3.1. Introduction

Nowadays, the treatment of infectious diseases by various antibiotics is thought to be highly promising and the people across the globe expect that those agents which do not offer any resistance to microorganisms will cure the infections. The society and the researchers now have time to heave after the development of vast number of these agents which are playing paramount role in fighting against those bacterial species which were a major threat. Despite all these advantages, people are losing their lives due to innumerable resistance of microbes to these antibiotics.

As a consequence, the demand is on the higher side to widen novel efficient and most promising antimicrobial agents having noteworthy structural modifications, which may exert their effect via different mode/mechanism of action. Favorable pharmacokinetic properties, low incidence of side effects and biocompatibility are other characteristics that would make a new drug suitable for extensive use.

There appears a new class of antibiotics called **cationic antimicrobial peptides** which is growing at a faster pace and has been identified as to provide a central role in the prevention of onset of different diseases. Since antimicrobial peptides are biocompatible and possess activity towards a broad spectrum of microbial cells, the mechanism of action of these drug molecules is considered to be different from those of the existing antibiotics.

3.1.1. Antimicrobial peptides (AMPs): *A solution for multidrug resistance*

The production of a large gamut of gene encoded antimicrobial peptide (AMPs) which are found in all living species and have exhibited the property to serve as part of their innate immunity to microbial invasion.[1-2]

Probably one of the fastest in the entire field of peptide science would be the research on antimicrobial peptides which is experiencing spectacular growth. Initially reports on eukaryotic peptides from plants with antibacterial or antifungal properties

date from the 1970s, but it was not until the next decade that a consistent body of knowledge began to accumulate. In the early 1990s, only a few peptide structures were known for which antimicrobial activity had been described. Later, the number of sequences has increased almost tenfold, and a paradigm has emerged on the role of antimicrobial peptides in host immunity and certain diseased conditions. Along with this has come a renewed awareness of the potential therapeutical applications of these peptides or their analogues at a time when the emergence of pervasive microbial resistance is raising serious concerns about the future of antibiotic therapy.

Irrespective of their origin, spectrum of activity and structure, most of these peptides share several properties. They are generally composed of less than 60 amino acid residues [mostly common *L*-amino acids], their net charge is positive, they are hydrophobic and/or amphiphilic, and in most cases they are membrane active. Further, they have an ability to kill or neutralize gram-negative, gram-positive bacteria, fungi (including yeasts), parasites (including planaria and nematodes), cancer cells, and even enveloped viruses like HIV and herpes simplex.

3.1.2. Mechanism of selectivity of antimicrobial peptides:

Many studies aimed at understanding the mode of action of antimicrobial peptides have been described in the past few years.[3-11] Antimicrobial peptides have targeted a surprising but clearly fundamental difference in the design of the membranes of microbes and multicellular animals best understood for bacterial targets. Bacterial membranes are organized in such a way that the outermost leaflet of the bilayer, the surface exposed to the outer world, is heavily populated by lipids with negatively charged phospholipids head groups. In contrast, the outer leaflet of the membranes of plants and animals is composed principally of lipids with no net charge; most of the lipids with negatively charged head groups are segregated into the inner leaflet, facing the cytoplasm [**Figure-3.1**]. Cationic antimicrobial peptides selectively bind to the bacterial molecular basis for membrane discrimination by antimicrobial peptides.[7] Cationic peptides preferentially bind to bacterial membranes with abundant acidic phospholipids by the aid of electrostatic interactions. The outer leaflets of mammalian cell membranes

are exclusively composed of zwitter ionic phospholipids for which the peptides show only low affinity. The presence of cholesterol also contributes to the resistance of the membranes against the peptides.

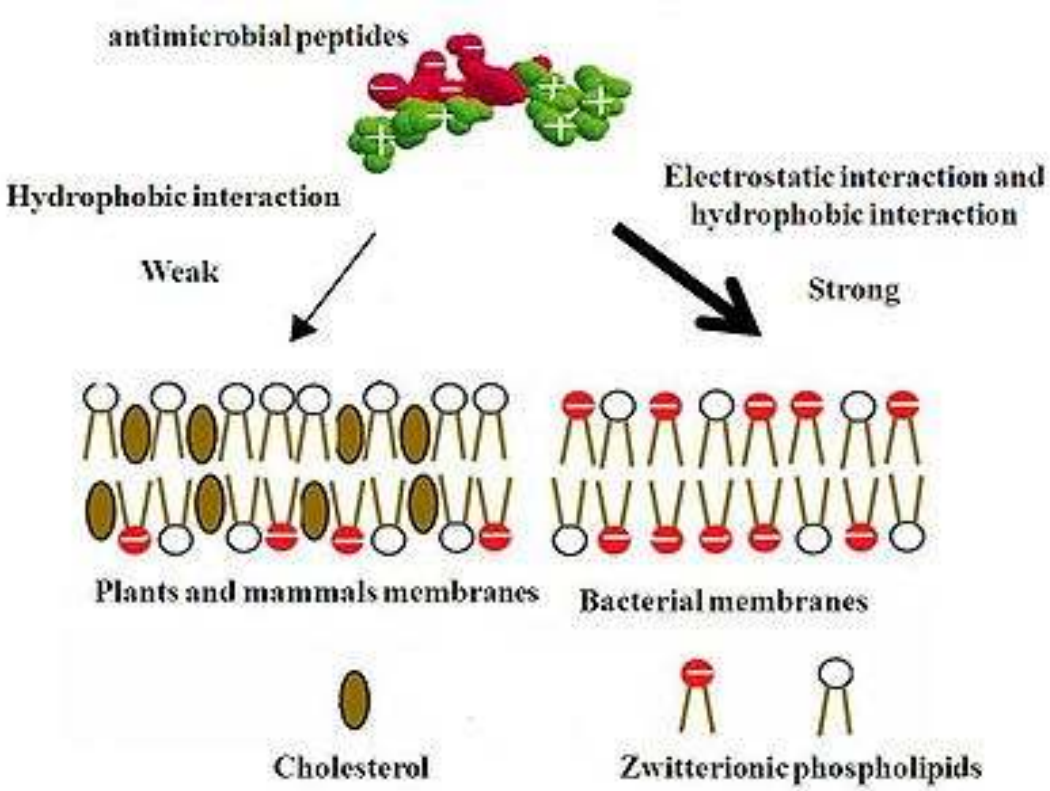


Figure 3.1: Molecular Basis of Cell Selectivity of Antimicrobial Peptide.

3.1.3. Bactenecins

AMPs are an important component of innate defenses of all species of life, which has been made clear now. The versatile peptides are those which possess good activities against a broad range of bacterial and fungal strains. On the other hand, to enable the use of these peptides for parental applications, it is remarkable to gain ample knowledge on peptides structure and properties. In this context, bactenecins are considered as model antimicrobial peptide for understanding the structure- activity relationship.

Bactenecins belongs to the family of cathelicidins and found in the granules of bovine neutrophils.[12,13] Independently of their composition and activity, these cationic polypeptides have been collectively called bactenecins from the Latin words bacterium

and necare (to kill). The first bactenecins purified and characterized was a dodecapeptide, which is very active against both *Escherichia coli* and *Staphylococcus aureus.*[14] Further, two additional classes of bactenecins are designated as bactenecin5 (Bac5) and bactenecin7 (Bac7) and examined for antimicrobial activity.[12,13] The primary structure determination using plasma desorption-mass spectrometry has shown Bac5 and Bac7 sequences to contain 42 and 59 residues with the molecular masses 5kDa and 7kDa respectively,[15] hence the suffix 5 and 7. Bactenecins exert a potent bactericidal activity toward several gram-negative organisms such as *Enterobacter clocae* and *Klebsiella pmeumoniae*.[12] The mechanism of cidal action of Bactenecins involves their interaction with the outer membrane of susceptible microorganisms followed by a rapid translocation to the inner membrane and impairment of energy-dependent membrane activities.[16-18]

3.1.4. Bactenecin7

Bactenecin7 is a cationic, proline- and arginine-rich, cathelicidin-derived peptide from large granules of bovine neutrophils, belonging to this distinctive group that also includes the closely related Bac5, PR-39 peptides obtained from bovine and pig neutrophils respectively. [12, 19, 20] Bac7 has been characterized by high contents of Arg and Pro (>60%) and by a unique sequence with an Arg clustered region and three tandem repeats of a tetradecamer. Further, it consists of twelve Pro-Arg-Pro triplets spaced by a single hydrophobic amino acid residue **(Table 3.1 and Figure 3.2).**

Table 3.1: Amino Acid composition of Bactenecin7[a]

Amino Acid	Bac7
Pro	28.9(28)
Gly	2.6(3)
Ile	4.1(4)
Leu	4.2(40)
Phe	3.3(3)
Arg	16.3(17)
(Total)	(59)

[a] Values are mol-mol and the numbers in parentheses are from sequencing data.

