plasmepsins Inhibitors

Plasmepsins I and II, found in the malarial parasite Plasmodium falciparum, are aspartic proteases that are believed to be essential for the degradation of its major food source, human hemoglobin [172]. Inhibition of these enzymes, which have 73% and 35% sequence homology with human cathepsin D, is therefore considered to be a viable therapeutic strategy for the treatment of malaria. Both plasmepepsin I and II are believed to initially cleave the Phe33-Leu34 peptide bond of the α-chain of hemoglobin, followed by cleavage of the polypeptides into smaller fragments which are subsequently processed by the cysteine protease falcipain. SC-5003, the first peptidomimetic inhibitor reported to selectively inhibit plasmepsin I and II, was active in vitro against the live parasite preventing hemoglobin degradation [173]. X-ray crystallographic structure of plasmepepsin II complexed to pepstatin A have been used to develop peptidic inhibitors which starves the live parasite in vitro as well as inhibits human cathepsin D.

Interestingly, a combination of cysteine and aspartic protease inhibitors was recently found to be more effective than either compound alone in inhibiting Plasmodium-mediated hemoglobin degradation in both culture and a murine malaria model [174]. This synergy suggests that combination therapy may be a viable strategy for antimalarial treatment regimes of the future.

Cathepsin D Inhibitors

Human cathepsin D is an intracellular aspartic protease mainly found in lysosomes. It has a number of "house-keeping" functions including degradation of cellular and phagocytosed proteins for reprocessing. The enzymes may be involved in a variety of disease states, including cancer and Alzheimer's disease. Clinical studies have shown that cathepsin D is overexpressed in breast cancer cells, and this seems to be associated with an increased risk of metastasis due to enhanced cell growth. Cathepsin D or a similar aspartic protease is also thought to be involved in formation of β-amyloid peptide in Alzheimer's disease [175]. The availability of selective and potent inhibitors will help to further define the role of cathepsin D in disease and possibly lead to therapeutic agents. Relatively few inhibitors of cathepsin D have been reported, partly because of its uncertain role as a viable target for therapeutic intervention. Although a general inhibitor of aspartic proteases, pepstatin A remains the most potent inhibitor known. There have been reports of cyclic inhibitors designed from the X-ray structures using the fact that the enzyme-bound conformation of the P2 and P3' residues of pepstatin are in close proximity to each other [176]. This allows cyclization of the inhibitor thereby increasing the proteolytic stability of the three-amide bonds in the cycle. Combinatorial approaches have been carried out for the development of inhibitors to prove the methodology for the optimized specificity against other aspartic proteases.

Secreted Aspartic Protease Inhibitors

The Candida yeast strains C. albicans, C. tropicalis, and C. parapsilosis exist in small quantities in a healthy intestinal tract but become a health problem when the immune system is compromised. Such opportunistic infections arise in AIDS patients where C. albicans is a serious pathogen of the mucous membranes [163]. It is also the major cause

of vaginitis and has been implicated in liver toxicity and in development of multiple chemical allergies. C. tropicalis is the predominant cause of fungal infections in neutropenic cancer patients. These organisms have the ability to secrete into the host [177] several aspartic proteases (SAP, secreted aspartic protease) of broad specificity. These proteases are thought to be linked to the virulent effects of Candida strains in humans as protease-deficient mutants reduce the virulence. The HIV-1 PR inhibitor indinavir is a weak but specific inhibitor of SAP and greatly reduces the viability and growth of C. albicans [178]. These enzymes are therefore becoming attractive targets for therapeutic attack. Nine SAPs have been identified in the genome of C. albicans to date (SAP1-9). From mutation experiments, SAP2 seems to be the dominant isoenzyme for the normal progression of systemic infection, while SAP1 and 3, are also important for overall virulence of C. albicans [179]. SAP4-6 appears to play a role in the process of induction of SAP2. X-ray crystal structures have been determined for SAP2 complexed to pepstatin, a close homologue SAP2X bound to the same inhibitor, and a SAP enzyme of C. tropicalis. Very little inhibitor design has been reported for SAP2. A-70450 was originally designed to inhibit rennin and later found to be nonselective inhibitor of the SAP of C. albicans. This inhibitor incorporates the (S)- hydroxylethylene isostere with the hydroxyl group positioned in the crystal structure between two catalytic aspartate residues.

Future perspectives

Past drug development failures provide not only invaluable lessons but are also a useful resource. Many of these failed compounds, especially specific inhibitors, could still be used as powerful tools in the target validation process and in the evaluation and discovery of protease signaling pathways by both academic and industrial researchers. The major areas of interest for protease-targeted therapies are likely to remain the cardiovascular, inflammatory and infectious diseases areas, but discovery efforts will probably increase for cancer and neurodegenerative disorders. A more detailed look at the proteases currently considered as potential targets shows that endogenous proteases are often linked to chronic diseases, and are therefore attractive to pharmaceutical companies. However, there has recently been a revived interest in protease-targeted drugs for infectious

diseases, although only a few (AIDS, hepatitis C) were considered seriously until recently. With the emergence of new technologies, and as the structure and physiological function of more proteases are revealed and updated, it should hopefully become easier both to identify and validate proteases as relevant drug targets and to develop effective and safe drugs against them.

Present Investigation

Like other mussels, *Perna viridis* is a cilliary-mucus suspension feeder. Firmly attached to a substrate, immobile mussels use their incurrent siphon to draw in food-laden water that is then carried by ciliary action to the branchial chamber. Green mussels are coastal bivalves, typically occurring at depths of less than 10 m, and shown to be tolerant of a wide range of turbidity and pollution. However, it has not been chemically examined for its bioactive constituents. In this endeavor, we have undertaken systematic activity guided isolation of bioactive constituents from this mussel for protease inhibitor. The objective of present investigation was to search a potent low molecular weight protease inhibitor, natural and synthetic, in order to combat the pathogens by targeting their proteases which are responsible for pathogenicity. In this endeavor we have chosen marine source as natural source of inhibitor since marine invertebrates are rich sources of small molecules with unique chemical skeletons and potent bioactivities. We here also screened several analogs based on different scaffolds for protease inhibitors and checked their antibacterial and antifungal properties. A thermodynamic study was done to examine the mode of binding of these inhibitors with their respective proteases. The SAR studies of these inhibitors with their proteases have been analyzed. We have carried some *in silico* studies to characterize our compounds as possible potent antibacterial drugs.

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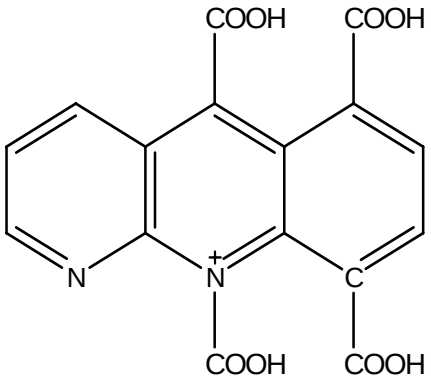
Chapter 2

Purification and characterization of serine protease inhibitor (SPI) from hemolymph of green mussel, *Perna viridis*

SUMMARY

Bioactivity guided fractions of cell free hemolymph of bacterially challenged marine mussel, *Perna viridis* led to the isolation of a novel quaternary alkaloid **1** by silica gel column and RP-HPLC chromatography, which was identified by its LC-MS, FTIR, ^{1}H NMR, ^{13}C NMR, ^{13}C DEPT , HMBC ^{1}H-^{13}C spectral data. The Compound **1** was obtained as an amorphous solid, *m/z* 355.0279 ($[M]^{+}$), $C_{16}H_9O_8N_2$. The isolated molecule **1** has been found to be a potent serine protease inhibitor (SPI) showing IC_{50} and K_i values of 102.5 μM and 97.1- 104.68 μM, respectively. During the initial kinetic analysis, the inhibitor showed competitive inhibition against trypsin *in vitro*. The E_t/K_i value of SPI is 6.3 whereas E_t/K_M value is 1.04. The Van't Hoff analysis showed that the value of K_i decreases with increase in temperature and the binding of the inhibitor is entropically driven. The inhibitor was found to be specific for trypsin and did not show any inhibition against chymotrypsin. It also did not show any activity against other classes of proteases.



Structure of isolated bioactive compound **1**

INTRODUCTION

Nature continues to be one of the most important sources of pharmacologically active compounds in the quest for drugs against life threatening diseases such as microbial infections, diseases of the heart and the circulatory system, cancer etc. Marine natural

products provide a rich source of chemical diversity that can be used to design and develop new, potentially useful therapeutic agents. Invertebrates possess a specific and innate immune mechanism, and lack an immune memory following the first encounter with a pathogen. Molluscs have humoral and cellular immunity, and the humoral system is constituted by lysosomal enzymes, agglutinins, lectins and antimicrobial peptides. Nevertheless, cellular immunity seems to perform the main role in shellfish immune processes [1]. Historically, one abundant source of novel therapeutic agents has been natural products [2-3]. Many of these have acted as pharmacophores or templates from which therapeutically useful agents have been designed. In fact, the first reported bioactive marine natural products, spongouridine and spongothymidine, served as templates for the development of cytosine arabinoside, an anticancer agent [4]. Halisulfate and suvanine another examples of novel therapeutic agents isolated from marine animals that inhibit serine proteases thrombin and trypsin [5].

In vertebrates, serine protease inhibitors have been studied extensively and they are known to be involved in phagocytosis, coagulation, complement activation, fibrinolysis, blood pressure regulation, *etc.* In the last decade, it became obvious that even in invertebrates, serine proteases and their inhibitors are involved in parallel physiological processes e.g. the blood clotting cascade in *Limulus* [7] and the innate immune response [7]. Moreover, some of the protease inhibitors isolated from invertebrate sources are quite specific towards individual mammalian serine proteases.

Perna viridis, the Asian green mussel, is a large (> 80 mm) bivalve, with a smooth, elongate shell. The native range of the Asian green mussel broadly encompasses the Asia-Pacific and Indo-Pacific regions [8]. Primarily, the green mussel has been utilized as a pollution control indicator [9-11]. However, it has not been chemically examined for its bioactive constituents. In this endeavor, we have undertaken systematic activity guided isolation of bioactive constituents from this mussel for the presence of protease inhibitor activity.

MATERIALS

Microbes *Staphylococcus aureus* (ATCC-25923); *Proteus vulgaris* (ATCC-29905); *Group D Streptococci* (ATCC-12959); *Bacillus spp.* (ATCC-14593); *Escherichia coli* (ATCC-25922); *Klebsiella pneumonia* (ATCC-13883) ; *Pseudomonas aeruginosa* (ATCC-27853); *Serratia morganii* (ATCC- 31665), obtained from Goa Medical college, Goa, India. Trypsin (EC 3.4.21.4) and Bz-DL-Arg-pNA (BAPNA) were purchased from Sigma Chemical Co., St. Louis, MO, USA. RP-HPLC (C_{18} symmetry) column was from Waters. All solvents and chemicals were of analytical grade and were used as obtained from Merck and Sigma-Aldrich.

METHODS

Isolation of Hemolyph from *Perna viridis*

The mussels were collected from western coast of Goa, India and were maintained under laboratory condition in sea water in an aerated glass aquarium. The mussels were acclimatized for 15 days before the start of experiment. Mussels (80-100 mm in length) were used as a source of hemolymph. Humoral immune responses were elicited by injecting a combination of G^+ and G^- human pathogenic bacteria in 0.85% brine solution into the posterior adductor muscles. The bacteria used were G^+ (*S. aureus, S.* Group D) and G^- *(P. vulgaris, E. coli, P. aeruginosa, K. pneumonie, S. morganii).* The bacteria were grown separately in the LB broth till the OD 0.6 at 660 nm was reached. Grown culture (4ml) was taken and bacterial cells were pelleted by centrifugation at 12,000 rpm at 4^0 C for 15 min. These pellets were washed twice with 0.85% NaCl (saline buffer). The pellets of different bacteria were mixed and suspended in 1500 µl saline buffer. Animals were injected with 100 µl of a cocktail of bacteria into the posterior adductor muscles. Hemolymph collection was done by slightly opening the animals with the help of forceps so that adductor muscle does not break and then hemolymph was collected from posterior adductor muscles. Hemolymph was collected into Alsevier solution and immediately

centrifuged at 1000 rpm at 4^0 C for 15min and the supernatant collected pooled and stored at -80^0 C for further use.

Purification of serine protease inhibitors from immune hemolymph

Frozen cell-free immune hemolymph (500 ml) was thawed and subjected to ultrafilteration by using a 3KDa membrane. The filtrate below 3KDa was lyophilized to dryness and subjected to organic solvent extraction. The lyophilized hemolymph (42 gm) was exhaustively partitioned between petroleum ether (3x300 ml), chloroform (3x300 ml), ethyl acetate (3x300 ml), and MeOH (3x300 ml). Each step of fractionation was monitored by the *in vitro* serine protease assay. The MeOH fraction (3 g) was loaded on silica gel column and eluted with $CHCl_3$: MeOH (0→5%). Fractions eluting with 3% MeOH-$CHCl_3$ showing bioactive violet spots on TLC were pooled together and subjected to preparative TLC (0.25 mm, 20 x 20 cm, SiO_2 $GF_{254,}$ Merck India) and developed in the solvent system of butanol: acetic acid: water (4:4:2). The crude product so obtained was finally purified (10 mg) by RP-HPLC (fig 1) (C_{18} symmetry, MeCN (5→95%): H_2O R_t 10 min, detection at 214 nm and 280 nm).

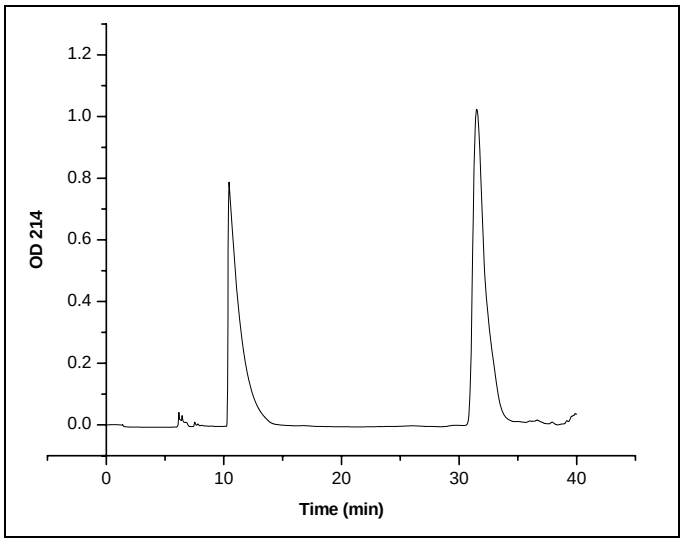


Fig 1 HPLC profile of compound 1

Structure elucidation

Compound **1** was obtained as a amorphous solid, *m/z* 355.0279 ([M] $^{+}$) (Fig 2), $C_{16}H_9O_8N_2$. The infrared **(IR)** spectrum (Fig 3) of **1** suggested the presence of aromatic C-H stretching (2925, 2854 cm^{-1}), tertiary amine, C=N stretching (1687.48, 1666.80 cm^{-1}) and carbonyls (1687, 1666 cm^{-1}). The ^{1}H NMR spectrum (Fig 4) of **1** showed the presence of five aromatic protons at δ 7.59 dd (2H, J=8.5, 7.0 Hz), δ 7.69 dd (1H, J=7.6, 7.2Hz) and δ 7.79 d (2H, J=7.6Hz) and a D_2O exchangeable broad singlet at 9.15 ppm integrating for four protons corresponding to four carboxylic protons. The ^{13}C NMR spectrum (Fig 5) of **1** showed 9 carbon signals at 116.0, 118.0, 128.5, 128.8, 129.5, 134.2, 158.0, 159.0 and 166.2 ppm. The carbon signals appearing at 116.0, 118.0, 128.8, 158.0, 159.0 and 166.3 ppm were found to be of quaternary nature by its ^{13}C DEPT (Fig 6). The proton signal appearing at δ 7.79 in the ^{1}H-^{1}H-COSY (Fig 7) spectrum showed the connectivity with the proton signal at δ 7.59 and 7.69. The structure was further confirmed by its HMBC ^{1}H-^{13}C spectrum (Fig 8) and the connectivities have been shown in Fig 9. The multiplet at 7.59, 7.69 and 7.77 ppm showed the correlations with the carbon signals appearing at 128.5, 129.5 ppm

