SYNTHESIS, CHARACTERIZATION AND ANTI-BACTERIAL STUDIES OF SOME CYCLIC PEPTIDE DERIVATIVES

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Abstract

The present research was designed to synthesize a series of cyclic peptides with their anti-bacterial activity using culture method and gatifloxacin and gentamycin as the reference drug. The research was envisaged to incorporate cyclic and acyclic amino acids through peptide bonds into the basic skeleton for synthesis of final compounds and to explore the effect of these substitutions on the *in-vitro* activity. All the synthesized compounds have been evaluated against different bacterial strains e.g. *Staphylococcus aureus*, *Bacillus subtilis*, *Streptococcus faecalis*, *Escherichia coli*, *Klebsiella pneumonia* and fungal strain (*Pseudomonas aeruginosa*). Finally, it can be concluded that, in scheme, KLP1 and KLP2 showed some activity as compare to standard drugs against gram positive and gram negative bacteria but cyclization of all linear peptides lead to decline in antibacterial activity.

Key words: Anti-microbial Agents, Cyclic Peptides, Gatifloxacin and Gentamycin.

Introduction

Cyclic peptides containing cyclic ring structure are generally polypeptide chains, that can be synthesized by joining one end of the peptide with the other through an amide, lactone, ether, thioether and disulfide bond etc (Claro *et al.*, 2018; Kates *et al.*, 1994; Joo, 2012; Woodburn *et al.*, 2019). Cyclic peptides (CPs) have advantages of having lesser degree of freedom within the ring for each constituent, reduced conformational flexibility and stabilized secondary structures (Andreev, 2017; Bastos *et al.*, 2009; Claro *et al.*, 2018; Corless, 2019; McHugh *et al.*, 2016). Another benefit is the resistance to hydrolysis by exopeptidases due to the absence of both carboxyl and amino termini (Estiarte *et al.*, 2013).

Generally CPs composed of an even number of alternating D- and L-amino acids adopt a planar ring conformation or cyclic structures (Lucke *et al.*, 2003). This conformation orients the amino acid side chains to the outside of the ring structure and the amide groups perpendicular to the plane of the ring. The amide groups are responsible for the hydrogen bond-directed stacking of cyclic peptide subunits in environments that favor hydrogen bond formation, such as lipid bilayers (Dahiya, 2008).

The self-assembling properties of individual cyclic D, L-peptides result in the formation of multimeric, hollow tubular structures also called peptide nanotubes. The interaction of these supramolecular structures with biological membranes is highly dependent upon the amino acid composition of the D, L-peptides and the chemical properties of the residues that are in contact with the components of the cell membrane (Chatterjee *et al.*, 2013; Marasco *et al.*, 2008; Pasupuleti *et al.*, 2012). Peptide nanotubes formed from amphipathic cyclic peptides adopt an orientation parallel to the membrane plane, where the hydrophobic side chains are inserted into the lipidic components of the membrane and the hydrophilic residues remain exposed to the hydrophilic components of the cell membrane (Lopez *et al.*, 2016).

In this format, peptide nanotubes are believed to permeate membranes through a carpet-like mechanism, collapse transmembrane potential and/or gradient, and cause rapid cell death (Dellai *et al.*, 2010). CPs may have altered pharmacological profile but reduced side

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effects with respect to their linear analogs. Incorporation of cyclic structures into numerous bioactive peptides lead to highly potent selective analogs (Dixon *et al.*, 2009). Different cyclization strategies include connection of amino terminus to carboxyl terminus, amino terminus to side chain, side chain to carboxyl terminus and side chain to another side chain. Moreover the degradation products of the same are simply amino acids, that would not induce toxicity (Roxin and Zheng, 2012).

The incidence of community-acquired and nosocomially acquired infections due to the bacterium *Staphylococcus aureus* is rising. From 1990 to 1992, this microorganism was the most common cause of nosocomial pneumonias and surgical wound infections (Hruby and Balse, 2000). The overall growing crisis in antibiotic resistance and the rise in the incidence of methicillin-resistant *S. aureus* (MRSA) strains have emphasized the need for therapeutic alternatives to currently available antibiotics. Multidrug resistance (MDR) in bacteria is usually mediated by the expression of efflux pumps or porins involved in transport, by the expression of mutated genes coding for specific drug targets or specific enzymatic barriers. As a matter of fact, MDR remains a major obstacle hindering successful antibacerial chemotherapy. One way of tackling the emergence of MDR is to diversify the chemical structures of anti-microbial drugs to which resistance has developed in order to extend their lifespan (Amaral and Fanning, 2011; Bhardwaj and Mohanty, 2012).

CPs are available in minor concentrations in various plants, marine and marine plant sources. Their isolation from the natural sources not only makes them expensive drugs but also can cause a severe ecological imbalance (Jackson *et al.*, 2018; Kaur *et al.*, 2012). They are found to be indispensable part of the drug discovery and drug development. In order to produce such biologically active CPs at commercial scale, synthetic strategies are developed. These include solid phase synthesis and solution phase synthesis (Kaur *et al.*, 2012).

Some examples of natural CPs synthesized through these strategies include cherimolacyclopeptide G, argifin, cyclosquamosin D, dichotomin A analogs, NMe-IB-01212 and tunicyclins C & D (Dahiya and Kumar, 2008; Fang *et al.*, 2016; Kumar *et al.*, 2017; Wele *et al.*, 2004). The major challenges in synthesizing CPs include synthesis of linear peptide through several protection, coupling and deprotection steps (usually 30 for a pentapeptide) which is subsequently cyclised through esterification (Malipeddi *et al.*, 2010; Marcucci *et al.*, 2012).

Even a minor error at any stage of this long sequential synthetic process makes the chemist to stop the process and start from the beginning. So far, various research groups have synthesized CPs composed of 5-8 amino acids (Brea *et al.*, 2017; Hamada and Shioiri, 2005; Perlman *et al.*, 2005; Tanaka, 2007). Most of them are mainly used to treat cancer, infection, and the diseases associated with metabolic disorders (Cheneval *et al.*, 2014; Ermert *et al.*, 2019; Meena *et al.*, 2020). At present, there are still hundreds of cyclic peptides at the stages of clinical and preclinical studies. It can be predicted that more and more cyclic peptide drugs will constantly be approved into the market in the near future (Roxin and Zheng, 2012). Till now, several classical reported natural cyclic peptides with antibacterial, anticancer and other biological activities are discussed thoroughly. Therefore the scientist is thought to exploit the antibacterial activity of cyclopeptides by synthesizing it. Because the emergence of many medically relevant resistant strains of bacteria today is a major issue in human health. It is therefore becoming essential that new therapeutic agents be developed to combat microorganisms resistant to traditional antibiotics.