Arg-Arg-Ile-Arg-Pro-Arg-Pro-Pro-Arg-Leu-Pro-Arg-Pro-Arg-Pro-Arg-Pro-Leu-Pro-Phe-Pro-Arg-Pro-Gly-Pro-Arg-Pro-Ile-Pro-Arg-Pro-Leu-Pro-Phe-Pro-Arg-Pro-Gly-Pro-Arg-Pro-Ile-Pro-Arg-Pro-Leu-Pro-Phe-Pro-Arg-Pro-Gly-Pro-Arg-Pro-Ile-Pro-Arg-Pro

Figure 3.2 Amino Acids Sequence of Bactenecin7

The Characteristic features of Bac7 sequence are:

(i) The invariable presence of proline every second residue, from position 10 to the *C*-terminus, and

(ii) The presence of three tandemly repeated tetra, decamers in the region between residue 15 and 56, with the three *C*-terminal residues 57-59 matching the first three amino acids of the repeat.

3.1.5. Antimicrobial activities of *Bac7*

The antimicrobial properties of the two bactenecins were investigated with an array of gram-positive and gram-negative microorganisms.[12] At the maximal concentration of Bac5 and Bac7 employed (200 μg/ml), *Staphyllococcus aureus* and *Streptococcus agalactiae*, as well as *Proteus vulgaries*, were not susceptible to the Bactenecins. Finally, *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* were resistant to Bac5 but susceptible to Bac7.

3.1.6. Peptides from synthetic mode:

To allow full utilization of peptides as new antimicrobial agents, it is important to determine their mode of action. To this end, synthetic peptides have been made by systematic variation of naturally occurring peptide, it is possible to improve antibacterial activity and at the same time give insight into the mechanism of action.

Numerous investigations were made on the synthesis of antimicrobial peptide analogues in view of understanding their mode of action and also to establish the structural requirements for optimal activity.[21-26] These structure activity relationship

studies have indicated at least seven parameters that can influences the potency and spectrum of activity of antimicrobial peptides: (1) the size, (2) the sequence, (3) the degree of structuring (% helical content), (4) the charge, (5) the overall hydrophobicity, (6) the amphipathicity and (7) respective widths of the hydrophobic and hydrophilic faces of the helix. These parameters are intimately related, so that modification aimed at altering one can also resulting the significant changes to one or more of the others. Understanding these inter- relationships is the key to design novel peptides with increased potency and directed activity.

Bessalle *et al.,*[27] synthesized a number of peptides named "modellins" of different lengths and hydrophobicities. They found that amphipathic peptides composed of 16 and 17 amino acids with highly hydrophobic (Trp and Phe) and hydrophilic (Lys) amino acids on opposite faces had high antibacterial and hemolytic activities. By replacing Trp or Phe with Leu, thereby reducing the hydrophobic nature of the peptide, a drastic reduction in hemolytic activity was seen, but bioactivity as on slightly decreased.

Synthetic combinatorial libraries allow the systematic examination of millions of peptides. This approach has brought a new dimension to the design of novel biologically active compounds. Thus, a set of peptide analogues were generated based on the screening of a library built around an existing lytic peptide, and on a deconvolution strategy directed toward activity specificity.[28] These peptide analogues also served as model systems to further study the effect of biomembrane mimetic systems on the peptides structural behavior relevant to their biological activities. Synthetic peptides can also be designed to improve factors such as specificity, stability and toxicity.

3.1.7. Synthesis and structure-activity studies of analogues of Bac7:

The spectrum of activity of native peptide Bac7 is very broad, killing most of the gram-ve bacteria, fungi and also inactivates human simplex virus types 1 and 2.[12,29,30] They have low toxicity and most normal mammalian cells and therefore are good candidates for development as therapeutic agents. However, it appears that these

peptides have evolved to work at high concentrations at local sites and do not exhibit a very low MIC value which is important characteristics of most active antibiotics. Therefore, SAR studies have been initiated to improve the antimicrobial activity of the native peptides while retaining their good therapeutic index. With this intention, some of the synthetic fragments of Bac7 were obtained, and found to be capable of translocation across the plasma membrane of mammalian cells without cytotoxic effects.

The synthesis of natural Bac7 was not carried by any researchers due to its longer chain length. But in an attempt to investigate the structure function relationship of Bac7, Benincas *et al*.,[31] have synthesized fragment of Bac7 containing (1-35) residues in length, to identify the domain responsible for antimicrobial activity. The fragments of Bac7 (1-35) and to a lesser extent, Bac7 (1-16) provide against a panel of antibiotic resistant clinical isolates of gram –ve bacteria.

In another attempt, synthesis of fragments of Bac-7 was explored for bacterial activity. Longer fragments of Bac7(1-24)[32] containing both regions were bactericidal and cell permeable, where as short fragments with only cations are hydrophobic region were well cell-permeate without the attendant microbicidal activity. And also, Tani *et al*., [33] synthesized two fragments of Bac7 which corresponds to residues 1-17(An Arg clustered region) and 46-59, (one of the three tandem repeats) respectively. They found that fragment 1-17 showed weak antimicrobial activity against several bacteria, but fragment 46-59 was almost inactive. These peptides can bind to acidic phospholipid bilayer without marked conformational changes. When acidic phospholipid bilayer entrapping 5,6-carboxyfluorescein was treated with peptides, no trace of dye leaked out indicating that the peptides lack the ability to disrupt the lipid membranes.

Abiraj *et al.,*[35-37] have synthesized a series of tetrapeptide fragments by substituting different *D* and *L*- amino acid residue of varying hydrophobicity at the *N*-terminal of Pro-Arg-Pro triplet. It was noticed that, the change in peptide parameters such as chain length, hydrophobicity, cationic charge or change in position of the amino acid residues could optimize their antimicrobial activity.

3.2. Present Work

3.2.1. Selection and Design of Peptides:

Bac7 comprises 59 residues and includes three-tandem repeats of a tetradecamer characterized by several Pro-Arg-Pro triplets spaced by single hydrophobic amino acids. Proline is known as a strong α-helix broken which can induce kinks in an α-helix of proteins,[34] suggesting that the repeating region of Bac7 may play an important role in antibacterial activity and/or in maintaining peptide conformation. In this connection, in order to investigate the structure-function relationship of Bac7, two peptide fragments, which correspond to residues 1-17 (an Arg-clustered region) and 46-59 (one of three tandem repeats) were synthesized by Tani *et al.*,[33] The fragment 1-17 showed weak antimicrobial activity against several bacteria, but fragment 46-59 was almost inactive. Therefore, a detailed study on the property and function of the repeating regions, which extend from the near *N*-terminal to the *C*-terminal portions of the peptides were of interest.

Thus, considering the vital role exhibited by Pro-Arg-Pro triplets in sustaining antimicrobial potency of Bac7, in our research laboratory we have been working towards the structure-activity relationship studies of shorter peptide analogues of Bac7. We have already reported antimicrobial evaluation of various series of tetrapeptide fragments by substituting different *D* and *L*- amino acid residue of varying hydrophobicity at the *N*-terminal of Pro-Arg-Pro triplet.[35-37] It was noticed that, the change in peptide parameters such as chain length, hydrophobicity, cationic charge or change in position of the amino acid residues could optimize their antimicrobial activity.

On the other hand, recently we have synthesized heterocyclic conjugated peptides by coupling heterocyclic precursor quinazolinone with inactive peptide sequences of elastin such as VP, GVP, VGVP and GVGVP peptides.[38] It was noticed that the conjugation of quinazolinone heterocycle with VGVP and GVGVP peptides showed increase in activity by nearly two fold compared to conventional antimicrobials. All the quinazolinone

conjugated peptides showed enhanced activity, even though peptides and quinazolinone moiety which when taken in isolation were inactive towards those bacterial strains.

Based on the above facts, we decided to design and synthesize peptides conjugated heterocycles by coupling heterocyclic precursors such as benzhydrilpiperazine, benzylpiperazine and 4-benzylpiperidine with shorter analogues of Bac7 peptides such as RP, PRP and GPRP.