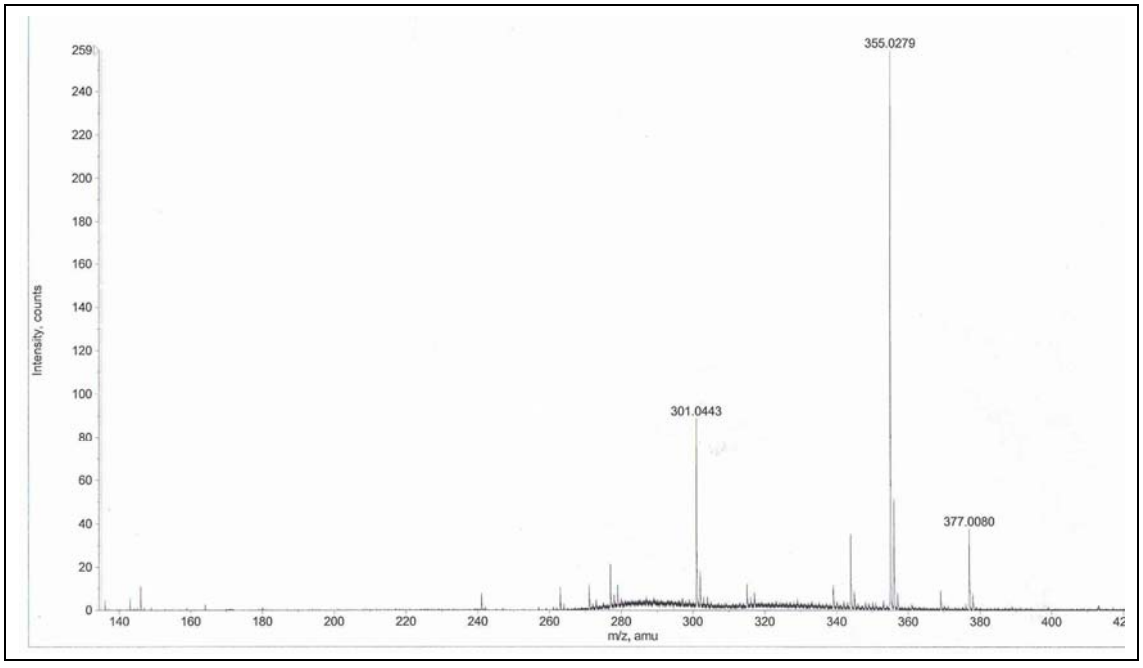


Fig 2 LC-MS spectrum

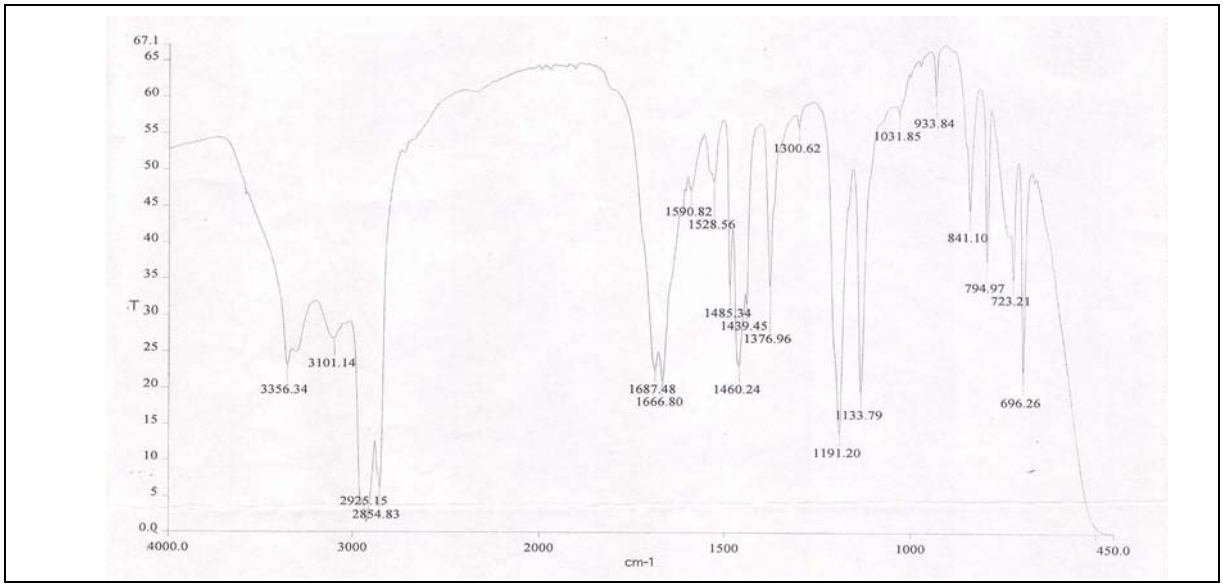


Fig 3 FTIR Spectrum

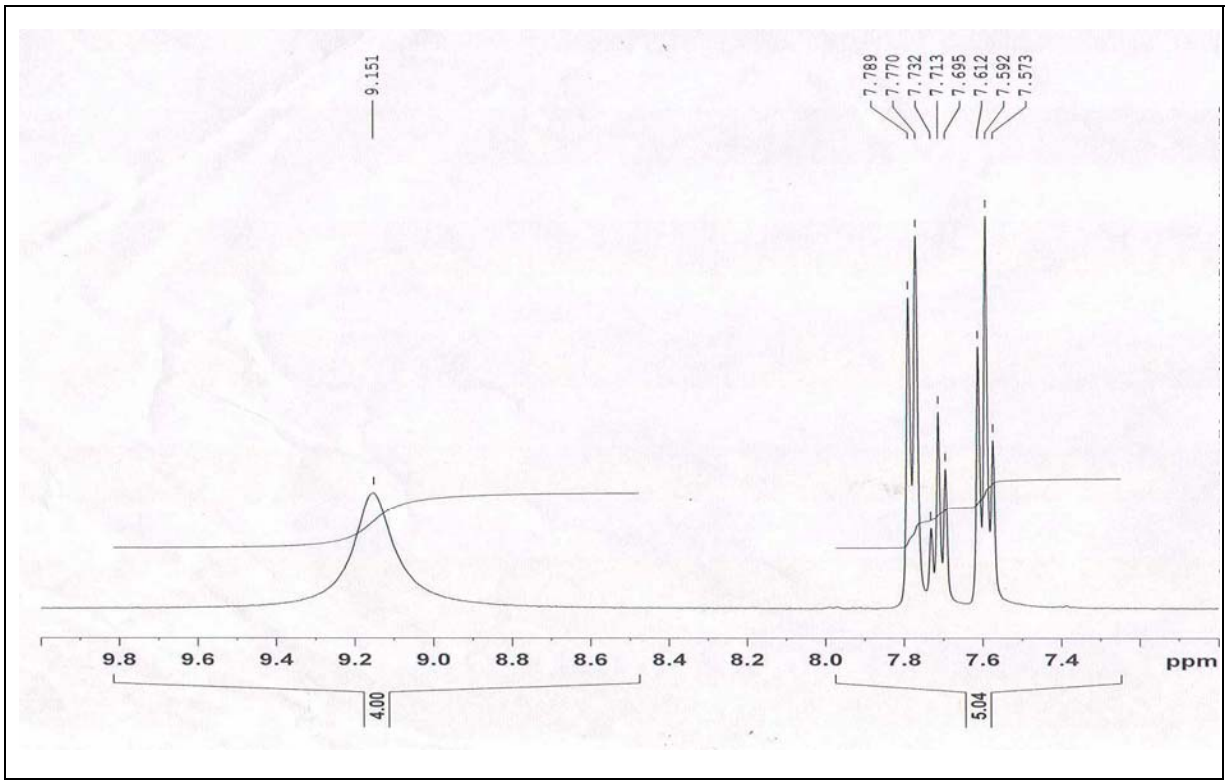


Fig 4 ^{1}H-NMR Spectra

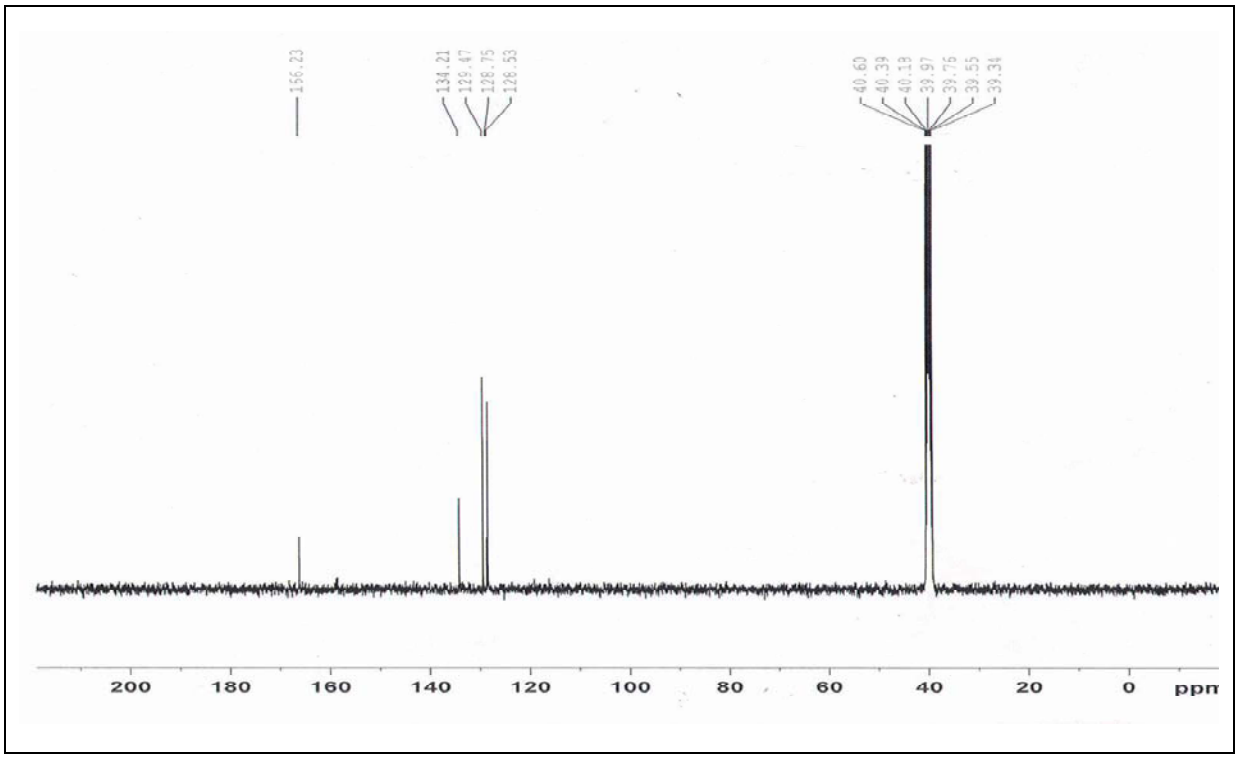


Fig 5 ^{13}C –NMR Spectrum

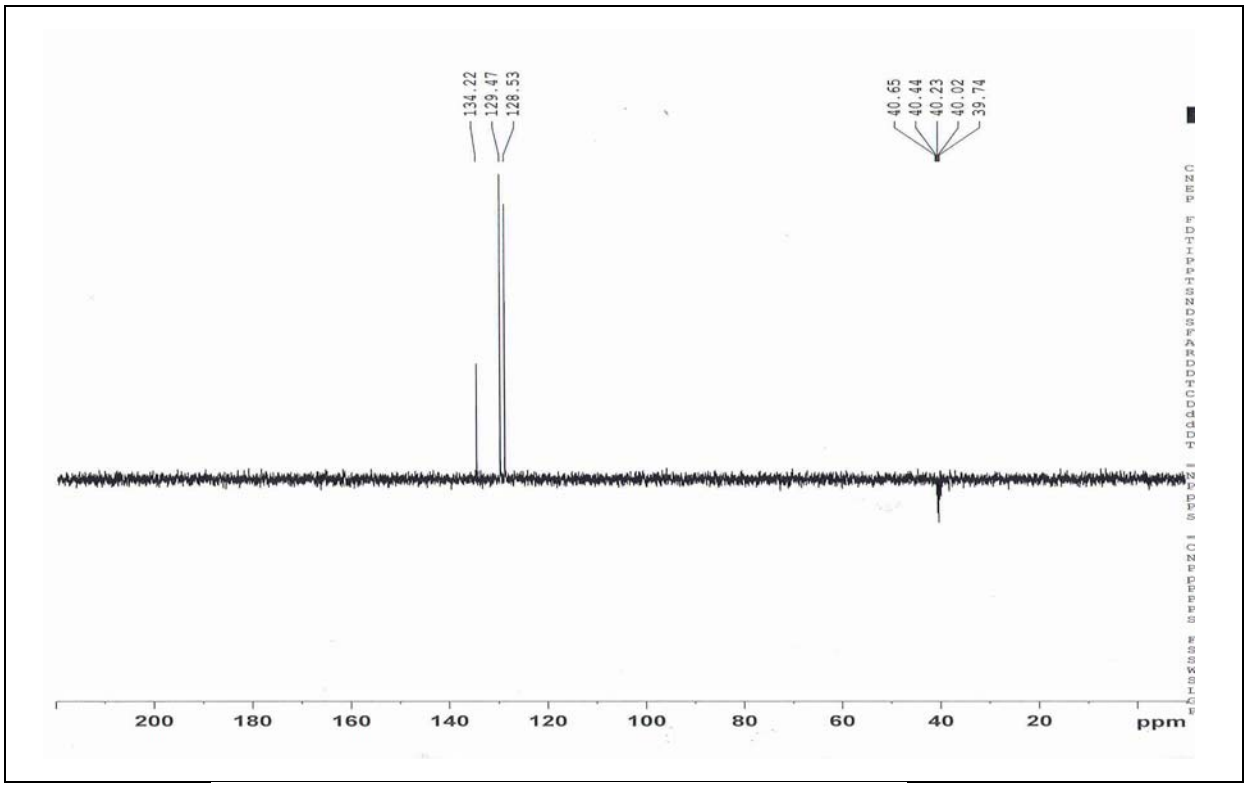


Fig 6 ^{13}C-DEPT

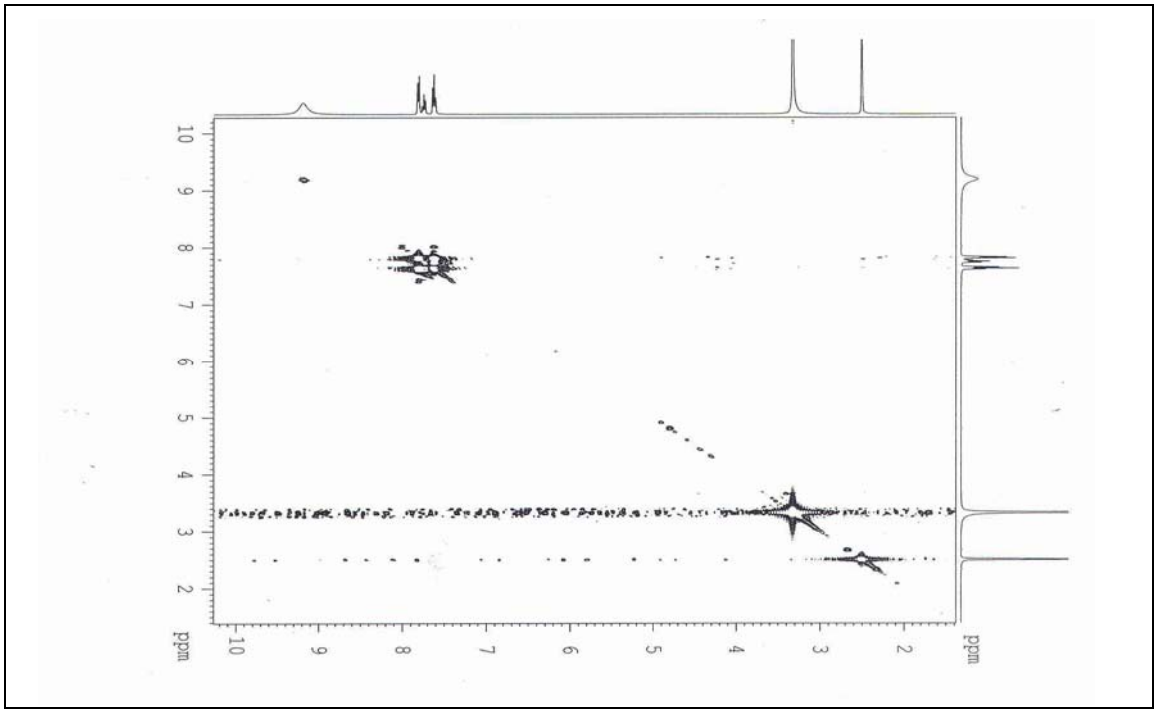


Fig 7 COSY

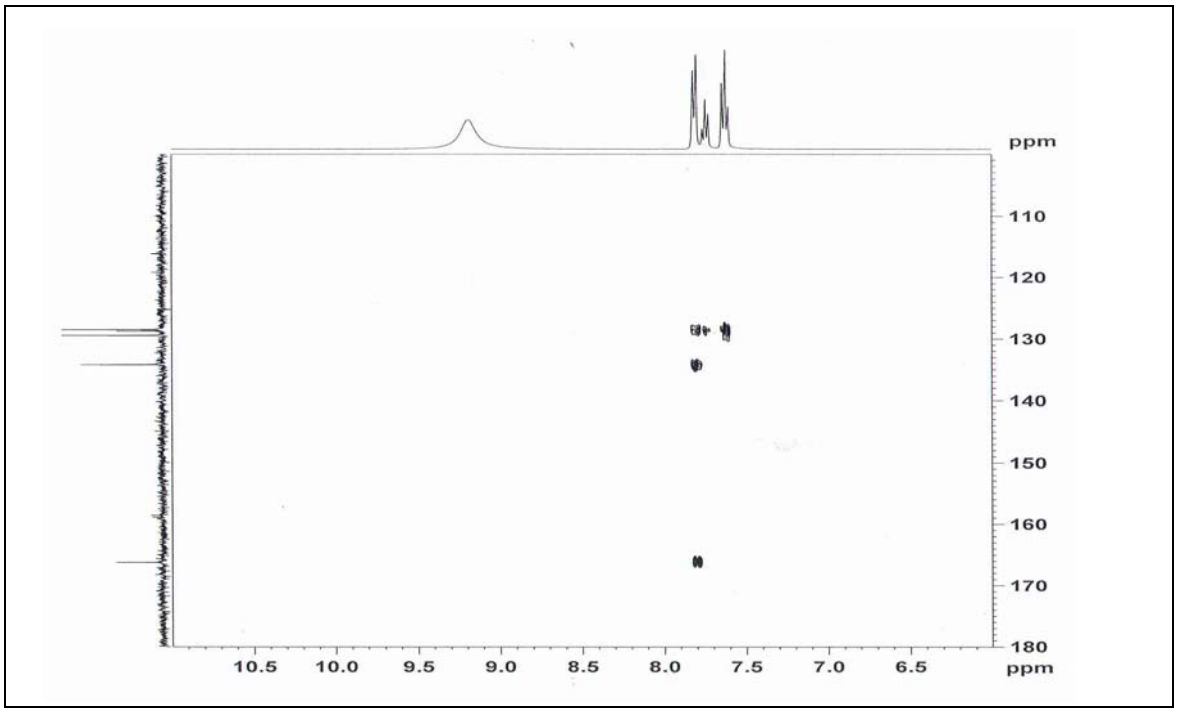


Fig 8 HMBC

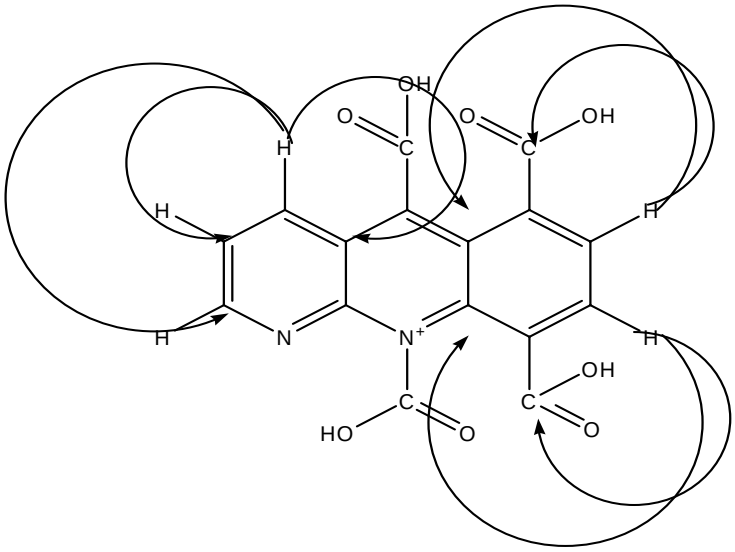


Fig 9 Complete HMBC correlations in compound

and specially the proton signals at 7.77 ppm showed further correlation with 134.2 and a carbonyl frequency appeared at 166.2.

On the basis of ^{13}C NMR, DEPT, and 2D NMR and LC-MS spectra, the isolated compound has been identified as **1** [Fig 10].

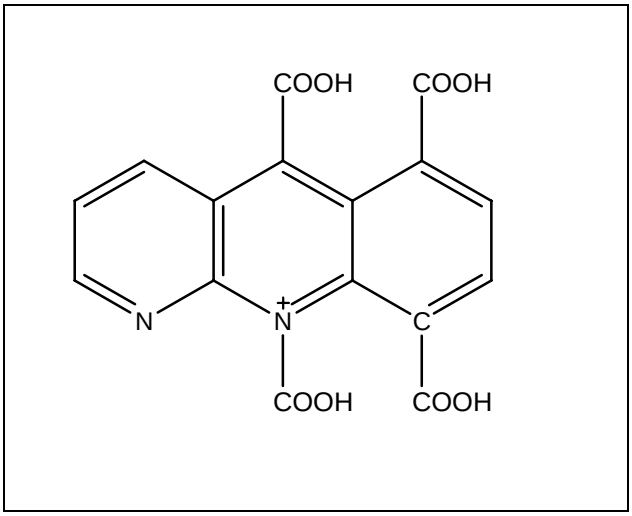


Fig 10 Structure of isolated bioactive compound **1**

Assay for inhibitory activity towards trypsin for purification of serine protease inhibitor

10µl of trypsin (100µg/ml) and 100µl inhibitor solution from each step of purification were incubated at 37 ^{0}C for 10min in tris–HCl buffer, pH 8.0. Then 0.5ml of hemoglobin (0.6 %) dissolved in the same buffer was added and incubated at 37 ^{0}C for 30min. The reaction was stopped by the addition of 5% trichloro acetic acid (TCA) acidified with 2.25% HCl. One unit of trypsin was defined as the amount of enzyme that produced an increase in absorbance of 0.001 at 280nm per minute under the conditions of the assay. One protease inhibitor unit is defined as the amount of inhibitor that inhibited one unit of trypsin activity [19].

Assay for inhibitory activity towards trypsin for inhibition kinetics

The enzyme activity was measured by using N-α-benzoyl-L-arginine p-nitroanilide (BAPNA) as the substrate. The standard assay mixture contained 50 mM Tris hydrochloride buffer (pH 8.5), 20 mM $CaCl_2$, and 0.2 mM BAPNA in a final volume of 2 ml. The enzyme activity was measured at 37^{0}C by recording the rate of the increase in the A_{410} [20] with a Gilford model 240 spectrophotometer. One unit of activity is defined as the amount of enzyme required to release 1 µmol of p-nitroaniline per min (the molar extinction coefficient of p-nitroaniline at 410 nm is 8,800 M^{-1} cm^{-1}). The rate of hydrolysis of N-α-benzoyl-L-arginine ethyl ester (BAEE) was determined by recording the change of the A_{255} in the same reaction mixture by using a molar absorption difference of 808 M^{-1} cm^{-1} [21]. The hydrolysis of N-α-p-tosyl-L-arginine methyl ester and N-α-p-tosyl-L-lysine methyl ester was followed at an A_{247} [22]. For the kinetic analysis and rate constant determinations, the assays were carried out in triplicates, and the average value was considered throughout this work.

Initial kinetic analysis for the determination of K_M and K_i

The kinetic parameters for the substrate hydrolysis were determined by measuring the initial rate of enzymatic activity. The inhibition constant K_i was determined by Dixon[12] method and also by the Lineweaver–Burk equation. The K_M value was also calculated from the double-reciprocal equation by fitting the data in to the computer software Origin 6.1. For the Lineweaver–Burk analysis of trypsin (612 μM) was incubated with 60 μM and 120 μM inhibitor and assayed at increasing concentration of BAPNA [13-16] (300–1250 μM) at 37 ^{0}C for 30 min. The reciprocals of substrate hydrolysis (1/v) for each inhibitor concentration were plotted against the reciprocals of the substrate concentrations, and the K_i was determined by fitting the resulting data. In Dixon's method, hydrolytic activity of trypsin (612 μM) was measured in the presence of 400 μM and 800 μM BAPNA, at concentrations of inhibitor ranging from 60 μM to 140 μM at 37 ^{0}C for 30min. The reciprocals of substrate hydrolysis (1/v) were plotted against the inhibitor concentration and the K_i was determined by fitting the data using Origin 6.1. The inhibitor was found to inhibit trypsin with an IC_{50} value (50% inhibitory concentration) of 102.5 μM (Fig. 11). The inhibition of trypsin followed a hyperbolic pattern with increasing concentrations of the inhibitor. However, the secondary plot (the slope of inhibition graph versus inhibitor concentration) was not linear, suggesting that the application of Michaelis–Menten inhibition kinetics was not appropriate in this study. The inhibition constant Ki, determined by the classical double reciprocal plot was 104.68 μM and by Dixon plot was 97.1 μM (Fig. 12), which is almost equal to the IC_{50} value of the inhibitor. The Line Weaver–Burk reciprocal plot (Fig. 13) showed that the inhibitor was a competitive inhibitor of trypsin and the K_M value for the trypsin with BAPNA was 588 μM and K_i for the inhibitor was 104.68 μM. For the kinetic analysis and rate constant determinations, the assays were carried out in triplicates, and the average value was considered throughout this work.

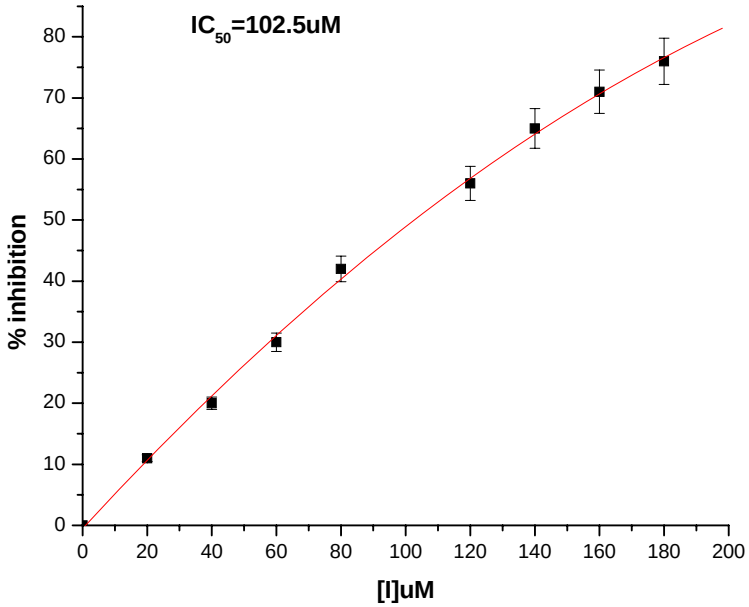


Fig 11. The sigmoidal curve indicates the best fit for the percentage inhibition data obtained, and the IC_{50} value was calculated from the graph.

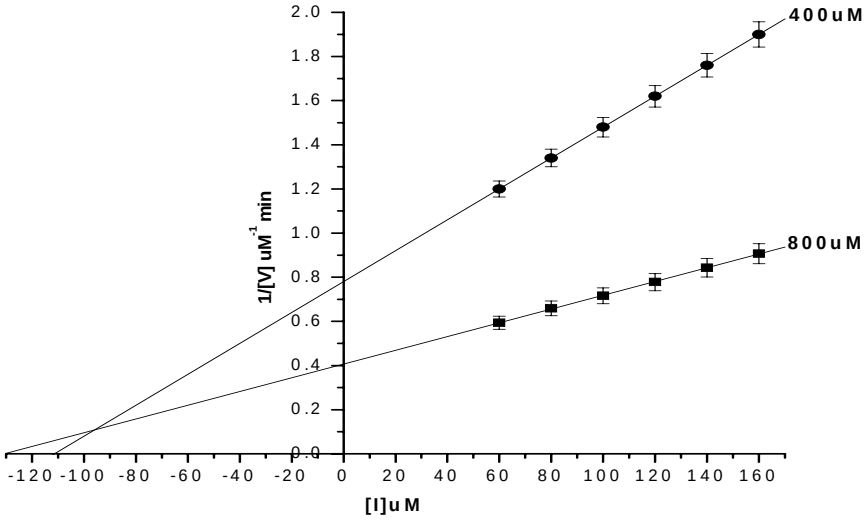


Fig 12. Enzymatic activity of the trypsin (612 µM) was estimated using the substrate BAPNA= 400 µM (●) and 800 µM (■) at different concentrations of SPI. Reciprocals of the reaction velocity were plotted versus the SPI concentration. The straight lines indicated the best fit of the data obtained. The inhibition constant Ki was calculated from the point of the intersection of the plots.