Cyclic peptides are one of the underexplored classes of bioactive peptides with a marine origin that have great promise in pharmaceutical areas. These compounds have garnered increased interest because of their significant bioactivities. Cyclic peptides originating from marine organisms have increased our understanding of potent new anticancer, antibacterial, ion channel-specific blockers, and antifungal properties of novel chemical structures related to the mechanisms of pharmacological activity. This information demonstrates that marine cyclic peptides are a novel alternative for biological and biomedical research (Fernandez-Lopez *et al.*, 2001).

CPs are available in minor concentrations in various plants, marine and marine plant sources. Their isolation from the natural sources not only makes them expensive drugs but also can cause a severe ecological imbalance (Ghadiri *et al.*, 1994). In order to produce such biologically active CPs at commercial scale, synthetic strategies are developed. These include solid phase synthesis and solution phase synthesis. Taking into consideration the importance of CPs and their synthetic complications, the objectives of the present study are focused to synthesize selected natural CPs and its analogs and their evaluation for the antibacterial activity.

Materials and methods

Chemistry

Melting points and Infrared (IR) spectra were determined on a Veego melting point apparatus and a

Shimadzo (Japan) 8400 S FT-IR spectrophotometer model respectively. Proton-NMR spectra were recorded on Brucker multinuclear FT-NMR spectrophotometer model AV-400, 400 MHz using deuterated-chloroform containing tetramethylsilane (Me$_4$Si) as internal standard (chemical shift in δ, ppm). Iodine was used to develop the TLC plates. All the solvents were distilled prior to use according to standard procedures. Anhydrous sodium sulphate was used as drying agent. A detailed procedure for synthesis of cyclic peptides is given below:

Synthesis of linear pentapeptides methyl ester HCl

Boc-Gly-Phe-Ser-Tyr-Arg OMe (KLP)

20 mililitres CHCl$_3$ was employed to dissolve the compound dipeptide Tyr-Arg OMe (3.50 gram, 10 milimole) trailed by addition of 2.3 mililitres NMM (21 milimole) at 0°C and was kept for 15 minutes under stirring. On the other hand, tripeptide was synthesized by dissolving Boc-Gly-Phe-Ser OH (3.90 gram, 10 milimole) in 20 mililitres CHCl$_3$ trailed by addition of 2.1 gram DCC (10 milimole) under the influence of continuous stirring to the above slurry.

The reaction slurry was sifted after 36 hour to obtain the remnant and then washed with 30 mililitres CHCl$_3$, added to filtrate, that was further washed with 5% NaHCO$_3$ (25 mililitres) and saturated solution of NaCl (25 mililitres) in the ratio of 1:1. Over anhyd. Na$_2$SO$_4$, the non-aqeous layer was dried, which was dissipated constrained to give unrefined item, that was recrystallised with CHCl$_3$ and pet ether trailed by cooling the same at 0°C to afford **KLP**.

Boc-Ala-Phe-Ser-Tyr-Arg

20 mililitres CHCl$_3$ was employed to dissolve the compound dipeptide Tyr-Arg OMe (3.50 gram, 10 milimole) was solubilzed in 20 mililitres CHCl$_3$ trailed by addition of 2.3 mililitres NMM (21 milimole) at 0°C and was kept for 15 minutes under stirring. Compound tripeptide Boc-Ala-Phe-Ser OH (4.04 gram, 10 milimole) was solubilzed in 20 mililitres of CHCl$_3$ trailed by addition of 2.1 gram DCC (10 milimole) under stirring to the above slurry.

The reaction slurry was sifted after 36 hour to obtain the remnant and then washed with 30 mililitres CHCl$_3$, added to filtrate, that was further washed with 5% NaHCO$_3$ (25 mililitres) and saturated solution of NaCl (25 mililitres) in the ratio of 1:1. Over anhyd. Na$_2$SO$_4$, the non-aqeous layer was dried, which was dissipated constrained to give unrefined item, that was recrystallised with CHCl$_3$ and pet ether trailed by cooling the same at 0°C to afford KLP1.

Boc-Gly-Tyr-Ser-Phe-Arg

20 mililitres CHCl$_3$ was employed to dissolve the compound dipeptide Phe-Arg OMe (3.34 gram, 10 milimole) trailed by addition of 2.3 mililitres NMM (21 milimole) at 0°C and was kept for 15 minutes under stirring. Compound tripeptide Boc-Gly-Tyr-Ser OH (4.06 gram, 10 milimole) was solubilzed in 20 mililitres of CHCl$_3$ trailed by addition of 2.1 gram DCC (10 milimole) under stirring to the above slurry.

The reaction slurry was sifted after 36 hour to obtain the remnant and then washed with 30 mililitres CHCl$_3$, added to filtrate, that was further washed with 5% NaHCO$_3$ (25 mililitres) and saturated solution of NaCl (25 mililitres) in the ratio of 1:1. Over anhyd. Na$_2$SO$_4$, the non-aqeous layer was dried, which was dissipated constrained to give unrefined item, that was recrystallised with CHCl$_3$ and pet ether trailed by cooling the same at 0°C to afford KLP2.

Boc-Ala-Tyr-Ser-Phe-Arg

20 mililitres CHCl$_3$ was employed to dissolve the compound dipeptide Phe-Arg OMe (3.34 gram, 10 milimole) followed by addition of 2.3 mililitres NMM (21 milimole) at 0°C and was kept for 15 minutes under stirring. Compound tripeptide Boc-Ala-Tyr-Ser OH (4.20 gram, 10 milimole) was solubilzed in 20 mililitres of CHCl$_3$ trailed by addition of 2.1 gram DCC (10 milimole) under stirring to the above slurry.

The reaction slurry was sifted after 36 hour to obtain the remnant and then washed with 30 mililitres CHCl$_3$, added to filtrate, that was further washed with 5% NaHCO$_3$ (25 mililitres) and saturated solution of NaCl (25 mililitres) in the ratio of 1:1. Over anhyd. Na$_2$SO$_4$, the non-aqueous layer was dried, which was dissipated constrained to give unrefined item, that was recrystallised with CHCl$_3$ and pet ether trailed by cooling the same at 0°C to afford KLP3.