3.2.2. Peptide Synthesis:

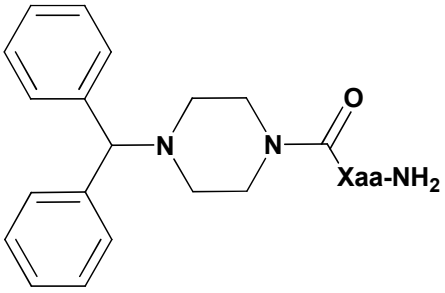
The peptide synthesis was carried out by the stepwise solution phase method. The Boc group was chosen for N^{α}-protection and its removal was achieved with 4*N* HCl in dioxane. The *C*-terminus carboxyl group was protected by the benzyl ester and its removal was achieved by hydrogenolysis using $HCOONH_4$/Pd-C (10%) or by saponification using 1*N* NaOH. The N^{g}- of Arg was protected by nitro group and its removal was effected by hydrogenolysis using $HCOONH_4$/Pd-C (10%). All the coupling reactions were achieved with IBCF. The protected peptides were characterized by standard physical and analytical techniques.

The tetrapeptide Gly-Pro-Arg-Pro was synthesized by stepwise approach as shown in **Scheme 3.1.** In this stepwise approach, Boc-Arg(NO_2)-Pro-OBzl was synthesized by the mixed anhydride method in the presence of HOBt, Boc group was deblocked and then coupled with Boc-Pro to obtain Boc-Pro-Arg(NO_2)-Pro-OBzl. Further the Boc group was deblocked and coupled with Boc-Gly to obtain Boc-Gly- Pro-Arg(NO_2)-Pro-OBzl.

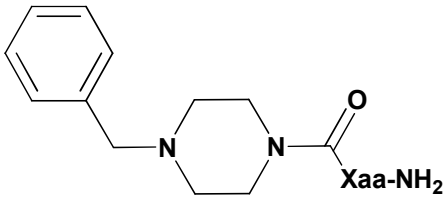
In the present study, the peptides Boc-Arg(NO_2)-Pro-OH, Boc-Pro-Arg(NO_2)-Pro-OH and Boc-Gly-Pro-Arg(NO_2)-Pro-OH are coupled to heterocycles such as benzhydrilpiperazine, benzylpiperazine and 4-benzylpiperidine. The synthesized Boc protected peptide conjugated heterocycles have been characterized by standard physical and analytical techniques.

The synthesized peptide conjugated heterocycles were deblocked with 4*N* HCl in dioxane to remove Boc group and the nitro group protection of N^{g} of Arg was removed by hydrogenolysis using $HCOONH_4$/Pd-C (10%). The deblocked compounds were used for

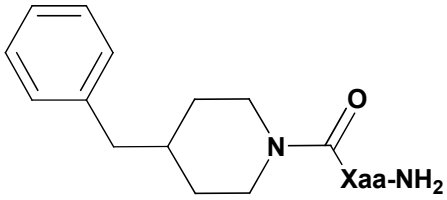
antibacterial and antifungal activities. The representative structures of peptide conjugated heterocycles are presented below.



Analogues-IV (XLV - XLVII)



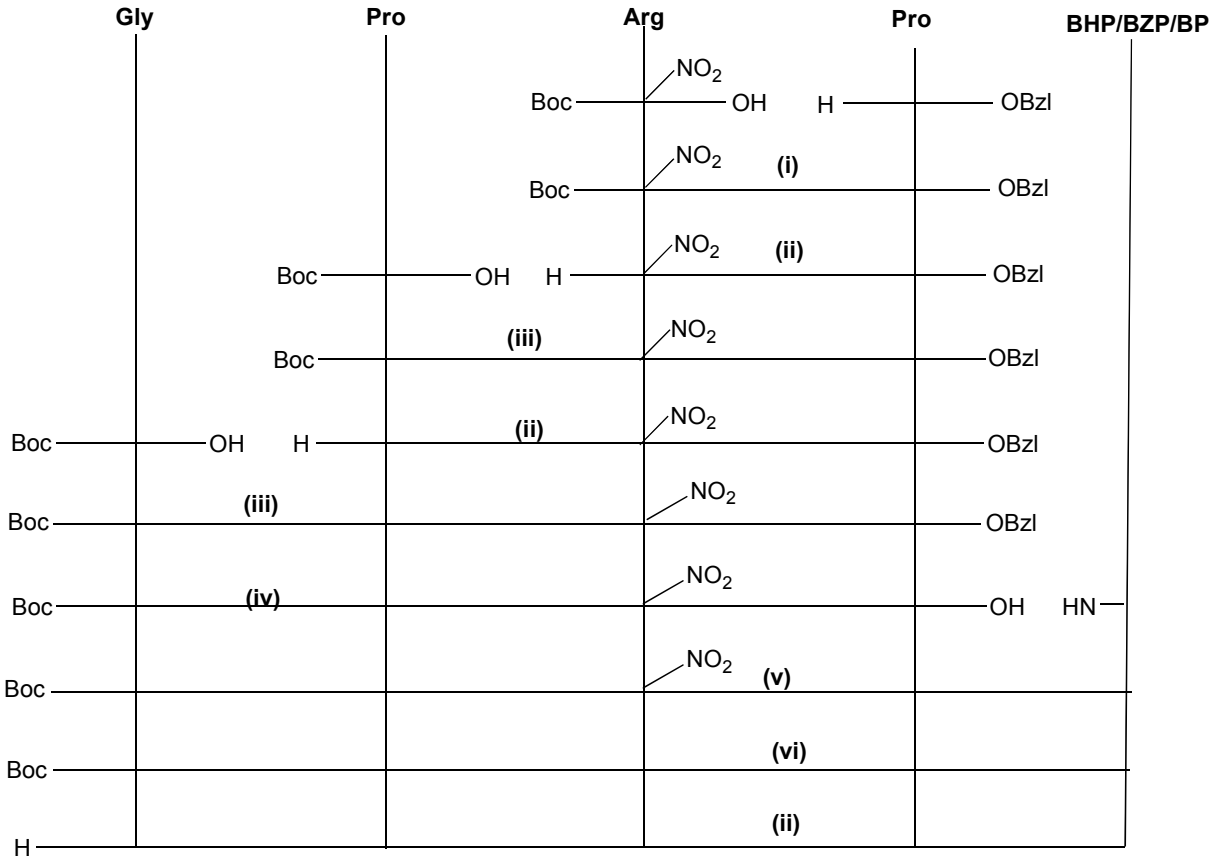
Analogues-V (XLVIII - L)



Analogues-VI (LI - LIII)

Where Xaa= RP, PRP and GPRP

Figure 3.3. The representative structures of peptide conjugated heterocycles



(i) IBCF/HOBt/NMM

(ii) HCl/Dioxane

(iii) IBCF /NMM

(iv) 1*N* NaOH/MeOH

(v) EDCI/HOBt/NMM

(vi) 10% Pd-C/$HCOONH_4$

BHP=Benzhydrilpiperazine

BZP = Benzylpiperazine

BP = 4-Benzylpiperidine

Scheme- 3.1: Synthesis of Peptide Conjugated Heterocycles (**Analogues IV, V and VI**)

3.3. Experimental

3.3.1. General:

All the amino acids used were of *L*-configuration unless otherwise specified. All tert-butyloxycarbonyl (Boc) amino acid derivatives and 1-hydroxybenzotriazole (HOBt) were purchased from Advanced Chem. Tech., (Louisville, Kentucky, USA). Isobutylchloroformate (IBCF), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI) and *N*-methyl morpholine (NMM) were purchased from Sigma Chemicals Co. (St. Louis, USA). All solvents and reagents were of analytical grade or were purified according to standard procedure recommended for peptide synthesis. The melting points were determined with open capillary and are uncorrected. ^{1}H NMR spectra were obtained on a 300 MHz Bruker FT-NMR Spectrometer instrument by using $CDCl_3$/DMSO as solvent and TMS as an internal standard.

The thin layer chromatography (TLC) was carried out on precoated silica gel plates prepared in laboratory with the following solvent systems:

R_f^1: $CHCl_3$-MeOH-HOAc (95:5:3)

R_f^2: $CHCl_3$-MeOH-HOAc (90:10:3)

R_f^3: $CHCl_3$-MeOH-HOAc (85:15:3)

The chromatograms were developed by employing one or more of the following methods.

a) TLC plates were exposed to iodine vapours for 5 min.

b) The chromatograms of free peptides were sprayed with the ninhydrin reagent (0.1% ninhydrin in n-BuOH: HOAc-99:1) and heated for 5 min in an air oven as 100-105 ^{0}C. In the case of Boc protected peptides the TLC plates were exposed to hydrogen chloride vapours for 15 min before spraying with ninhydrin reagent.

c) Sakaguchi reagent was employed for peptides having free guanidino group (Arg) as follows: the chromatograms were sprayed with 0.1% solution of 8-

hydroxyquinoline in acetone, dried and then sprayed with a 2% solution of bromine in 1*N* NaOH.

