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ANTIMICROBIAL ACTIVITY OF OCTAPEPTIDES RELATED TO HUMAN HISTATIN 8

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ABSTRACT

Objective: To Synthesize, characterize and antimicrobial evaluation of octapeptides related to human histatin 8. **Method**: Solid phase peptide synthesis (SPPS) by Fluorenyl methyl oxycarbonyl (Fmoc) chemistry protocol. **Results:** The N-terminal portion of HH-1, HH-2 and HH-3 showed high potential against gram negative bacteria. The peptides were evaluated for antimycobacterial activity against *Mycobacterium tuberculosis* H_{37} RV and Clinical isolates of *M. tuberculosis* resistant strain by Luciferase Reporter Phage assay (LRP). Among the tested peptides, the peptide HH3 has been showed maximum percentage of reduction in relative light units (RLU) against *Mycobacterium tuberculosis*- H37 Rv and clinical isolate of *M. Tuberculosis* resistant at the concentration of 100 µg/mL. **Conclusion:** octapeptides related to human histatin 8 were synthesized, characterized and exhibited promising antibacterial, antifungal and antimycobacterial activity.

KEYWORDS: Antimicrobial peptides; Solid Phase Peptide Synthesis; Human histatin 8; Luciferase Reporter Phage assay; *Mycobacterium tuberculosis*.

1. INTRODUCTION

An emerging current issue is the multiple drug resistant pathogen, nowadays increasing its occurrence. The most six scariest microbes are *Staphylococcus aureus, Escherichia coli, Klebsiella, Acinetobacter baumanni, Aspergillus, Pseudomonas aeruginosa*. The Infectious Diseases Society of America, having more than 8,000 infectious-disease specialists, has

announcing a hit list of these six most worrisome germs facing in their clinical practice. Tuberculosis (TB) is a most treacherous disease caused by *Mycobacterium tuberculosis.* Right now the emergence of multiple drug resistance and human immune virus (HIV) associated tuberculosis. According to World Health Organization (WHO) more than 13 million deaths related to infectious diseases, including tuberculosis have been reported worldwide. As per the global tuberculosis scenario 2013[1] 3.5% of new and 20.5% of previously treated TB cases were estimated to have had MDR-TB in 2013. This translates into an estimated 480 000 people having developed MDR-TB in 2013. On average, an estimated 9.0% of patients with MDRTB had extensively drug resistant TB (XDR-TB). If all notified TB patients (6.1 million, new and previously treated) had been tested for drug resistance in 2013, an estimated 300 000 cases of MDR-TB would have been detected, more than half of these in three countries alone: India, China and the Russian Federation. In 2013, 136000 of the estimated 300000 MDR-TB patients who could have been detected were diagnosed and notified. This was equivalent to almost one in two (45%) and up from one in six in 2009. Progress in the detection of drug-resistant TB has been facilitated by the use of new rapid diagnostics *Mycobacterium tuberculosis* affects about one third of the human population and represents the infectious condition causing more death worldwide, particularly in India nearly one third of the global population of tuberculosis about 2 million people acquire TB every year.[2]

Peptides are an alternative for heterocyclic compounds and other type of antibiotics. The antimicrobial peptides[3] are an essential part of innate immunity of the resistance microorganisms. These are found in plants, animals and humans containing 6 to 60 amino acids present in the sequence of the peptides.[4-5] The sequence of the peptides consists of cationic amino acids like arginine or lysine or histidine and hydrophobic amino acids like leucine, alanine etc. The basic mechanism of these molecules is killing or inhibition by pore forming or channel forming or translocation. Human Histatins are a group of cationic, histidine-rich antimicrobial peptides secreted from human saliva.[6] These are secreted in the subcellular location and tissue specificity of submandibular-sublingual and parotid glands. These are found many antimicrobial activity against broad spectrum bacteria and fungus.[7] The mechanism of action of these peptides likes nonlytic ATP efflux, protease activity, neutralization of lipopolysaccharides. Human histatin 8 consist of 12 amino acid in the sequence of the peptide having active against gram positive and gram negative and fungi.

Solid phase peptide synthesis[8-12] was introduced by Bruce Merrifield in 1963**.** The most of the peptide synthesized by solid phase peptide synthesis, usually containing less than 20 amino acids. Synthesis of such peptides is a routine and straightforward without significant complications. Solid phase peptide synthesis is based on the sequential addition of amino acid to an insoluble polymer bound resin and the Fluorenyl methyl oxy-carbonyl (FMOC) group which is used for n-alpha protection. The protected amino acid is added using either a coupling reagent or pre-activated mixer and the resulting peptide is attached to the resin through C terminals and cleaved to yield an amide. The cleavage of the peptide from resin and side chain deprotection should be done simultaneously. Deprotection of the FMOC protecting group by 20% piperidine in DMF. N-N-dimethyl formamide (DMF) are the primary solvent used for resin deprotection, coupling and washing of the peptide. Synthesis can be carried out in a continuous flowing manner and the resin is contained in a column through which reagents and solvents are pumped continuously again under manual or automatic control. FMOC strategy is fully compatible with the continuous flow method which, depending on the instrument used allows for real time spectral photo metric monitoring of the progress of coupling and deprotection. Final cleavage of peptide resin and side chain deprotection requires strong acid, such as trifluoro acetic acid (TFA) in FMOC chemistry.

The molecular docking studies were completed against DNA Gyraze and 14 α Demethylase and the work was published.[13] Very short peptide[14-15] residues have been identified and reported to be active against bacteria. Some of them even show broad spectrum activity.[15] Ionic/charge interactions and hydrophobicity only play a major role in eliciting the antimicrobial activity. Several lipopeptides have been shown to exhibit both antibacterial and antifungal properties.[16] Fatty acylation of linear peptides has, generally, resulted in enhanced membrane association of the peptides.

The goal of the present research is to synthesize an N terminal portion, modified N terminal portion and acyl octapeptide related to human histatin 8. Characterize by amino acid sequence analysis and mass spectroscopy using LC-MS-MS. Investigate and compare the antibacterial and antifungal activity (*in vitro*) of the peptides by minimum inhibitory concentration and zone of inhibition. Evaluate the *in vitro* anti-mycobacterial activity of the peptides by percentage of reduction in relative light units against *Mycobacterium tuberculosis*

H_{37}Rv and clinical isolate of *M. tuberculosis* (Streptomycin (S), Isoniazide(H), Rifampicin (R) and Ethambutol (E) resistant).

2. MATERIALS AND METHODS

2.1 Chemicals and solvents

Chemicals, amino acids, coupling reagents, polymer bound resin and scavengers were received from SIGMA ALDRICH Pvt Ltd. Solvents were received from S D Fine Chem, Mumbai. Nutrient broth (NB), Nutrient Agar (NA), Peptone water and antibiotics amikacin, Ciprofloxacin were procured from Hi-media laboratories, Mumbai, India. Sabouraud Dextrose Broth (SDB), Sabouraud Dextrose Agar (SDA), Peptone water and antibiotic Ketoconazole were procured from Hi-media laboratories, Mumbai, India. DMSO was procured from E. Merck Ltd., Mumbai, India. The microbial cultures *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa and Candida albicans* were procured from National Centre for Industrial Microorganisms (NCIM), Pune, India. *Mycobacterium tuberculosis* H_{37}Rv and Clinical isolate of *M.tuberculosis* (Streptomycin (S), Isoniazid (H), Rifampicin (R) and Ethambutol (E) resistant) from Department of Bacteriology, National Institute for Research in Tuberculosis (ICMR), Chetput, Chennai, Tamilnadu, India.

2.2 Instruments

The manual Peptide synthesizer was purchased from R.K Scientific, Chennai. Mass spectrum and amino acid sequence analysis were recorded on true analytical LC-MS-MS**.** Rotavapor Buchi Rotavapor R-114 and Centrifuge - Eppendorf centrifuge 5424. HPLC – AGILENT 1200 series, binary pump. MS –ABI 3000 SCIEX. Column C18-AGILENT XDB 150 mm, 4.6mm, 5 micron. The incubator was recorded from TECHNICHO Ltd.

2.3 Method

The peptides were synthesized (fig no 2) by solid phase peptide synthesis using FMOC chemistry protocols.[17-18]

2.4 Procedure for synthesis

2.4.1 Synthesis of octapeptide related to human histatin-8 (HH1)

2.4.1.1 Chemicals

The sequence of the peptide (K F H E K H H S - CONH2) contains eight amino acid and the chemicals were used in the synthesis of HH1 is shown in table no 1.

2.4.1.2 Resin activation

About 250 mg of Rink amide methyl benzhydryl amine (MBHA) resin was allowed to swell in dry dimethyl formamide (DMF) for about an hour and then the excess DMF was decanted. To the resin was added 10 mL 20% piperidine (figure no 1) in DMF swirled for 8 minutes and then decanted. This procedure was repeated once again to ensure complete removal of the FMOC protective group. The resin was washed with 6 mL of dry DMF (six times with 2 minute interval between the successive washes) before attaching the first amino acid to the resin.

2.4.1.3 First amino acid attachment

FMOC-L-serine was mixed with tetra-methyl uranium hexa-fluoro phosphate (HBTU) and hydroxyl benzotriazole (HOBT) in 1 mL dimethyl formamide (DMF). Di-isopropyl ethyl amine (DIPEA) was added to the milky colloidal solution and sonicated to get a clear solution (figure no 1). The mixture became a transparent yellow solution after one minute. Then the contents were added to the activated resin and gently shaken for 90 minutes continuously. Then the resin was washed with 6 mL of dry DMF (six times with 2 minutes interval between the successive washes) to remove the excess active ester. The negative response to ninhydrin tests indicated 100% attached to the resin.