Synthesis of Cyclic Pentapeptides

Cyclo(Gly-Phe-Ser-Tyr-Arg)KCP

Compound LINEAR PEPTIDE Boc-Gly-Phe-Ser-Tyr-Arg OMe (3.70 gm, 5 mmol) was dissolved in 50 ml of chloroform at 0°C. To The above solution, 0.94 gm of pnp (6.7 mmol) was added and stirred at room temperature for 12 hr. The reaction mixture was filtered and the filtrate was washed with 15 ml of 10% sodium hydrogen carbonate solution until excess of pnp was removed and finally washed with 5% hydrochloric acid (2 x 10 ml) to get the corresponding p-nitrophenyl ester Boc-Gly-Phe-Ser-Tyr-Arg-O-pnp.

The resulting compound (3.49 gm, 4 mmol) was dissolved in 35 ml of chloroform, 0.91 gm of TFA (8 mmol) was added, stirred at room temperature for 1 hr and washed with 10% sodium bicarbonate solution (2 x 25 ml). The organic layer was dried over anhydrous sodium sulphate to get Gly-Phe-Ser-Tyr-Arg-pro-O-pnp, which was dissolved in 25 ml of chloroform and 2.3 ml of NMM (21 mmol) was added. Then all the contents were kept at 0°C for 7 days. The reaction mixture was washed with 10% sodium hydrogen carbonate solution until the byproduct p-nitrophenol was removed completely and finally washed with 5% hydrochloric acid (3 x 15 ml). The organic layer was dried over anhydrous sodium sulphate. Finally, chloroform was distilled off and the crude cyclized product was recrystallized from chloroform and n-hexane to give yellow solid of compound (86%), m.p. 177-180°C.

Spectral and elemental analyses

FT-IRí$_{max}$ (KBr): 3197.98 (N-H stretch), 3167.12, 3055.24 (C-H stretch, aromatic), 2980.02 (C-H stretch, aliphatic), 1655.72 (C=O stretch, amide), 1544.98 (N-H bend), 1492.90 (C=C stretch, aromatic), 1280.73 (C-N stretch, aliphatic) and 798.53 cm^{-1} (out-of-plane N-H wagging).

^{1}H NMR (DMSO-d_6, 400 MHz): δ 1.64 (m, 6H, -CH_2), 2.35 (s, 2H, -CH_2), 3.21 (m, 2H, -CH_2), 3.42 (m, 3H, -CH_2),3.65 (m, 2H, -CH_2), 3.91 (m, 3H, -CH), 4.54 (m, 3H, >NH), 4.63 (s, 3H, >NH), 4.78 (m, 3H, >NH),7.55 (m, 2H, ArH), 7.57 (m, 2H, ArH), 7.61 (m, 3H, ArH),7.86 (m, 2H, ArH), 8.30 (s, 1H, -OH) and 8.87 (s, 1H, -OH).

ESI-MS: *m/z* 392.1 [M+H]$^+$

Cyclo (Ala-Phe-Ser-Tyr-Arg) KCP1

Compound LINEAR PEPTIDE KLP1 (3.77 gm, 5 mmol) was dissolved in 50 ml of chloroform at 0°C. To The above solution, 0.94 gm of pnp (6.7 mmol) was added and stirred at room temperature for 12 hr. The reaction mixture was filtered and the filtrate was washed with 15 ml of 10% sodium hydrogen carbonate solution until excess of pnp was removed and finally washed with 5% hydrochloric acid (2 x 10 ml) to get the corresponding p-nitrophenyl ester Boc-Ala -Phe-Ser-Tyr-Arg-O-pnp).

The resulting compound (3.51 gm, 4 mmol) was dissolved in 35 ml of chloroform, 0.91 gm of TFA (8 mmol) was added, stirred at room temperature for 1 hr and washed with 10% sodium bicarbonate solution (2 x 25 ml). The organic layer was dried over anhydrous sodium sulphate to get Ala-Phe-Ser-Tyr-Arg-pro-O-pnp, which was dissolved in 25 ml of chloroform and 2.3 ml of NMM (21 mmol) was added. Then all the contents were kept at 0°C for 7 days. The reaction mixture was washed with 10% sodium hydrogen carbonate solution until the byproduct p-nitrophenol was removed completely and finally washed with 5% hydrochloric acid (3 x 15 ml). The organic layer was dried over anhydrous sodium sulphate. Finally, chloroform was distilled off and the crude cyclized product was recrystallized from chloroform and n-hexane to give yellow solid of compound (84%), m.p. 170-173 °C.

Spectral and elemental analyses

FT-IRí$_{max}$ (KBr): 3073.40 (C-H stretch, aromatic), 2980.00 (C-H stretch, aliphatic), 1627.38 (C=C stretch, aromatic), 1587.10 (C=N stretch), 1474.22 (C-H bend, CH_2), 1383.00 (C-N stretch, aromatic), 1190.62 (C-N stretch, aliphatic) and 1095.00 cm^{-1} (C-Cl stretch).

^{1}H NMR (DMSO-d_6, 400 MHz): δ 1.64 (m, 6H, -CH_2), 2.35 (s, 2H, -CH_2), 3.21 (m, 2H, -CH_2), 3.42 (m, 3H, -CH_2), 3.65 (m, 2H, -CH_2), 3.91 (m, 3H, -CH_3), 4.54 (m, 3H, >NH), 4.53 (s, 3H, >NH), 4.92 (m, 3H, >NH), 5.35 (m, 2H, -CH_2), 7.55 (m, 2H, ArH), 7.57 (m, 2H, ArH), 7.61 (m, 3H, ArH), 7.86 (m, 2H, ArH), 8.30 (s, 1H, -OH) and 8.87 (s, 1H, -OH).

Cyclo(Gly-Tyr-Ser-Phe-Arg) KCP2

Compound KLP2 (3.73 gm, 5 mmol) was dissolved in 50 ml of chloroform at 0°C. To The above solution, 0.94 gm of pnp (6.7 mmol) was added and stirred at room temperature for 12 hr. The reaction mixture was filtered and the filtrate was washed with 15 ml of 10% sodium hydrogen carbonate solution until excess of pnp was removed and finally washed with 5% hydrochloric acid (2 x 10 ml) to get the corresponding p-nitrophenyl ester Boc-Gly-Tyr-Ser-Phe-Arg-O-pnp.