3.3.2. Synthesis:

3.3.2.1. Peptide synthesis:

Boc-Arg-(NO_2)-Pro-OBzl (XV):

To Boc-Arg(NO_2)-OH (12.72 g, 40 mmol) dissolved in acetonitrile (120 mL) and cooled to 0 ^{0}C, was added NMM (4.4 ml, 40 mmol). The solution was cooled to -15 ± 1 ^{0}C and IBCF (5.45 mL, 40 mmol) was added under stirring, while maintaining the temperature at -15 ^{0}C ± 1 ^{0}C. After stirring the reaction mixture for 10 min at this temperature, a pre-cooled solution of HOBt (6.12 g, 40 mmol) in DMF (60 mL) was added. Then, the reaction mixture was stirred for additional 10 min and a pre-cooled solution of HCl.H-Pro-OBzl (9.7 g, 40 mmol) and NMM (4.4 mL, 40 mmol) in DMF (100 mL) was added slowly. After 20 min, the pH of the solution was adjusted to 8 by the addition of NMM and the reaction mixture was stirred overnight at rt. The acetonitrile was removed under reduced pressure and the residual DMF solution poured into about 600 mL of ice cold 90% saturated $KHCO_3$ solution and stirred for 30 min. The precipitated peptide was filtered, washed with water, 0.1*N* cold HCl, water and dried. The crude peptide was recrystallized from ether and petroleum ether to obtain the desired product, Yield (88%), M. P. 44-45 ^{0}C (lit: 45-46 ^{0}C).[37]

Boc-Pro-Arg(NO_2)-Pro-OBzl (XVI):

11.5 g of **XV** was deblocked with 4*N* HCl/dioxane (115 mL) for 1.5 hr. Excess HCl and dioxane were removed under reduced pressure, triturated with ether, filtered, washed with ether and dried (Yield, 100%). The HCl.H-Arg(NO_2)-Pro-OBzl (10 g, 22.5 mmol) in DMF (100 mL) was neutralized with NMM (2.5 mL, 22.5 mmol) and coupled to Boc-Pro-OH (4.85 g, 22.5 mmol) in acetonitrile (50 mL) and NMM (3 mL, 22.5mmol) using IBCF (2.93 mL, 22.5 mmol) and HOBt (3.44 g, 22.5 mmol) worked up the same as **XV** to obtain **XVI**. The sample was recrystallized from ether/petroleum ether, Yield (87%), M. P. 46-47 ^{0}C (lit. 47 ^{0}C).[37]

Boc-Gly-Pro-Arg(NO_2)-Pro-OBzl (XVII):

8.0 g of **XVI** was deblocked with 4*N* HCl/dioxane (80 mL) for 1.5 hr. Excess HCl and dioxane were removed under reduced pressure, triturated with ether, filtered, washed with ether and dried (Yield, 100%). The HCl.H-Pro-Arg(NO_2)-Pro-OBzl (7.0 g, 13.0 mmol) in DMF (70 mL) was neutralized with NMM (1.4 mL, 13.0 mmol) and coupled to Boc-Gly-OH (2.27 g, 13.0 mmol) in acetonitrile (30 mL) and NMM (1.4 mL, 13.0 mmol) using IBCF (1.7 mL, 13.0 mmol) and worked up the same as **XV** to obtain **XVII**. The sample was recrystallized from ether/petroleum ether, Yield (90%), M.P. 47-48 ^{0}C (lit. 48-49 ^{0}C).[37]

Boc-Arg(NO_2)-Pro-OH (XVIII):

XV (6.10 g, 12.0 mmol) was dissolved in methanol (60 mL) and cooled to 0 ^{0}C. Then added a cooled solution of 1*N* NaOH (30 mL, 30 mmol) slowly and stirred the solution for about 1.5 hours. When the reaction get completed (monitored by TLC), evaporated the methanol, cooled and neutralized with 1*N* HCl (cold). Then, extracted with chloroform (3 X 25 mL) and washed with 1*N* HCl (1 X 25 mL), water (1 X 25 mL) and dried over Na_2SO_4. The solvent was removed under reduced pressure to get Boc-Arg(NO_2)-Pro-OH (**XVIII**), Yield (85%).

Boc-Xaa-Arg(NO_2)-Pro-OH (**Xaa** is **Pro** for **XIX**, **Gly-Pro** for **XX**)**:**

Each peptide (**XVI & XVII**, 10 mmol) was dissolved in methanol (10 mL/g of peptide) and cooled to 0 ^{0}C. Then, added a cooled solution of 1*N* NaOH (20 mL, 20 mmol) slowly and stirred the solution for about 1.5 hours. When the reaction get completed (monitored by TLC), evaporated the methanol, cooled and neutralized with 1*N* HCl (cold). Then, extract with chloroform (3 X 25 mL) and washed with 1*N* HCl (1 X 25 mL), water (1 X 25 mL) and dried over Na_2SO_4. The solvent was removed under reduced pressure and dried over vacuum to get debenzylated peptide {**XVIII**, yield (88%) & **XIX,** yield (88%)}.

The above synthesized peptidic precursors (**XVIII to XX**) were divided into two parts. One part of which is used for further coupling with heterocycles and other part was used for the antimicrobial studies after removing side chain protections completely.

Boc-Arg-Pro-OH (XXI):

The guanidine nitro group of **XVIII** (0.6g, 1.4 mmol) was hydrogenolysed in methanol (5 mL) using ammonium formate (2 eq.) and 10% Pd/C (0.1 g) for 30 min at rt. The catalyst was filtered and washed with methanol. The combined filtrate was evaporated under reduced pressure and the residue taken into chloroform, washed with water and dried over Na_2SO_4. The solvent was removed under reduced pressure and triturated with ether, filtered washed with ether and dried, yield (**XXI**, 90%).

Boc-Xaa-Arg-Pro-OH (**Xaa** is **Pro** for **XXII**, **Gly-Pro** for **XXIII**)**:**

The guanidine nitro group of each peptide (**XIX & XX**, 1.5 mmol) was hydrogenolysed in methanol (5 mL) using ammonium formate (2 eq) and 10% Pd/C (0.1 g) for 30 min at room temperature. The catalyst was removed by filtration and washed with methanol. The combined filtrate was evaporated under reduced pressure and the residue taken into chloroform, washed with water and dried over Na_2SO_4. The solvent was removed under reduced pressure and triturated with ether, filtered washed with ether and dried, yield (**XXII** & **XXIII,** 90%).

HCl.NH_2-Arg-Pro-OH (XXIV):

0.5 g of **XXI** was deblocked by stirring with 5 mL of 4*N* HCl/dioxane for 1.5 hr. Excess HCl and dioxane were removed under reduced pressure, triturated with ether, filtered, washed with ether and dried, Yield (**XXIV** ,100%).

HCl.NH_2-Xaa-Arg-Pro-OH (**Xaa** is **Pro** for **XXV**, **Gly-Pro** for **XXVI**)**:**

0.5 g of each peptide **(XXII & XXIII**) was deblocked by stirring with 5 mL of 4*N* HCl/dioxane for 1.5 hr. Excess HCl and dioxane were removed under reduced pressure, triturated with ether, filtered, washed with ether and dried, Yield (**XXV & XXVI,** 100%).

3.3.2.2. Synthesis of Peptide Conjugated Heterocycles:

3.3.2.2.1. General procedure for coupling of Boc protected peptides with heterocyclic precursors:

To the stirred solution of Boc-peptide (**XVIII - XX**, 2 mmol) and HOBt (0.31 g, 2 mmol) in DMF (10 mL) cooled to 0 ^{0}C, added NMM (0.22 mL, 2 mmol). The reaction mixture was further cooled to -15 ^{0}C ± 1 ^{0}C and added EDCI (0.39 g, 2 mmol) and heterocycles (**IV, IX, XII,** 2 mmol). After 20 minutes, the pH of solution was adjusted to 8 by the addition of NMM and the reaction mixture was stirred overnight while slowly warming to rt. The reaction mixture was quenched with water (2 mL) and the solvent was condensed. The residue was dissolved in chloroform (25 mL), washed with 5% $NaHCO_3$ (3 x 20 mL), H_2O (1 x 20 mL) followed by 0.1*N* cold HCl (3 x 20 mL) and brine solution (3 x 20 mL), dried over anhydrous Na_2SO_4. The chloroform was removed under reduced pressure to obtain the desired products (**XXVII - XXXV**). Yield, melting point and ^{1}H NMR data of these compounds are presented in **Table-3.2**.