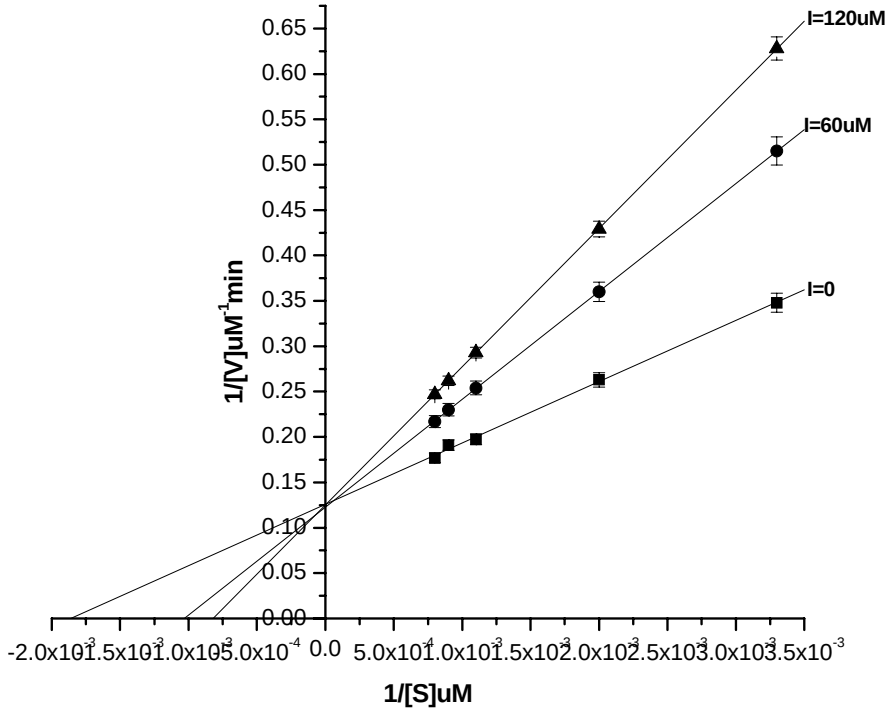


Fig 13. Trypsin (612 μM) was incubated without (■) or with SPI at 60 μM (●) and 120 μM (▲) concentrations and assayed at increasing concentrations of the substrate. The reciprocals of the rate of substrate hydrolysis for each inhibitor concentration were plotted against the reciprocals of the substrate concentrations. K_i was determined from the formula as per the competitive type of inhibition.

The inhibitor was found to be specific for trypsin and did not show any inhibition against chymotrypsin. It also did not show any activity against other classes of proteases. Goldstein (1944) showed that the ratio E_t/K_i should be 0.01 or less for a Michaelis-Menten analysis to be valid at sub-saturating concentration of inhibitor. If E_t/K_i exceeds 100 then virtually all of the added inhibitor molecules become bound to the enzyme and at intermediate E_t/K_i values, the total inhibitor is distributed between molecules free in solution and those complexed with the enzyme [17]. By a similar argument, the ratio E_t/K_m reveals the validity of the Michaelis- Menten treatment for rates obtained with substrate alone in the system [17-19]. The E_t/K_i value of SPI is 6.3 whereas value for E_t/K_m of SPI is 1.04.

Thermodynamics analysis of SPI

Free energy changes of inhibition of Trypsin against SPI (ΔG) were determined by the equation,

$$\Delta G = -RT\ In K_a \qquad (1)$$

Temperature dependence of the inhibition constants was used to determine the thermodynamic parameters. Changes in enthalpy (ΔH) were determined from the Van't Hoff plots by using the equation,

$$In\ K_a = (-\Delta H/RT) + \Delta S/R \qquad (2)$$

Where ΔH is enthalpy change, R is gas constant, ΔS is entropy change and T is the absolute temperature. The entropy change was obtained from the equation,

$$\Delta G = \Delta H - T\Delta S \qquad (3)$$

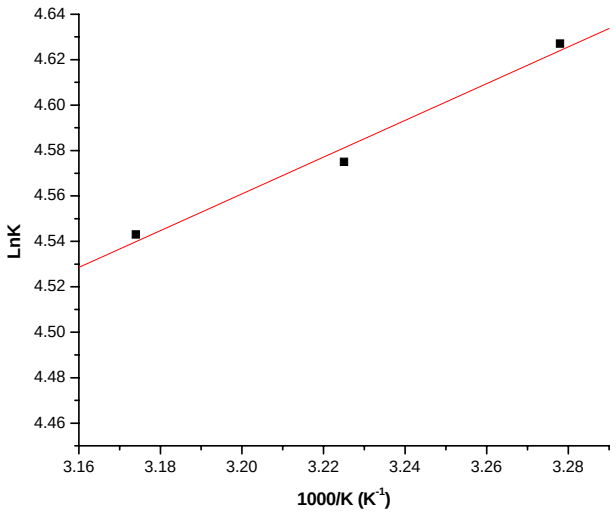


Fig 14. Van't Hoff Plot of the effect of temperature on the inhibition constant of Trypsin against SPI

Table1: Inhibition constant and thermodynamic parameter for inhibition of Trypsin against SPI at different temperatures

Temperature	Ki (μM)	ΔG ($KJmol^{-1}$)	ΔH ($KJmol^{-1}$)	ΔS ($Jmol\text{-}K^{-1}$)
32^0C	102.3(±5.1)	-11.7(±0.5)	-2.0(±0.08)	31.9(±1.4)
37^0C	97.1(±4.3)	-11.7(±0.4)	-2.0(±0.09)	31.1(±1.2)
42^0C	94.0(±4.4)	-11.8(±0.5)	-2.1(±0.08)	31.0(±1.4)

(±) Standard deviation n=3

As can be seen from Van't Hoff plot (Fig 14) that the inhibition of trypsin increases with increase in temperature and binding is endothermic. The free energy (ΔG) of binding of the inhibitor is negative (Table 1) and therefore the reaction is spontaneous. However if the enthalpy (-ΔH) and entropy (ΔS) is taken into consideration, the major contribution to free energy (-ΔG) comes from entropy (ΔS or -TΔS) and therefore the binding of the inhibitor is entropically driven.

CONCLUSION

In conclusion, we have isolated a new quaternary alkaloid 1 from the green mussel, *Perna viridis,* which was found to be an serine protease inhibitor with an IC_{50} value of 102.5μM and K_i 97.1–104.68 μM. During the initial kinetic analysis, the inhibitor showed competitive inhibition against trypsin *in vitro*. The inhibitor was found to be specific for trypsin and did not show any inhibition against chymotrypsin. It also did not show any activity against other classes of proteases.

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Chapter 3

Synthesized 2,4,5-trisubstituted imidazole derivatives: A candidature towards antibacterials

SUMMARY

Some novel chemically synthesized 2,4,5-trisubstituted imidazoles from aryl aldehydes and 1,2-diketones or α–hydroxyketones were screened against eight different human pathogenic bacteria and fungi. Seven compounds were found to be active against several Gram positive and Gram negative bacteria. These compounds showed variation in activity and were found to be active against Gram-positive as well as Gram-negative bacteria. Compound 4-(4,5-Diphenyl-*1H*-imidazol-2-yl)-phenol, **3d** was the only compound which showed activity against *Klebsiella pneumoniae* while rest of the compounds did not show significant activity against this microorganism. MICs of the compounds were in the range of 0.50 µg/ml to 6.1 µg/ml and MBC ranges were from 1.11 µg/ml to 12.9 µg/ml. The candidature of active compounds to be an effective and novel drug was examined based on Lipinski's rule of Five which explained ClogP, LogS, H-bond acceptors, H-Bond donors and Rotational Bonds. Compounds **3a-d** and **3f** satisfy Lipinski's rule of Five and could be proposed as potent new antibacterial drugs.

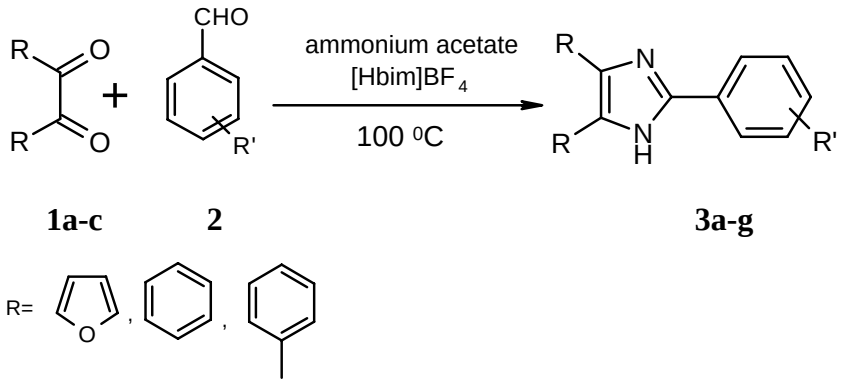
INTRODUCTION

Different imidazoles have been reported to show biological activities such as anti-inflammatory activity inhibiting cytokine release or inhibiting the p38 MAP kinase [1], anti-allergic activity [2] and analgesic activity [3]. They are also sensitizers of multi-drug-resistant cancer cells [4], pesticides [5], antibiotics [6] or sodium-channel modulators [7]. Some imidazole containing drugs have surface activity and are able to damage membranes directly when used in a high concentration for a very short time, independently of the culture medium and growth rate [8]. When in direct contact [9] imidazoles interact directly with the lipid bilayer [10] of the plasma membrane, probably by binding to the unsaturated fatty acid part of the phospholipid components of the membrane. The imidazoles, as a group of antifungal drugs (e.g. ketoconazole), act by inhibiting the 14α-demethylation of sterols. This is a key step in the conversion of lanosterol to ergosterol, an essential component of fungal cell wall. The demethylation is a multistep, enzyme-mediated pathway involving a series of oxidation steps catalysed by

the iron haem protein cytochrome P-450 [11]. The imidazoles coordinate to, and therefore block, the haem iron atom (in Cyt-P450). The binding is fairly unspecific and thus imidazole fungicides also inhibit the activities of a broad spectrum of other cytochrome P-450 dependent enzymes, including key enzymes involved in biosynthesis and metabolism of steroids, e.g. CYP19 aromatase [12]. This is a site normally occupied by O_2 and is a key part of the activation of molecular oxygen for the oxidation process that the enzyme mediates. Inhibition of these steps leads to a build up of 14α-methyl sterols, which replace ergosterol in the cell membrane. Ergosterol is quasi-planar and stabilizes the phospholipid membrane, but the 14α-methyl sterols are non-planar. This results in a series of changes in the fungal cell including change in cell membrane, increase in cell volume, abnormalities in cell division and cell function. Several imidazole drugs have been reported for example Prochloraz an imidazole fungicide that antagonizes the androgen and the oestrogen receptor, agonizes the Ah receptor and inhibits aromatase activity [13]. Miconazole has broad-spectrum activity against microbes, fungi and gram-positive bacteria [14]. It interacts with the cell wall [15], changes permeability barrier [16] and inhibits biosynthesis of ergosterol [17]. Clotrimazole is a promising imidazole antimycotic active against a wide range of pathogenic fungi. It causes leakage of various small ions and small compounds namely, K^+, inorganic phosphates, amino acids and nucleotides [18]. Azomycin inhibits ribonucleotide reductase and is active against Gram-positive, Gram-negative bacteria, mycobacteria and protozoa [19]. Metronidazole (MTZ) and related N-1 substituted 5-nitroimidazoles like ornidazole, secnidazole and tinidazole are antibacterial and antiprotozoal drugs [20]. MTZ is active only against anaerobic organisms. It is activated when reduced through electron donation from ferredoxin or flavodoxin [21]. Activated MTZ is thought to interact directly with DNA and the resultant complex can no longer function as an effective primer for DNA and RNA polymerases. In view of this, it was of considerable interest to synthesize the trisubstituted imidazoles, target compounds of the present work and screen them for antibacterial and antifungal activity with respect to their functional moieties with a hope to obtain potent biologically active compounds which could be proposed as new potent drugs.

Chemical Synthesis of Biologically active compounds

2,4,5-trisubstitued imidazoles were synthesized by one-pot condensation of 1,2-diketones or α-hydroxy ketone, aromatic aldehydes and ammonium acetate in the (IL) ionic liquid, 1-butyl imidazolium tetrafluoroborate ([Hbim]BF4) according to Siddiqui *et al* [22].



1a-c **2** **3a-g**

R=

R' = *p*-OMe; *m*-OMe; *p*-OH; *o*-OH; *p*-NO_2; 

Scheme 1

MATERIALS

Microbes *Staphylococcus aureus* (ATCC-25923; *Proteus vulgaris* (ATCC-29905); *Group D Streptococci* (ATCC-12959); *Bacillus spp.* (ATCC-14593); *Escherichia coli* (ATCC-25922); *Klebsiella pneumonia* (ATCC-13883) ; *Pseudomonas aeruginosa* (ATCC-27853); *Serratia morganii* (ATCC- 31665) were obtained from Goa Medical college, Goa, India. All solvents and chemicals were of analytical grade and were used as obtained from Merck and Sigma-Aldrich.

METHODS

Determination of Minimum Inhibitory Concentration (MIC)

The disk diffusion method [23] was used for the preliminary antibacterial evaluation of 2,4,5-trisubstituted imidazoles**.** The MICs (Table 1) of 2,4,5-trisubstituted imidazoles, showing inhibition in the preliminary tests, were determined by the microtitre plate technique using micro dilution method [24]. Briefly, *Staphylococcus aureus* (ATCC 25923); *Proteus vulgaris* (ATCC 29905); *Group D Streptococci* (ATCC 12959); *Bacillus spp.* (ATCC 14593); *Escherichia coli* (ATCC 25922); *Klebsiella pneumoniae* (ATCC 13883) ; *Pseudomonas aeruginosa* (ATCC 27853) and *Serratia morganii* (ATCC 31665), obtained from Goa Medical college, Goa, India and grown to mid-logarithmic phase were harvested by centrifugation, washed with 10mM sodium phosphate buffer (SPB) at pH 7.4, and diluted to 2×10^5 colony forming units (CFU)/ml in SPB containing 0.03% Luria–Bertani (LB) broth. Imidazole derivatives were serially diluted in 50µL of LB medium in 96-well microtitre plates to achieve the desired concentrations (0.1-15µg/ml) with bacterial inoculum (5×10^4 CFU per well). After incubation at 37 ^oC overnight, the MIC was taken as the lowest imidazole derivative concentration at which growth was inhibited. For the agar plate count method [25], a 25µL aliquot of bacteria at 1×10^5 CFU/ml in SPB containing 0.03% LB broth was incubated with 25 µL of diluted compounds for 2 h at 37 ^oC [26]. The mixtures of bacteria and compounds were serially diluted 10-fold with SPB and plated on LB plates that were incubated at 37 ^oC overnight. Bacterial colonies were enumerated the following day.

Determination of minimum bactericidal concentration (MBC)

For the determination of the minimum bactericidal concentrations (MBC) the following strains were assayed: *Staphylococcus aureus* (ATCC 25923) MIC 4.7-5.6 µg/ml; *Proteus vulgaris* (ATCC 29905) MIC 2.3-2.6µg/ml; *Group D Streptococci* (ATCC 12959) MIC 0.5-0.7 µg/ml; *Bacillus spp.* (ATCC 14593) MIC 2.6-3.2 µg/ml; *Escherichia coli* (ATCC 25922) MIC 5.1-6.1µg/ml; *Klebsiella pneumoniae* (ATCC 13883) MIC 0.5 µg/ml;

Pseudomonas aeruginosa (ATCC-27853) MIC 2.1-3.0 µg/ml; *Serratia morganii* (ATCC- 31665) MIC 1.6 -3.0 µg/ml (Table 1).

After having determined the MIC of bacteria from the wells of the micro titer plate with no visible bacterial growth, samples were removed for serial sub cultivation of 2µl into microtiter plates containing 100µl of broth per well and further incubated for 24h. The lowest concentration with no visible growth was defined as MBC, indicating 99.5% killing of the original inoculum. The optical density of each well was measured at a wavelength of 620 nm by microtiter plate manager 4.0 (Bio-Rad laboratories) and compared with a blank. Solvent (DMSO) was used as a negative control. Two replicates were done for each compound and the experiment was repeated two times.

RESULTS AND DISCUSSION

Compounds **3a-g** (Table 2) were evaluated for antibacterial and/or antifungal activity against representative bacteria/ fungi — *S. aureus* (ATCC 25923); *P. vulgaris* (ATCC 29905); *Group D Streptococci* (ATCC 12959); *Bacillus spp.* (ATCC 14593); *E. coli* (ATCC 25922); *K. pneumoniae* (ATCC 13883) ; *P. aeruginosa* (ATCC 27853); *S. morganii* (ATCC 31665) and fungi *Aspergillus oryzae* (NCIM 635); *Aspergillus niger* (NCIM 773); *Fusarium moniliforme* (NCIM 1100); *Helminthosporium spp.* (NCIM 1079) and *Claviceps purpurea* (NCIM 1046) obtained from NCIM, National Chemical Laboratory, Pune, India.

As can be seen in Figure 1, compound **3a** was active against *P. aeruginosa* and *S. morganii,* **3b** was active against *Group D Streptococci*, *Bacillus spp*. and *E. coli.* Compound **3c** showed activity against *S. aureus, Group D Streptococci* and *P. aeruginosa*, **3d** and **3e** were active against all cultures of *S. aureus, P. vulgaris, Group D Streptococci*, *Bacillus spp.*, *E. coli*, *K. pneumoniae*, *P. aeruginosa* and *S. morganii* with the exception of *K. pneumoniae* against which compound **3e** was not active, Compound **3f** showed activity against *S. aureus, P. vulgaris, Group D Streptococci*, *Bacillus spp., E. coli* and *S. morganii* and **3g** was found to be active against *S. aureus, P. vulgaris, Group D Streptococci*, *Bacillus spp., E. coli, P. aeruginosa* and *S. morganii.*

The antibacterial activity of the compounds has been attributed to the presence of imidazole ring. It is unknown whether the electrophilic centers of the imidazole ring [27] or the metabolically activated side chains act as the binding sites [28] but the secondary nitrogen of the imidazole, bearing active hydrogen (1-unsubstituted) and acting as a nucleophilic centre has been found to be essential for activity [29]. Second nitrogen may also be involved in H-bonding [30]. However, it has been proven that the side chains bind covalently to SH-groups of proteins containing cysteine but not to nucleic acids [28]. It may be concluded [28-30] that compounds have broad spectrum activity and may interact with enzymes required for cell membrane synthesis or enzymes present inside the cell. Compounds **3a-b** have same furanyl moiety at C-4 and C-5 position whereas C-2 position has methoxy phenyl and 2- methoxy phenol moieties, respectively. It could be inferred from data shown in Table 1 that among C-2 aryl substituted imidazole, a 4-substitution on aryl ring is preferred and more polar substituent makes the compound more active. Hence compound **3b** is more active than **3a**. Among compounds **3c-e** (Table 2), the compounds having more polar substituent at 4^{th} position of C-2 aryl ring are more active whereas substituent at 2-position makes compound less active (Table 1). Compound **3f** has been found to be more active than **3g** due to substitution at 2^{nd} position on C-2 aryl ring whereas **3f** is having a different furanyl moiety which is more active than **3g** (Table 1) but less active than **3d**. It has been shown that at C-4 position, presence of aromatic ring is required for the activity as it binds with hydrophobic pocket of enzymes [31] and increasing either the size or the polarity of substituents leads to diminished potency [32]. The C-5 moieties may involve in polar interaction [31] depending upon the enzymes they are interacting with.

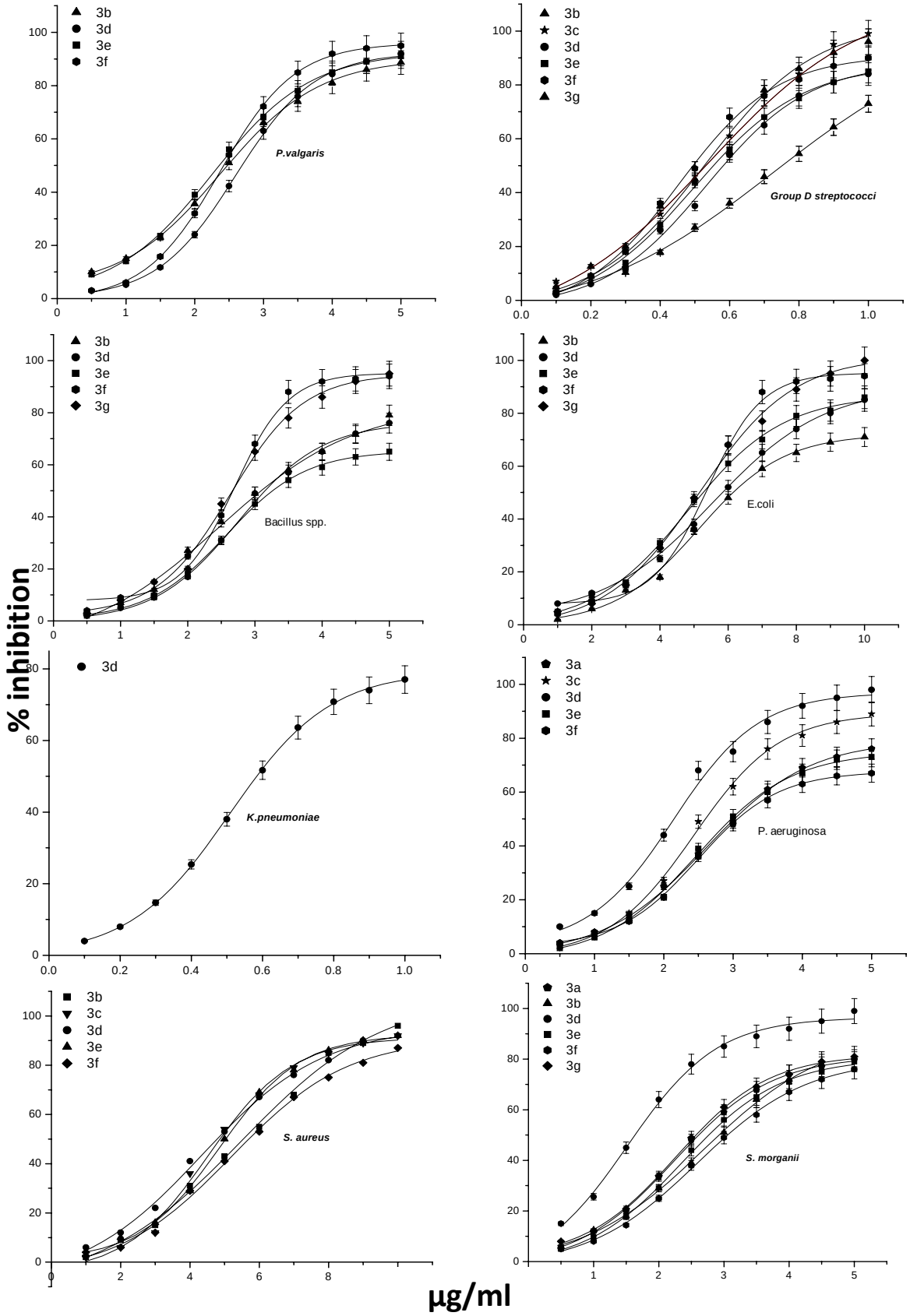


FIG. 1. Growth inhibition of the bacterial strains at different concentrations of synthesized 2,4,5-triaryl imidazole derivatives was recorded after 10-12 hrs.