The resulting intermediate (3.46 gm, 4 mmol) was dissolved in 35 ml of chloroform, 0.91 gm of TFA (8 mmol) was added, stirred at room temperature for 1 hr and washed with 10% sodium bicarbonate solution (2 x 25 ml). The organic layer was dried over anhydrous sodium sulphate to get Gly-Tyr-Ser-Phe-Arg-pro-O-pnp, which was dissolved in 25 ml of chloroform and 2.3 ml of NMM (21 mmol) was added. Then all the contents were kept at 0°C for 7 days. The reaction mixture was washed with 10% sodium hydrogen carbonate solution until the byproduct p-nitrophenol was removed completely and finally washed with 5% hydrochloric acid (3 x 15 ml). The organic layer was dried over anhydrous sodium sulphate. Finally, chloroform was distilled off and the crude cyclized product was recrystallized from chloroform and n-hexane to give yellow solid of compound (92%), m.p. 190-193 °C.

7[1]H NMR (DMSO-d_6, 400 MHz): δ 1.64 (m, 6H, -CH_2), 2.35 (s, 2H, -CH_2), 3.21 (m, 2H, -CH_2), 3.42 (m, 3H, -CH_2), 3.65 (m, 2H, -CH_2), 3.91 (m, 2H, -CH), 4.54 (m, 3H, >NH), 4.53 (s, 3H, >NH), 4.92 (m, 3H, >NH), 5.35 (m, 1H, -CH_2), 7.55 (m, 2H, ArH), 7.57 (m, 2H, ArH), 7.61 (m, 3H, ArH), 7.86 (m, 2H, ArH), 8.30 (s, 1H, -OH) and 8.87 (s, 1H, -OH).

Cyclo(Ala-Tyr-Ser-Phe-Arg) KCP3

Compound KLP3 (3.77 gm, 5 mmol) was dissolved in 50 ml of chloroform at 0°C. To The above solution, 0.94 gm of pnp (6.7 mmol) was added and stirred at room temperature for 12 hr. The reaction mixture was filtered and the filtrate was washed with 15 ml of 10% sodium hydrogen carbonate solution until excess of pnp was removed and finally washed with 5% hydrochloric acid (2 x 10 ml) to get the corresponding p-nitrophenyl ester Boc-Ala -Tyr-Ser-Phe-Arg-O-pnp.

The resulting intermediate (3.51 gm, 4 mmol) was dissolved in 35 ml of chloroform, 0.91 gm of TFA (8 mmol) was added, stirred at room temperature for 1 hr and washed with 10% sodium bicarbonate solution (2 x 25 ml). The organic layer was dried over anhydrous sodium sulphate to get Ala-Tyr-Ser-Phe-Arg-pro-O-pnp, which was dissolved in 25 ml of chloroform and 2.3 ml of NMM (21 mmol) was added. Then all the contents were kept at 0°C for 7 days. The reaction mixture was washed with 10% sodium hydrogen carbonate solution until the byproduct p-nitrophenol was removed completely and finally washed with 5% hydrochloric acid (3 x 15 ml). The organic layer was dried over anhydrous sodium sulphate. Finally, chloroform was distilled off and the crude cyclized product was recrystallized from chloroform and n-hexane to give yellow solid of compound (77%), m.p. 143-145 °C.

Spectral and elemental analyses

FT-IRí$_{max}$ (KBr): 3187.76 (N-H stretch), 3107.36 (C-H stretch, aromatic), 2920.23 (C-H stretch, aliphatic), 1697.36 (C=C stretch, aromatic), 1546.91 (C=N stretch), 1492.90 (C-H bend, CH$_2$) and 1139.93 (C-N stretch, aliphatic) cm^{-1}.

^{1}H NMR (DMSO-d_6, 400 MHz): δ 1.64 (m, 6H, -CH_2), 2.35 (s, 2H, -CH_2), 3.21 (m, 2H, -CH_2), 3.42 (m, 3H, -CH_2), 3.65 (m, 2H, -CH_2), 3.91 (m, 3H, -CH_3), 4.54 (m, 3H, >NH), 4.53 (s, 3H, >NH), 4.92 (m, 3H, >NH), 5.35 (m, 2H, -CH_2), 7.55 (m, 3H, ArH), 7.57 (m, 2H, ArH), 7.61 (m, 2H, ArH), 7.86 (m, 2H, ArH), 8.30 (s, 1H, -OHadjacent to methyl group) and 8.87 (s, 1H, -OHadjacent to phenyl ring).

Pharmacological studies

All the compounds synthesized were evaluated using the disc-diffusion methods for their anti-bacterial performance.

Antimicrobial activity

In vitro antibacterial studies of all the synthesized compounds were carried out against Gram negative and positive bacterial strains by disk-diffusion assay.[12] The gram negative bacterial strains were *Escherichia coli, Pseudomonas aeruginosa* and *Klebsiella pneumonia*, whereas the gram positive ones were *Staphylococcus aureus, Bacillus subtilis, Streptococcus faecalis*. In disk-diffusion assay, the antimicrobial activity was performed using different concentrations of the test compounds. Sample solutions of desired concentrations (100 5Øßg/disc, 200 5µg/disc, 400 5µg/disc and 500 5µg/disc) were applied in the disc with the help of the micropipette in an aseptic condition. The bacterial isolate was inoculated uniformly on to the surface of mullar hungton agar (MHA) plate and then filter disk impregnated with a known amount of compound was applied to the surface of the plate. These discs were left for a few minutes in aseptic condition for complete evaporation of the solvent. Standard discs were used to compare the antibacterial activity of the test material. After 24 h of incubation at 37 ºC, the diameter of growth inhibition zones was measured. The size of the zone obtained at a particular concentration is directly proportional to the growth inhibition of the organism to the compound.

The activity of compounds was determined in comparison to standard antibiotic discs of Gentamycin and Gatifloxcin. Zone of inhibition of compounds exhibiting considerable activity (Table 1) was determined by using *in vitro* disc diffusion assay method.[13] The initial optical density (OD) of the medium was measured by spectrophotometer at 600 nm. All the synthesized cyclic peptides, exhibit good to moderate antibacterial activity with observable variations due to different amino acids. The obtained results revealed that some of the compounds possess excellent antibacterial activity against selected strain. They were found to posess significant activity in comparison to standards gatifloxacin and gentamycin at a dose of 10 µg/ml. The growth inhibition zone was measured ranged from 8.87 to 18.71 mm for all the sensitive bacteria. Whereas KLP-3 and KCP-3 do not possess any antibacterial activity.