General procedure for the catalytic transfer hydrogenation of peptide conjugated heterocycles:

The guanidine nitro group of each peptide conjugated heterocycles (**XXVII - XXXV,** 1.5 mmol) was hydrogenolysed in methanol (10 mL/ g of peptide) using ammonium formate (2 eq.) and 10% Pd/C (0.1 g/1.0 g of compound) for 30 min at rt. The catalyst was filtered and washed with methanol. The combined filtrate was evaporated in vacuum and the residue taken into chloroform, washed with water, and dried over Na_2SO_4. The solvent was removed under reduced pressure and triturated with ether, filtered, washed with ether and dried to get desired compounds (**XXXVI - XLIV**), yield (90%).

General procedure for the deprotection of N^{α}-Boc of peptide conjugated heterocycles:

0. 5 g of each peptide conjugated heterocycles (**XXXVI - XLIV**) was deblocked by stirring with 5 mL of 4*N* HCl/dioxane for 1.5 hr. Excess HCl and dioxane were removed

under reduced pressure, triturated with ether, filtered, washed with ether and dried get hydrochloride salts of peptide conjugated heterocycles (**XLV - LIII**), Yield (100%). These compounds were used for antimicrobial study (**Table-3.3 & 3.4**).

Table 3.2: Characterization of synthesized peptides conjugated heterocycles

Entry	Peptide Conjugated Heterocycles	Yield (%)	M.P. ^0C	^1H NMR Data	
				Component	**Chemical Shift (in ppm)**
XXVII	**Boc-RP-BHP**	88	52	Arg^2 Pro^1 BHP	4.50[1H, t, $^{\alpha}CH$], 2.17 [2H, m, $^{\beta}CH_2$], 1.84[2H, m, $^{\gamma}CH_2$], 3.49[2H, m, $^{\delta}CH_2$], 8.18-8.28 [2H, s, NH], 1.45[9H, s, Boc] 4.32[1H, t,$^{\alpha}CH$], 3.22 [2H, m, $^{\beta}CH_2$], 2.62[2H, m, $^{\gamma}CH_2$], 3.55[2H, t, $^{\delta}CH_2$] 7.10-7.35 [10H, m, ArH], 3.24 [4H, t, -CH_2-piperazine], 2.32[4H, t, -CH_2-piperazine], 4.68 [1H, s, $^{\alpha}CH$]
XXVIII	**Boc-PRP-BHP**	90	54-55	Pro^3 Arg^2 Pro^1 BHP	4.23[1H, m, $^{\alpha}CH$], 3.17 [2H, m, $^{\beta}CH_2$], 2.59 [2H, m, $^{\gamma}CH_2$], 3.50[2H, t, $^{\delta}CH_2$], 1.45 [9H, s, Boc]. 4.47[1H, t, $^{\alpha}CH$], 2.13 [2H, m, $^{\beta}CH_2$], 1.82 [2H, m, $^{\gamma}CH_2$], 3.43[2H, m, $^{\delta}CH_2$], 8.14-8.25 [2H, s, NH]. 4.34[1H, m, $^{\alpha}CH$], 3.23[2H, m, $^{\beta}CH_2$], 2.64[2H, m, $^{\gamma}CH_2$],3.58[2H, t, $^{\delta}CH_2$] 7.10-7.30[10H, m, ArH], 3.24[4H, t, -CH_2-piperazine], 2.28[4H, t, -CH_2-piperazine], 4.70[1H, s, $^{\alpha}CH$]
XXIX	**Boc-GPRP-BHP**	85	55-56	Gly^4 Pro^3 Arg^2 Pro^1 BHP	4.15[2H, s, CH_2], 8.0 [1H, s, NH], 1.43[9H, s, Boc]. 4.33[1H, m, $^{\alpha}CH$], 3.19[2H, m, $^{\beta}CH_2$],2.63[2H, m, $^{\gamma}CH_2$], 3.56[2H, t, $^{\delta}CH_2$] 4.49[1H, t, $^{\alpha}CH$], 2.14[2H, m, $^{\beta}CH_2$], 1.80[2H, m, $^{\gamma}CH_2$], 3.45[2H, m, $^{\delta}CH_2$], 8.10-8.20[2H, s, NH] 4.34[1H, m, $^{\alpha}CH$], 3.23[2H, m, $^{\beta}CH_2$],2.64[2H, m, $^{\gamma}CH_2$], 3.58[2H, t, $^{\delta}CH_2$] 7.10-7.32[10H, m, ArH], 3.24[4H, t, -CH_2-piperaizne], 2.30[4H, t, -CH_2-piperazine], 4.60[1H, s, $^{\alpha}CH$]
XXX	**Boc-RP-BZP**	90	50-51	Arg^2 Pro^1 BZP	4.46[1H, t, $^{\alpha}CH$), 2.12[2H, m, $^{\beta}CH_2$], 1.90[2H, m, $^{\gamma}CH_2$], 3.47[2H, m, $^{\delta}CH_2$], 8.16-8.28 [2H, s, NH], 1.45[9H, s, Boc] 4.30[1H, t,$^{\alpha}CH$], 3.17[2H, m, $^{\beta}CH_2$], 2.62[2H, m, $^{\gamma}CH_2$], 3.55[2H, t, $^{\delta}CH_2$] 7.25-7.40[5H, m, ArH], 3.15[4H, t, -CH_2-piperazine], 2.25[4H, t, -CH_2-piperazine], 4.1[2H, s, $ArCH_2$]
XXXI	**Boc-PRP-BZP**	90	53-54	Pro^3	4.23[1H, t, $^{\alpha}CH$], 3.12[2H, m, $^{\beta}CH_2$], 2.58[2H, m, $^{\gamma}CH_2$], 3.50[2H, t, $^{\delta}CH_2$], 1.45[9H, s, Boc]

				Arg^2 Pro^1 BZP	4.47[1H, t, $^{\alpha}CH$], 2.11[2H, m, $^{\beta}CH_2$], 1.89[2H, m, $^{\gamma}CH_2$], 3.54[2H, m, $^{\delta}CH_2$], 8.14-8.28 [2H, s, NH] 4.33[1H, t, $^{\alpha}CH$], 3.17[2H, m, $^{\beta}CH_2$], 2.66[2H, m, $^{\gamma}CH_2$], 3.55 [2H, t, $^{\delta}CH_2$] 7.30-7.47 [5H, m, ArH], 3.12 [4H, t, -CH_2-piperazine], 2.17 [4H, t, -CH_2-piperazine], 4.1 [1H, s, ArCH_2]
XXXII	**Boc-GPRP-BZP**	85	55	Gly^4 Pro^3 Arg^2 Pro^1 BZP	4.10[2H, s, $^{\alpha}CH$], 8.1[1H, s, NH], 1.43 [9H, s, Boc] 4.33[1H, t, $^{\alpha}CH$], 3.15 [2H, m, $^{\beta}CH_2$], 2.64[2H, m, $^{\gamma}CH_2$], 3.55[2H, t, $^{\delta}CH_2$] 4.49[1H, t, $^{\alpha}CH$], 1.98 [2H, m, $^{\beta}CH_2$], 1.89 [2H, m, $^{\gamma}CH_2$], 3.43[2H, m, $^{\delta}CH_2$], 8.12-8.22 [2H, s, NH] 4.33[1H, t, $^{\alpha}CH$], 3.17 [2H, m, $^{\beta}CH_2$], 2.66[2H, m, $^{\gamma}CH_2$], 3.56[2H, t, $^{\delta}CH_2$] 7.2-7.3 [5H, m, ArH], 3.12 [4H, t, -CH_2-piperazine], 2.31[4H, t, -CH_2-piperazine], 4.00 [2H, s, ArCH_2]
XXXIII	**Boc-RP-BP**	92	50	Arg^2 Pro^1 BP	4.50[1H, t, $^{\alpha}CH$), 2.00[2H, m, $^{\beta}CH_2$], 1.82[2H, m, $^{\gamma}CH_2$], 3.29[2H, m, $^{\delta}CH_2$], 8.20-8.26 [2H, s, NH], 1.45[9H, s, Boc] 4.26[1H, t,$^{\alpha}CH$], 3.16[2H, m, $^{\beta}CH_2$], 2.62[2H, m, $^{\gamma}CH_2$], 3.55[2H, t, $^{\delta}CH_2$] 7.13-7.29[5H, m, ArH], 2.45 [2H, s, ArCH_2], 2.85[4H, t, -CH_2-piperidine], 1.75[4H, t, -CH_2-piperidine], 1.90[1H, s, CH]
XXXIV	**Boc-PRP-BP**	89	51-52	Pro^3 Arg^2 Pro^1 BP	4.24[1H, t, $^{\alpha}CH$], 3.13[2H, m, $^{\beta}CH_2$], 2.62[2H, m, $^{\gamma}CH_2$], 3.54[2H, t, $^{\delta}CH_2$], 1.45[9H, s, Boc] 4.49[1H, t, $^{\alpha}CH$], 1.97[2H, m, $^{\beta}CH_2$], 1.80[2H, m, $^{\gamma}CH_2$], 3.31[2H, m, $^{\delta}CH_2$], 8.15-8.24 [2H, s, NH] 4.26[1H, t, $^{\alpha}CH$], 3.17[2H, m, $^{\beta}CH_2$], 2.64[2H, m, $^{\gamma}CH_2$], 3.56 [2H, t, $^{\delta}CH_2$] 7.20-7.32[5H, m, ArH], 2.5[2H, s, ArCH_2], 2.80[4H, t, -CH_2-piperidine], 1.75[4H, t, -CH_2-piperidine], 1.94[1H, s, CH]
XXXV	**Boc-GPRP-BP**	91	53	Gly^4 Pro^3 Arg^2 Pro^1 BP	4.16[2H, d, $^{\alpha}CH$], 8.1[1H, s, NH], 1.43[9H, s, Boc]. 4.28[1H, m, $^{\alpha}CH$], 3.17[2H, m, $^{\beta}CH$], 2.68[2H, m, $^{\gamma}CH$], 3.56[2H, t, $^{\delta}CH$]. 4.51[1H, m, $^{\alpha}CH$], 1.98[2H, m, $^{\beta}CH$], 1.82[2H, m, $^{\gamma}CH$], 3.33[2H, m, $^{\delta}CH$], 8.17-8.30 [2H, s, NH] 4.28[1H, m, $^{\alpha}CH$], 3.17[2H, m, $^{\beta}CH$], 2.69[2H, m, $^{\gamma}CH$], 3.57[2H, t, $^{\delta}CH$]. 7.2-7.3[5H, m, ArH], 2.52 [2H, s, ArCH_2] 2.82[4H, t, -CH_2-piperidine], 1.75[4H, t, -CH_2-piperidine], 1.88[2H, s, CH]