2.4.1.4 FMOC deprotection and coupling of remaining amino acid

After washing the resin with dry DMF, about 10 mL of 20% piperidine was added and shaken for 8 minutes and the operation was repeated for one more time . Finally the resin was washed with 6 mL of dry DMF (figure no 1) (six times with 2 minutes interval between the successive washes). The positive response to ninhydrin tests indicated 100% detachment of FMOC from the resin. HH1 was synthesized by solid Phase Peptide Synthesis (SPPS) using a manual peptide synthesizer. The flow chart protocol for the synthesis of HH1 is listed in table no 2.

2.4.1.5 Cleavage of the peptide HH1 from resin

The peptide bound resin was extensively washed with 10 mL of dichloromethane (DCM) for two times, then 10 mL of acetic acid followed by 10 mL of DCM twice and finally with 10 mL of solvent ether for two times. Then peptide bound resin was treated with a mixture of 0.2 mL ethanedithiol, 0.2 mL M-cresol, and 7 mL TFA (figure no 1). The contents were occasionally shaken for one hour and filtered through sintered crucible, washed the resin with TFA and the filtrate was concentrated under high vacuum to remove the residual TFA to get

slurry mass. The slurry mass was triturated with ice-cold ether and centrifuged to decant ether. The white precipitate obtained was dried under the stream of nitrogen and stored in a refrigerator until further study.

2.4.2 Synthesis of modified octapeptide related to Human histatin-8 (HH2)

2.4.2.1 Chemicals

The sequence of the peptide (K F L K K L L K- CONH2) contains eight amino acid and the chemicals were used in the synthesis of HH2 is shown in table no 3.

2.4.2.2 Resin activation

About 500 mg of Rink amide MBHA Resin was allowed to swell in dry DMF for about an hour and then the excess DMF was decanted. To the resin was added 10 mL 20% piperidine (figure no 2) in DMF swirled for 8 minutes and then decanted. This procedure was repeated once again to ensure complete removal of the FMOC protective group. The resin was washed with 6 mL of dry DMF (six times with 2 minutes the interval between the successive washes) before attaching the first amino acid to the resin.

2.4.2.3 First amino acid attachment

FMOC-L-Lysine was mixed with HBTU and HOBT in 1 mL DMF (figure no 2). DIPEA was added to the milky colloidal solution and sonicated to get a clear solution. The mixture became a transparent yellow solution after one minute. Then the contents were added to the activated resin and gently shaken for 90 minutes continuously. Then the resin was washed with 6 mL of dry DMF (six times with 2 minutes interval between the successive washes) to remove the excess active ester. The negative response to ninhydrin tests indicated 100% attached to the resin.

2.4.2.4 FMOC deprotection and coupling of remaining amino acid

After washing the resin with dry DMF, about 10 mL of 20% piperidine was added (figure no 2) and shaken for 8 minutes and the operation was repeated for one more time. Finally the resin was washed with 6 mL of dry DMF (six times with 2 min. interval between the successive washes). The positive response to ninhydrin tests indicated 100% detachment of FMOC from the resin. HH2 was synthesized by Solid Phase Peptide Synthesis (SPPS) using a manual peptide synthesizer. The flow chart protocol for the synthesis of HH2 is listed in table no 4.

2.4.2.5 Cleavage of the peptide HH2 from resin

The peptide bound resin was extensively washed with 10 mL of dichloromethane (DCM) for two times, then 10 mL of acetic acid followed by 10 mL of dichloromethane twice and finally with 10 mL of solvent ether for two times. Then peptide bound resin was treated with a mixture of 0.2 mL ethanedithiol, 0.2 mL M-cresol, and 7 mL TFA (figure no 2). The contents were occasionally shaken for one hour and filtered through sintered crucible, washed the resin with TFA and the filtrate was concentrated under high vacuum to remove the residual TFA to get slurry mass. The slurry mass was triturated with ice-cold ether, and centrifuged to decant ether. The white precipitate obtained was dried under the stream of nitrogen and stored in a refrigerator until further study.

2.4.3 Synthesis of acyl octapeptide related to human histatin-8 (HH3)

2.4.3.1 Chemicals

The sequence of the peptide (FA-K F L K K L L K- CONH2) contains eight amino acid along with fatty acid (lauric acid) and the chemicals were used in the synthesis of HH3 is shown in the table no 5.

2.4.3.2 Synthesis of acyl octapeptide

About 400 mg of resin bound octapeptide was added 10 mL 20% piperidine in DMF swirled for 8 minutes and then decanted. This procedure was repeated once again to ensure complete removal of the FMOC protective group. The resin was washed with 6 mL of dry DMF (six times with 2 minutes the interval between the successive washes) before attaching the lauric acid to the resin. Lauricacid was mixed with HBTU in 1 mL DMF (figure no 3). DIPEA was added to the milky colloidal solution and sonicated to get a clear solution. The contents were added to the modified octapeptide bound resin and gently shaken for 60 minutes continuously. Then the resin was washed with 6 mL of dry DMF (six times with 2 minutes interval between the successive washes) to remove the excess active ester.

2.4.3.3 Cleavage of the peptide HH3 from resin

The peptide bound resin was extensively washed with 10 mL of dichloromethane for two times, then 10 mL of acetic acid followed by 10 mL of dichloromethane twice and finally with 10 mL of solvent ether for two times. Then peptide bound resin was treated with a mixture of 0.2 mL ethanedithiol, 0.2 mL M-cresol, and 7 mL TFA (figure no 3). The contents were occasionally shaken for one hour and filtered through sintered crucible, washed the resin with TFA and the filtrate was concentrated under high vacuum to remove the residual

TFA to get slurry mass. The slurry mass was triturated with ice-cold ether, and centrifuged to decant ether. The white precipitate obtained was dried under the stream of nitrogen and stored in a refrigerator until further study.

2.5 Characterization

2.5.1 Amino acid sequence analysis

0.3 mg of sample taken in 10 mL volume of container and adds 6N HCl solution in 5 ml and passes small volume of nitrogen gas and close the container immediately with the cap sealed and kept in hot air oven for 23 hours. After completion take the container out and kept for cooling at room temperature**.**

2.6 *In-Vitro a*ntimicrobial activity

2.6.1 Antifungal activity-zone of inhibition (well diffusion method)

The antifungal activity of the synthesized compounds was studied systematically against C*andida albicans*. The standard procedure was followed from Fan SR, Liu XP., 2008.[19]

2.6.2 Antibacterial activity[20]

The antibacterial activity of the synthesized compounds was studied systematically against four different strains of bacteria *Staphylococcus aureus, Bacillus subtillis* (Gram-positive), and *Escherichia coli, Pseudomonas vulgaris* (Gram-negative). The standard procedure was followed from Satyajit *et al.*, 2007.

2.6.3 Antifungal activity[21]

The antifungal activity of the synthesized compounds was studied systematically against C*andida albicans.* The standard procedure was followed from Gibson *et al*., 2002.

2.6.4 Antimycobacterial activity by Luciferase reporter phage assay (LRP)[22]

Antimycobacterial activity was evaluated by Luciferase Reporter Phage assay (LRP) against *M. tuberculosis* H_{37}Rv, Clinical isolate (S, H, R and E resistant). The standard procedure was followed from Sivakumar PM *et al*., 2007.

3. RESULTS

Synthesized the antimicrobial octa-peptides related to human histatin 8 by solid phase peptide synthesis (SPPS) using fluorenyl methyl oxycarbonyl (FMOC) chemistry protocol. The structural integrity of the peptides and the amino acid sequence analysis of the peptides were confirmed by LC-MS-MS method. It is clear from the mass spectral data that the chemical