Results and discussion

An amino acid is an organic molecule that is made up of a basic amino group (-NH$_2$), an acidic carboxyl

Table 1: Zone of Inhibition (Dose 1mg/ml)

	Zone of Inhibition (mm)					
	Staphylococcus aureus (G+)	*Bacillus subtilis* (G+)	*Streptococcus faecalis* (G+)	*Escherichia coli* (G-)	*Pseudomonas aeruginosa* (G-)	*Klebsiella pneumonia* (G-)
KLP	12.29±0.72	13.87±0.59	12.75±0.84	16.08±1.44	14.09±0.78	15.15±1.35
KLP-1	18.71±1.01	19.98±0.83	21.81±0.91	19.97±0.52	19.51±1.24	20.09±1.10
KLP-2	17.37±0.98	16.05±0.79	19.82±0.37	17.12±0.93	19.25±1.14	18.67±0.71
KLP-3	Fail	Fail	Fail	Fail	Fail	Fail
KCP	10.12±1.38	10.72±1.02	11.67±0.97	10..56±0.78	11.81±1.11	10.62±1.26
KCP-1	09.34±0.71	09.45±0.93	10.19±0.66	09.32±0.56	08.45±0.68	09.15±0.47
KCP-2	08.87±0.58	09.52±1.09	09.33±0.82	08.10±0.65	09.90±0.72	9.61±0.46
KCP-3	Fail	Fail	Fail	Fail	Fail	Fail
Gentamycin (10µg/ml)	25.47±1.52	19.75±1.65	22.83±1.15	25.56±2.08	24.64±1.52	23.31±0.57
Gatifloxacinc (10µg/ml)	26.61±1.45	24.52±0.98	23.82±1.02	27.73±1.52	30.91±1.00	27.56±1.23

Values are expressed as mean ± standard deviation of the three replicates, Diameter of the well is not included in Zone of inhibition.

group (-COOH), and an organic R group (or side chain) that is unique to each amino acid. Due to both reactive groups at each end, it is required to block one end for enlarging the length of peptide at another one. There are two ways to protect the amino acids by either the formation of tert-butyloxycarbonyl at the amino end or the synthesis of methyl ester on the carboxyl end.

Synthesis of BOC-amino acids

Amino acid was dissolved in sodium hydroxide and isopropanol. To the resulting solution, Di-*tert*-butylpyrocarbonate in isopropanol was added followed by sodium hydroxide. The solution was stirred at room temperature for 2 hr, further was washed with light petroleum ether (b.p. 40-60°C), acidification was carried out with sulphuric acid to pH 3.0 and extraction was carried out using chloroform. The organic layer was dried over anhydrous sodium sulphate and evaporated under reduced pressure to give crude product. The purification of the same was carried out by recrystallization using methanol and ether at 0°C to give protected amino acid.
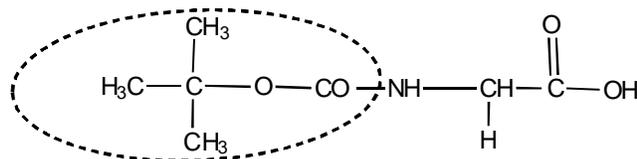
tert-Butyloxycarbonyl

Synthesis of L-amino acid methyl ester hydrochlorides

Amino acid was added to the solution of thionyl chloride and methanol at 0°C and the resulting mixture was refluxed for about 10-15 hr at 110°C. After the completion of reaction, methanol was evaporated and the residue was triturated with ether at 0°C until excess dimethyl sulphite was removed. The recrystallization was carried out using the mixture of methanol and ether at 0°C to give the desired protected compound by converting the carboxylic group into ester as depicted in the fig.

Conversion of carbo-xylic into methyl ester
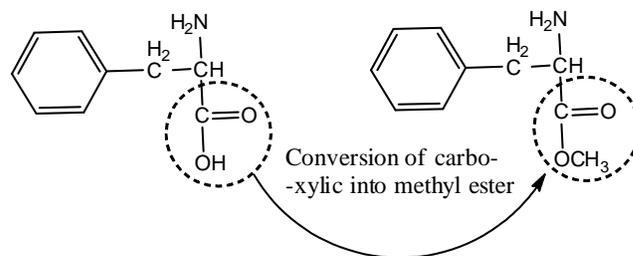
Synthesis of boc-dipeptide methyl esters

Boc-dipeptide methyl esters was prepared by the admixture of both amino acids (boc and methyl ester protected) in chloroform. The linear monomers or amino acids were linked through the formation of peptide bond using *N*-Methylmorpholine and *N, N'*-dicyclohexylcarbodiimide under stirring. Usually these types of reaction were carried out at room temperature and or below 0°C.

After shaking for a particular time, the reaction mixture was filtered and the residue was washed with chloroform and added to the filtrate. The filtrate was washed with 5% sodium bicarbonate and saturated sodium chloride solution. The organic layer was dried over anhydrous sodium sulphate, filtered and evaporated in vacuum. The crude product was recrystallized from mixture of chloroform and light petroleum ether (b.p. 40-60°C) followed by cooling at 0°C to yield the intermediate.

Boc-amino acid

Amino acid methyl ester

Formation of Dipeptide through peptide bond
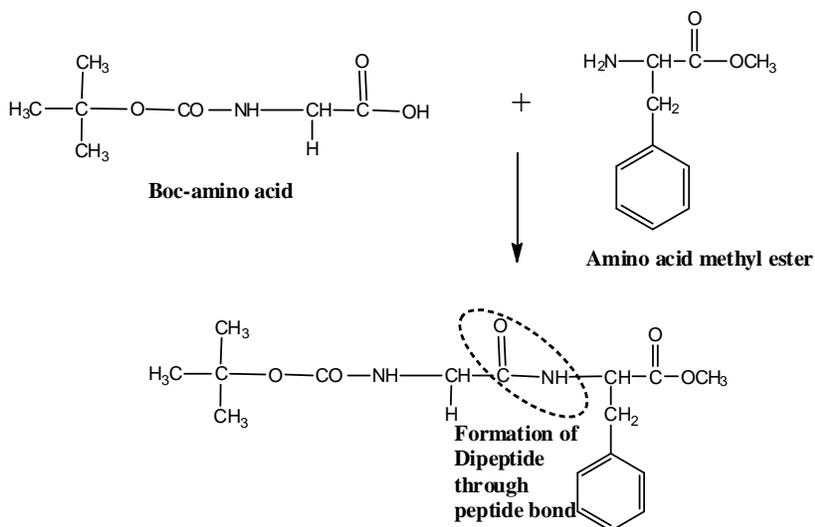
The monitoring of reactions was carried out using thin layer chromatography.