Table 1. Antimicrobial activity of compounds 3a-g (MIC µg/ml)

	Microorganism[a](MIC µg/ml)							
Compound	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
3a	NA	NA	NA	NA	NA	NA	3(±0.15)	2.5(±0.12)
3b	5.5(±0.27)	2.6(±0.13)	0.5(±0.02)	2.6(±0.13)	5.1(±0.25)	NA	NA	2.5(±0.12)
3c	4.7(±0.23)	NA	0.5(±0.02)	NA	NA	NA	2.5(±0.12)	NA
3d	5(±0.25)	2.4(±0.12)	0.5(±0.02)	3.1(±0.15)	5.8(±. 29)	0.5(±0.02)	2.1(±0.10)	1.6(±0.08)
3e	4.8(±0.24)	2.4(±0.12)	0.5(±0.02)	2.6(±0.13)	5.3(±0.26)	NA	3(±0.15)	3(±0.15)
3f	5.6(±0.28)	2.3(±0.11)	0.7(±0.03)	3(±0.15)	6.1(±0.30)	NA	2.9(±0.14)	2.9(±0.14)
3g	NA	NA	0.5(±0.02)	3.2(±0.16)	5.2(±0.26)	NA	NA	2.7(±0.13)
Ceftriaxone	4.0(±0.20)	0.5(±0.02)	0.08(±0.004)	0.9(±0.04)	0.06(±0.003)	0.05(±0.002)	1.6(±0.08)	0.8(±0.04)
Clotrimazole	0.00074	0.00074	NA	NA	50	50	50	ND

NA- Not active.

(±) Shows 5% error

1. *S. aureus*, 2.*P. vulgaris*, 3. *Group D Streptococci*, 4. *Bacillus spp.*, 5. *E. coli*,

6. *K. pneumoniae*, 7. *P. aeruginosa*, 8. *S. morganii*

Table 3. Prediction of antibacterial compounds as drugs based on Lipinski's rule of Five

S.No.	Compound	[a]ClogP	[b]LogS	H-bond acceptors	H-Bond donors	[c]MWT	Rotational Bond
1	3a	3.2	-4.85	5	1	306.3	4
2	3b	2.9	-4.55	6	2	322.3	4
3	3c	4.89	-5.84	3	2	312.4	3
4	3d	4.89	-5.84	3	2	312.4	3
5	3e	5.2	-6.64	3	1	341.4	4
6	3f	4.88	-6.17	3	1	314.4	3
7	3g	5.52	-6.52	3	2	340.4	3

[a] compounds to have a reasonable propability of being well absorbt their logP value must not be greater than 5.0

[b] more than 80% of the drugs on the market have a (estimated) logS value greater than -4.

[c] more than 80 % of all traded drugs have a molecular weight below 450.

CLogP and LogS Values obtained from http://www.organic-chemistry.Org/prog/peo/portal

HBA; HBD; MWT and RB values obtained from ChemDraw (ChemOffice 2005 Package).

Table 4. Antimicrobial activity of compounds 3a-g (MBC µg/ml)

Compound	Microorganism[a] (MBC µg/ml)							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
3a	NA	NA	NA	NA	NA	NA	5.8(±0.29)	5.1(±0.25)
3b	10.2(±0.51)	5.4(±0.27)	1.3(±0.06)	5.5(±0.27)	11.2(±0.56)	NA	NA	5.2(±0.26)
3c	8.1(±0.40)	NA	1.4(±0.07)	NA	NA	NA	5.1(±0.25)	NA
3d	9.3(±0.46)	5.6(±0.28)	1.4(±0.07)	6.4(±0.32)	12.0(±0.6)	1.1(±0.05)	4.2(±0.21)	3.3(±0.16)
3e	9.1(±0.45)	5.7(±0.28)	1.2(±0.06)	4.9(±0.24)	11.2(±0.56)	NA	6.2(±0.31)	6.2(±0.31)
3f	11.3(±0.56)	5.6(±0.28)	1.8(±0.09)	5.8(±0.29)	12.9(±0.64)	NA	6.1(±0.3)	6.0(±0.3)
3g	NA	NA	1.3(±0.06)	6.2(±0.31)	10.3(±0.51)	NA	NA	5.8(±0.29)

NA- Not active.

(±) Shows 5% error

1. *S. aureus*, 2.*P. vulgaris*, 3. *Group D Streptococci*, 4. *Bacillus spp.*, 5. *E. coli*,

6. *K. pneumoniae*, 7. *P. aeruginosa*, 8. *S. morganii*

We compared the MICs (Table 1) of our compounds with a standard antibiotic, ceftriaxone. None of our compounds showed better activity than ceftriaxone, only comparable against *S. aureus* (Table 1). Data presented in Table 1 clearly shows that our compounds are more effective than clotrimazole [37] against *E. coli*, *K. pneumoniae* and *P. aeruginosa* whereas less effective than *S. aureus* and *P. vulgaris.* Compound **3d,** 4-(4, 5-Diphenyl-*1H*-imidazol-2-yl)-phenol is the only compound which showed inhibition against *K. pneumoniae* while other compounds were not active against this microbe. The most potent compounds in this series are **3b-e** and **3g** which have MIC (0.50 µg/ml) against *Group D Streptococci* whereas 2-furan-2-yl-4,5-di-*p*-tolyl-*1H*- imidazole phenol, **3f** was found to be least active having MIC 6.1 µg/ml against *Escherichia coli*. MIC of the compounds from this series ranges from 0.50 µg/ml to 6.1 µg/ml and MBC ranges from 1.11 µg/ml to 12.9 µg/ml. This study revealed that compounds **3a-d** and **3f** satisfies Lipinski's rule of Five [33, 34] whereas **3e** has nitro group, a tumorogenic moiety [35], and **3g** has ClogP [36] value more than 5 (Table 3). Compounds **3a-d** and **3f** could be proposed as new potent antibacterial agents. None of the compounds except **3e** is mutagenic, tumorigenic, irritant or have any reproductive effect [36]. None of the compounds showed activity against any fungus at the concentration tested but may show activity at higher concentration.

CONCLUSION AND FUTURE DIRECTIONS

This study reports antibacterial activity of 2, 4, 5-trisubstituted imidazoles. It reveals that among C-2 aryl substituted imidazole, a 4-substitution on aryl ring is preferred and more polar substituent makes the compound more active whereas polar substituents at 2-position on C-2 aryl ring makes the compound less active. Based on Lipinski's rule of Five we propose **3a-d** and **3f** as potent antibacterial agents. At C-4 position presence of aromatic ring is required for activity. None of the tested compounds showed significant activity against any fungus when tested at 15µg/ml concentration. However compounds may show activity at higher concentrations. These compounds could be further used for ADME and Docking studies to make them potential functional drugs.

Chemistry

NMR spectra were recorded on a Bruker AC-200 spectrometer in $CDCl_3$/DMSO-d_6 with TMS as an internal standard. Infra red spectra were recorded with ATI-MATT-SON RS-1 FTIR spectrometer. Polarity of ILs was recorded on Lambda EZ 201, using Reichardt's dye. Melting points were recorded in open capillary and were uncorrected. All solvents and chemicals were of research grade and were used as obtained from Merck and Lancaster.

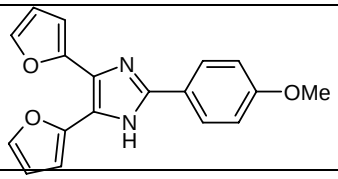
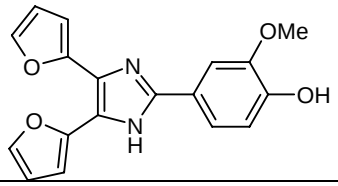
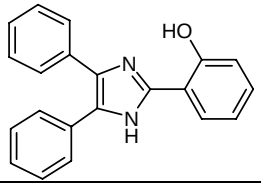
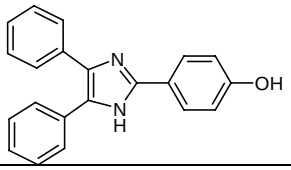
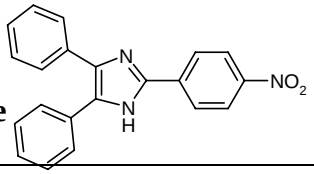
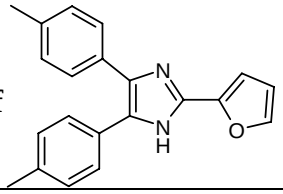
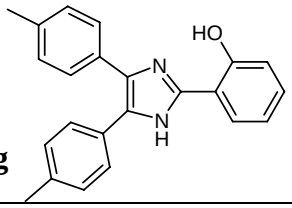
4,5-Difuran-2-yl-2-(4-methoxy-phenyl)-*1H*-imidazole (3a)

Mp 198 ^{0}C; IR (Nujol, cm^{-1})**:** υ_{max} 635, 708, 880, 1050, 1230, 1508, 1570, 1610, 3105, 3419; ^{1}H NMR ($CDCl_3$/DMSO-d_6, 200 MHz) δ 3.75 (s, 3H), 6.14-6.19 (d, *J*= 8 Hz, 2H), 6.83-6.86 (d, *J*= 8.5 Hz, 2H), 7.16 (s, 2H), 7.41 (d, *J*= 8 Hz, 2H), 7.79-7.82 (d, *J*= 8 Hz, 2H), 12.53 (brs, 1H); ^{13}C NMR ($CDCl_3$/DMSO-d_6, 200 MHz) δ 55.2, 107.3, 111.6, 114.1, 121.9, 127, 141.2, 146.4, 147, 160.3; Elemental Analysis ($C_{18}H_{14}N_2O_3$) Calcd. C, 70.58; H, 4.61; N, 9.15. Found C, 70.22; H, 4.32; N, 9.02.

4-(4, 5-Difuran-2-yl-*1H*-imidazol-2-yl)-2-methoxy phenol (3b)

Mp 238 ^{0}C; IR (Nujol, cm^{-1})**:** υ_{max} 715, 864, 910, 1210, 1416, 1575, 1610, 3130, 3420, 3600; ^{1}H NMR ($CDCl_3$/DMSO-d_6, 200 MHz) δ 3.65 (s, 3H), 6.30-6.33 (m, 4H), 7.31-7.40 (m, 2H), 7.55-7.58 (m, 3H), 12.71 (brs, 1H); ^{13}C NMR ($CDCl_3$/DMSO-d_6, 200 MHz) δ 105.2, 111.6, 115.5, 120.2, 122.6, 124.2, 127.1, 140.1, 152.3, 156.2; Elemental Analysis ($C_{18}H_{14}N_2O_4$) Calcd. C, 67.08; H, 4.34; N, 8.69. Found C, 66.68; H, 4.09; N, 8.32.

Table 2: Name and structure of compounds

Name of Compounds	Structure of compounds
4,5-Difuran-2-yl-2-(4-methoxy-phenyl)-*1H*-imidazole(3a)	**3a** 
4-(4, 5-difuran-2-yl-*1H*-imidazol-2-yl)-2-methoxy phenol (3b)	**3b** 
2-(4,5-Diphenyl-*1H*-imidazol-2-yl)-phenol (3c)	**3c** 
4-(4,5-Diphenyl-*1H*-imidazol-2-yl)-phenol (3d)	**3d** 
2-(4-Nitro-phenyl)-4,5-diphenyl-*1H*-imidazole (3e)	**3e** 
2-furan-2-yl-4,5-di-*p*-tolyl-*1H*-imidazole (3f)	**3f** 
2-(4,5-di-*p*-tolyl-*1H*-imidazol-2-yl)-phenol (3g)	**3g** 

2-(4,5-Diphenyl-*1H*-imidazol-2-yl)-phenol (3c)

Mp 205 8°C; IR (Nujol, cm^{-1})**:** υ_{max} 1216, 1638, 2465, 2998, 3432, 3596; ^1H NMR ($CDCl_3$/DMSO-d_6, 200 MHz) δ 6.87–6.95 (d, *J*= 7.5 Hz, 2H), 6.97–7.01 (d, *J*= 8.06 Hz, 2H), 7.17–7.23 (m, 10H), 12.74 (brs, 1H); ^{13}C NMR ($CDCl_3$/DMSO-d_6, 200 MHz) δ 112.7, 116.4, 118.1, 124.8, 126.8, 127.4, 127.8, 129.1, 145.7, 156.6; Elemental Analysis $C_{21}H_{16}N_2O$ (312): calcd C, 80.75, H, 5.16, N, 8.97; found C, 80.62, H, 5.08, N, 8.85.

4-(4,5-Diphenyl-*1H*-imidazol-2-yl)-phenol (3d)

Mp 233 8°C; IR (Nujol, cm^{-1})**:** υ_{max} 1216, 1638, 2465, 2998, 3432, 3596; ^1H NMR ($CDCl_3$/DMSO-d_6, 200 MHz) δ 6.93–6.97 (d, *J*= 8 Hz, 2H), 7.52–7.87 (m, 10H), 7.88–7.92 (d, *J*= 8.5 Hz, 2H), 12.58 (brs, 1H); ^{13}C NMR ($CDCl_3$/DMSO-d_6, 200 MHz) δ 113.7, 119.9, 125.1, 125.3, 126.1, 126.5, 144.7, 159.2; Elemental Analysis $C_{21}H_{16}N_2O$ (312): calcd C, 80.75, H, 5.16, N, 8.97; found C, 80.68, H, 5.05, N, 8.90.

2-(4-Nitro-phenyl)-4,5-diphenyl-*1H*-imidazole (3e)

Mp 196 8°C (decomposes); IR (Nujol, cm^{-1})**:** υ_{max} 845, 1443, 1522, 1540, 1602, 3056; ^1H NMR ($CDCl_3$/DMSO-d_6, 200 MHz) δ 7.25–7.57 (m, 10H), 7.78 (d, *J*= 9 Hz, 2H), 8.50 (d, *J*= 9 Hz, 2H), 12.59 (brs, 1H); ^{13}C NMR ($CDCl_3$/DMSO-d_6, 200 MHz) δ 122.7, 124.2 127.3, 127.6, 132.8, 146.7, 160.8; Elemental Analysis $C_{21}H_{15}N_3O_2$ (341): calcd C 73.88, H 4.43, N 12.31; found C 73.85, H 4.38, N 12.25.

2-Furan-2-yl-4,5-di-*p*-tolyl-*1H*-imidazole (3f)

Mp 185 ^0C; IR (Nujol, cm^{-1})**:** υ_{max} 845, 1443, 1522, 1540, 1602, 3056; ^1H NMR ($CDCl_3$/DMSO-d_6, 200 MHz) δ; ^{13}C NMR ($CDCl_3$/DMSO-d_6, 200 MHz) Elemental Analysis $C_{21}H_{18}N_2O$ (314): calcd C 80.23, H 5.77, N 8.91; found C 79.85, H 5.38, N 8.45.

2-(4,5-di-*p*-tolyl-*1H*-imidazol-2-yl)-phenol (3g)

Mp 223 ^{0}C; IR (Nujol, cm^{-1})**:** υ_{max} 1216, 1555, 1638, 2465, 2998, 3432, 3596; ^{1}H NMR ($CDCl_3$/DMSO-d_6, 200 MHz) δ; 2.36 (s, 6H), 6.85-6.90 (t, *J*= 8.3 Hz, 1H), 6.95-6.98 (d, *J*= 8.06 Hz, 1H), 7.14-7.17 (m, 4H), 7.21-7.23(d, *J*= 7.33 Hz, 1H), 7.43-7.46 (m, 4H), 7.96-7.99 (d, *J*= 8.06 Hz, 1H), 12.84 (brs, 1H); ^{13}C NMR ($CDCl_3$/DMSO-d_6, 200 MHz) δ; 19.7, 114.2, 118.5, 126.1, 126.5, 126.8, 127.6, 127.7, 127.9, 127.9, 129.7, 135.5, 144.4, 157.2; Elemental Analysis $C_{23}H_{20}N_2O$ Calcd C, 81.15; H, 5.92; N, 8.23. Found C, 80.85; H, 5.61; N, 7.78.

2-(4,5-di-*p*-tolyl-*1H*-imidazol-2-yl)-phenol (3g)

Mp 223 ^{0}C; IR (Nujol, cm^{-1})**:** υ_{max} 1216, 1555, 1638, 2465, 2998, 3432, 3596; ^{1}H NMR ($CDCl_3$/DMSO-d_6, 200 MHz) δ; 2.36 (s, 6H), 6.85-6.90 (t, *J*= 8.3 Hz, 1H), 6.95-6.98 (d, *J*= 8.06 Hz, 1H), 7.14-7.17 (m, 4H), 7.21-7.23(d, *J*= 7.33 Hz, 1H), 7.43-7.46 (m, 4H), 7.96-7.99 (d, *J*= 8.06 Hz, 1H), 12.84 (brs, 1H); ^{13}C NMR ($CDCl_3$/DMSO-d_6, 200 MHz) δ; 19.7, 114.2, 118.5, 126.1, 126.5, 126.8, 127.6, 127.7, 127.9, 127.9, 129.7, 135.5, 144.4, 157.2; Elemental Analysis $C_{23}H_{20}N_2O$ Calcd C, 81.15; H, 5.92; N, 8.23. Found C, 80.85; H, 5.61; N, 7.78.

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Chapter 4

Antibacterial activities of Cysteine protease inhibitors, 1-substituted pyridylimidazo [1,5-a] pyridine derivatives

SUMMARY

Papain family cysteine proteases are key factors in the pathogenesis of microbial infections, cancer invasion, arthritis and osteoporosis. Targeting this enzyme family is one of the strategies in the development of new chemotherapy for a number of diseases. The disparate nature of parasite cysteine protease compared to the host orthologous proteins has opened novel opportunities for chemotherapy. We report here a new group of synthetic cysteine protease inhibitors, 1-substituted pyridylimidazo [1,5-a] pyridine derivatives, whose K_i and IC_{50} values ranges from 13.75μM to 99.3 μM and 13.4 μM to 96.5 μM, respectively. These inhibitors have been shown to inhibit the replication of seven different clinically important G^+ & G^- bacteria, their MIC_{50} range from 0.6-1.4 μg/ml, though it is not been directly demonstrated that the effects are due to protease inhibitory capacity of these compounds. The Van't Hoff analysis showed that the value of K_i decreases with increase in temperature and the binding of the inhibitor is entropically driven and hydrophobic in nature. These compounds showed good agreement with drug criteria and scored fairly high values for drug likeness. Based on Lipinski's rule of Five we proposed these compounds as potent antibacterial drugs. The most active antibacterial compound was found to be 1-(2-Pyridyl)-3-(2-hydroxyphenyl) imidazo [1,5-*a*] pyridine.

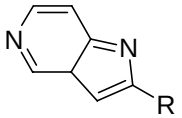
INTRODUCTION

Interest in papain family cysteine proteases as targets derives from the recognition that they are critical to the life cycle or pathogenicity of many parasites e.g. *Streptococcal* cysteine protease [1], *Staphylococcal* cysteine protease [2], falcipain-1, -2, and -3 [3] and Cruzipain [4]. The recently described cystatin superfamily of proteins comprises both eukaryotic and prokaryotic cysteine protease inhibitors [5]. Human cystatins C, D and S, rat cystatins A and S, chicken cystatin and oryzacystatin have been reported to inhibit the replication of certain viruses and bacteria [6] although it has not yet been directly demonstrated that these effects are due to the protease inhibitory capacity of the cystatins [7,8]. Also it is now established that many parasite proteases are promising chemotherapeutic or vaccine targets [9,10,11]. The key role of cysteine proteases in parasitic infections, coupled with the relative lack of redundancy compared to

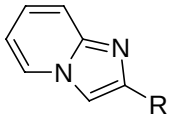
mammalian systems has made parasite proteases attractive targets for the development of novel chemotherapeutic approaches [12,13]. The treatment of experimental models of parasitic diseases with cysteine protease inhibitors has confirmed the use of cysteine protease inhibitors *in vivo*. Cysteine protease inhibitors can selectively arrest replication of a microbial pathogen without untoward toxicity to the host. The functional diversity is in turn derived from their unique nucleophilicity, adaptability to different substrates and stability in different biological environments [14]. Parasite derived cysteine proteases play key roles in immunoevasion, enzyme activation, virulence, tissue and cellular invasion as well as excystment, hatching and moulting. Although host homologues exist, parasite cysteine proteases have distinct structural and biochemical properties including pH optima and stability, diverse substrate specificity and cellular location [15]. In African trypanosomes, three major cysteine proteases have been studied: rhodesain from *T. b. rhodesiense* and its equivalent form in cattle, congopain from *T. congolense* and brucipain, from *T. brucei*. All three enzymes promote lysosomal activities and have been identified in all the stages of the life-cycle of the protozoa, particularly during the infective stage of parasite development [16-18]. Promising experiments using cysteine protease inhibitors have validated the suitability of these proteases as drug target candidates for antiparasitic chemotherapy [19-24]. An attractive potential target for new chemotherapies is a family of cathepsin L- and B-like proteases found in all species of *Leishmania* [25,26].

Fused bicyclic heterocycles with nitrogen atom in the five-membered ring, are of interest because of the occurrence of their saturated and partially saturated derivatives in biologically active compounds. Imidazopyridine ring systems represent an important class of compounds not only for their theoretical interest but also from a pharmacological point of view. They have been shown to possess a broad range of useful pharmacological activities [27] including antigastric, antisecretory [28], local anesthetic [29], antiviral [30], antianxiety [31], antibacterial, antifungal, antihelminthic, antiprotozoal, anticonvulsant, gastrointestinal, antiulcer (Zolmidine), anxiolytic (Alpidem), hypnotic (Zolpidem) and immunomodulatory [32]. The nature and the position of the substituents on the pyridinic moiety influence these pharmacological activities. These imidazo pyridine heterocyclic structures form part of the skeleton of natural alkaloids [33],

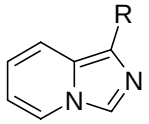
neuromuscular blocking agents [34], reversible inhibitors of the H^+, K^+-ATPase enzyme [35] with a potent antisecretory activity [36] and of sedative hypnotics of the nervous system [37]. There are several imidazo pyridines which are present as natural products:



imidazo[1,4*c*]pyridine



imidazo[1,2*a*]pyridine



imidazo[1,5*a*]pyridine

We have synthesized some 1-substituted pyridylimidazo [1,5-a] pyridine derivatives [38] and checked for their antibacterial and protease inhibitor activities.