entity (HH1) was present as indicated by the appearance of a M+1 peak at 1049.9 (table no 6). The sequence analysis of the histidine rich octa peptide (HH1) was present in the analyte peak named as glutamic acid (E), histidine (H), lysine (K), phenylalanine (F), serine (S) at the counts of analyte peak area 1410000, 42300000, 9270000, 163000, 1540000 and the retention time of analyte was shown at 4.70, 4.23, 4.16, 6.35, 4.35 (minutes). The concentration of the amino acids was found to be at 3.78 µg/mL (E), 28.1 µg/mL (H), 10.2 µg/mL (K), 2.32 µg/mL (F), 4.36 µg/mL (S) is shown in the table no 7. The mass spectral data of the modified octapeptide (HH2) were indicated by the appearance of a M+1 peak at 1017.2 (table no 6). The sequence analysis of the modified octapeptide was present in the analyte peak named as lysine (K), phenylalanine (F) and leucine (L) at the counts of analyte peak area likes 5100000, 2800 and 6650000 and the retention time of analyte was found to be 4.18, 6.41, 6.42 (minutes). The concentration of the analytes was established at 5.59 µg/mL (K), 0.0314 µg/mL (F), 0.291 µg/mL (L) is listed in the table no 7. The mass spectral data of the acyl octapeptide (HH3) were indicated by the appearance of a M+1 peak at 1199.2 (table no 6). The sequence analysis of the acyl octapeptide was present as the analyte peak named as lysine (K), phenylalanine (F) and leucine (L) at the counts of analyte peak area likes 6560000, 39000 and 8860000 and the retention time of analyte was found to be 4.18, 6.86, 6.26 (minutes). The concentration of the analytes was shown at 7.20 µg/mL (K), 0.547 µg/mL (F), 0.391 µg/mL (L) is revealed in the table no 7. The antifungal activity (zone of inhibition) of the compounds were evaluated against *Candida albicans* by the well diffusion method. The zone of inhibition of the compound HH1, HH2 and HH3 was found to be 3 mm, 3 mm and 8 mm at the concentration of 100 µg/mL. The compound HH1, HH2 and HH3 was found to be 12 mm, 13mm and 10 mm at the concentration of 200 µg/mL as compared with the standard is shown in the figure no 4. The antibacterial activity (minimum inhibitory concentration) of the compounds was evaluated against *Staphylococcus aureus, Bacillus subtilis* (gram positive) and *Escherichia coli, Pseudomonas aeruginosa* (gram negative) by 96 well plate method. The minimum inhibitory concentration of the compounds HH1 and HH2 was found to be at 50 µg/mL against both gram positive and gram negative organisms. The compound HH3 was shown the complete inhibition at the concentration of 50 µg/mL against gram negative organisms and 100 µg/mL against gram positive organisms as compared with the standard is shown in the figure no 5. The antifungal activity (minimum inhibitory concentration) of the compounds was investigated against C*andida albicans* by 96 well plate method. The minimum inhibitory concentration of the compounds HH1, HH2 and HH3 was found to be at 200 µg/mL against C*andida albicans* as compared with the standard

is shown in the figure no 6. Anti-mycobacterial activity were evaluated against *M.tuberculosis* H_{37}Rv and clinical isolates of *M.tuberculosis* (resistant to Streptomycin (S), Isoniazid (H), Rifampicin (R) and Ethamputol (E)) by Luciferase Reporter Phage assay (LRP). The percentage of reduction in relative light units (RLU) of the compounds HH3 and HH2 were found to be potent antimycobacterial activity of 52.12% and 45.72% at the concentration of 100 µg/mL against *Mycobacterium tuberculosis* H_{37} Rv as compared with control is shown in the figure no 7. The compound HH3 was found to be extremely potential reduction of 60.50% in the concentration of 100 µg/mL against S, H, R and E resistant as compared with control. The compound HH2 was found potential reduction of 46.11% in the concentration of 100 µg/mL against clinical isolates of *M.tuberculosis* S, H, R and E resistant. The compound HH3 and HH2 were found to be a moderate reduction of 44.87 % and 37.23% at the concentration of 50 µg/mL against clinical isolates S, H, R and E resistant as compared with control is shown in the figure no 8.

4. DISCUSSION

Short peptides have been identified and reported to be active against bacteria and some of them exhibit broad spectrum activity.[15] Ionic/charge interactions and hydrophobicity only play a major role in eliciting the antimicrobial activity. HH2 is a short peptide having 8 amino acid present in the sequence of the peptide. The potent antibacterial, antifungal and antimycobacterial activity of the peptide due to the presence of hydrophobic amino acid and cationic amino acid equally nearby in the sequence of the peptide. The peptide has 4 positively charged lysine residues and it can tightly bound to the negatively charged bacterial membranes and bring out antimicrobial activity. Compare the antibacterial and antifungal activity of these three peptides, HH2 was more potent than HH1 and HH3 due to the presence of hydrophobic and cationic amino acid present in the sequence of the peptides. Several lipopeptides have been shown to exhibit both antibacterial and antifungal properties (24). Fatty acylation of linear peptides has, generally, resulted in enhanced membrane association of the peptides. Acyl peptide (HH3) is more hydrophobic than HH2 due to the N-terminal charge is covered by the acylation using lauric acid. However, the increased hydrophobicity of the peptide would help to raise the potency of the entire peptide. The acylation at the N-terminus of the peptide can increase the overall hydrophobicity of the peptide and improve the binding affinity for lipid membranes particularly in the cell wall of the *Mycobacterium tuberculosis*. The outer layer of the Mycobacterium cell wall is surrounded by mycolic acid which is made up of long chain fatty acid. The mycolic acid is covalently linked with

arabinogalactan.[1] The peptide having binding affinity with the mycolic acid due to the presence of lauric acid and four hydrophobic amino acid (leucine). The four cationic amino acid (lysine) which is strongly bound with the negative charge of the bacterial membrane. HH3 can be expected to behave as a non-lytic antimicrobial lipopeptide due to the attachment of lauric acid in the N terminus of the peptide. It is significance here that non-lytic short antimicrobial peptides are better for pharmaceutical applications.[26] The binding affinity of cationic amino acid, hydrophobic amino acid and N-terminal acylation with the cell wall of the mycobacteria can retain the antimycobacterial activity.

Table No 1. Chemicals for the synthesis of the HH1

S.No	Chemicals Quantity	Quantity (mmole)
01	Rink amide MBHA resin	250 mg (0.2)
02	Fmoc-Ser (TBU) -OH	383.44mg (1.0)
03	Fmoc-His (TRT) -OH	3X309.85mg (0.5)
04	Fmoc-Lys (B0C) -OH	2x234.27mg (0.5)
05	Fmoc-Giu (Otbu) -OH	212.73mg (0.5)
06	Fmoc-Phe-OH	193.71mg (0.5)
07	HBTU (First Amino acid)	371.5mg (0.98)
08	HBTU (Remaining Amino acid)	180mg (0.48)
09	HOBT (First Amino acid)	153mg (1.0)
10	HOBT (Remaining Amino acid)	76.5mg (0.5)
11	DIPEA (First Amino acid)	0.348 mL (2.0)
12	DIPEA (Remaining Amino acid)	0.174mL (1.0)
13	DMF	500 mL
14	20% Piperidine in DMF	300 mL
15	Ninhydrin Solution	50 mL

Table No 2. Flow chart protocol for the synthesis of HH1

Deprotection		Ninhydrin Test	Amino acid	Amino Acid Substitution		Ninhydrin Test
20% Piperidine	DMF Wash			Activated Amino acid	DMF wash	
2 X 10 mL (2 X 8 min)	6 X 6 mL (6 X 2 min)	Positive	Fmoc-Ser (TBU) -OH	1.5- 2.0 mL (90 min)	6 X 6 mL (6 X 2min)	Negative
2 X 10 mL (2 X 8 min)	6 X 6 mL (6 X 2 min)	Positive	Fmoc-His (TRT) -OH	1.5- 2.0 mL (90 min	6 X 6 mL (6 X 2min)	Negative
2 X 10 mL (2 X 8 min)	6 X 6 mL (6 X 2 min)	Positive	Fmoc-His (TRT) -OH	1.5- 2.0 mL (90 min	6 X 6 mL (6 X 2min)	Negative
2 X 10 mL (2 X 8 min)	6 X 6 mL (6 X 2 min)	Positive	Fmoc-Lys (B0C) -OH	1.5- 2.0 mL (90 min	6 X 6 mL (6 X 2min)	Negative
2 X 10 mL (2 X 8 min)	6 X 6 mL (6 X 2 min)	Positive	Fmoc-Giu (Otbu) -OH	1.5- 2.0 mL (90 min	6 X 6 mL (6 X 2min)	Negative
2 X 10 mL (2 X 8 min)	6 X 6 mL (6 X 2 min)	Positive	Fmoc-His (TRT) -OH	1.5- 2.0 mL (90 min	6 X 6 mL (6 X 2min)	Negative
2 X 10 mL	6 X 6 mL	Positive	Fmoc-Phe-OH	1.5- 2.0 mL	6 X 6 mL	Negative

(2 X 8 min)	(6 X 2 min)			(90 min	(6 X 2min)	
2 X 10 mL (2 X 8 min)	6 X 6 mL (6 X 2 min)	Positive	Fmoc-Lys (Boc) -OH	1.5- 2.0 mL (90 min	6 X 6 mL (6 X 2min)	Negative
2 X 10 mL (2 X 8 min)	6 X 6 mL (6 X 2 min)	Positive				

Table No 3. Chemicals for the synthesis of the HH2

S.No	Chemicals Quantity	Quantity(mmole)
01	Rink amide MBHA resin	500 mg (0.4)
02	Fmoc-Lys (Boc) -OH	937.08 mg (2.0)
03	Fmoc-Leu-OH	3X353.4mg (1.0)
04	Fmoc-Lys (B0C) -OH	3x468.54mg (1.0)
05	Fmoc-Phe-OH	387.43mg (1.0)
06	HBTU (First Amino acid)	743.4 mg (1.96)
07	HBTU (Remaining Amino acid)	360.3 mg (0.95)
08	HOBT (First Amino acid)	306.3 mg (2.0)
09	HOBT (Remaining Amino acid)	153.1 mg (1.0)
10	DIPEA (First Amino acid)	0.696 mL (4.0)
11	DIPEA (Remaining Amino acid)	0.348 mL (2.0)
12	DMF	500 mL
13	20% Piperidine in DMF	200 mL
14	Ninhydrin Solution	50 mL