Furthermore, the concept of deprotection is same for the dipeptide as that of single amino acid is very crucial to understand. Because for addition of new amino acids to the same chain, we need to deprotect the peptide chain also.

Deprotection of dipeptides

The dipeptides were deprotected by two ways: Boc and methyl ester as depicted in the figure given below.

METHYL ESTER

BOC
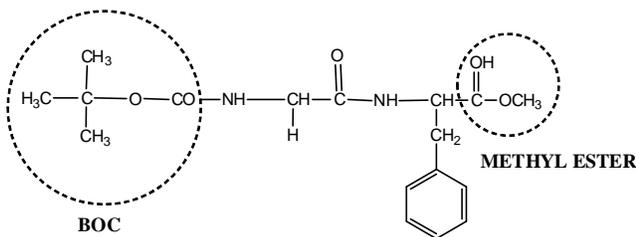
pressure and the crude product was recrystallized from methanol and ether to give final compound.
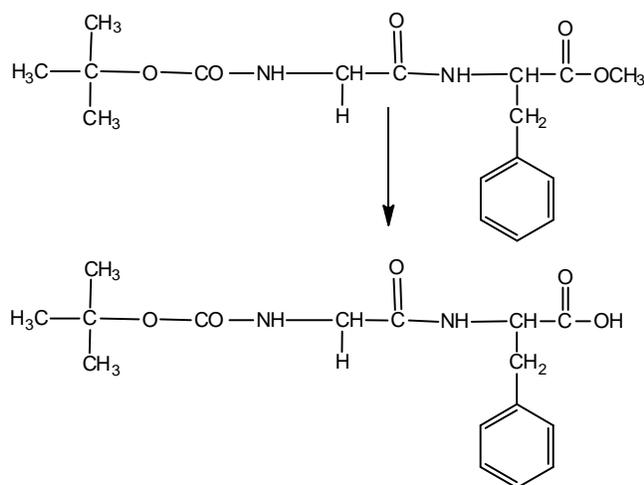
The detailed procedure and discussion behind it is given in the proceeding paragraphs.

Deprotection of dipeptides at carboxyl end

At carboxyl end, deprotection was done using the mixture of tetrahydrofuran and water solvent with ratio of 1:1 and lithium hydroxide at 0°C. The mixture was stirred at room temperature for 1 hr, and acidified to pH 3.5 with sulphuric acid The extraction of aqueous layer was carried out thrice with diethyl ether. The organic extracts were combined and dried over anhydrous sodium sulphate. It was concentrated under reduced
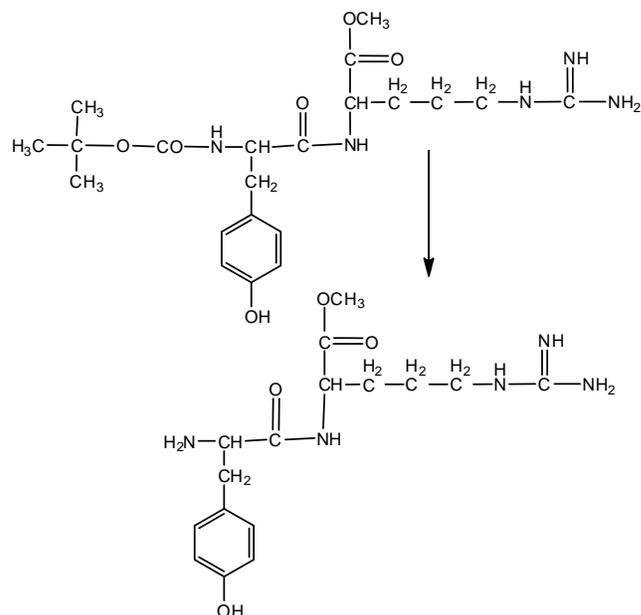
Deprotection of dipeptides at amino end

Compound (DIPEPTIDE) was dissolved in chloroform and treated with trifluoroacetic acid. The resulting solution was stirred at room temperature for 1 hr and washed with saturated sodium bicarbonate solution. The organic layer was dried over anhydrous sodium sulphate and concentrated under reduced pressure. The crude product was recrystallized from mixture of chloroform and light petroleum ether (b.p. 40-60°C) to give brown semisolid mass of compound. The deprotected dipeptides were further used for the synthesis of tripeptides.

Synthesis of boc-tripeptide methyl esters

Tripeptides were prepared in the same way as that of dipeptides. Compound (METHYL ESTER) (2.05 gm,

10 mmol) was dissolved in 20 ml of chloroform and 2.3 ml of NMM (21 mmol) was added at 0°C. The reaction mixture was stirred for 15 min. Compound (DIPEPTIDE) (1.75 gm, 10 mmol) in 20 ml of chloroform and 2.1 gm of DCC (10 mmol) were added under stirring to the above mixture. After 36 hr, the reaction mixture was filtered and the residue was washed with 30 ml of chloroform and added to the filtrate. The filtrate was washed with 25 ml of 5% sodium hydrogen carbonate and 25 ml of saturated sodium chloride solution. The organic layer was dried over anhydrous sodium sulphate, filtered and evaporated in vacuum. The crude product was recrystallized from mixture of chloroform and light petroleum ether (b.p. 40-60°C) followed by cooling at 0°C to give yellow orange semisolid mass of compound.

Continuing on the same line of thought, tetrapeptides, pentapeptides, hexapeptides were synthesized in the similar manner as described above.

Synthesis of linear peptides

Here, for the synthesis if cyclic peptides, two types of linear peptides were first formed:

· Linear pentapeptides methyl ester

· Linear hexapeptides methyl ester

Synthesis of linear pentapeptides methyl ester

Compound dipeptide (10 mmol) was dissolved in 20 ml of chloroform and *N*-methylmorpholine (21 mmol) was added at 0°C. The reaction mixture was stirred for 15 min. Protected tripeptide (10 mmol) in chloroform and *N,N*2 - Dicyclohexylcarbodiimide (10 mmol) were added under stirring to the above mixture. After 36 hr, the reaction mixture was filtered and the residue was washed with 30 ml of chloroform and added to the filtrate. The filtrate was washed with 25 ml of 5% sodium bicarbonate and 25 ml of saturated sodium chloride solution. The organic layer was dried over anhydrous sodium sulphate, filtered and evaporated in vacuum to afford the final product.