Table-3.3: Antibacterial Activity of Peptide Conjugated Heterocycles:

Entry	Compound [a]	Inhibitory Zone (diameter) mm[b]			
		Escherichia coli	***Klebesiella pneumoniae***	***Pseudomonas auregenosa***	***Staphylococcus aureus***
IV	BHP	03	03	04	04
IX	BZP	02	02	03	03
XII	BP	02	02	02	01
XXIV	RP	00	00	00	00
XXV	PRP	01	01	00	00
XXVI	GPRP	02	02	02	01
XLV	BHP-RP	06	06	05	05
XLVI	BHP-PRP	07	07	06	05
XLVII	BHP-GPRP	12	09	11	08
XLVIII	BZP-RP	05	05	05	05
XLIX	BZP-PRP	06	06	05	05
L	BZP-GPRP	10	08	09	07
LI	BP-RP	04	03	03	02
LII	BP-PRP	05	04	03	03
LIII	BP-GPGP	09	07	08	06
	Streptomycin	12	10	11	12

[a] Concentration of compounds and reference drug: 10 µg/well.

[b] Values are means of three determinations, the ranges of which are less than 5% of the mean in all cases.

Table-3.4: Antifungal Activity of Peptide Conjugated Heterocycles:

Entry	Compounds [a]	Inhibitory Zone (diameter) mm [b]		
		Aspergillus niger	***Aspergillus flavus***	***Fusarium monoliforme***
IV	BHP	02	04	05
IX	BZP	02	03	04
XII	BP	02	02	02
XXIV	RP	00	00	00
XXV	PRP	00	00	00
XXVI	GPRP	03	03	04
XLV	BHP-RP	06	06	07
XLVI	BHP-PRP	06	06	07
XLVII	BHP-GPRP	09	09	10
XLVIII	BZP-RP	04	05	06
XLIX	BZP-PRP	05	05	06
L	BZP-GPRP	08	08	09
LI	BP-RP	03	03	04
LII	BP-PRP	04	04	05
LIII	BP-GPRP	07	07	08
	Bavistin	09	10	09

[a] Concentration of compounds and reference drug: 10 µg/mL

[b] Values are means of three determinations, the ranges of which are less than 5% of the mean in all cases.

Fig 3.4: Antibacterial activity of Peptides and Peptide Conjugated Heterocycles

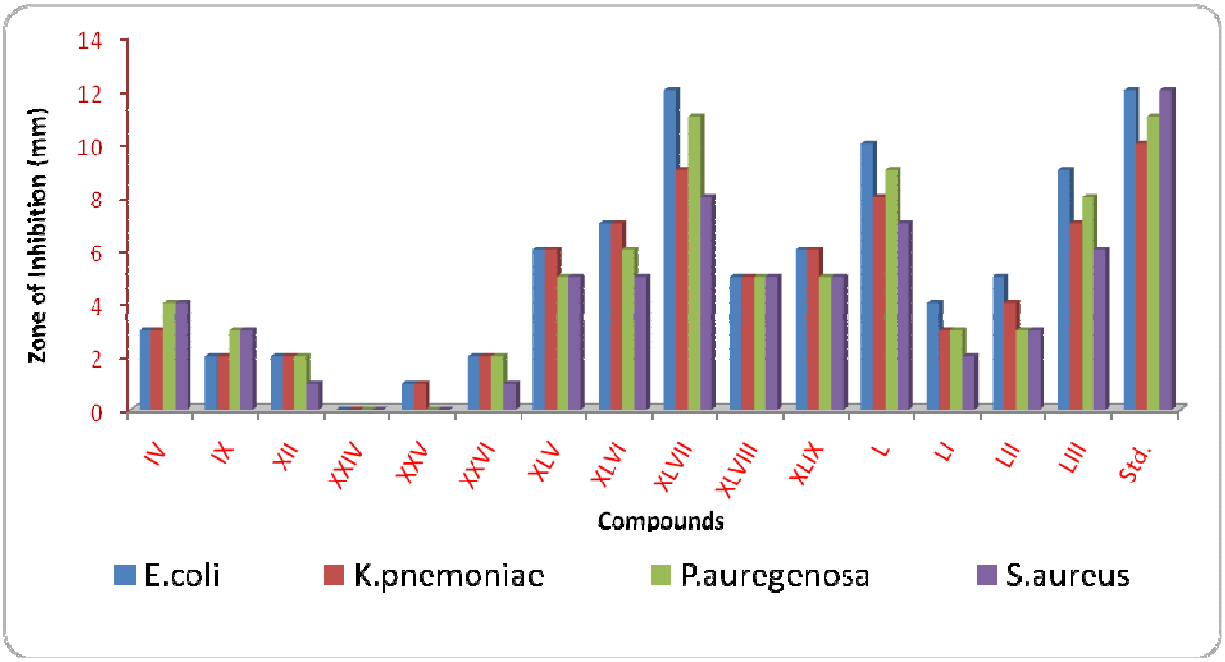
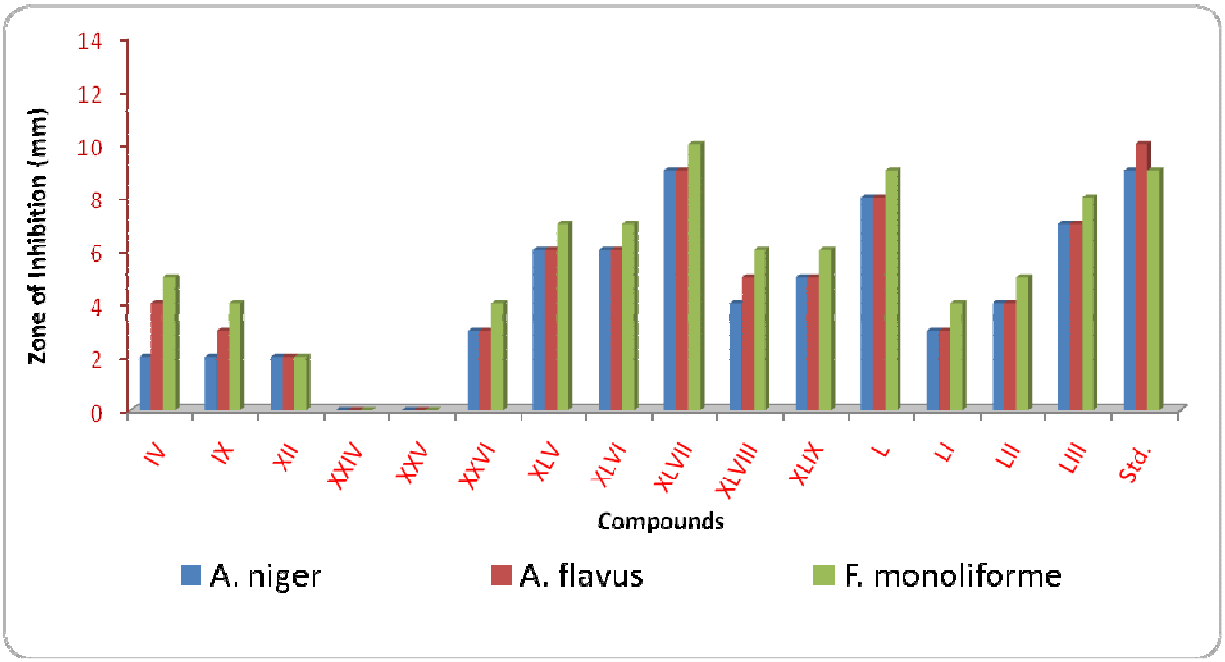