Table No 4. Flow chart protocol for the synthesis of HH2

Deprotection		Ninhydrin Test	Amino acid	Amino Acid Substitution		Ninhydrin Test
20% Piperidine	DMF Wash			Activated Amino acid	DMF wash	
2 X 10 mL (2 X 8 min)	6 X 6 mL (6 X 2 min)	Positive	Fmoc-Lys (B0C) -OH	1.5- 2.0 mL (90 min)	6 X 6 mL (6 X 2min)	Negative
2 X 10 mL (2 X 8 min)	6 X 6 mL (6 X 2 min)	Positive	Fmoc-Leu-OH	1.5- 2.0 mL (90 min	6 X 6 mL (6 X 2min)	Negative
2 X 10 mL (2 X 8 min)	6 X 6 mL (6 X 2 min)	Positive	Fmoc-Leu-OH	1.5- 2.0 mL (90 min	6 X 6 mL (6 X 2min)	Negative
2 X 10 mL (2 X 8 min)	6 X 6 mL (6 X 2 min)	Positive	Fmoc-Lys (B0C)-OH	1.5- 2.0 mL (90 min	6 X 6 mL (6 X 2min)	Negative
2 X 10 mL (2 X 8 min)	6 X 6 mL (6 X 2 min)	Positive	Fmoc-Lys (B0C)-OH	1.5- 2.0 mL (90 min	6 X 6 mL (6 X 2min)	Negative
2 X 10 mL (2 X 8 min)	6 X 6 mL (6 X 2 min)	Positive	Fmoc-Leu-OH	1.5- 2.0 mL (90 min	6 X 6 mL (6 X 2min)	Negative
2 X 10 mL (2 X 8 min)	6 X 6 mL (6 X 2 min)	Positive	Fmoc-Phe-OH	1.5- 2.0 mL (90 min	6 X 6 mL (6 X 2min)	Negative
2 X 10 mL (2 X 8 min)	6 X 6 mL (6 X 2 min)	Positive	Fmoc-Lys (Boc)-OH	1.5- 2.0 mL (90 min	6 X 6 mL (6 X 2min)	Negative
2 X 10 mL (2 X 8 min)	6 X 6 mL (6 X 2 min)	Positive				

Table No 5. Chemicals for the synthesis of the HH3

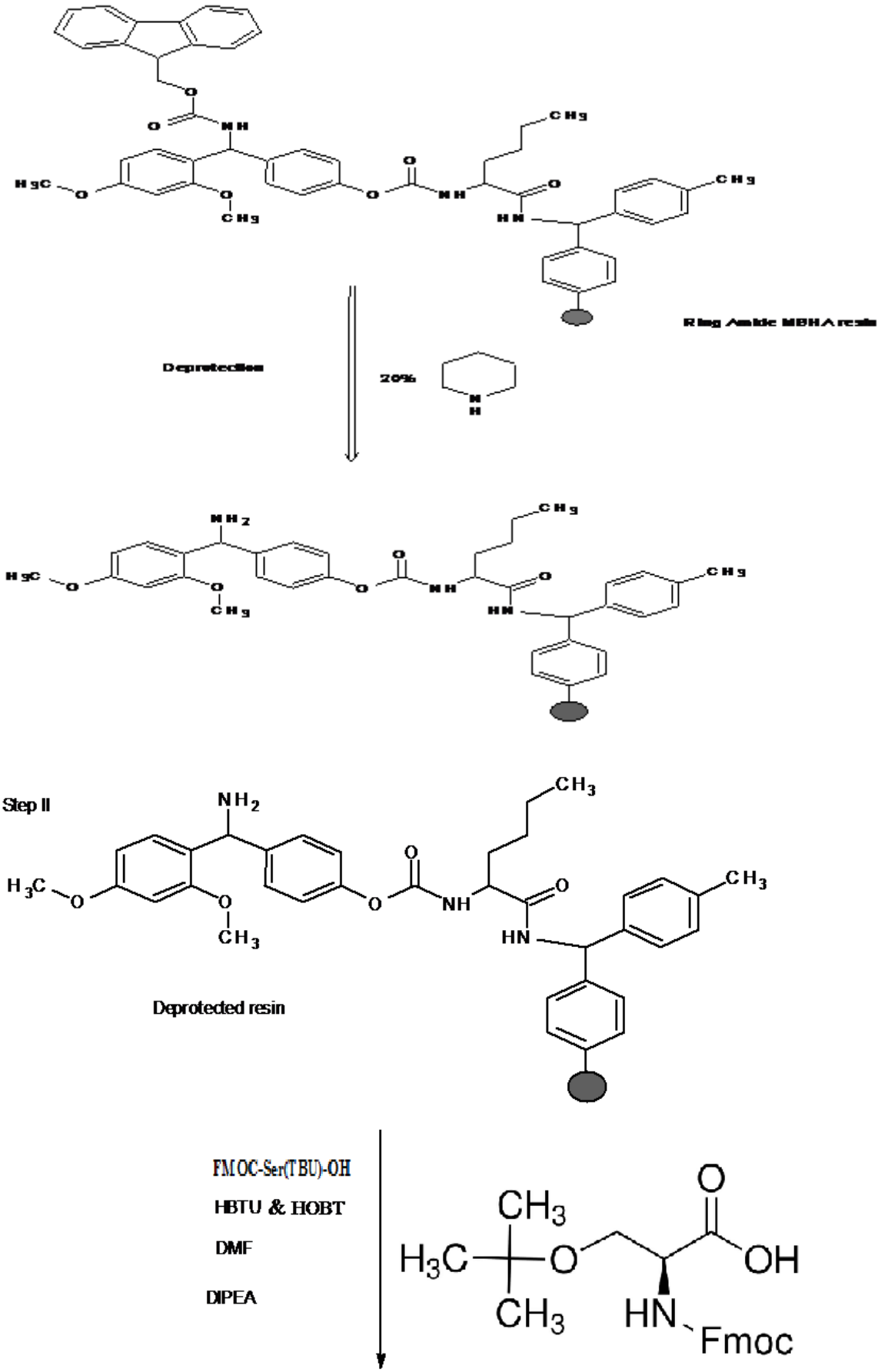
S.No	Chemicals Quantity	Quantity(mmole)
1.	Resin with pep	400 mg
2.	Lauric acid	200mg(1.0)
3.	HBTU	180.2 mg (1.96)
4.	DIPEA (First Amino acid)	0.174mL (1.0)
5.	DMF	75mL
6.	20% Piperidine in DMF	20 mL
7.	Ninhydrin Solution	2 mL

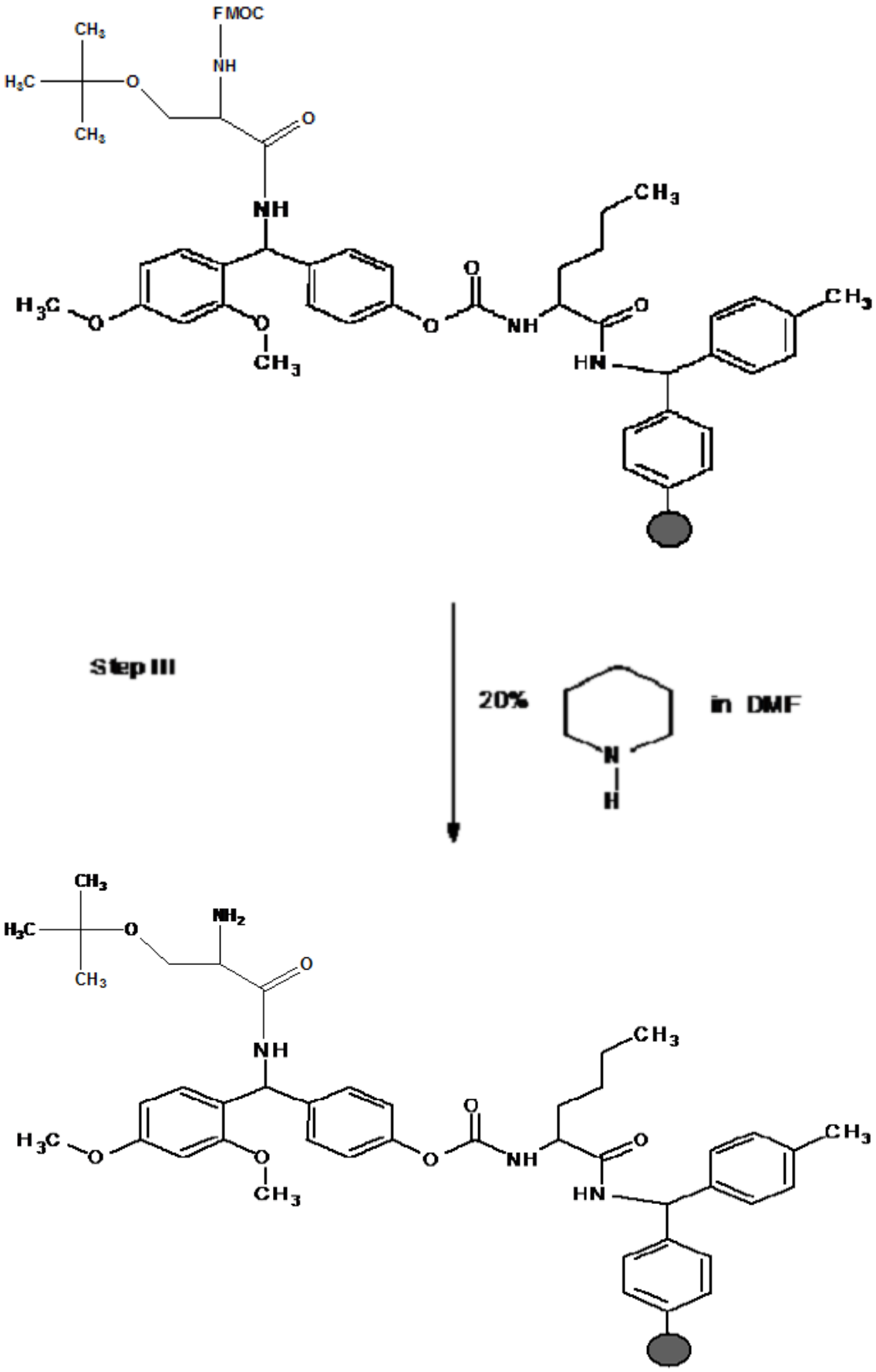
Table No 6. Mass spectrum of the octapeptides related human histatin 8