Synthesis of linear hexapeptides methyl ester

The procedure is same for the linear

Linear Peptide

Cyclic Peptide

Boc-Gly-Phe-Ser OH + Tyr-Arg OMe

KLP

KCP Cyclo (Gly-Phe-Ser-Tyr-Arg)

Boc-Ala-Phe-Ser OH   +   Tyr-Arg OMe

KLP1

KCP1 Cyclo(Ala-Phe-Ser-Tyr-Arg)

Boc-Gly-Tyr-Ser OH   +   Phe-Arg OMe

KLP2

KCP2 (Cyclo(Gly-Tyr -Ser-Phe-Arg)
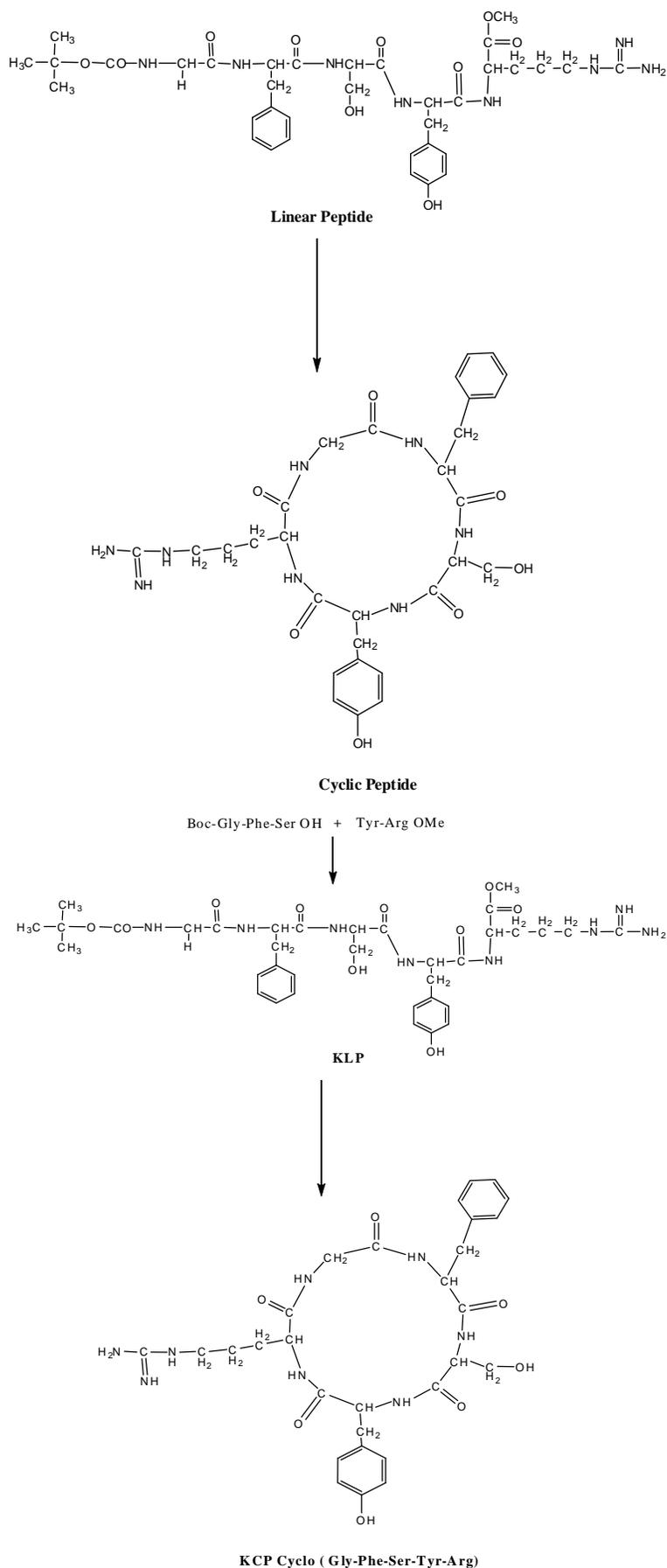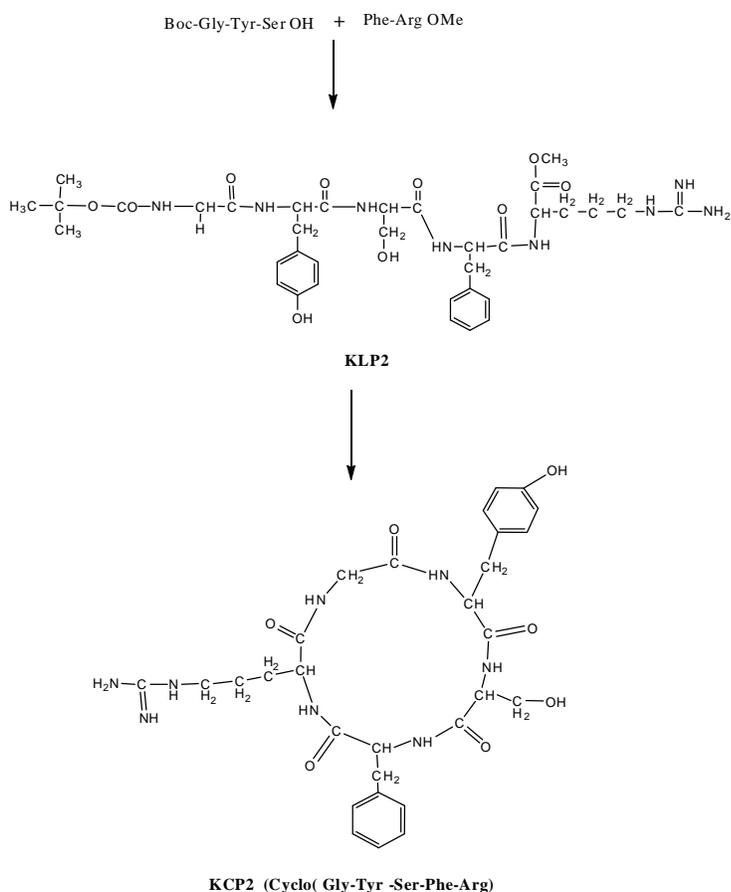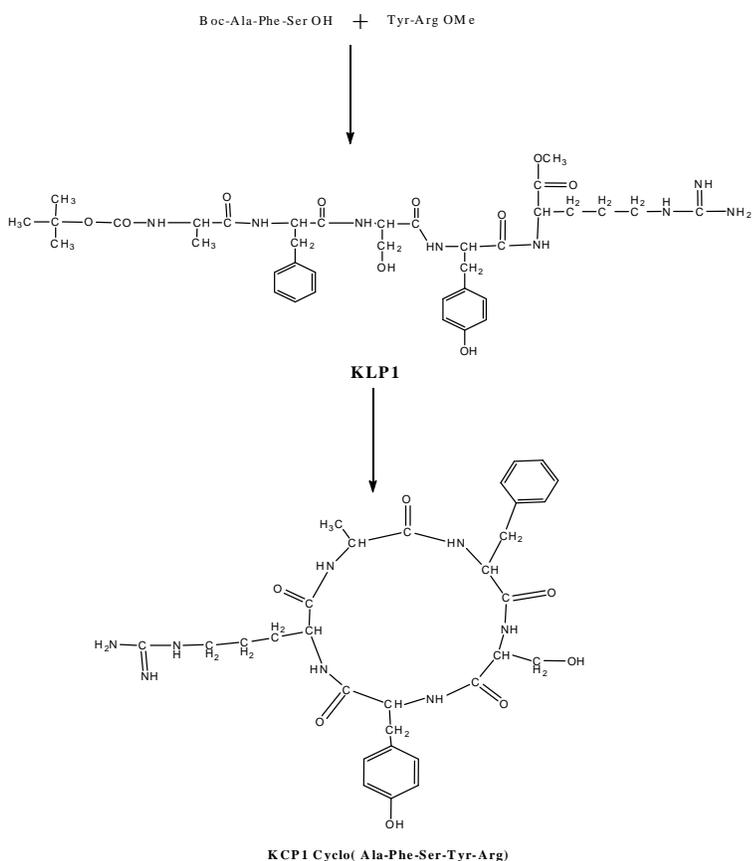
hexapeptides as that of pentapeptides except the type of peptides, here tripeptides were used instead of dipeptides.