Fig 3.5: Antifungal activity of Peptides and Peptide Conjugated Heterocycles



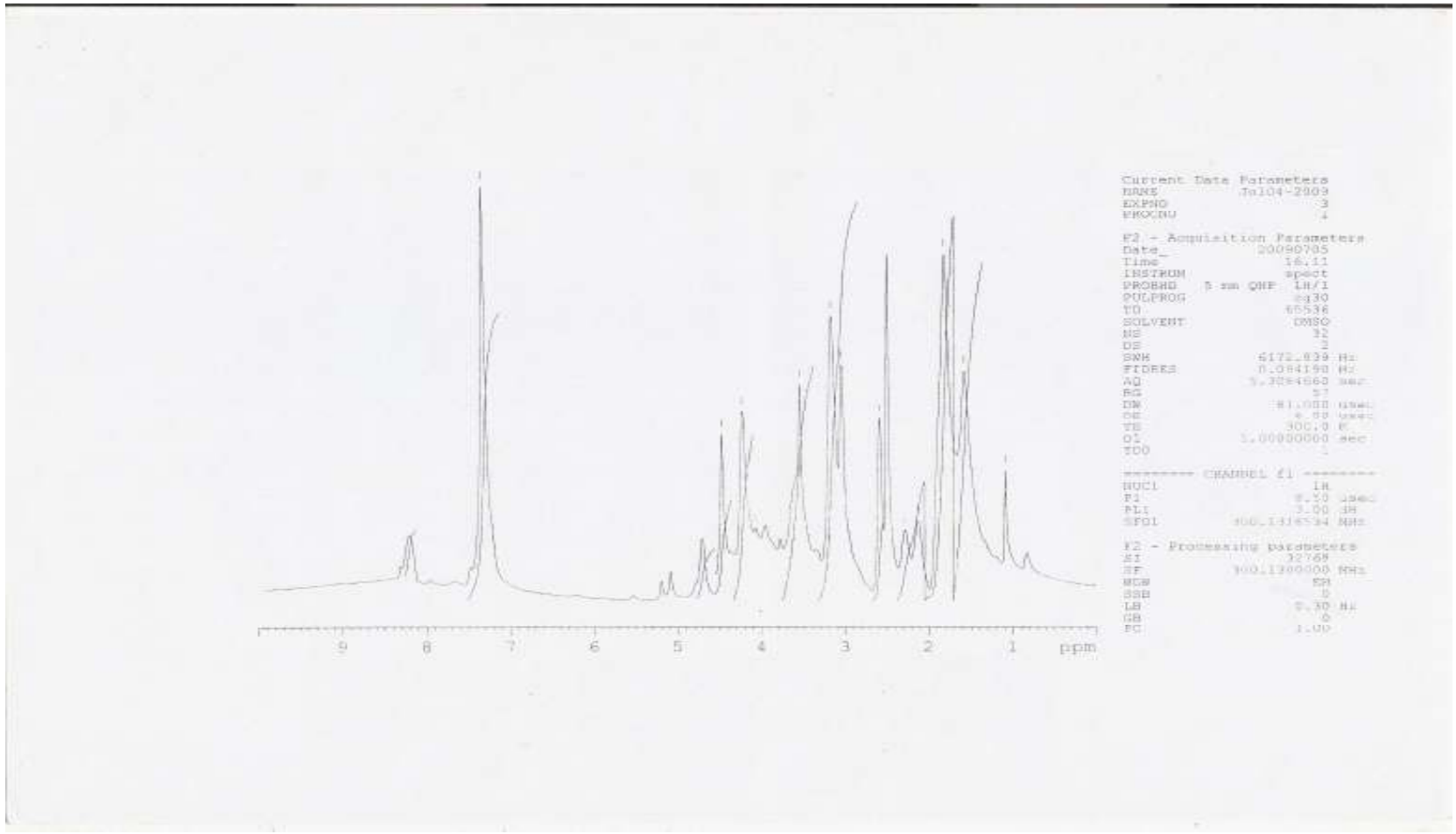


Figure-3.6. ^{1}H NMR Spectra of Boc-PRP-BHP (XLVI, Analogues-IV)

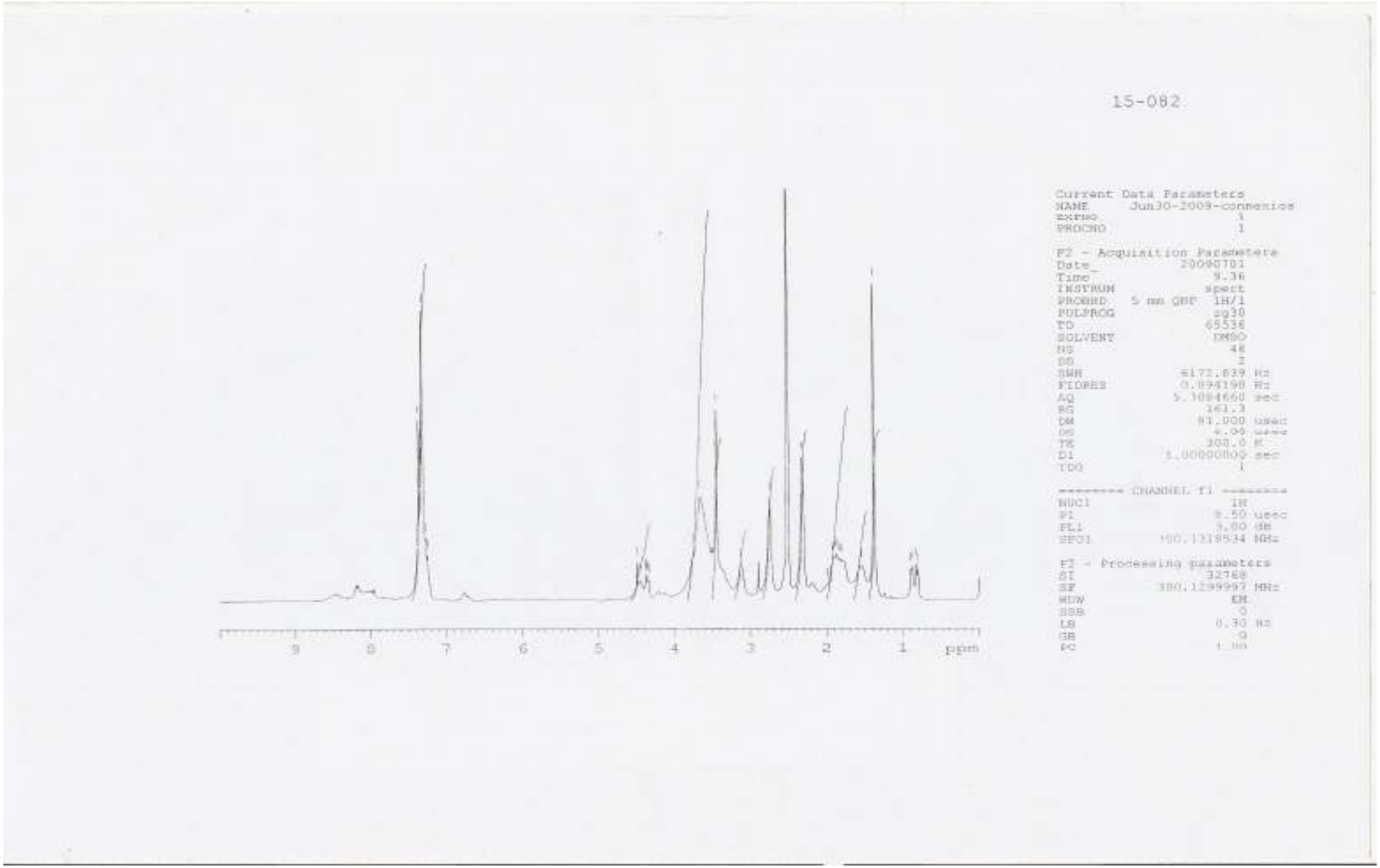


Figure-3.7. ^{1}H NMR Spectra of Boc-GPRP-BZP (L, Analogues-V)