S.No	Compound code	Molecular formula	Molecular weight	Mass peak
01	HH1	$C_{47}H_{72}N_{17}O_{11}$	1048	1049.9
02	HH2	$C_{51}H_{93}N_{13}O_{8}$	1016	1017.2
03	HH3	$C_{63}H_{115}N_{13}O_{9}$	1198	1199.2

Table No 7. Amino acid sequence analysis of octapeptides related human histatin 8

S.No	Sample name	Analyte peak name	Analyte peak area	Analyte retention time	Calculated concentration(µg/mL)
01	HH1	Glutamic acid	1410000	4.70	3.78
02	HH1	Histidine	42300000	4.23	28.1
03	HH1	Lysine	9270000	4.16	10.2
04	HH1	Phenylalanine	163000	6.35	2.32
05	HH1	Serine	1540000	4.35	4.36
06	HH2	Lysine	5100000	4.18	5.59
07	HH2	Phenylalanine	2800	6.41	0.0314
08	HH2	Leucine	6650000	6.42	0.291
08	HH3	Lysine	6560000	4.18	7.20
09	HH3	Phenylalanine	39000	6.86	0.547
10	HH3	Leucine	8860000	6.26	0.391


Deprotection
20%
Step II
Deprotected resin
FMOC-Ser(TBU)-OH
HBTU & HOBT
DMF
DIPEA
Fmoc


FMOC
NH
CH3
H3C
O
CH3
O
NH
CH3
H3C
O
O
CH3
O
NH
O
HN
CH3
Step III
20%
in DMF
N
H
NH2
NH
HN

Repeat step II & III for each amino acid additon

(coupling and deprotection)