MATERIALS

1-substituted pyridylimidazo [1,5-a] pyridine derivatives 1-(2-Pyridyl)-3-(2-hydroxyphenyl) imidazo [1,5-*a*] pyridine (**3a);** 1-(2-Pyridyl)-3-(4-methoxyphenyl) imidazo [1,5-*a*] pyridine (**3b);** 1-(2-Pyridyl)-3-(2-hlorophenyl) imidazo [1,5-*a*] pyridine (**3c)** and 1-(2-Pyridyl)-3-(3,5-di-*tert*-butyl-4-hydroxyphenyl) imidazo [1,5-*a*] pyridine (**3d)** were synthesized according to Siddiqui et al [38]. Microbes *Staphylococcus aureus* (ATCC-25923; *Proteus vulgaris* (ATCC-29905); *Group D Streptococci* (ATCC-12959); *Bacillus spp.* (ATCC-14593); *Escherichia coli* (ATCC-25922); *Klebsiella pneumonia* (ATCC-13883) ; *Pseudomonas aeruginosa* (ATCC-27853); *Serratia morganii* (ATCC-31665), obtained from Goa Medical college, Goa, India. Papain (EC 3.4.22.2) and Bz-DL-Arg-pNA (BAPNA) were purchased from Sigma Chemical Co., St. Louis, MO, USA. All solvents and chemicals were of analytical grade and were used as obtained from Merck and Sigma-Aldrich.

METHODS

Determination of Minimum Inhibitory Concentration (MIC)

The disk diffusion method [39] was used for the preliminary antibacterial evaluation of 1-substituted pyridylimidazo [1,5-a] pyridine derivatives. The MIC_{50} and MIC_{90} (Table 1)

of 1-substituted pyridylimidazo [1,5-a] pyridine derivatives, showing inhibition in the preliminary tests, were determined by the microtitre plate technique using micro dilution method [40]. Briefly, *Staphylococcus aureus* (ATCC-25923); *Proteus vulgaris* (ATCC-29905); *Group D Streptococci* (ATCC-12959); *Bacillus spp.* (ATCC-14593); *Escherichia coli* (ATCC-25922); *Klebsiella pneumoniae* (ATCC-13883) ; *Pseudomonas aeruginosa* (ATCC-27853) and *Serratia morganii* (ATCC- 31665), obtained from Goa Medical college, Goa, India and grown to mid-logarithmic phase were harvested by centrifugation, washed with 10mM sodium phosphate buffer (SPB) at pH 7.4, and diluted to 2×10^5 colony-forming units (CFU)/ml in SPB containing 0.03% Luria–Bertani (LB) broth. Pyridylimidazo [1,5-a] pyridine derivatives were serially diluted in 50µL of LB medium in 96-well microtitre plates to achieve the desired concentrations (0.1-10µg/ml) with bacterial inoculum (5×10^4 CFU per well). After incubation at 37 ^{0}C overnight, the MICs were taken as the lowest concentration of Pyridylimidazo [1,5-a] pyridine derivatives at which growth was inhibited. For the agar plate count method [41], a 25µL aliquot of bacteria at 1×10^5 CFU/ml in SPB containing 0.03% LB broth was incubated with 25 µL of diluted compounds for 2 h at 37 ^{0}C [42]. The mixtures of bacteria and compounds were serially diluted 10-fold with SPB and plated on LB plates that were incubated at 37 ^{0}C overnight. Bacterial colonies were enumerated the following day.

Determination of minimum bactericidal concentration (MBC)

For the determination of the minimum bactericidal concentrations (MBC) the following strains were assayed: *Staphylococcus aureus* (ATCC-25923) MIC_{50} 0.9-1.2 µg/ml; *Proteus vulgaris* (ATCC-29905) MIC_{50} 0.8-1.0µg/ml; *Group D streptococci* (ATCC-12959) MIC_{50} 0.7-1.0 µg/ml; *Bacillus spp.* (ATCC-14593) MIC_{50} 0.6-1.4 µg/ml; *Escherichia coli* (ATCC-25922) MIC_{50} 0.6-1.2 µg/ml; *Pseudomonas aeruginosa* (ATCC-27853) MIC_{50} 0.6-1.2 µg/ml; *Serratia morganii* (ATCC- 31665) MIC_{50} 0.8-1.1 µg/ml.

After having determined the MICs of *S. aureus* (ATCC-25923); *P. vulgaris* (ATCC-29905); *Group D Streptococci* (ATCC-12959); *Bacillus spp.* (ATCC-14593); *E. coli* (ATCC-25922); *K. pneumoniae* (ATCC-13883) ; *P. aeruginosa* (ATCC-27853); *S. morganii* (ATCC- 31665) from the wells of the micro plate with no visible bacterial growth, samples were removed for serial sub cultivation of 2µl into microtiter plates

containing 100µl of broth per well and further incubated for 24h. The lowest concentration with no visible growth was defined as MBC, indicating 99.5% killing of the original inoculum. The optical density of each well was measured at a wavelength of 620nm by microplate manager 4.0 (Bio-Rad laboratories) and compared with a blank. Solvent (DMSO) was used as a negative control. Three replicates were done for each compound and experiment was repeated two times.

Cysteine protease inhibitory activity

The capacity of the 1-substituted pyridylimidazo [1,5-a] pyridine derivatives (**3a-d)** to inhibit cysteine proteases was tested using papain as the model enzyme. The proteolytic activity of the reaction mixtures was determined using Bz-DL-Arg-pNA as the chromogenic substrate [43, 44]. To solutions of active papain (final concentration: 0.05 mM) in Tris-HCl buffer, pH 7.2 were added concentrated solutions of the 1-substituted pyridylimidazo [1,5-a] pyridine derivatives **(3a-d)** to make final concentration of 0.2 mM. After incubation for 30 min at 37^0C, the substrate solution was added and after a further incubation for 20 min the reaction was stopped and the absorbance of the reaction mixture was determined at a wavelength of 410 nm by microplate manager 4.0 (Bio-Rad laboratories). The same procedure was used at 32^0C and 42^0C for thermodynamic studies. The kinetic parameters for the substrate hydrolysis were determined by measuring the initial rate of enzymatic activity. The inhibition constant K_i was determined by Dixon method [45] and also by the Lineweaver–Burk equation. The K_M value was calculated from the double-reciprocal equation by fitting the data into the computer software Origin 6.1. The Lineweaver–Burk plot was used to determine the types of inhibition. For the kinetic analysis and rate constant determinations, the assays were carried out in triplicates, and the average value was considered throughout this work.

Thermodynamic analysis of CPI:

Free energy changes of inhibition of Papain against 1-substituted pyridylimidazo [1,5-a] pyridine derivatives **(3a-d)** (ΔG) were determined by the equation,

$$\Delta G = -RT\ In K_a \qquad (1)$$

Temperature dependence of the inhibition constants was used to determine the thermodynamic parameters. Changes in enthalpy (ΔH) were determined from the Van't Hoff plots by using the equation,

$$\text{In } K_a = (-\Delta H/RT) + \Delta S/R \qquad (2)$$

Where Δ*H* is enthalpy change, R is gas constant, ΔS is entropy change and T is the absolute temperature. The entropy change was obtained from the equation,

$$\Delta G = \Delta H - T\Delta S \qquad (3)$$

The assay was done at different temperatures calculating various K_i of 1-substituted pyridylimidazo [1,5-a] pyridine derivatives with papain as model enzyme.

Computational parameters for a compound to be a good drug

Lipinski's rule of Five [46] states that poor absorption or permeation is more likely when:

(1) There are more than 5 H-bond donors (expressed as the sum of OHs and NHs)

(2) The MWT is over 500

(3) The Log P is over 5 (or MLogP is over 4.15)

(4) There are more than 10 H-bond acceptors (expressed as the sum of Ns and Os)

Compounds that are the substrates for biological transporters are exceptions to the rule. There are certain necessary features which help to predict the candidature of a compound to be a good drug. The prediction process relies on a precomputed set of structural fragments that give rise to toxicity alerts in case they are encountered in the structure currently drawn [47]. The compound should have a reasonable probability of being well absorbed and their logP value must not be greater than 5.0. Typically, a low solubility goes along with a bad absorption and therefore the general aim is to avoid poorly soluble compounds. LogS value is a unit stripped logarithm (base 10) of the solubility measured

in mol/liter. More than 80% of the drugs in the market have an (estimated) logS value greater than –4 [47]. Compounds with higher molecular weights are less likely to be absorbed and therefore less likely to ever reach the place of action. Thus, trying to keep molecular weights as low as possible should be the desire of every drug forger. The drug likeness is calculated by score values of those fragments that are present in the molecule under investigation. The drug score combines drug likeness, cLogP, logS, molecular weight and toxicity risks in one handy value that may be used to judge the compound's overall potential to qualify for a drug. Toxicity risk alerts are an indication that the drawn structure may be harmful concerning the risk category specified. However, risk alerts are by no means meant to be a fully reliable toxicity prediction, nor it should be concluded from the absence of risk alerts that a particular substance is completely free of any toxic effect [47].

RESULTS AND DISCUSSION

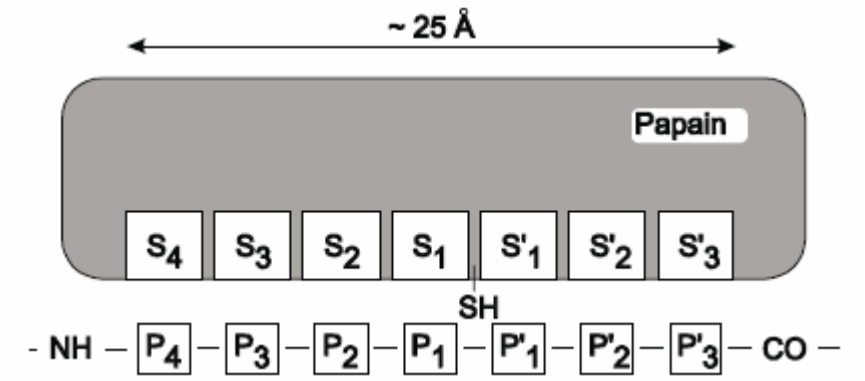
Compounds (**3a-3d)** (Table 1) were evaluated for antibacteria activity against representative bacteria — *S. aureus, P. vulgaris, Group D streptococci*, *Bacillus sp.*, *E. coli*, *P. aeruginosa* and *S. marganii.*

As can be seen in Table 2, Table 3 and Fig 1, compounds **3a, 3c** and **3d** were active against all tested bacterial cultures whereas compound **3b** was active against all tested bacterial cultures except *Group D streptococci* and *S. morganii.* The cysteine protease papain catalyzes the hydrolysis of amide and ester substrates by a process that can be minimally represented by three steps:

$$E + S \rightleftharpoons ES \longrightarrow ES' \longrightarrow E + P_2$$
$$+$$
$$P_1$$

The michaelis complex ES gives rise to an acyl enzyme derivative (ES'), formed by reaction at the thiol group of cysteine 25, and the first product P_1 (either an amine or an alcohol). Deacylation with base-catalysed addition of water gives second product P_2, a carboxylic acid. Both acylation and deacylation are thought to proceed via a tetrahedral

intermediate (a mechanistic feature that is shared by serine proteases) and thought to be stabilized by hydrogen bonding of the oxyanion to proximate amide functions .



Turk *et al.* have proposed, on the basis of kinetic and structural studies, that only 5 subsites are important for substrate binding. The S2, S1, and $S1^{l}$ pockets are important for both backbone and side-chain binding, whereas S3 and $S2^{l}$ are crucial only for amino acid side-chain binding. The S2 site of the enzyme is a hydrophobic pocket, formed by the amino acid residues Trp69, Tyr67, Phe207, Pro68, Ala160, Val133, and Val157.

A variety of intermediates are generated when papain reacts with a substrate or an inhibitor [22-26]. Like serine proteases, cysteine proteases tend to have relatively shallow, solvent-exposed active sites that can accommodate short substrate/inhibitor segments of protein loops (e.g. from endogenous inhibitors such as cystatins) or strands. The possibility of conformational flexibility of the enzyme induced by substrate or inhibitor binding should not be overlooked. The inhibitor molecule is bound with a combination of hydrogen bonds and hydrophobic interactions. The results indicate that all the molecules that were tested contain 1-pyridylimidazo [1,5-*a*] pyridine moiety which differ at C-5 position (Table1). 1-pyridylimidazo [1,5-*a*] pyridine moiety contains three Nitrogen atoms, out of which, two are having lone pair of electrons available for substitution reaction. Imidazo [1,5-a] pyridine (Table1) has two Nitrogen atoms and only one Nitrogen atom is involved in aromatic system while another remains free with the

Table 2: Antimicrobial activity of compounds (3a-d)

Name of Compounds		Microorganism[a]						
		1	2	3	4	5	6	7
3a	***MIC$_{50}$**	0.9(±0.04)	0.8(±0.04)	0.7(±0.03)	0.6(±0.03)	0.6(±0.03)	0.6(±0.03)	0.8(±0.04)
	***MIC$_{90}$**	1.7(±0.08)	1.6(±.08)	1.2(±0.06)	1.2(±0.06)	0.99(±0.04)	1.09(±0.05)	1.8(±0.09)
	***MBC$_{50}$**	1.8(±0.09)	1.6(±0.08)	1.3(±0.06)	1.2(±0.06)	1.3(±0.06)	1.1(±0.05)	1.6(±0.08)
	***MBC$_{90}$**	3.5(±0.17)	3(±0.15)	2.4(±0.12)	2.5(±0.12)	1.5(±0.07)	1.9(±0.09)	3.6(±0.18)
3b	**MIC$_{50}$**	1(±0.05)	0.8(±0.04)	NA	0.9(±0.05)	1(±0.05)	1(±0.05)	NA
	MIC$_{90}$	2.04(±0.1)	1.7(±.08)	NA	1.7(±0.08)	1.2(±0.06)	1.8(±0.09)	NA
	MBC$_{50}$	2.3(±0.11)	1.6(±0..08)	NA	1.7(±0.08)	1.9(±0.09)	2.1(±0.1)	NA
	MBC$_{90}$	4.3(±0.21)	3.4(±0.17)	NA	3.5(±0.17)	2.3(±0.11)	3.5(±0.17)	NA
3c	**MIC$_{50}$**	1.2(±0.06)	1(±0.05)	0.9(±0.04)	0.9(±0.05)	0.6(±0.03)	1.2(±0.06)	1(±0.05)
	MIC$_{90}$	2.6(±0.13)	2.1(±0.1)	1.5(±0.07)	1.7(±0.08)	1.6(±0.08)	2.2(±0.11)	1.9(±0.09)
	MBC$_{50}$	2.4(±0.12)	2.2(±0.11)	1.8(±0.09)	1.9(±0.09)	1.2(±0.06)	2.4(±0.12)	2.1(±0.1)
	MBC$_{90}$	4.7(±0.23)	4.1(±0.2)	3.3(±0.16)	3.8(±0.19)	3(±0.15)	4(±0.2)	4.1(±0.2)
3d	**MIC$_{50}$**	1.2(±0.06)	0.9(±0.05)	1(±0.05)	1.4(±0.07)	1.2(±0.06)	1.2(±0.06)	1.1(±0.05)
	MIC$_{90}$	3.1(±0.15)	2(±0.1)	1.8(±0.09)	2.2(±0.11)	2.3(±0.11)	2(±0.1)	2.1(±0.1)
	MBC$_{50}$	2.6(±0.13)	1.7(±0..08)	2.3(±0.11)	2.5(±0.12)	2.5(±0.12)	2.4(±0.12)	2.4(±0.12)
	MBC$_{90}$	5.5(±0.27)	4.4(±0.22)	3.9(±0.19)	4.7(±0.23)	4.9(±0.24)	4.4(±0.22)	4.4(±0.22)
ceftriaxone	**MIC$_{50}$**	4.0(±0.20)	0.5(±0.02)	0.08(±0.004)	0.9(±0.04)	0.06(±0.003)	1.6(±0.08)	0.8(±0.04)
	MIC$_{90}$	6(±0.30)	0.8(±0.03)	1.4(±0.05)	1.6(±0.08)	0.12(±0.005)	3(±0.14)	1.5(±0.07)
	MBC$_{50}$	7(±0.33)	0.9(±0.04)	1.5(±0.07)	1.7(±0.07)	0.14(±0.005)	3.2(±0.16)	1.7(±0.08)
	MBC$_{90}$	10(±0.42)	1.2(±0.05)	1.9(±0.09)	3.1(±0.14)	0.24(±0.012)	5.5(±0.23)	3.3(±0.13)

[a] *1. S. aureus, 2. P. vulgaris, 3. Group D streptococci, 4. Bacillus, 5. E.coli, 6. Pseudomonas aeruginosa, 7. Serratia margarsi*

NA- Not active

(±) standard deviation; n=3

* Values in µg/ml

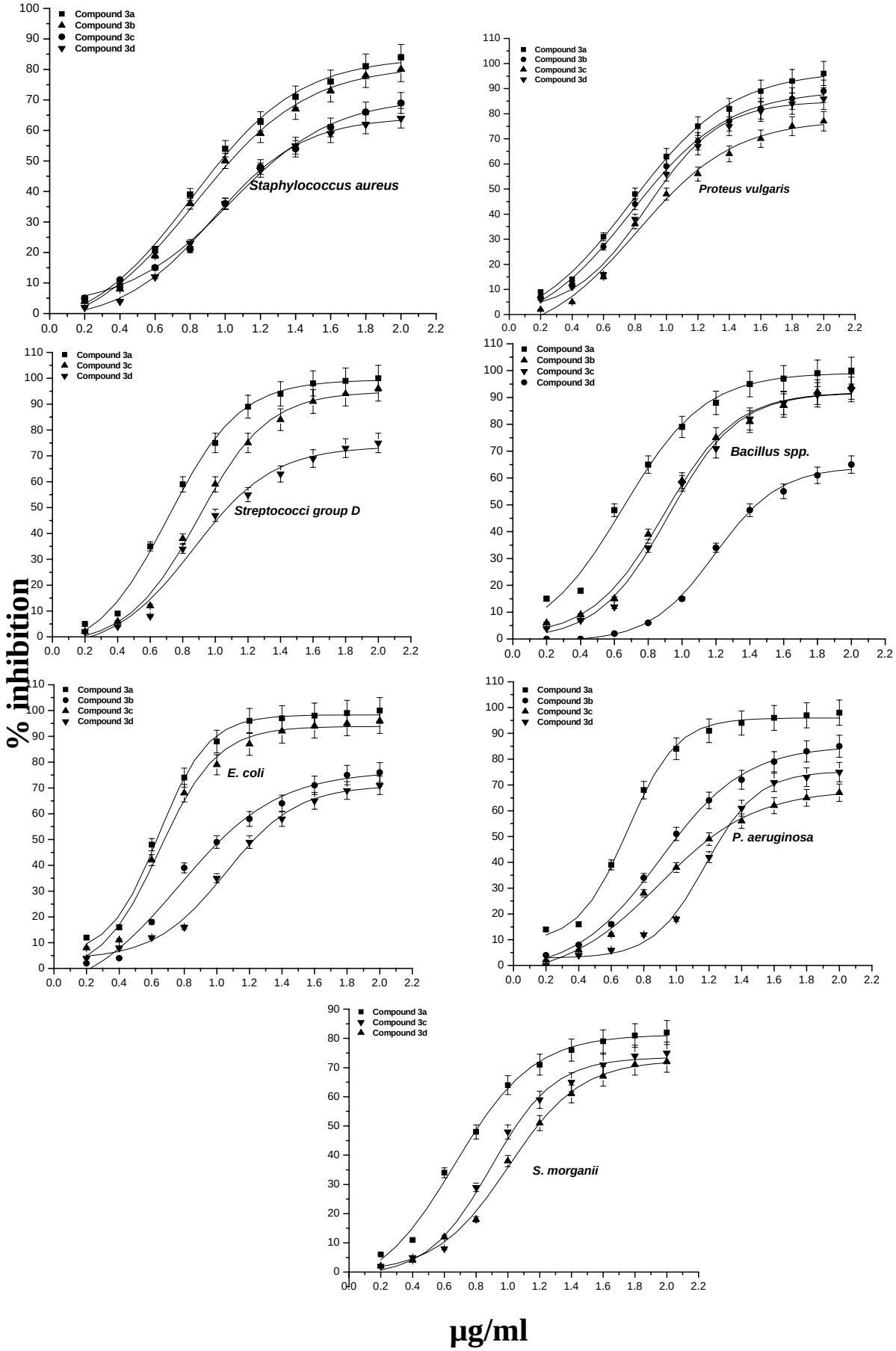


Fig. 1. Growth inhibition of compounds (**3a-d**) at different concentrations was recorded after 10-12hrs.

lone pair of electrons and this nitrogen may take part in hydrogen bonding for activity. Pyridine is a modest base (pK_a=5.2) and the basic unshared electron pair is not part of the aromatic sextet. Pyridinium species produced by N-substitution retain the aromaticity of pyridine and may be involved in hydrogen bonding. Substitution at C-5 position is responsible for the variation in antimicrobial activity. Polar substitution at 2nd position of C-5 aryl ring enhances the activity whereas non polar substitution decreases (**3a & 3c**) the activity. The polar substitution at 4 th position of C-5 aryl ring decreases the activity (**3b & 3d**). In molecule **3d** the 3rd & 5th position of C-5 aryl ring bear bulky groups (isobutyl group) which cause steric hindrance and make the molecule less active

Table 3: K_i and IC_{50} of compounds (3a-d) against cysteine protease, Papain

Compound	Type of inhibition	Ki (µM)	IC_{50} (µM)
3a	Non Competitive	13.75	13.4
3b	Competitive	23.21	21.17
3c	Non Competitive	90	94.5
3d	Non Competitive	99.3	96.5

Since MICs (Table 2) of compounds **(3a-d)** are directly proportional to the K_i (Table 3) against all tested bacteria except *E. coli* and *P. vulgaris* which clearly indicates that these compounds inhibit cysteine proteases of these pathogens. The thermodynamic parameters viz. free energy (ΔG), enthalpy (ΔH) and entropy (ΔS) of binding, determined by K_i at different temperatures, are given in Table 4.