Synthesis of cyclic peptides

Compound LINEAR PEPTIDE (5 mmol) was dissolved in chloroform at 0°C and 6.7 mmol of *p*-nitrophenol was added to it, stirred at room temperature for 12 hr. The resulting mixture was filtered and the filtrate was washed with 10% sodium bicarbonate solution until excess of *p*-nitrophenol was removed and finally washed with 5% hydrochloric acid (2 x 10 ml) to get the corresponding p-nitrophenyl ester Boc-peptide-O-pnp.

The resulting protected peptide (4 mmol) was dissolved in chloroform and trifluoroacetic acid (8 mmol) was added, stirred at room temperature for 1 hr followed by washing with 10% sodium bicarbonate solution (2 x 25 ml). The organic layer was dried over anhydrous sodium sulphate to get peptide-O-pnp, which was further dissolved in chloroform and NMM (21 mmol). Then all the contents were kept at 0°C for 7 days. The *p*-nitrophenol was obtained by washing the reaction mixture with 10% sodium bicarbonate solution and then was finally washed with 5% hydrochloric acid (3 x 15 ml). The organic layer was dried over anhydrous sodium sulphate. Finally, chloroform was distilled off and the crude cyclized product was recrystallized from chloroform and *n*-hexane. The detailed reactions of the final compounds are given below in the schemes.

Conclusion

Novel derivatives of cyclic peptide have been synthesized following protection and deprotection techniques in which tetrapeptides, pentapeptides, hexapeptides were joined through peptide bonds. Acyclic and cyclic amino acids were chosen depending upon the importance of the same in the biological system. The derivatives were characterized by physicochemical and spectral techniques such as IR, ^{1}H NMR, and Mass. The spectral data obtained was in full agreement with the proposed structures. The *in vitro* evaluation of the newly synthesized compounds has been done through culture media. Finally, it can be concluded that, in scheme, KLP1 and KLP2 showed some activity as compare to standard drugs against gram positive and gram negative bacteria but cyclization of all linear peptides lead

Boc-Ala-Tyr-Ser OH   +   Phe-Arg OMe

KLP3

KCP3 (Cyclo(Ala-Tyr -Ser-Phe-Arg)
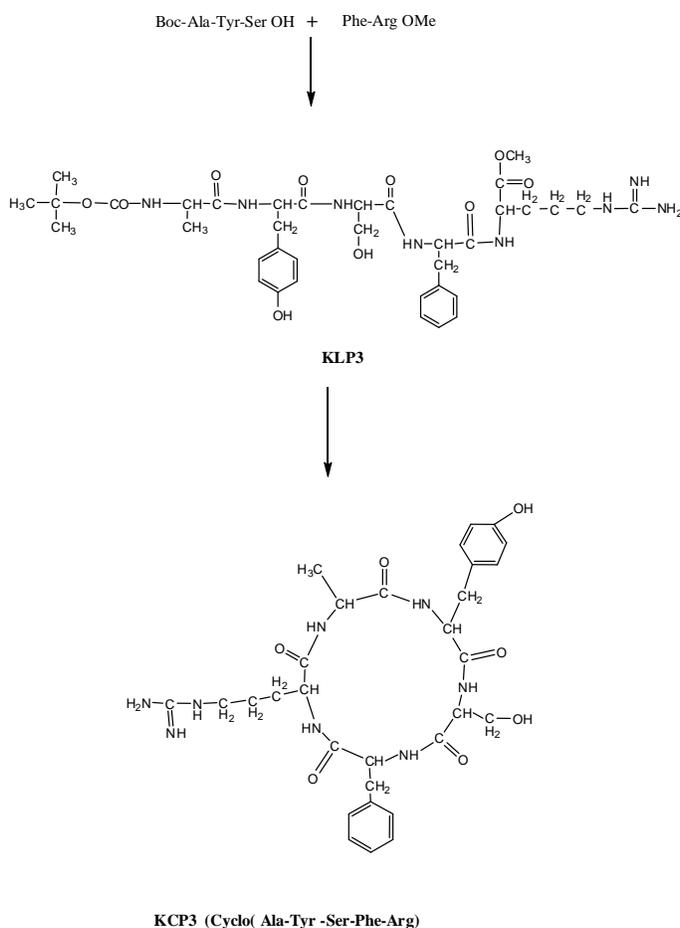
to decline in antibacterial activity.

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