Fmoc-His(TRT)-OH

Fmoc-His(TRT)-OH

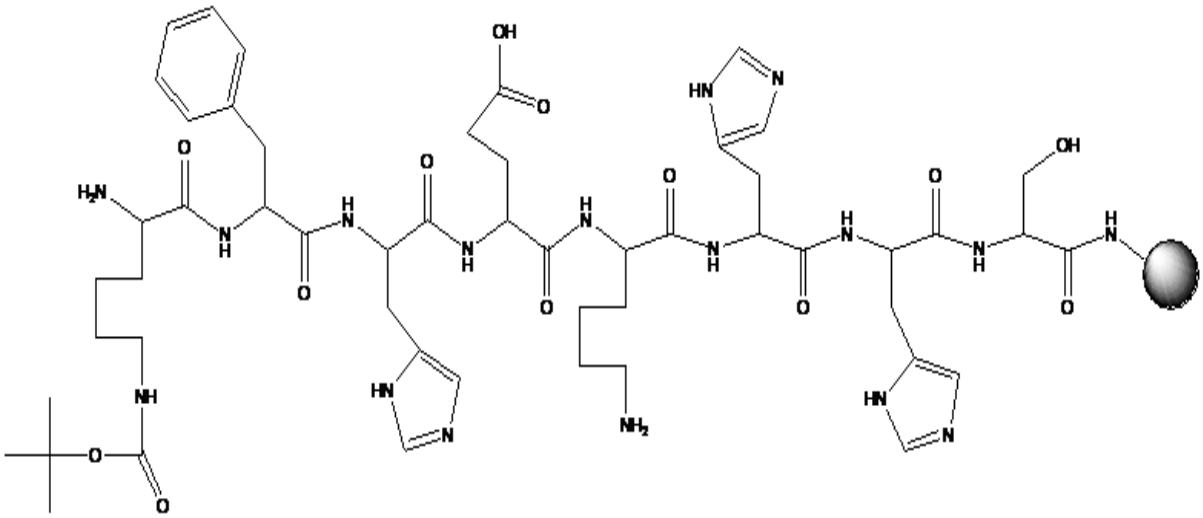
Fmoc-Lys(B0C)-OH

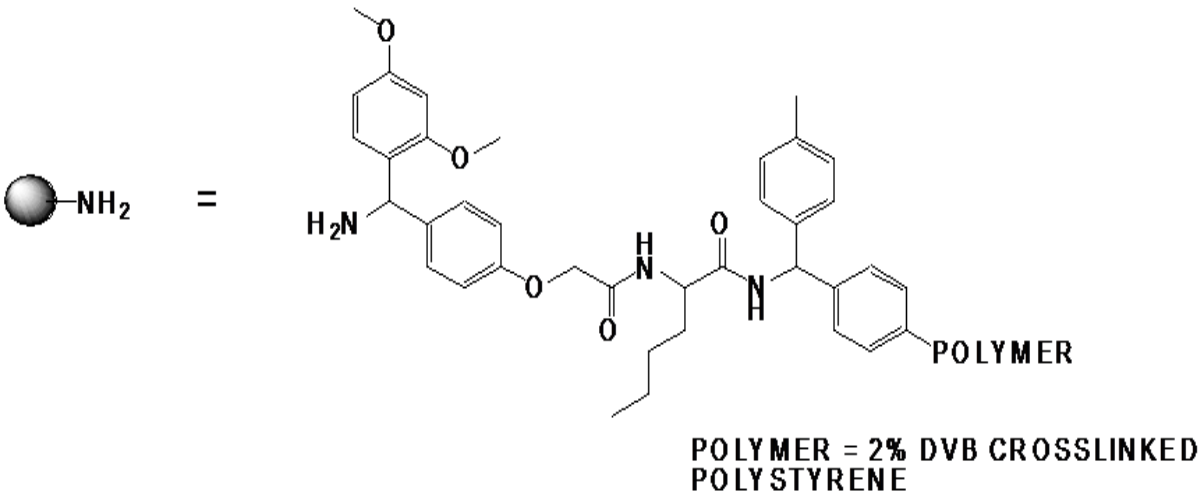
Fmoc-Giu(Otbu)-OH

Fmoc-His(TRT)-OH

Fmoc-Phe-OH

Fmoc-Lys(B0C)-OH

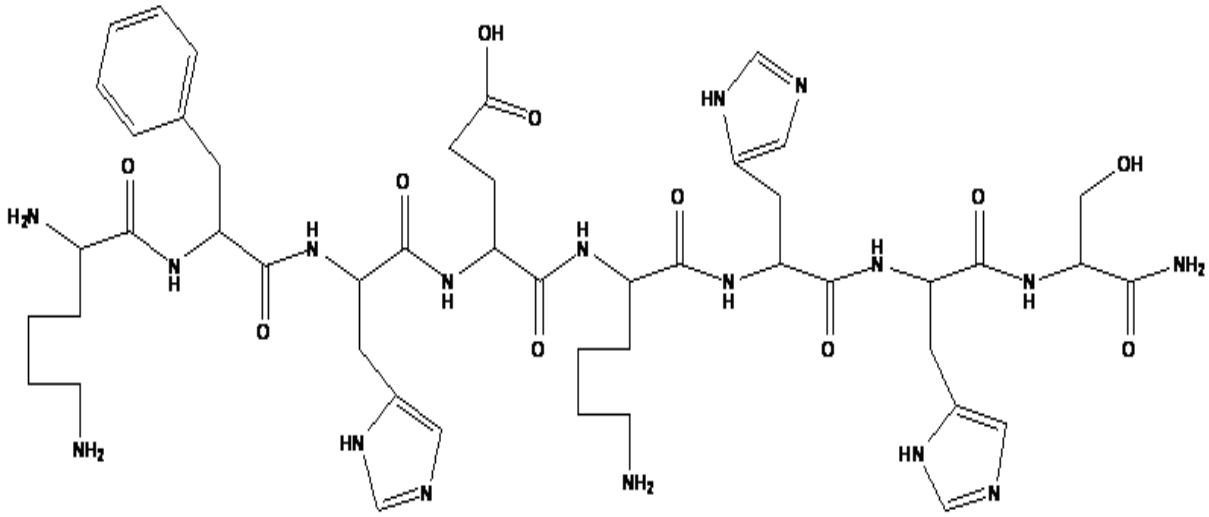




M-Cresol

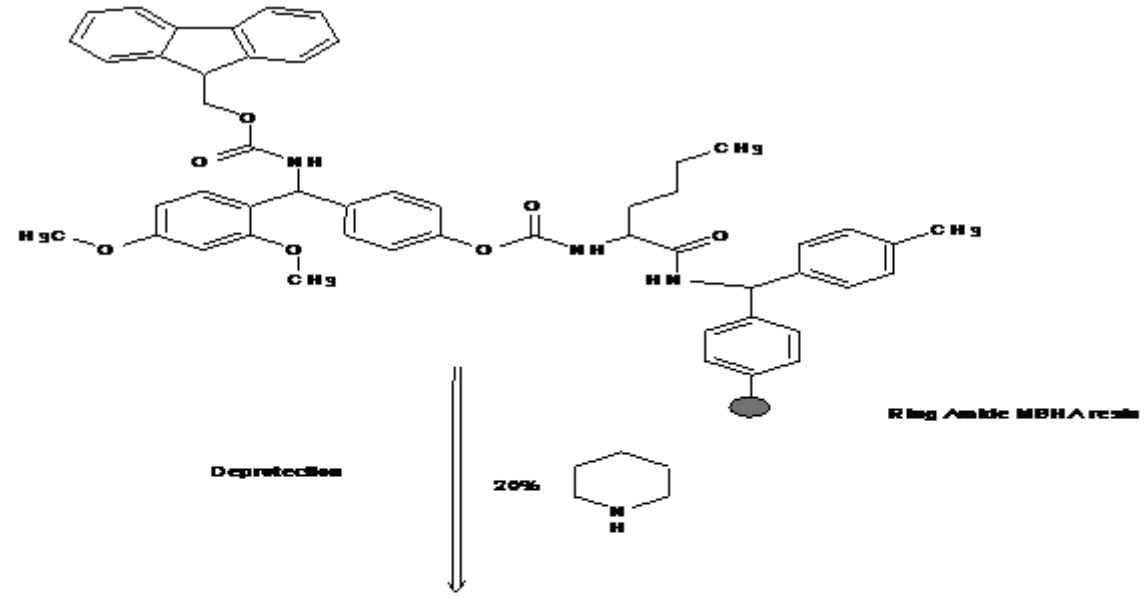
Ethane di-thiol **Trifluoroaceticacid**

Thioanisole

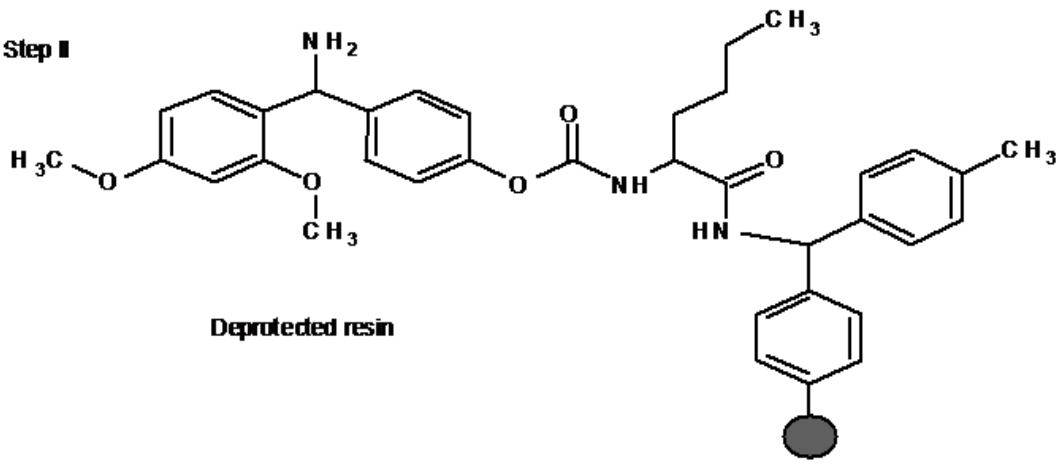


HH1

Figure No 1. Synthetic scheme of the hexapeptide related to Human histatin 8 (HH1).



Step II



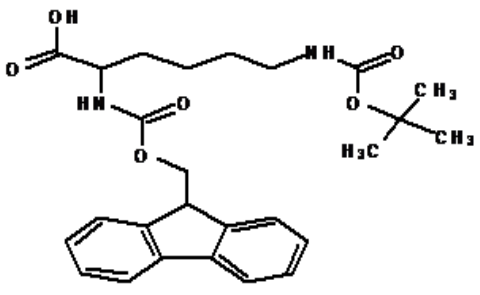
Deproteded resin

FMOC LYS(BOC)-OH

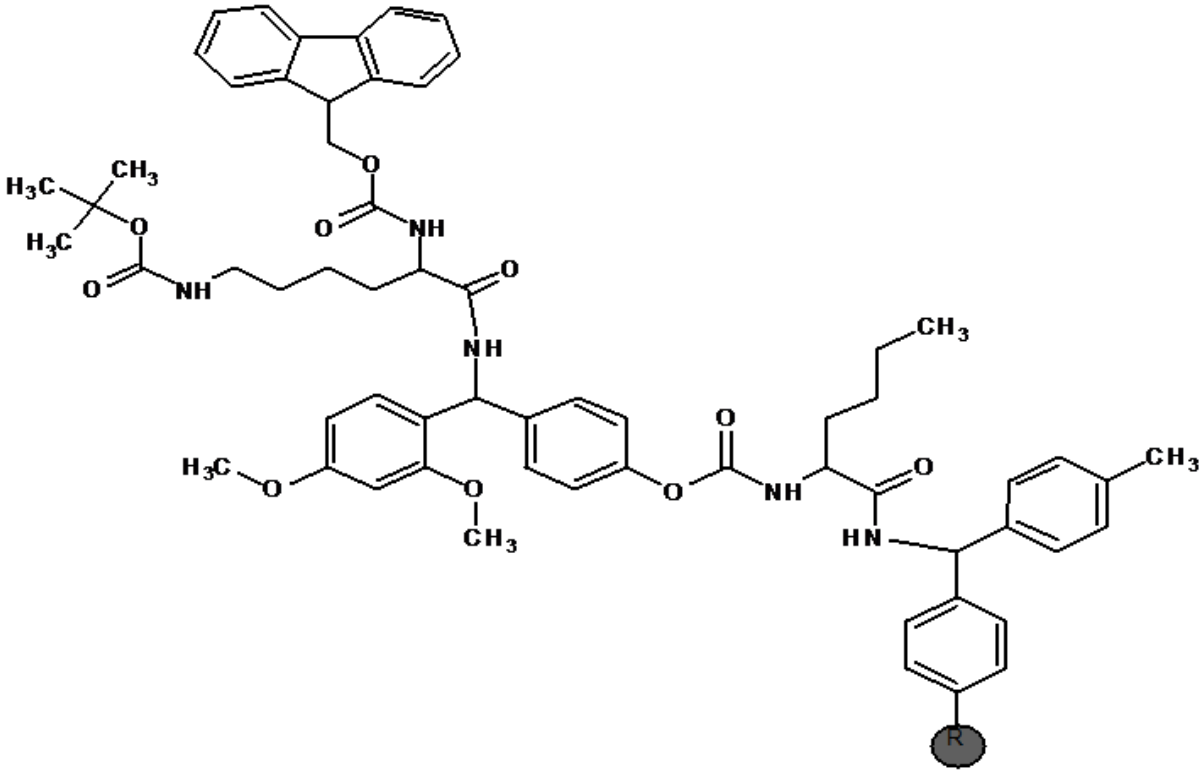
HBTU

DMF

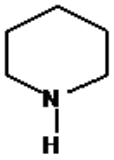
DIPEA

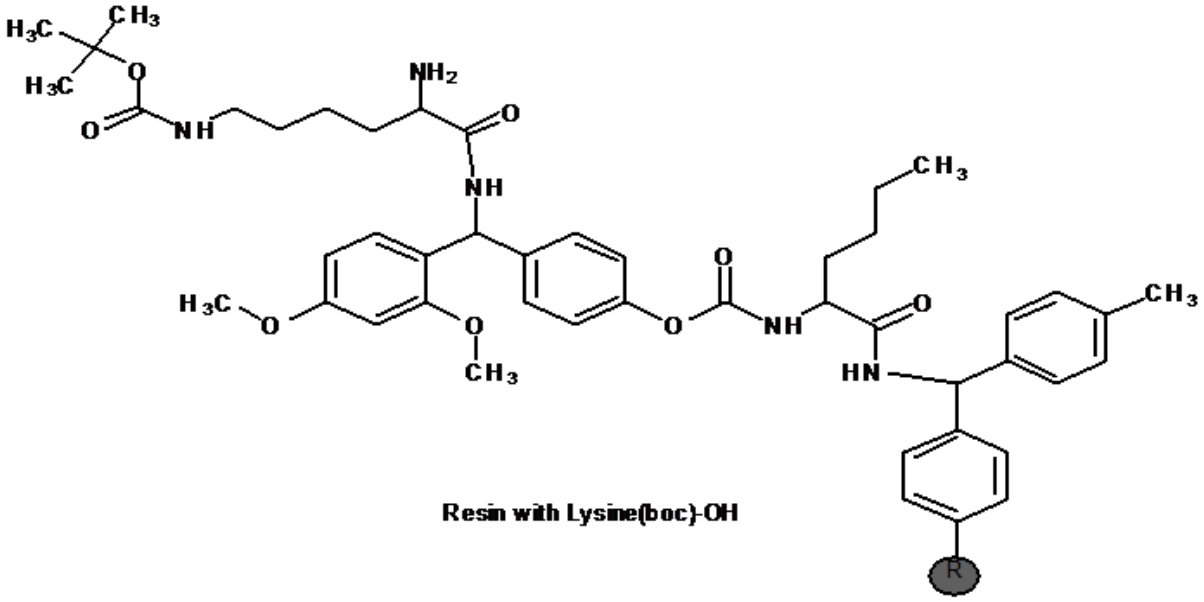


Resin containing FMOC-Lysine



Step III

20%  in DMF



Resin with Lysine(boc)-OH

Repeat step II & III for each amino acid additon

(coupling and deprotection)