The Van't Hoff plots were linear ($r > 0.9$ and $n=3$) for all inhibitors in the temperature range studied (Fig. 2). Free energy of the binding (ΔG) was negative at all temperatures, suggesting the spontaneous binding. Enthalpy change (ΔH) of binding is negative and entropy (ΔS) change of binding is positive which indicates exothermic and entropically

driven nature of binding. This pattern of temperature dependence is characteristic of hydrophobic interaction. Thermodynamic study reveals that hydrophobic interactions favour binding of inhibitor with protease. The partition coefficient (logP) is a well-established measure of the compound's lipophilicity. The distribution of calculated logP (cLogP) values of a majority of drugs in the market is in the range of 0–5 [46].

Lipophilicity (Table 5) clearly showed relationship of the compounds with their activity. It probably helps the compound to penetrate inside the bacteria. All the compounds studied except **3d**, showed good agreement with the criteria laid down for the prediction [46-47] of a molecule to be a potential drug.

Fig 2: Van't Hoff Plot of the effect of temperature on the inhibition constant of Papain against 1-pyridylimidazo [1,5-*a*] pyridines derivatives

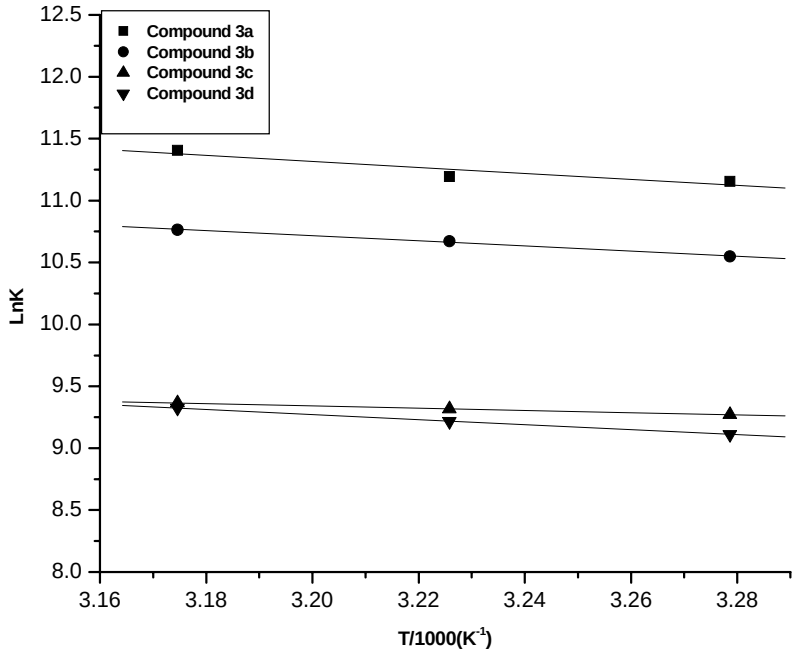


Table 4: Inhibition constant and thermodynamic parameter for inhibition of Papain against 1-substituted pyridylimidazo [1,5-a] pyridine derivatives at different temperatures

Name of compounds	Ki (μM) Temperature			*ΔG	*ΔH	*ΔS
	32^0C	37^0C	42^0C	(KJmol^{-1})	(KJmol^{-1})	(Jmol-K^{-1})
3a	14.3 (±0.71)	13.7 (±0.57)	11.1 (±0.52)	-28.8 (±1.26)	-6.2 (±0.88)	72.92 (±2.33)
3b	26.2 (±1.10)	23.2 (±1.09)	21.1 (±0.92)	-27.5 (±1.37)	-5.3 (±0.80)	71.4 (±2.46)
3c	94.1 (±4.42)	90.0 (±4.50)	85.6 (±3.59)	-24.0 (±1.12)	-2.3 (±0.31)	69.88 (±2.65)
3d	110.2 (±4.85)	99.3 (±4.36)	89.2 (±3.74)	-23.7 (±0.99)	-5.2 (±0.84)	59.73 (±1.93)

*Value at 37 °C
(±) standard deviation; n=3

Table 5: Prediction of antibacterial compounds as drugs (http://www.organic-chemistry.org)

Sr. No.	Name of compounds	[a]cLogP prediction	[b]solubility prediction	[c]Mw	[d]Drug Likeness prediction	[e]Overall Drug Likeness Score	[*]M	[#]T	[‡]Irritating Effect	[¶]Reproductive Effect
1	**3a**	3.84 (4.13) [3.2]	-5.16 (-4.11)	287	0.71	0.41	N	N	N	N
2	**3b**	4.04 (4.05) [4.19]	-5.48 (-4.64)	301	-0.23	0.41	N	N	N	N
3	**3c**	4.75 (4.58) [4.64]	-6.19 (-5.23)	305	0.16	0.35	N	N	N	N
4	**3d**	6.88 (6.57) [7.15]	-7.48 (-5.72)	399	-16.3	0.09	N	Y	N	Y

[*]Mutagenicity
[#]Tumorigenicity

[a] compounds to have a reasonable propability of being well absorbt their logP value must not be greater than 5.0

[b] more than 80% of the drugs on the market have a (estimated) logS value greater than -4.

[c] more than 80 % of all traded drugs have a molecular weight below 450.

[d] 80% of the drugs have a positive druglikeness value.

[e] drug score combines druglikeness, cLogP, logS, molecular weight and toxicity risks

[f] The prediction process relies on a precomputed set of structural fragment that give rise to toxicity alerts in case they are encountered in the structure currently drawn.

( ) (Tetko et al., 2005)

[ ] Chemdraw

Table 5 (Cont.): Prediction of antibacterial compounds as drugs (Chemdraw)

S.No.	Compound	H-bond accaeptor[g]	H-bond donor[h]	No. of rotational bonds
1	**3a**	4	1	2
2	**3b**	4	0	3
3	**3c**	3	0	2
4	**3d**	4	1	4

[g] H-bond acceptors should be less than 10
[h] H-bond donors should be less than 5

All molecules do not show any threat against toxicity risk assessment except molecule **3d** which showed threat as tumorogenic and endrogenic effect due to the presence of isobutyl group. All molecules have H-bond donors less than 5 and H-bond acceptor less than 10 whereas the number of rotational bonds lie in the moderate range i.e. 2-4. Among all tested molecules, molecule **3a** is the most potent molecule whose MIC is the lowest among all molecules (Table 2) and showed maximum drug score and positive values for drug likeness.

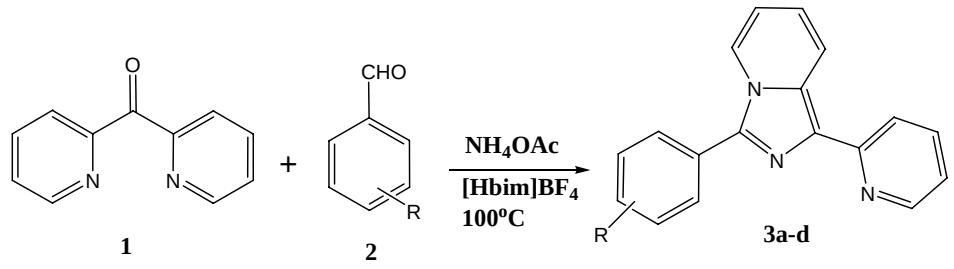
CONCLUSION

This study reports the antimicrobial activity of a class of substituted 1-pyridylimidazo [1,5-*a*] pyridines **(3a-d)** which are also cysteine protease inhibitors. Substitution at C-5 position is responsible for the variation in antimicrobial activity. Polar substitution at 2nd position of C-5 aryl ring enhances the activity whereas non polar one reduces whereas the polar substitution at 4th position of C-5 aryl ring decreases the activity **(3b & 3d).** MICs (Table 2) of compounds **(3a-d)** are directly proportional to the K_i (Table 3) against all tested bacteria except *E. coli* and *P. vulgaris* which clearly indicate that these compounds inhibit cysteine proteases of these pathogens which are directly or indirectly involve into pathogenicity of microbes. Thermodynamic study reveals that binding of inhibitors with enzyme is hydrophobic. We propose these antibacterial compounds as new potent

antibacterial drug as they showed good agreement with the computational criteria for drug prediction and Lipinski's rule of Five. The most active compound is 1-(2-Pyridyl)-3-(2-hydroxyphenyl) imidazo [1,5-*a*] pyridine.

CHEMISTRY

[Hbim]BF4 (IL) was used as the reaction medium and promoter to generate a variety of 1-substituted imidazo[1,5-*a*]pyridines **3a**–**d** by the reaction of 1,2-dipyridylketone (**1**), 2-benzoyl and 2-acetyl pyridine with aryl aldehydes **2** and ammonium acetate, respectively, at 100 °C (Scheme 1).The 1-pyridylimidazo[1,5-*a*]pyridines have been obtained in excellent isolated yields in relatively short reaction times. It can be observed that the process tolerates both electron-donating and electron withdrawing substituents on the aldehyde.



1 **2** **3a-d**

R=2-OH; 4-OMe; 2-Cl; 6-t-Bu-4-OH

Scheme 1

1-(2-Pyridyl)-3-(2-hydroxyphenyl)imidazo[1,5-*a*]pyridine (3a)

Pale yellow solid; mp 195–196 °C, 1H NMR (CDCl3–DMSO-*d*6, 200 MHz): d = 9.12 (d, *J* = 9.3 Hz, 1H), 9.00 (d, *J* = 4.5 Hz, 1 H), 8.66 (d, *J* = 6.8 Hz, 1 H), 8.52 (d, *J* =7. 8 Hz, 1 H), 8.16 (d, *J* = 7.9 Hz, 1 H), 7.76 (t, *J* = 8.0 Hz, 1 H),7.54–7.34 (m, 4 H), 7.18 (t, *J* = 6.8 Hz, 1 H), 13C NMR (CDCl3–DMSO-*d*6, 50 MHz): d = 156.5, 153.3, 147.3, 136.4, 134.8, 129.4, 128.6, 128.2, 120.6, 119.9, 118.9, 118.0, 117.3, 114.8, 113.8, 112.5.
LC-MS: *m*/*z* = 288 (M+), Anal. Calcd for C18H13N3O (287.32): C, 75.25; H, 4.56; N, 14.63, Found: C, 74.71; H, 4.32; N, 14.48.

1-(2-Pyridyl)-3-(4-methoxyphenyl)imidazo[1,5-*a*]pyridine (3b)

Yellow solid; mp 120–121 °C, 1H NMR (CDCl3, 200 MHz): d = 8.61 (d, *J* = 9.3 Hz, 1 H), 8.56 (d,*J* = 5.9 Hz, 1 H), 8.17 (dd, *J* = 8.2, 7.0 Hz, 2 H), 7.69–7.59 (m, 3H), 7.04–6.96 (m, 3 H), 6.85 (dd, *J* = 9.0, 6.4 Hz, 1 H), 6.57 (t, *J* =7.7 Hz, 1 H), 3.80 (s, 3 H), 13C NMR (CDCl3, 50 MHz): d = 160.0, 154.9, 148.8, 138.0, 136.2,130.0, 129.8, 129.7, 122.6, 122.4, 121.5, 120.8, 120.2, 119.8, 114.4,113.6, 55.3, LC-MS: *m*/*z* = 302 (M+), Anal. Calcd for C19H15N3O (301.34): C, 75.73; H, 5.02; N, 13.94, Found: C, 75.66; H, 4.59; N, 13.72.

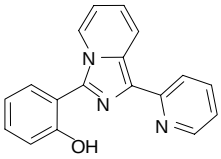
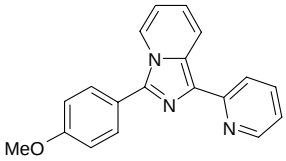
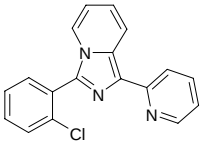
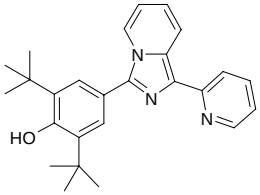
1-(2-Pyridyl)-3-(2-chlorophenyl)imidazo[1,5-*a*]pyridine (3c)

Bright yellow solid; mp 178–179 °C, 1H NMR (CDCl3, 200 MHz): d = 8.74–8.69 (m, 1 H), 8.63–8.60 (m,1 H), 8.24–8.19 (m, 1 H), 7.72–7.37 (m, 6 H), 7.10–7.16 (m, 1 H),6.98–6.90 (m, 1 H), 6.67–6.60 (m, 1 H), 13C NMR (CDCl3, 50 MHz): d = 154.8, 148.9, 136.1, 135.5, 134.3,133.2, 130.8, 129.8, 129.7, 129.1, 127.2, 122.2, 121.4, 121.1, 120.3, 119.7, 113.4. LC-MS: *m*/*z* = 306 (M+), Anal. Calcd for C18H12N3Cl (305.76): C, 70.71; H, 3.96; N, 13.74; Cl, 11.6. Found: C, 70.59; H, 3.71; N, 13.59; Cl, 11.5.

1-(2-Pyridyl)-3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)imidazo[1,5-*a*] pyridine (3d)

Yellow solid; mp 212–213 °C, 1H NMR (CDCl3, 200 MHz): d = 8.71–8.60 (m, 2 H), 8.28–8.24 (d, *J* = 8.5 Hz, 1 H), 8.16–8.12 (d, *J* = 7.3 Hz, 1 H), 7.74–7.66 (m, 1H), 7.58 (s, 2 H), 7.10–7.03 (m, 1 H), 6.92–6.84 (m, 1 H), 5.45 (br s, 1 H), 1.50 (s, 18 H), 13C NMR (CDCl3, 50 MHz): d = 136.2, 125.6, 121.7, 120.7, 120.2,119.9, 113.5, 30.2,LC-MS: *m*/*z* = 401 (M+), Anal. Calcd for C26H29N3O (399.53): C, 78.16; H, 7.32; N, 10.52. Found: C, 78.04; H, 7.10; N, 10.20.

Table 1: Structure of compounds and their names

Compound	Structure
1-(2-Pyridyl)-3-(2-hydroxyphenyl) imidazo [1,5-*a*] pyridine **3a**	
1-(2-Pyridyl)-3-(4-methoxyphenyl) imidazo [1,5-*a*] pyridine **3b**	
1-(2-Pyridyl)-3-(2-hlorophenyl) imidazo [1,5-*a*] pyridine **3c**	
1-(2-Pyridyl)-3-(3,5-di-*tert*-butyl-4-hydroxyphenyl) imidazo [1,5-*a*] pyridine **3d**	

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Chapter 5

Study of synthesized compounds as aspartic protease inhibitors and Cysteine protease inhibitors

ABSTRACT

2,4,5-triaryl imidazole derivatives and 4-substituted aryl 3,4-dihydro-2-pyridones were screened as inhibitors of the aspartic protease pepsin and cysteine protease papain as model enzymes, respectively. These inhibitors were kinetically and thermodynamically characterized. The binding of inhibitors with proteases was explained by using Van't Hoff plot and thermodynamic parameters viz. free energy (ΔG), Entropy (ΔS) and Enthalpy (ΔH). 2,4,5-triaryl imidazoles act as potent aspartic protease inhibitors with K_i ranges from 37.2 μM to 167.16 μM while 4-substituted aryl 3,4-dihydro-2-pyridones showed K_i ranges from 0.51 μM - to 1.1 μM. Strong hydrophobic groups at C-4 & C-5 position in 2,4,5-triaryl imidazole derivatives favour binding of inhibitors with protease. Experiments also showed that among C-2 aryl substituted imidazole, a 4-substitution on aryl ring is preferred and a less polar substituent makes the molecule more active whereas polar substituents at 2-position on C-2 aryl ring makes the molecule less active. In 4-substituted aryl 3,4-dihydro-2-pyridones hydrophobic substitution decreases the potency of compounds. In both cases the Van't Hoff analysis showed that the value of K_i decreases with increase in temperature and the binding of the inhibitor are entropically driven.

INTRODUCTION

There is a need for small non peptide inhibitors which are easy to synthesize and exhibit good bioavailability. We have designed and screened new templates which would straddle the active site of porcine pepsin and Papain, act as a scaffold upon which to build. Proteases play a key role in a variety of biological processes both at the physiological level and in infection. Their interaction with inhibitors is the subject of intense investigation also with the view of finding lead compounds for drugs that can prevent or treat pathologies dependent on specific protein processing or degradation. Aspartate and cystein proteases have attracted much attention for their role in pathogenicity. Aspartic family includes mammalian proteins such as the digestive enzyme pepsin and renin which is involved in control of blood pressure and lysosomal cathepsin D. Proteases are also key players in determining the infectiveness of various

pathogens, ranging from retroviruses to fungi and protozoa. Cysteine family found to be critical for pathogens life cycle e.g. cruzipain [1], falcipain [2], *Streptococcal* [3] and *Staphylococcal* [4] proteases. Cysteine protease inhibitors can selectively arrest replication of a microbial pathogen without untoward toxicity to the host. Their inhibition thus has therapeutic value in conditions varying from hypertension, inflammation, tumour metastasis and Alzheimer's disease to fungal or viral infections and malaria.

A number of aspartyl proteinases including mammalian (renin, BACE, γ-secretase), viral (HIV-1 protease) and parasitic (plasmepsin) origin enzymes have been identified as useful targets for chemotherapeutic intervention [5–8]. A significant number of peptidomimetic inhibitors, designed as stable bisubstrate analogues (with statine, hydroxyethylene, or fluorostatone isosteres), have been developed. Some of these have even reached clinical trials, but were abandoned later due to a limited bioavailability or excessive production costs. New structural classes of nonpeptidic inhibitors with improved physicochemical properties were identified [9–13] through screening or rational design techniques for a number of therapeutically important aspartyl proteases. Three-dimensional crystal structures of some of these inhibitors bound to their target enzymes have been reported [9, 10], and this has aided lead optimization with respect to selectivity and affinity. However, attainment of structural information is not always straightforward and in these cases the inhibitor modality and binding site have to be inferred from kinetic analysis of enzyme inhibition.

Enzymes are the unquestioned protagonists of catalysis for virtually all the biochemical reactions occuring within cells. Inhibitors are usually proteins or other molecules specifically competing with enzyme substrates, hence exerting a regulatory mechanism on the biochemical pathway they belong to. Understanding the physico-chemical features of enzyme-inhibitor binding is fundamental, especially for designing specific molecules for therapeutic and biomedical purposes. In this study we have designed and screened several compounds in order to investigate a new potent scaffold which may be used to design some therapeutically important protease inhibitors.

MATERIAL

2,4,5-triaryl imidazole derivatives 2-(4,5 dip-tolyl-1H-imidazole-2-yl) Phenol **(3a)**, 2-(4-methoxyphenyl)-4,5 dip-tolyl-1H-imidazole **(3b)**, 2-Phenyl-4,5-dip-toly-1H-imidazole **(3c)**, 4-(4,5-dip-tolyl-1H-imidazole-2-yl)-2methoxyphenol **(3d)** and 2-(4-methoxyphenyl)-4,5-diphenyl-1H-imidazole **(3e)** were synthesized according to Siddiqui *et al* [14] and Methyl-2-methyl-6-oxo-4-phenyl-1,4,5,6-tetrahydropyridine-3-carboxylate **(4a)** & ethyl 4-(4-methoxyphenyl)-2-methyl-6-oxo-1,4,5,6-tetrahydropyridine-3-carboxylate **(4b)** were also synthesized according to Siddiqui *et al* [Manuscript under preperation]. N-acetyl-L-phenylalanyl-L-3,5-diiodotyrosine (APD), Pepsin (EC 3.4.23.1) , Papain (EC 3.4.22.2), Bz-DL-Arg-pNA (BAPNA), hemoglobin and other chromogenic substrates were from Sigma Chem. Co. USA. All solvents and chemicals were of analytical grade and were used as obtained from Merck and Sigma-Aldrich.

METHOD

Assay for inhibitory activity for screening of API and CPI

10 µl of pepsin or papain (100 µg/ml) and 100 µl inhibitor solution were incubated at 37 °C for 10 min in KCl–HCl buffer, pH 2.0 or Tris-HCl buffer, pH 7.2, respectively. Then 0.5 ml of hemoglobin (1%) dissolved in the same buffer was added and incubated at 37 °C for 30 min. The reaction was stopped by the addition of 5% trichloric acid (TCA) acidified with 2.25% HCl. One unit of pepsin was defined as the amount of enzyme that produced an increase in absorbance of 0.001 at 280 nm per minute under the conditions of the assay. One protease inhibitor unit was defined as the amount of inhibitor that inhibited one unit of pepsin or papain activity [15, 16].

Assay for inhibitory activity towards Pepsin for inhibition kinetics

Pepsin was assayed in the presence of the synthetic substrate N-acetyl-Lphenylalanyl-L-3, 5-diiodotyrosine (APD). To 0.25 ml of the enzyme solution (50 nM) at 37 °C is added 0.125 ml of 0.05N HCl. After 10 min 0.125 ml of APD solution is added to the test reaction and 0.5 ml of ninhydrin reagent is added to the blanks (final enzyme conc. 12.5

nM). After 30 min more, 0.5 ml ninhydrin reagent is added to the test reactions and at any time 0.125 ml of APD solution is added to the blanks. All the tubes are placed in a boiling water bath for exactly 15 min and are then cooled. The contents of these tubes are diluted with 2.5 ml of 60% (v/v) ethanol and the tubes are then shaken to mix the solutions. The absorbance of the solutions at 570 nm is read against water. The activity can be expressed in APD units. One APD unit is the quantity of enzyme which liberates 1 micromole of diiodotyrosine per minute under the above conditions [17]. For the kinetic analysis and rate constant determinations, the assays were carried out in triplicates, and the average value was considered throughout this work. The same procedure was used at 32^{o}C and 42^{o}C for thermodynamics studies.

Assay for inhibitory activity towards Papain for inhibition kinetics

The capacity of the 1-substituted pyridylimidazo [1,5-a] pyridine derivatives (**4a-b)** to inhibit cysteine proteases was tested using papain as the model enzyme. The proteolytic activity of the reaction mixtures was determined using Bz-DL-Arg-pNA as the chromogenic substrate [18]. To solutions of active papain (final concentration: 0.05 mM) in Tris-HCl buffer, pH 7.2 were added concentrated solutions of the 1-substituted pyridylimidazo [1,5-a] pyridine derivatives **(3a-d)** to final concentrations of 0.2 mM. After incubation for 30 min at 37^{0}C, the substrate solution was added and after a further incubation for 20 min the reaction was stopped and the absorbance of the reaction mixture was determined at a wavelength of 410 nm by microplate manager 4.0 (Bio-Rad laboratories). The same procedure was used at 32^{0}C and 42^{0}C for thermodynamics studies.