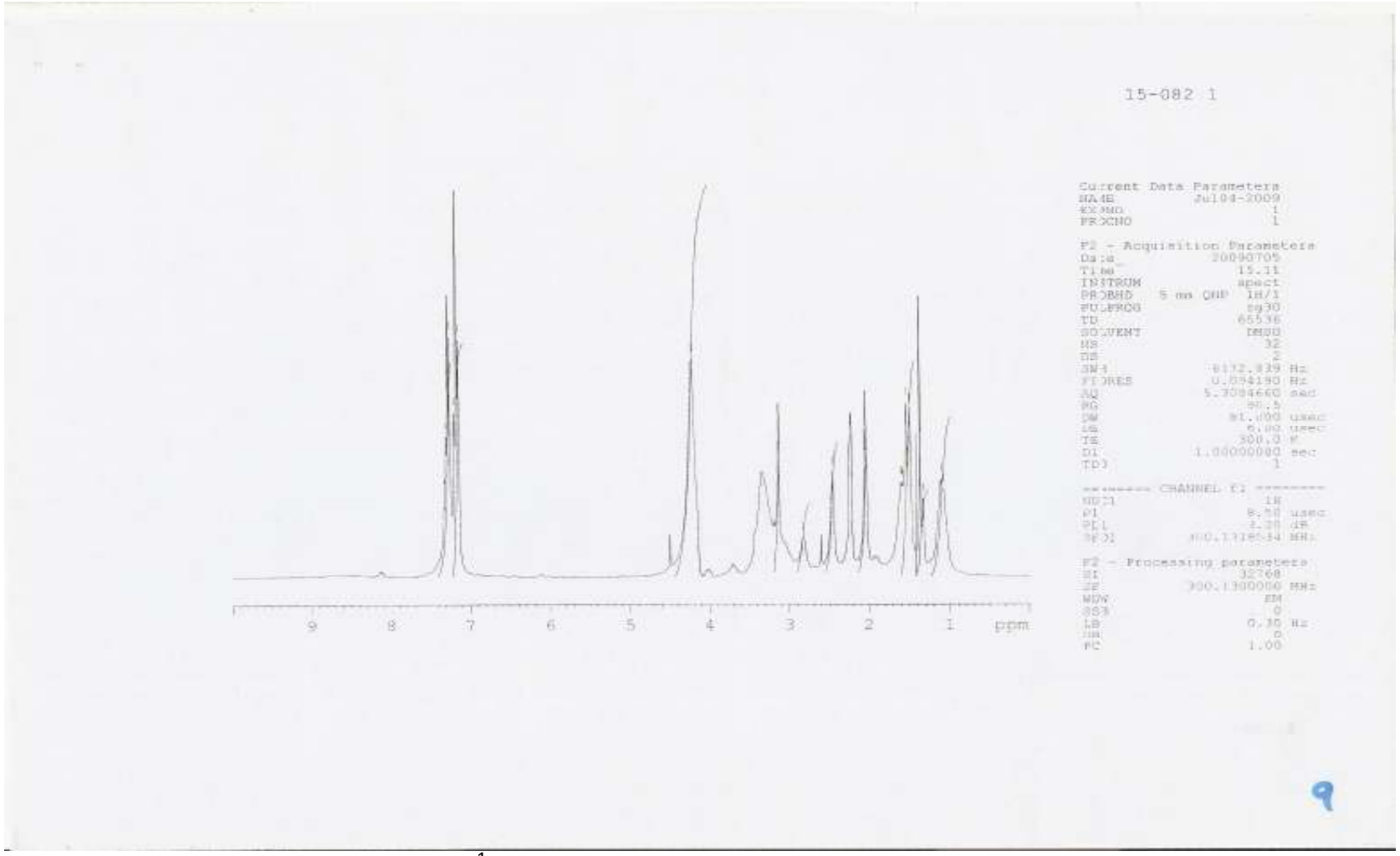


Figure-3.8. ^{1}H NMR Spectra of Boc-RP-BP (LI, Analogues-VI)

3.4. Results and Discussion

3.4.1. Antibacterial studies

The antibacterial efficacy of the above said compounds were tested against both gram+ve and gram-ve bacteria such as *Staphylococcus aureus, Escherichia coli*, *Klebesiella pneumoniae and Pseudomonas auregenosa* at the same concentration as that of the standard, streptomycin. The results are expressed as zone of inhibition measured in mm. The results are summarized in **Table-3.3.**

Among all the synthesized compounds, **GPRP-BHP** (**XLVII**) showed highest activity against all the bacterial strains tested which are equipotent with the standard drug. In the remaining two analogues, **GPRP-BZP** (**L**) and **GPRP-BP** (**LIII**) also showed moderate activity. On the whole, ***all the compounds showed good activity against the gram -ve bacteria when compared to gram +ve bacteria***. This may be due to the easy passage of tested compounds through thin peptidoglycan layer of gram-negative bacterial cell wall when compared to the gram-positive bacteria, which is very much thicker. In view of this, antibacterial activity of the synthesized compounds reveals that, in both heterocyclic conjugated peptides and peptides alone, activity increases from dipeptide to tetrapeptide *i.e.,* **as the chain length of the peptide increases the activity also increases**. Where as peptides tested alone, the dipeptide does not show any activity, but, tri and tetrapeptides showed some activity against all the bacterial strains tested. On the contrary, all the heterocycles taken in isolation have also been subjected to antibacterial studies which fetched the negligible results. Thus, it can be emphasized that the conjugation of peptides with that of heterocycles resulted in marked increase in the activity which can be viewed as lead forefront molecules for further investigations.

In the midst of two peptide conjugated piperazine derivatives, benzhydrilpiperazine derivatives showed the marginal antibacterial activity over the peptide conjugated benzylpiperazine derivatives. Here it may be looked that, the presence of an additional phenyl ring in the benzhydrilpiperazine moiety, which is thought to play a role in exerting more

activity. On the other hand, 4-benzylpiperidine conjugated peptides exhibit least antibacterial potency compared to the former analogues discussed which may be due to the lack of an extra phenyl ring as in the case of benzhydrilpiperazine and also the presence of only one basic nitrogen atom in comparison to two nitrogen atoms in benzylpiperazine.

3.4.2. Antifungal activity:

Antifungal potency of the above said compounds were tested against various fungal strains such as *Aspergillus niger, Aspergillus flavus and Fusarium monoliforme* at the same concentration as that of the standard, Bavistin and the results are expressed as zone of inhibition measured in mm. The results are summarized in **Table-3.4.**

Among all the compounds tested the tetrapeptide conjugated benzhydrilpiperazine derivatives (**XLVII**) showed maximum activity in the series equipotent with bavistin. The tetrapeptide coupled benzylpiperazine (**L**) and 4-benzylpiperidine (**LIII**) also showed good activity, but less compared to that of benzhydrilpiperazine counterparts. The attributes discussed under antibacterial activity holds good for the antifungal studies also. The remaining di and tripeptide conjugated heterocycles showed moderate to good activity compared to the peptide and heterocycles alone.

3.5. Conclusion

In an effort to explore peptide conjugated heterocycles as a new class of antimicrobial drugs, we found that compounds containing tetrapeptide fragments of benzhydrilpiperazine, benzylpiperazine and 4-benzylpiperidine showed good antibacterial activity as well as good antifungal activity and all other compounds showed a moderate activity. Hence, it can be inferred that conjugation of peptides of different chain length with heterocyclic motifs enhanced both the antibacterial and antifungal activities compared to their mother molecules. The results of the present study indicate that compounds **XLVII**, **L** and **LIII** might be of great interest to develop novel analogues for better antimicrobial potency.

3.6. References

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