Fmoc-Leu-OH

Fmoc-Leu-OH

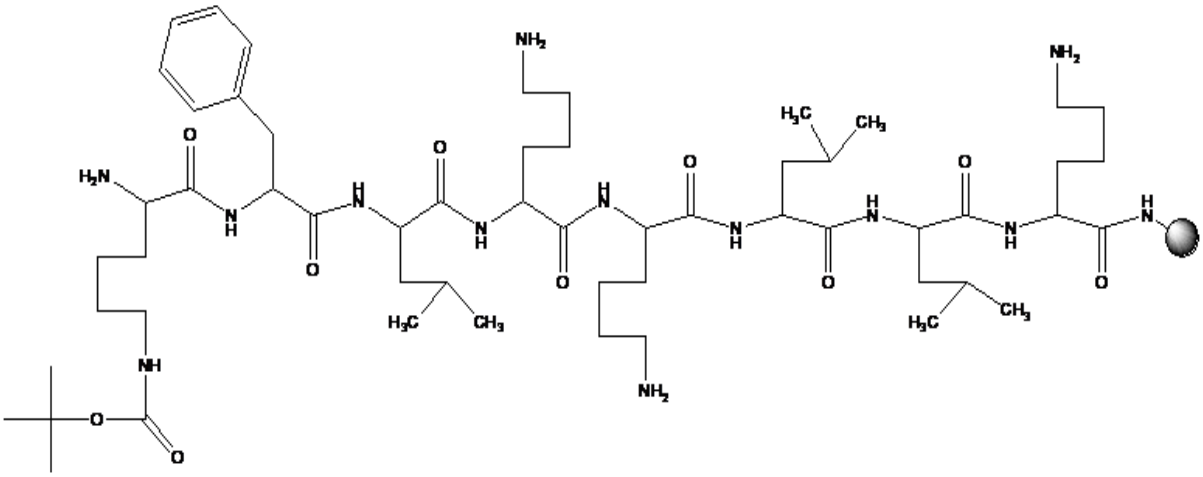
Fmoc-Lys(B0C)-OH

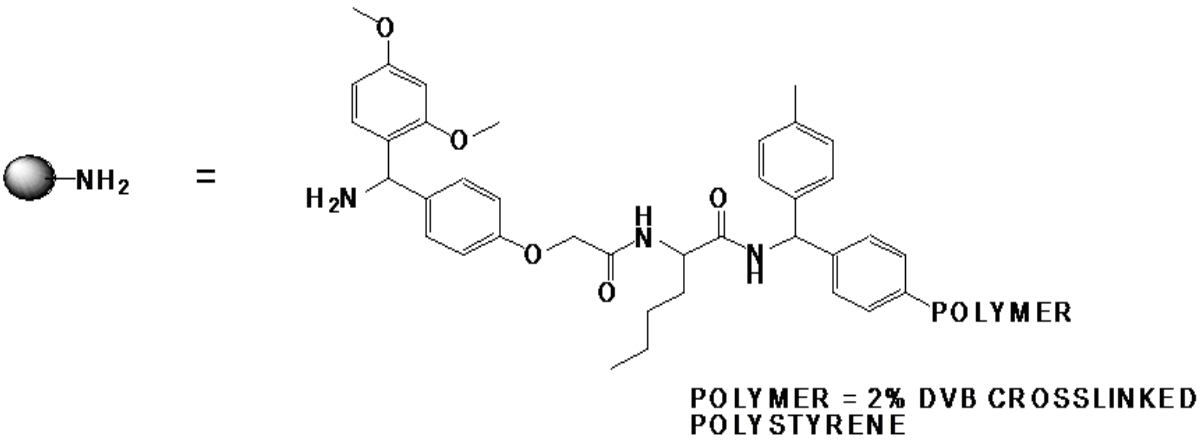
Fmoc-Lys(B0C)-OH

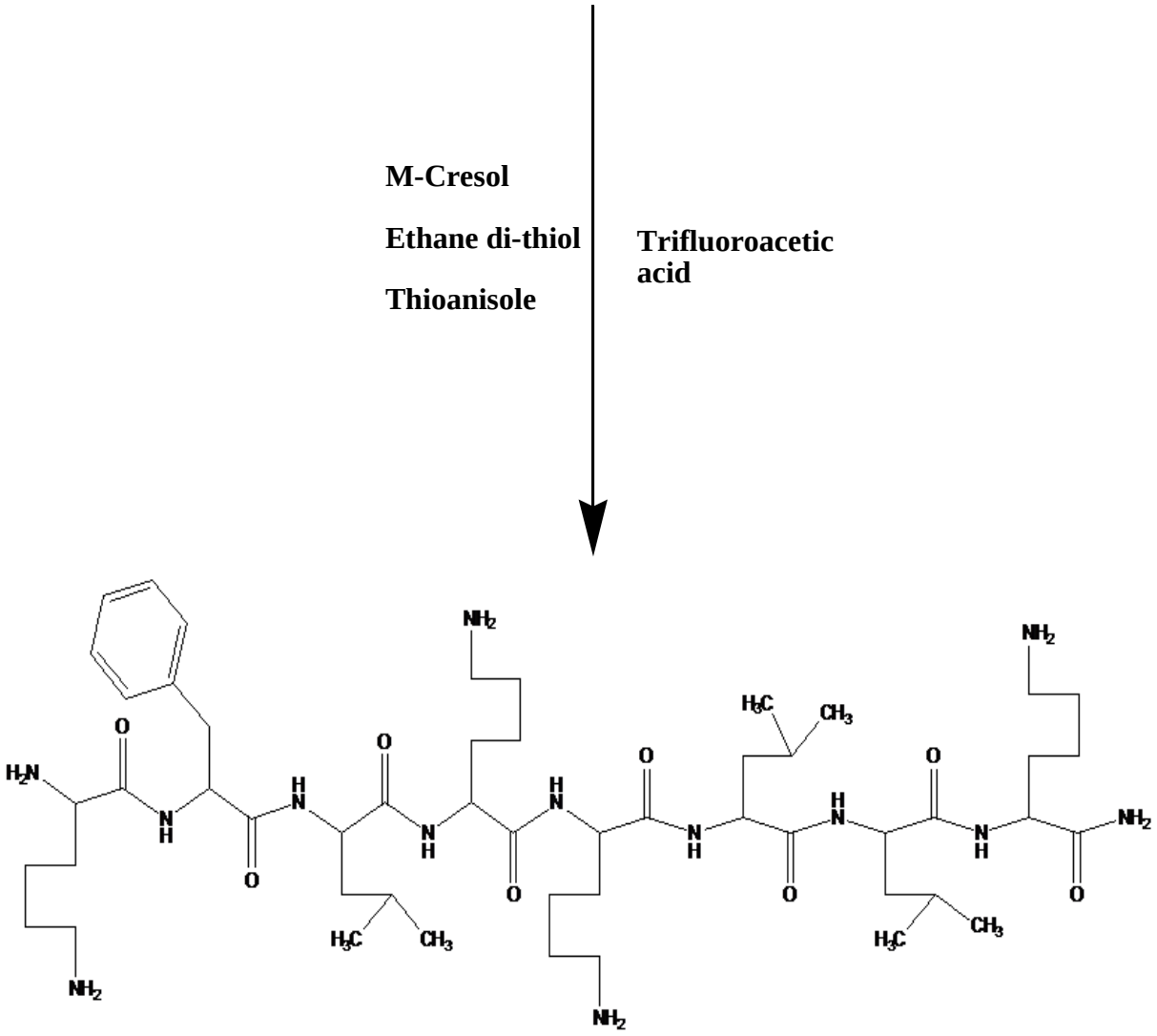
Fmoc-Leu-OH

Fmoc-Phe-OH

Fmoc-Lys(B0C)-OH

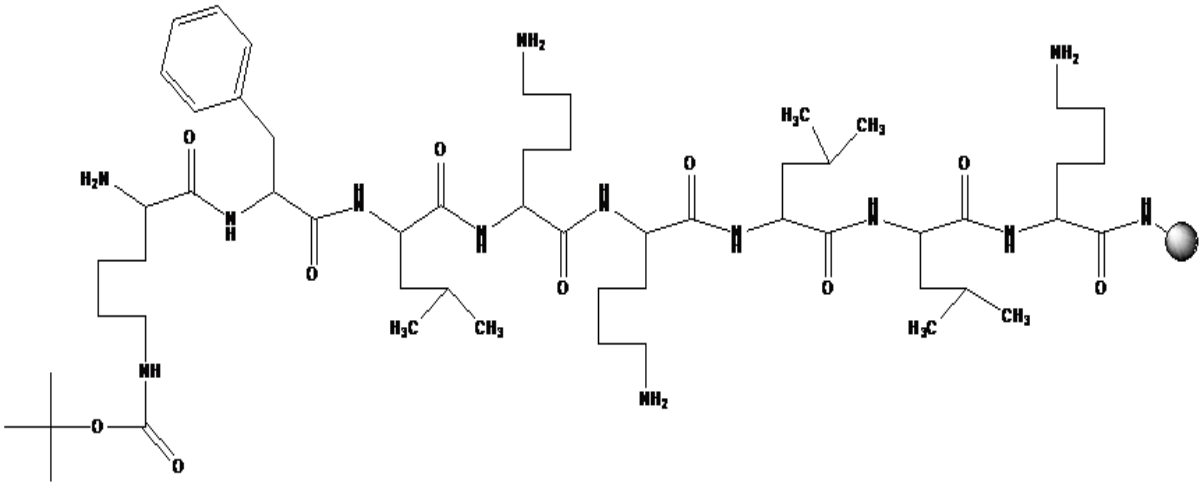


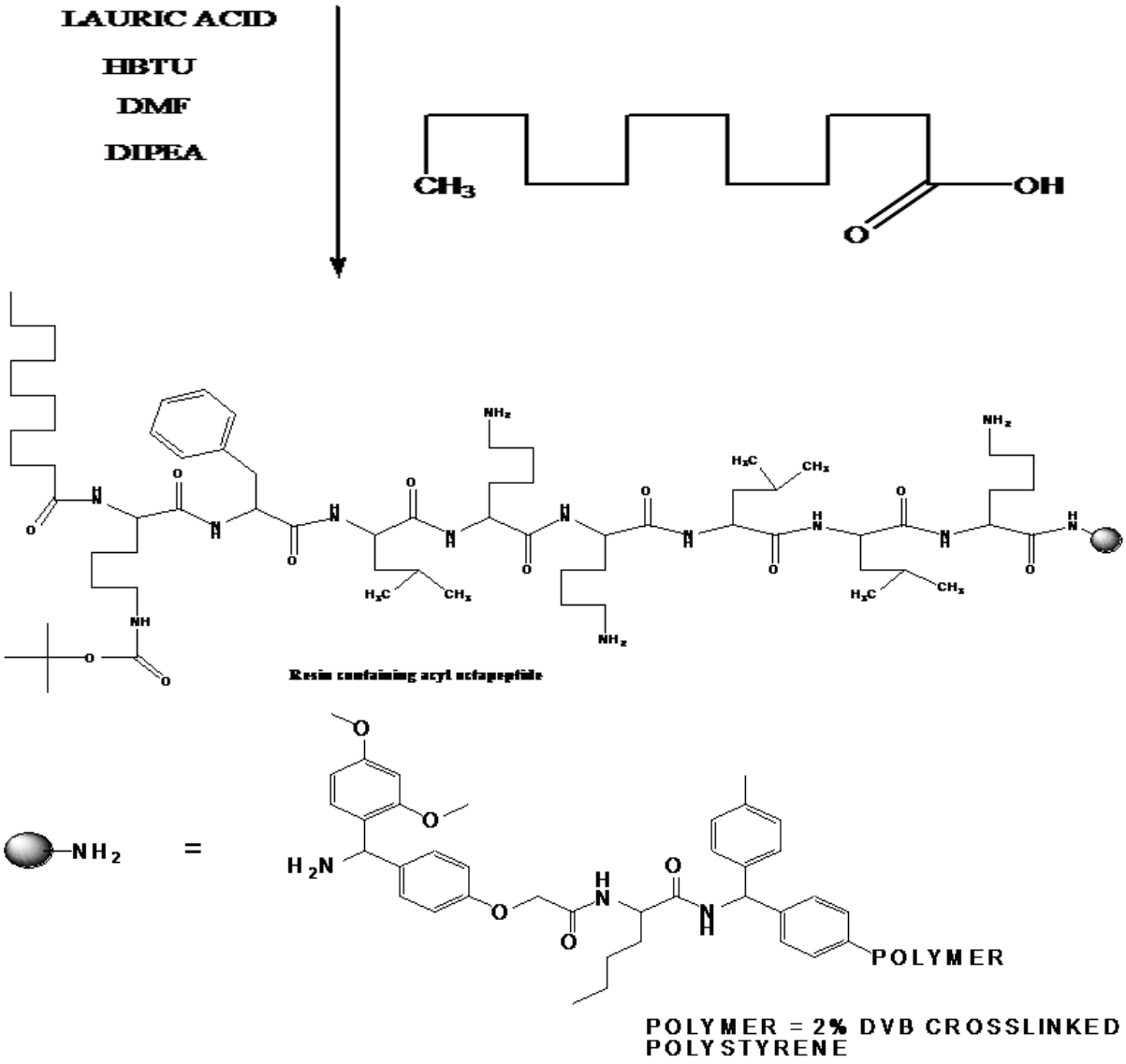




HH2

Figure No 2. Synthetic scheme of the modified hexapeptide related to Human histatin 8 (HH2).