Initial kinetic analysis for the determination of K_M and K_i

The kinetic parameters for the substrate hydrolysis were determined by measuring the initial rate of enzymatic activity. The inhibition constant K_i was determined by Dixon [19] method and also by the Lineweaver–Burk equation. The K_M value was also calculated from the double-reciprocal equation by fitting the data into the computer software Origin 6.1. For the Lineweaver–Burk analysis of pepsin (12.5nM) and Papain

(0.05mM) was incubated with API 30 μM to 200 μM and with CPI 0.5 μM to 1.5 μM, respectively and assayed at increasing concentration of APD (10–100μM) and BAPNA (1mM-5mM), respectively, at 37 °C for 30 min. The reciprocals of substrate hydrolysis (1/v) for each API and CPI concentration were plotted against the reciprocals of the substrate concentrations, and the K_i was determined by fitting the resulting data. Inhibitor was found to inhibit pepsin and papain with an IC_{50} value (50% inhibitory concentration) of 38.73 μM- 183 μM and 0.67 μM - 1.0 μM, respectively. The inhibition of pepsin and papain followed a hyperbolic pattern with increasing concentrations of the inhibitor. However, the secondary plot (the slope of inhibition graph versus inhibitor concentration) was not linear, suggesting that the application of Michaelis–Menten inhibition kinetics was not appropriate in this study. In Dixon's method, hydrolytic activity of pepsin (12.5nM) or Papain (0.05mM) was measured in the presence of 50 μM and 100 μM APD or 2mM and 4mM BAPNA, at concentrations of API ranging from 30 μM to 200 μM or CPI ranging from 0.5 μM to 1.5 μM at 37 °C for 30min. The reciprocals of substrate hydrolysis (1/v) were plotted against the API concentration and the Ki was determined by fitting the data using Origin 6.1

Thermodynamic analysis of API and CPI:

Free energy changes of inhibition of Pepsin against 2,4,5-trisubstitued imidazoles derivatives and 4-substituted aryl 3,4-dihydro-2-pyridones (ΔG) were determined by the equation,

$$\Delta G = -RT\, In K_a \qquad (1)$$

Temperature dependence of the inhibition constants was used to determine the thermodynamic parameters. Changes in enthalpy (ΔH) were determined from the Van't Hoff plots by using the equation,

$$In\ K_a = (-\Delta H/RT) + \Delta S/R \qquad (2)$$

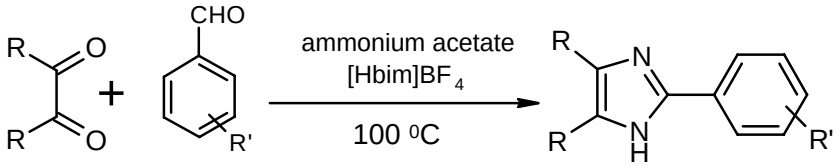
Where Δ*H* is enthalpy change, R is gas constant, ΔS is entropy change and T is the absolute temperature. The entropy change was obtained from the equation,

$$\Delta G = \Delta H - T\Delta S \qquad (3)$$

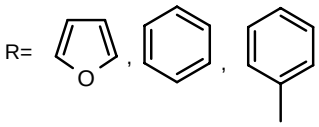
The assay was done at different temperatures calculating various K_i of 2,4,5-trisubstitued imidazoles derivatives and 4-substituted aryl 3,4-dihydro-2-pyridones with pepsin and papain as model enzymes respectively.

Chemical Synthesis of API:

2,4,5-trisubstitued imidazoles were synthesized by one-pot condensation of 1,2-diketones or α-hydroxy ketone, aromatic aldehydes and ammonium acetate in the (IL) ionic liquid, 1-butyl imidazolium tetrafluoroborate ([Hbim]BF4) according to Siddiqui *et al* [22].



3a-g **1a-c** **2**



R' = *p*-OMe; *m*-OMe; *p*-OH; *o*-OH; *p*-NO_2; 

Scheme 1

Chemistry

NMR spectra were recorded on a Bruker AC-200 spectrometer in $CDCl_3$/DMSO-d_6 with TMS as an internal standard. Infra red spectra were recorded with ATI-MATT-SON RS-1 FTIR spectrometer. Polarity of ILs was recorded on Lambda EZ 201, using Reichardt's dye. Melting points were recorded in open capillary and were uncorrected. All solvents and chemicals were of research grade and were used as obtained from Merck and Lancaster.

2-(4,5 dip-tolyl-*1H*-imidazole-2-yl) Phenol (3a)

Yellow solid, M. P. (°C) : 223 , IR (Nujol, cm^{-1}): υ_{max} 1216, 1555, 1638, 2465, 2998, 3432, 3596, ^{1}H NMR : δ 2.36 (s, 6H), 6.85-6.90 (t, *J*= 8.3 Hz, 1H), 6.95-6.98 (d, (200 MHz, $CDCl_3$/ DMSO-d_6) *J*= 8.0 Hz, 1H), 7.14-7.17 (m, 4H), 7.21-7.23(d, *J*= 7.3 Hz, 1H) 7.43-7.46 (m, 4H), 7.96-7.99 (d, *J*= 8.0 Hz, 1H), 12.84 (brs, 1H), ^{13}C NMR (50 MHz, $CDCl_3$/ DMSO-d_6) : δ 19.7, 114.2, 118.5, 126.1, 126.5, 126.8, 127.6, 127.7, 127.9, 127.9, 129.7, 135.5, 144.4, 157.2, LC-MS : 341.16 (M+H), 312.09, 298.12, 248.05, Elemental Analysis : C-$2_3H_{20}N_2O$ Calcd C, 81.15; H, 5.92; N, 8.23, Found C, 80.95; H, 5.71; N, 8.08.

2-(4-methoxyphenyl)-4,5 dip-tolyl-*1H*-imidazole (3b)

Pale yellow solid, M. P. (°C): 243-244, IR (Nujol, cm^{-1}) : υ_{max} 1216, 1560, 1638, 2475, 2988, 3430, ^{1}H NMR : δ 2.37 (s, 6H), 3.86 (s, 3H), 6.94-6.96 (d, *J*= 8.2 Hz, 2H) (200 MHz, $CDCl_3$/ DMSO-d_6) 7.13-7.15 (m, 4H), 7.46-7.48 (m, 4H), 8.03-8.05 (d, *J*= 8.2 Hz, 2H), 12.59 (brs, 1H), ^{13}C NMR : δ 20.2, 54.3, 112.9, 122.6, 126.1, 126.9, 128.1, 135.4, 145.1, (50 MHz,$CDCl_3$/ DMSO-d_6) 158.6, LC-MS: 355.18 (M+H), 329.12, 308.15, 289.09, Elemental Analysis: C-$2_4H_{22}N_2O$ Calcd. C, 81.33; H, 6.26; N, 7.90, Found C, 80.91; H, 5.88; N, 7.58.

2-Phenyl-4,5-dip-toly-*1H*-imidazole (3c)

Yellow solid, M. P. ^{0}C : 254-255 , IR (Nujol, cm^{-1}) : υ_{max} 629, 726, 878, 1216, 1570, 1638, 2465, 2998, 3432, ^{1}H NMR : δ 2.36 (s, 6H), 7.14-7.34 (m, 8H), 7.35-7.38 (m, 5H),

(200 MHz,$CDCl_3$/ DMSO-d_6) 12.56 (brs, 1H), ^{13}C NMR : δ 19.7, 124.1, 126.4, 127.1, 127.5, 127.7, 128.3, 129.1, 129.2, (50 MHz,$CDCl_3$/ DMSO-d_6) 135.1, LC-MS: 325.17 (M+H), 302.15, 285.14, Elemental Analysis: C-$2_3H_{20}N_2$ Calcd. C, 85.15; H, 6.21; N, 8.63, Found C, 84.92; H, 5.97; N, 8.51.

4-(4,5-dip-tolyl-*1H*-imidazole-2-yl)-2methoxyphenol(3d)

Pale yellow solid M.P 245 (^oC); IR (Nujol, cm^{-1}): υ_{max} 1216, 1638, 2475, 2988, 3422, 3616; 1H NMR (CDCl3/DMSO-d6, 200 MHz) d 2.37 (s, 6H), 3.80 (s, 3H), 6.75–6.69 (d, JZ8.22 Hz, 1H), 7.14–7.19 (m, 4H), 7.23–7.25 (d, JZ8.7 Hz, 1H), 7.43–7.47 (m, 4H), 7.56–7.57 (d, JZ8.3 Hz, 1H), 12.72 (brs, 1H); 13C NMR (CDCl3/DMSO-d6, 200 MHz) d 19.4, 55.1, 108.7, 114.7, 118.1, 121.2, 126.3, 127.3, 127.5, 132.3, 146.4, 146.9; C-$2_4H_{22}N_2O_2$ (370) : calcd C, 77.81, H, 5.99, N, 7.56; found C, 77.72; H, 5.89, N, 7.47.

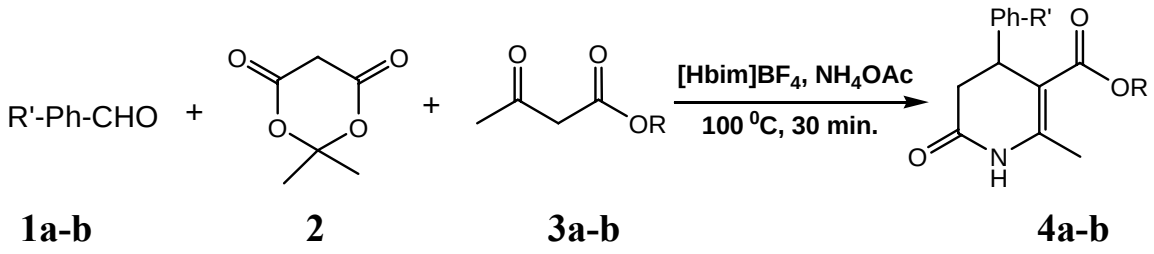
2-(4-methoxyphenyl)-4,5-diphenyl-*1H*-imidazole (3e)

Pale yellow solid, M. P. (°C) : 222-223 ,IR (Nujol, cm^{-1}) : υ_{max} 764, 829, 968, 1070, 1216, 1565,1636, 2465, 2893, 3428. ^1H NMR (200 MHz,$CDCl_3$/ DMSO-d_6) : δ 3.85 (s, 3H), 6.93-6.96 (d, J= 8.8 Hz, 2H), 7.25-7.59 (m, 10H), 8.02-8.05 (d, J= 8.8 Hz, 2H), 12.52 (brs, 1H), ^{13}C NMR (50 MHz,$CDCl_3$/ DMSO-d_6) : δ 54.6, 113.2, 122.7, 126.3, 126.5, 127.4, 127.6, 132.8, 145.7, 159.1, LC-MS : 327.15 (M+H), 314.10, 298.07, 233.05, Elemental Analysis : C-$2_2H_{18}N_2O$ Calcd. C, 80.96; H, 5.56; N, 8.58. Found C, 80.75; H, 5.38; N, 8.23.

Chemical synthesis of CPI

The reaction was performed with benzaldehyde (**1a-b**), meldrums acid **(2)**, methyl acetoacetate **(3a-b)** and ammonium acetate in ionic liquid ([Hbim]BF_4) at 100 ^0C and reaction was complete within 30 min (monitored by TLC). The reaction mixture was diluted with water and filtered off to give a solid product which was further purified by chromatography through a column of silica gel using 25% EtOAc in petroleum ether as

eluent to yield the desired 4-susbstituted aryl-3,4-dihydro-2-pyridones in excellent yields and were fully characterized [Manuscript under preperation].



Scheme 1

Methyl-2-methyl-6-oxo-4-phenyl-1,4,5,6-tetrahydropyridine-3-carboxylate (4a)

M. P. (^{0}C): 197-198, IR ($CHCl_3$, cm^{-1}) : υ_{max} 1215, 1676, 1700, 2400, 3019, 3210, ^{1}H NMR (200 MHz, $CDCl_3$): δ 2.44 (s, 3H), 2.68-2.77 (dd, *J*= 16.8, 1.8 Hz, 1H), 2.90-3.02 (dd, *J*= 16.4, 7.8 Hz, 1H), 3.68 (s, 3H), 4.24-4.30 (t, 1H), 7.22-7.31 (m, 5H), 8.11 (s, 1H), ^{13}C NMR : δ 18.8, 37.6, 38.1, 51.3, 106.8, 126.5, 128.7, 141.8, 146.8, 167.3, 50 MHz, $CDCl_3$ 171.4, Elemental Analysis : $C_{14}H_{15}NO_3$ Calcd. C, 68.56; H, 6.16; N, 5.71, Found C, 68.42; H, 5.98; N, 5.65.

Ethyl-4-(4-methoxyphenyl)-2-methyl-6-oxo-1,4,5,6-tetrahydropyridine-3-carboxylate (4b)

M. P. (^{0}C) : 148-195, IR ($CHCl_3$, cm^{-1}) : υ_{max} 1217, 1510, 1635, 1697, 3016, 3132, 3234, ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.16-1.23 (t, 3H), 2.39 (s, 3H), 2.62-2.71 (dd, *J*= 16.3, 2.1 Hz, 1H), 2.84-2.96 (dd, *J*= 16.6, 7.7 Hz, 1H), 3.76 (s, 3H), 4.06-4.14 (m, 2H), 4.17-4.22 (t, 1H), 6.77-6.82 (d, *J*= 6.7 Hz, 2H), 7.08-7.12 (d, *J*= 6.7 Hz, 2H), 7.54 (brs, 1H), ^{13}C NMR (50 MHz,$CDCl_3$) : δ 14.1, 19.1, 37, 38.1, 55.1, 60.1, 107.7, 114, 127.7, 134.2, 145.5, 158.4, 166.9, 170.9. Elemental Analysis : $C_{16}H_{19}NO_4$ Calcd. C, 66.42; H, 6.62; N, 4.84, Found C, 66.32; H, 6.53; N, 4.75.

RESULT AND DISCUSSION

This study revealed that 2,4,5-triaryl imidazole derivatives and 4-substituted aryl 3,4-dihydro-2-pyridones were found to be potent inhibitors of the aspartic protease pepsin (Table 1) and cysteine protease papain (Table 3), respectively. In 2,4,5-triaryl imidazole derivatives the scaffold, imidazole ring posses an electrophilic center and a secondary nitrogen bearing active hydrogen (1-unsubstituted) as a nucleophilic center. Compounds **3a-d** carries same toluene moiety at C-4 and C-5 position but they posses different moieties at C-2 positions. Results also showed that among C-2 aryl substituted imidazole, a 4-substitution on aryl ring is preferred **(3b & 3c)** and less polar substituent makes the molecule more active **(3b & 3d)** whereas polar substituent at 2-position on C-2 aryl ring makes the molecule less active **(3a & 3c)**. Compounds **3b & 3e** have same methoxy group at 4 position of C-2 aryl ring but they have different moieties at C-4 and C-5

Fig 1: Van't Hoff Plot of the effect of temperature on the inhibition constant of Pepsin against 2,4,5-triaryl imidazole derivatives

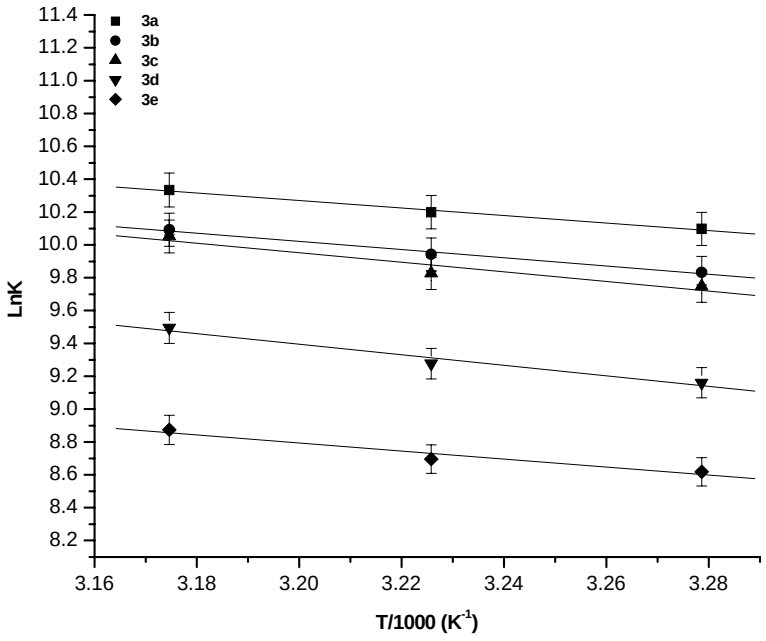


Table 1 : Names and structures of the API

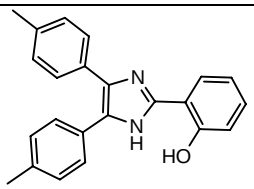
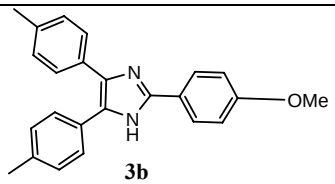
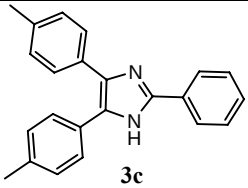
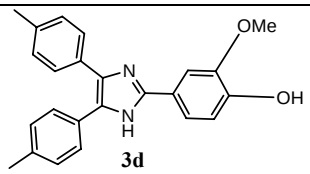
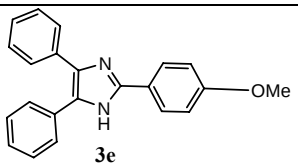
Name of compound	Structure	Types of Inhibition	K_i (μM)	IC-50 (μM)
2-(4,5 dip-tolyl-1H-imidazole-2-yl) Phenol (3a)	 **3a**	Non competitive	56.96	56.91
2-(4-methoxyphenyl)-4,5 dip-tolyl-1H-imidazole (3b)	 **3b**	Competitive	37.2	38.74
2-Phenyl-4,5-dip-toly-1H-imidazole (3c)	 **3c**	Competitive	48.05	50.79
4-(4,5-dip-tolyl-1H-imidazole-2-yl)-2 methoxyphenol (3d)	 **3d**	Non competitive	167.1	183
2-(4-methoxyphenyl)-4,5-diphenyl-1H-imidazole (3e)	 **3e**	Competitive	93.4	94.4

Table 2: Inhibition constant and thermodynamic parameters for inhibition of Pepsin against 2,4,5-triaryl imidazole derivatives at different temperatures.

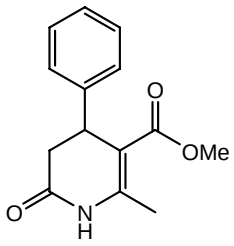
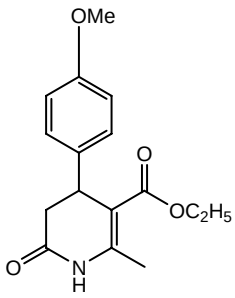
Name of Compounds	K_i (μM) Temperature			ΔG (K.Jmol^{-1})*	ΔH (K.Jmol^{-1})*	ΔS (Jmol-K^{-1})*
	32°C	37°C	42°C			
3a	41.2 (±1.23)	37.2 (±1.87)	32.5 (±1.8)	-26.2 (±0.71)	-5.8 (±0.1)	66.8 (±2.34)
3b	53.7 (±1.76)	48.05 (±2.01)	41.4 (±1.92)	-25.6 (±0.74)	-6.4 (±0.09)	61.8 (±2.71)
3c	58.4 (±1.45)	53.96 (±2.32)	43.1 (±1.82)	-25.3 (±0.79)	-7.5 (±0.12)	57.3 (±1.98)
3d	105.1 (±2.10)	93.5 (±3.43)	75.2 (±2.95)	-23.9 (±0.64)	-8.2 (±0.15)	50.3 (±1.76)
3e	180.6 (±4.34)	167.16 (±4.12)	140 (±2.34)	-22.4 (±0.68)	-6.2 (±0.16)	51.9 (±1.66)

*Value at 37 °C
(±) standard deviation; n=3

positions. Compound **3b** posses more hydrophobic moiety, Toluene, than compound **3e,** phenyl and it is more potent. It may be concluded that the hydrophobic moieties are required to interact with hydrophobic pockets [20] of aspartic proteases. The thermodynamic parameters viz. free energy (ΔG), enthalpy (ΔH) and entropy (ΔS) of binding, determined by K_i at different temperatures, are given in Table 2.

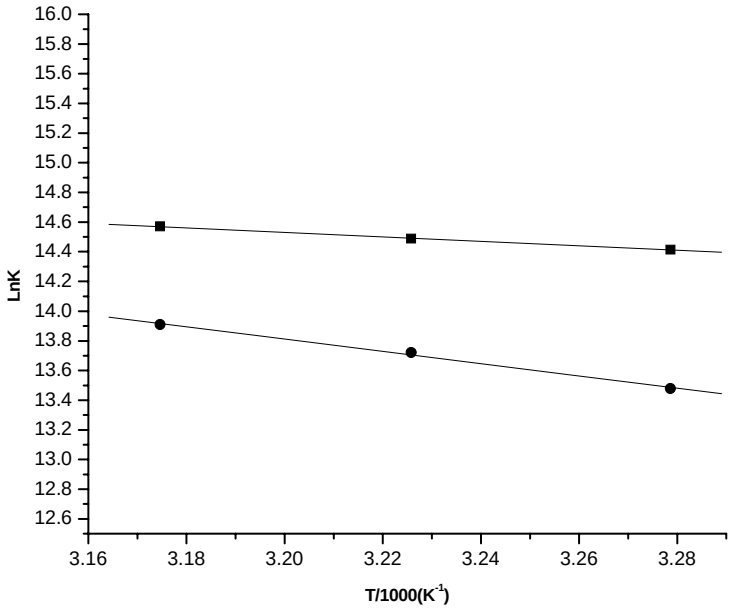
The Van't Hoff plots were linear ($r > 0.9$ and $n=3$) for all inhibitors in the temperature range studied (Fig. 1). Free energy of the binding (ΔG) was negative at all temperatures,

Table 3: Names and structures of the CPI

Name of compound	Structure	Types of Inhibition	K_i (μM)	IC-50 (μM)
Methyl 2-methyl-6-oxo-4-phenyl-1,4,5,6-tetrahydropyridine-3-carboxylate (4a)	 **4a**	Non competitive	0.51	0.67
Ethyl 4-(4-methoxyphenyl)-2-methyl-6-oxo-1,4,5,6-tetrahydropyridine-3-carboxylate (4b)	 **4b**	Non competitive	1.1	1.0

suggesting the spontaneous binding. Enthalpy change (ΔH) of the binding is negative and entropy (ΔS) change of the binding is positive which indicates the exothermic and entropically driven nature of binding. This pattern of temperature dependence is characteristic of hydrophobic interaction. Thermodynamics study reveals that hydrophobic interactions favour binding of inhibitor with protease. The scaffold methyl 2-methyl-6-oxo-4-phenyl-1,4,5,6-tetrahydro-3-pyridinecarboxylate of 4-substituted aryl 3,4-dihydro-2-pyridones bears two oxygen atoms and one nitrogen bearing active hydrogen (1-unsubstituted) as nucleophilic moieties. The result (Table 3) shows that

Fig 2: Van't Hoff Plot of the effect of temperature on the inhibition constant of Papain against 4-substituted aryl 3,4-dihydro-2-pyridones



when hydrophobic moieties like methyl and ethyl group are substituted, the potency of compound decreases **(4a & 4b)**.

The thermodynamic study was done at three different temperatures and Van't Hoff plot was studied. The calculated parameters viz. free energy (ΔG), enthalpy (ΔH) and entropy (ΔS) of binding, determined by K_i at different temperatures, are given in Table 4.

Table 4: Inhibition constant and thermodynamic parameters for inhibition of Papain against 4-substituted aryl 3,4-dihydro-2-pyridones at different temperatures.

Comp.	K_i (μM) Temperature			ΔG ($KJmol^{-1}$)*	ΔH ($KJmol^{-1}$)*	ΔS ($Jmol\text{-}K^{-1}$)*
	32°C	37°C	42°C			
1a	0.55 (±0.02)	0.51 (±0.02)	0.47 (±0.02)	-36.74 (±2.1)	-3.89 (±0.2)	105.95 (±5.1)
1b	1.4 (±0.06)	1.1 (±0.05)	0.91 (±0.04)	-34.79 (±2.1)	-1.06 (±0.1)	77.77 (±3.3)

Value at 37°C
(±) standard deviation; n=3

The Van't Hoff plots were linear ($r > 0.9$ and $n=3$) for all inhibitors in the temperature range studied (Fig. 2). Free energy of the binding (ΔG) was negative at all temperatures, suggesting the spontaneous binding. Enthalpy change (ΔH) of the binding is negative and entropy (ΔS) change of the binding is positive which indicates the exothermic and entropically driven nature of binding.

CONCLUSION

This study reports that 2,4,5-triaryl imidazole derivatives and 4-substituted aryl 3,4-dihydro-2-pyridones were found to be potent inhibitors of the aspartic protease pepsin and cysteine protease papain, respectively. 2,4,5-triaryl imidazole derivatives were found to be specific for aspartic proteases. It showed inhibition against HIV-I protease and didn't show any inhibition against other classes of proteases. Similarly 4-substituted aryl 3,4-dihydro-2-pyridones were also found specific for cysteine proteases and showed insignificant inhibitions against serine proteases. The binding of the inhibitors were found entropically driven and hydrophobic in nature. Imidazole ring for aspartic protease inhibitors and methyl 2-methyl-6-oxo-4-phenyl-1,4,5,6-tetrahydro-3-pyridinecarboxylate for cysteine protease inhibitors represent new scaffold to work on and to develop new potent inhibitors.

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CHAPTER 6

General discussion and Conclusion

DISCUSSION

Marine invertebrates are rich sources of small molecules with unique chemical skeletons and potent bioactivities. Target-oriented searches for bioactive substances have become a growing need for small-molecule inhibitors essential for studies of complex processes at the interface of chemistry and biology [1]. Inhibitors of specific serine proteases can be potential drug leads for many disease states [2]. Cyclotheonamides A and B from a sponge *T. swinhoei* inhibit thrombin, trypsin, and plasmin [3]. A series of ascidian alkaloids, the lamellarins, show selective inhibition against HIV integrase. Lamellarin R 20-sulfate demonstrates inhibition in the early steps of replication of HIV-1 in cell culture [4]. An acetylenic metabolite from Okinawan sponge *Hippospongia* sp., possesses HIV reverse transcriptase inhibitory activity [5]. Four cheilanthane sesterterpenoids, isolated from the sponge *Ircinia* sp. as inhibitors of MSK1 and MAPKAPK-2 [6]. Polycyclic bisindoles from a marine sponge *Dictyodendrilla* sp. strongly inhibit bovine lens aldose reductase [7]. Compounds with imidazole nucleus being significantly important in biochemical processes posses inhibitory activity against p38 MAP kinase, fungi, herbs and plant growth regulators [8]. The imidazo pyridine heterocyclic structures form part of the skeleton of natural alkaloids [9], neuromuscular blocking agents [10], reversible inhibitors of the H^+, K^+-ATPase enzyme [11] with a potent antisecretory activity [12] and of sedative hypnotics of the nervous system [13]. Milrinone, Amrinone [14] and their analogues [15] containing the 2-pyridone[16] scaffold are cardiotonic agents for the treatment of heart failure. Some 2-pyridones are also reported to possess antitumor [17], antibacterial [18], antimicrobial, antiviral, antifungal, antihypertensive, specific non-nucleoside reverse transcriptase inhibitor of human immunodeficiency virus-1 (HIV-1) [19] and other biological activities [20].

In the present work a serine protease inhibitor, an alkaloid, has been isolated from the hemolymph of green mussel, *Perna viridis.* Molluscs have humoral and cellular immunity, and the humoral system is constituted by lysosomal enzymes, agglutinins, lectins and antimicrobial peptides. Nevertheless, cellular immunity seems to perform the main role in shellfish immune processes [21]. Lysosomal enzymes (β-glucuronidase, acid and alkaline phosphatase, lipase, aminopeptidase and lysozyme) are comprised especially

in granular hemocyte lysosomes and their release into serum depends on cell degranulation during phagocytosis [22]. To elicit the immune response mussels were bacterially challenged by injecting the combination of G^+ and G^- human pathogenic bacteria in 0.85% brine solution into the posterior adductor muscles. The bacteria used were G^+ (*S. aureus, S.* Group D) and G^- (*P. vulgaris, E. coli, P. aeruginosa, K. pneumonie, S. morganii*). The combination of G^+ and G^- bacteria were used to maximally elicit the immune response and checked for secreted compounds. The hemolymph was collected from adductor muscle of *Perna viridis* after 24 hrs of bacterial challenge. The unchallenged hemolymph did not show any serine protease inhibitory activity while the challenged one showed maximum activity after 24 hrs of challenge since we isolated hemolymph after 6 hrs, 12 hrs, 18 hrs and 24 hrs. The mussel also showed seasonal variation in serine protease inhibitory activity because it is observed that mussels undergo dramatic seasonal changes in their physiological condition which are associated with changes in their nutritional requirements for energy and protein [21, 22]. The freshly isolated hemolymph was centrifuged immediately at 1000 rpm at 4^oC for 15min. *Perna viridis* has not been chemically examined for its bioactive constituents although primarily, it has been utilized as a pollution control indicator [23]. The serine protease inhibitor (SPI) was purified by silica gel column and RP-HPLC (C_{18} symmetry, MeCN (5→95%): H_2O R_t 10 min, detection at 214 nm and 280 nm). The structure of SPI was established on the basis of FTIR, ^1H NMR, ^{13}C NMR, DEPT, 2D NMR and LC-MS spectra. The established structure was found to be an alkaloid. The scaffold, 5,6,9,10-tetramethylbenzo [*b*][1,8] naphthyridin-10-ium, is absolutely new of its kind (an alkaloid). Alkaloids are common in marine organism [23] and have been found to be potent inhibitorsof various classes of enzymes. Alkaloids have been reported from different marine sources to have different activities like HIV integrase inhibitor, an ascidian alkaloid [24]; Pyrrole alkaloid as Raf/MEK-1/MAPK inhibitor from *Stylissa massa* [25]; bis-indole alkaloid (Ropaladins) as cyclin dependent kinase inhibitor from *Rhopalaea sp.*and many more yet to be described. This scaffold is new and can be modified in order to get more potent inhibitors. The inhibition constant K_i, determined by the classical double reciprocal plot was 104.68 μM and by Dixon plot was 97.1 μM

which is almost equal to the IC_{50} value of the inhibitor. The Line Weaver–Burk reciprocal plot showed that the inhibitor was a competitive inhibitor of trypsin and the K_M value for the trypsin with BAPNA was 588 μM. The Ki of SPI is comparable to other natural and synthetic heterocyclic protease inhibitors. The inhibitor was found to be specific for trypsin and did not show any inhibition against chymotrypsin. It also did not show any activity against other classes of proteases. Moreover, some of the protease inhibitors isolated from invertebrate sources are quite specific towards individual mammalian serine proteases. The specificity of the compound is another unique feature. The thermodynamics study was done to evaluate Van't Hoff plot which shows that binding is spontaneous and positive entropically driven. The value of enthalpy shows that the binding is exothermic in nature. Thermodynamic study would help a great deal in designing highly potent inhibitor as it dictates types of binding.

Imidazole derivatives have been found to be associated with several biological activities [26] like CNS depressant, monoamine oxidase (MAO) inhibitory, anticonvulsant [27], antibacterial activities [28] and others. This observation prompted us to design and synthesize imidazole derivatives. In this endeavor, we screened about 20 chemically synthesized 2,4,5-trisubstituted imidazole against eight different human pathogenic bacteria and fungi, out of which seven compounds were found to be active against different bacteria. These compounds showed variation in activity and were found to be active against both Gram-positive as well as Gram-negative bacteria. The SAR study of these compounds was performed which helped us to understand the mode of action of these compounds in a better manner. It was proved that the imidazole ring was required for activity as it provided a hydrophilic moiety to interact by forming hydrogen bonds. The variation in the substitution at C-2 or C-4 & C-5 position of imidazole ring was made in order to get best substituent among them. The same moieties were added at C-4 & C-5 positions whereas different substitutions at 2^{nd} and 4^{th} position at C-2 substituted aryl ring were made. It has been shown that at C-4 position, presence of aromatic ring is required for the activity as it binds with hydrophobic pocket of enzymes [29] and increasing either the size or the polarity of substituents leads to diminished potency [28]. The C-5 moieties may involve in polar interaction [29] depending upon the enzymes they are interacting

with. The compound should have hydrophobic moieties to interact with enzyme whereas hydrophilic moieties are required to establish the interaction of enzyme by establishing hydrogen bonding. Imidazole derived compounds are generally bioactive as imidazole ring readily forms hydrogen bonds with enzyme and establishes the interaction with the enzyme [30]. Imidazole ring acts by inhibiting the synthesis of bacterial or fungal cell wall. It may bind directly with unsaturated fatty acid part of the phospholipid components of the membrane [31] or may inhibit 14α-demethylation of sterols [32]. The imidazoles may coordinate with haem iron atom (in Cyt-P450) and their binding is fairly unspecific and thus imidazole fungicides also inhibit the activities of a broad spectrum of other cytochrome P-450 dependent enzymes [28]. We checked drug like properties of our compounds based on Lipinski's rule of Five (*in silico*) and calculated ClogP, LogS, H-bond acceptors, H-Bond donors and Rotational Bonds by using Chem Draw. Compounds were found to have good drug like properties. This study revealed that our these compounds satisfy Lipinski's rule of Five and do not have any threatening sign as mutagenic, tumorogenic, irritant or reproductive effect except the compound which has nitro group and likely to be a tumorogenic moiety [29]. Nitro compounds like Metronizadole (MTZ) get activated when reduced through electron donation from ferredoxin or flavodoxin that were themselves reduced by the pyruvate:ferredoxin oxidoreductase (POR), possibly forming an hydroxylamine,this process occurs only under strongly reducing conditions. MTZ is also activated in hypoxic cells of animals and hence it has been applied as a radio sensitizer of human tumors [33]. Activated MTZ is thought to interact directly with DNA and the resultant complex can no longer function as an effective primer for DNA and RNA polymerases. We compared our compounds with a commercially available drug, clotrimazole and an antibiotics, ceftriaxone. Prochloraz, inhibits aromatase activity [34], Miconazole changes cell wall permeability barrier [24], Azomycin inhibits ribonucleotide reductase [35] and Clotrimazole causes leakage of various small ions and small compounds namely K^+, inorganic phosphates, amino acids and nucleotides [36]. Ceftriaxone is a third-generation cephalosporin antibiotic. Ceftriaxone inhibits bacterial cell wall synthesis by binding to one or more of the penicillin-binding proteins (PBPs) which in turn inhibits the final transpeptidation step of

peptidoglycan synthesis in bacterial cell walls, thus inhibiting cell wall biosynthesis. Bacteria eventually lyse due to ongoing activity of cell wall autolytic enzymes (autolysins and murein hydrolases) while cell wall assembly is arrested. SAR study of our compounds showed that they interact with Cyt P-450 and inhibits the conversion of lanosterol to ergosterol. It was found that our compounds were more potent than the commercial drug clotrimazole and comparable with ceftriaxone.

The emergence of bacterial pathogen resistance to common antibiotics strongly supports the necessity to develop alternative mechanisms, interrupting metabolic pathways other than those that are known to be involved in cell wall biosynthesis, for combating drug-resistant forms of these infective organisms. Bacterial proteases serve as activators of cascade pathways in disruption of cytokine networks, excision of cell surface receptors and most importantly, inactivation of host proteinase inhibitors. Synthetic protease inhibitors have got invaluable success in developing synthetic inhibitors against the HIV aspartyl proteinase and ultimately in the treatment of AIDS patients [37]. Interest in papain family cysteine proteases as targets derives from the recognition that they are critical to the life cycle or pathogenicity of many parasites e.g. Streptococcal cysteine protease [38], Staphylococcal cysteine protease [39], falcipain-1, -2, and -3 [40] and Cruzipain [41]. We designed, synthesized and screened about nine 1-substituted pyridylimidazo [1,5-a] pyridine derivatives against seven different G^+ & G^- bacteria. Out of nine we found that four compounds were active against these bacteria and they were found to be potent inhibitor of papain, a cysteine protease (although it is not been directly demonstrated that the effects are due to protease inhibitory capacity of these compounds). The study showed that the MICs of these compounds are directly proportional to the K_i against all tested bacteria except *E. coli* and *P. vulgaris* which clearly indicates that these compounds inhibit cysteine proteases of these pathogens. Imidazopyridine ring systems possess a broad range of useful pharmacological activities [42] including antigastric, antisecretory [43], local anesthetic [44], antiviral [45], antianxiety [46], antibacterial, antifungal, antihelminthic, antiprotozoal, anticonvulsant, gastrointestinal, antiulcer (Zolmidine), anxiolytic (Alpidem), hypnotic (Zolpidem) and immunomodulatory [32]. The nature and the position of the substituents on the pyridinic moiety influence these

pharmacological activities. Pyridinium species produced by N-substitution retain the aromaticity of pyridine and may be involved in hydrogen bonding. Substitution at C-5 position is responsible for the variation in antimicrobial activity. Polar substitution at 2^{nd} position of C-5 aryl ring enhances the activity whereas non polar decreases and again the polar substitution at 4^{th} position of C-5 aryl ring decreases the activity. The SAR study infers that isobutyl, a bulky group, may decrease the activity as it does not interact properly with pockets present in the enzyme. The thermodynamic parameters viz. free energy (ΔG), enthalpy (ΔH) and entropy (ΔS) of binding was determined by K_i at different temperatures. Free energy of the binding (ΔG) was negative at all temperatures, suggesting the spontaneous binding. Enthalpy change (ΔH) of the binding is negative and entropy (ΔS) change of the binding is positive which indicates the exothermic and entropically driven nature of binding. This pattern of temperature dependence is characteristic of a hydrophobic interaction. The thermodynamic study helped us to design better inhibitors which could interact properly with the protease. We checked drug like properties of our compounds based on Lipinski's rule of Five (*in silico*) and calculated ClogP, LogS, H-bond acceptors, H-Bond donors and Rotational Bonds by using Chem Draw. We also calculated drug likeness prediction, overall drug likeness score, mutagenicity, tumorogenicity, irritating effect and reproductive effect by using www. Organic-chemistry.org web portal. The partition coefficient (logP) is a well-established measure of the lipophilicity of the compound. The distribution of calculated logP (cLogP) values of a majority of drugs in the market is in the range of 0–5 [45]. Lipophilicity clearly shows relation of compounds with their activity. It helps the compound to penetrate inside the bacteria. All the compounds studied except isobutyl containing compound, showed good agreement with the criteria laid down for the prediction [47] of a compound to be a potential drug. All the compounds tested do not show any threat against toxicity risk assessment except one which showed threat as tumorogenic and reproductive effect due to the presence of the isobutyl group. All molecules have H-bond donors less than 5 and H-bond acceptor less than 10 whereas number of rotational bonds lie in the moderate range i.e. 2-4. All molecules showed positive values for overall drug likeness score. We compared our compounds with the

commercially available drug, clotrimazole and the antibiotics, ceftriaxone. Our compounds were found to be more effective than clotrimazole and comparable with ceftriaxone. Clotrimazole causes leakage of various small ions and small compounds and Ceftriaxone inhibits bacterial cell wall synthesis whereas our compounds target cysteine proteases.

Aspartate and cysteine proteases have attracted much attention for their role in pathogenicity. Proteases are also key players in determining the infectiveness of various pathogens, ranging from retroviruses to fungi and protozoa. There is a need for small non peptide inhibitors which are easy to synthesize and exhibit good bioavailability. We designed and screened 2,4,5-triaryl imidazole derivatives and 4-substituted aryl 3,4-dihydro-2-pyridones as inhibitors of the aspartic protease pepsin and cysteine protease papain as model enzymes, respectively. These inhibitors are specific and did not show any inhibitory activity against any other group of proteases. The imidazole based aspartic protease inhibitors are very rare. In 2,4,5-triaryl imidazole derivatives the scaffold imidazole ring posses an electrophilic center and a secondary nitrogen bearing active hydrogen (1-unsubstituted) as a nucleophilic center which interacts with the hydrophilic chain of the enzyme whereas moieties, toluene or benzene, present at C-4 and C-5 position interact with hydrophobic pockets of protease. Compounds having strong hydrophobic moieties at C-4 and C-5 position are more active. It may be concluded that the hydrophobic moieties are required to interact with hydrophobic pockets [48] of aspartic proteases. SAR study showed that among C-2 aryl substituted imidazole, a 4-substitution on aryl ring is preferred and a less polar substituent makes the molecule more active whereas polar substituent at 2^{nd} position on C-2 aryl ring makes the molecule less active The thermodynamic parameters viz. free energy (ΔG), enthalpy (ΔH) and entropy (ΔS) of binding, determined by K_i at different temperatures and Van't Hoff plot was studied which explained the binding of inhibitors with proteases. Free energy of the binding (ΔG) was negative at all temperatures, suggesting the spontaneous binding. Enthalpy change (ΔH) of the binding is negative and entropy (ΔS) change of the binding is positive which indicates the exothermic and entropically driven nature of binding. This pattern of temperature dependence is characteristic of hydrophobic interaction.

Thermodynamics study reveals that hydrophobic interactions favour binding of the inhibitor with the protease.

Many naturally occurring and synthetic compounds containing the 2-pyridone scaffold possess interesting pharmacological properties as they are identified as specific non-nucleoside reverse transcriptase inhibitor of human immunodeficiency virus-1 (HIV-1) [49]. Milrinone, Amrinone and their analogues are cardiotonic agents for the treatment of heart failure. Some 2-pyridones are also reported to possess antitumor , antibacterial [50], antimicrobial, antiviral, antifungal, antihypertensive and other biological activities [51]. The scaffold methyl 2-methyl-6-oxo-4-phenyl-1,4,5,6-tetrahydro-3-pyridinecarboxylate of 4-substituted aryl 3,4-dihydro-2-pyridones bears two oxygen atoms and one nitrogen bearing active hydrogen (1-unsubstituted) as nucleophilic moieties. This scaffold has three atoms (2 Oxygen and 1 Hydrogen) which could make hydrogen bonding with hydrophilic pockets of the proteases. The study shows that when hydrophobic moieties like methyl and ethyl group are substituted, the potency of the compound decreases. This kind of structure activity relationship shows that the inhibitors interact primarily by hydrophilic interaction after forming hydrogen bonds and more hydrophobic moieties (substitution of methyl by ethyl) make compounds less active by increasing steric hindrance. But hydrophobic moieties are also mandatory to stabilize the interaction of the protease with inhibitors. The thermodynamic study was done at three different temperatures and Van't Hoff plot was studied. The calculated parameters viz. free energy (ΔG), enthalpy (ΔH) and entropy (ΔS) of binding were determined by K_i at different temperatures. The Van't Hoff plots were linear for all inhibitors in the temperature range studied. Free energy of binding (ΔG) was negative at all temperatures, suggesting the spontaneous binding. Enthalpy change (ΔH) of the binding is negative and entropy (ΔS) change of the binding is positive which indicates the exothermic and entropically driven nature of binding. The thermodynamic study shows that entropically driven binding favors hydrophobic interaction.

CONCLUSIONS

1. The serine protease inhibitor was secreted by *Perna viridis* after being injected with eight different pathogenic bacteria as a defense mechanism because the unchallenged one did not produce any SPI.

2. The secreted SPI is a tertiary alkaloid and exhibits a unique structure with specific activity against trypsin only and not against chymotrypsin or other class of proteases.

3. The SPI was purified from hemolymph by silica gel column and RP-HPLC.

4. The van't Hoff analysis showed that the interaction of SPI with trypsin is positively entropically driven.

5. The synthetic 2,4,5-trisubstituted imidazoles were found active against eight different human pathogenic bacteria. These compounds showed good agreement with Lipinski's rule of Five and did not show any physiological threat *in silico.*

6. These compounds are more active than Clotrimazole and comparable to Ceftriaxone.

7. The synthetic 1-substituted pyridylimidazo [1,5-a] pyridine derivatives were found to inhibit cysteine proteases of seven different human pathogenic bacteria and described *in silico* as potent antibacterial compounds.

8. The thermodynamic study of these compounds showed that the interaction with protease was hydrophobically favoured and they inhibit proteases (metabolic pathways) unlike Ceftriaxone and Clotrimazole which inhibit cell wall synthesis.

9. The 2,4,5-trisubstituted imidazole was identified as an aspartic protease inhibitor which is rare and their interaction with proteases was studied thermodynamically.

10. The 4-substituted aryl 3,4-dihydro-2-pyridones derivatives were tested as potent cysteine protease inhibitors as their K_i ranges from 0.5-1.0 μM.

List of publications

1. M. S. Khan, S. A. Siddiqui, M. S. Siddiqui, U.Goswami, K. V. Srinivasan, M.I. Khan (2008) "Antibacterial activity of Synthesized 2,4,5-trisubstituted imidazole derivatives" Chemical Biology & Drug Design doi: 10.1111/j.1747-0285.2008.00691.x.

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3. Muhammed Zafar Iqbal, A.N., Khan, M.S., Goswami, U. (2007) "Cytogenetic studies in green mussel, *Perna viridis* (Mytiloida: Pteriomorphia), from West Coast of India" Marine Biology: 153: 5, 987-993.

4. M.S.Khan, S.A.Siddiqui, M.S.Siddiqui, U.Goswami K. V. Srinivasan, M.I. Khan (2008) "Synthesized 1-substituted pyridylimidazo [1,5-a] pyridine derivatives, cysteine protease inhibitors as antibacterial drugs" (Communicated).

5. M.S.Khan, S.A.Siddiqui, M.S.Siddiqui, U.Goswami K. V. Srinivasan, M.I. Khan (2008) "Analog study of 2,4,5-trisubstituted imidazole deravatives as aspartic protease inhibitors" (Communicated).

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