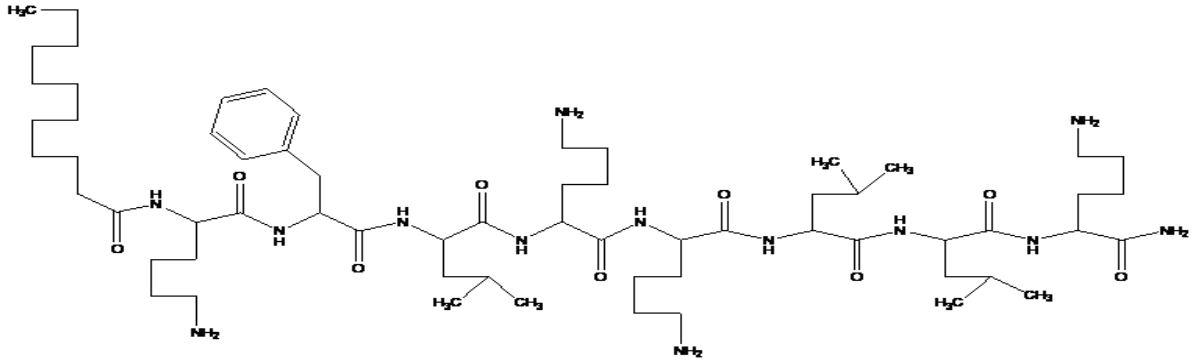


M-Cresol

Ethane di-thiol

Thioanisole

Trifluoroacetic acid



HH3

Figure No 3. Synthetic scheme of the acyl hexapeptide related to humanhistatin 8 (HH3).

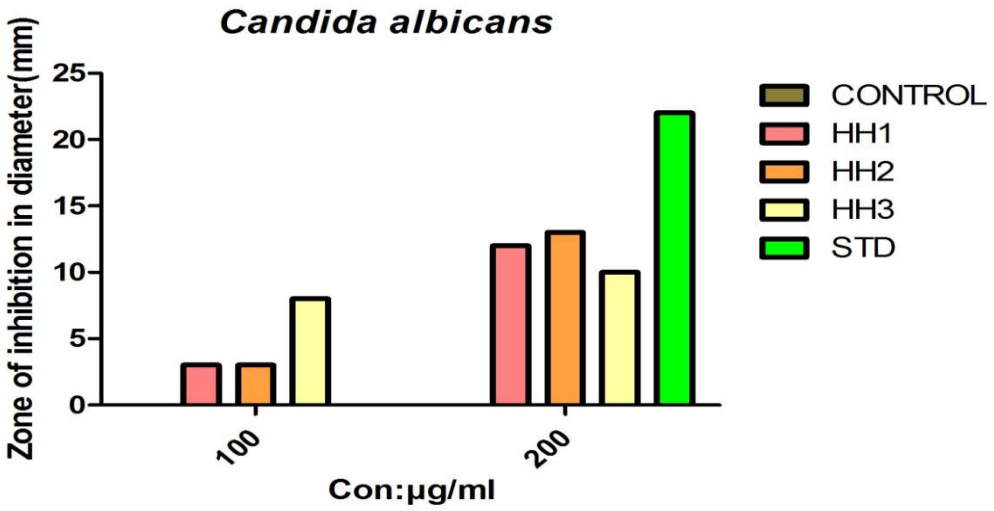


Figure No 4: **Antifungal activity of the octapeptides related Human histatin 8.**

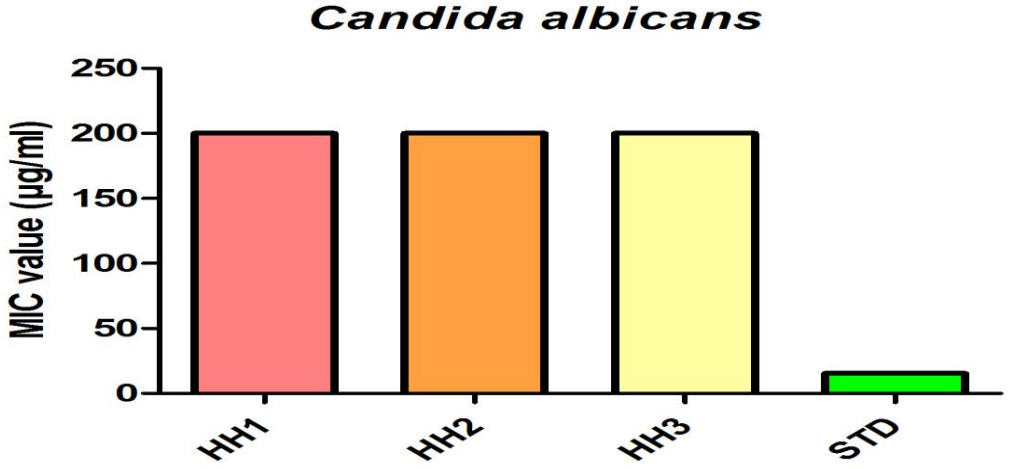


Figure No 5: Antibacterial activity of the octapeptides related Human histatin 8

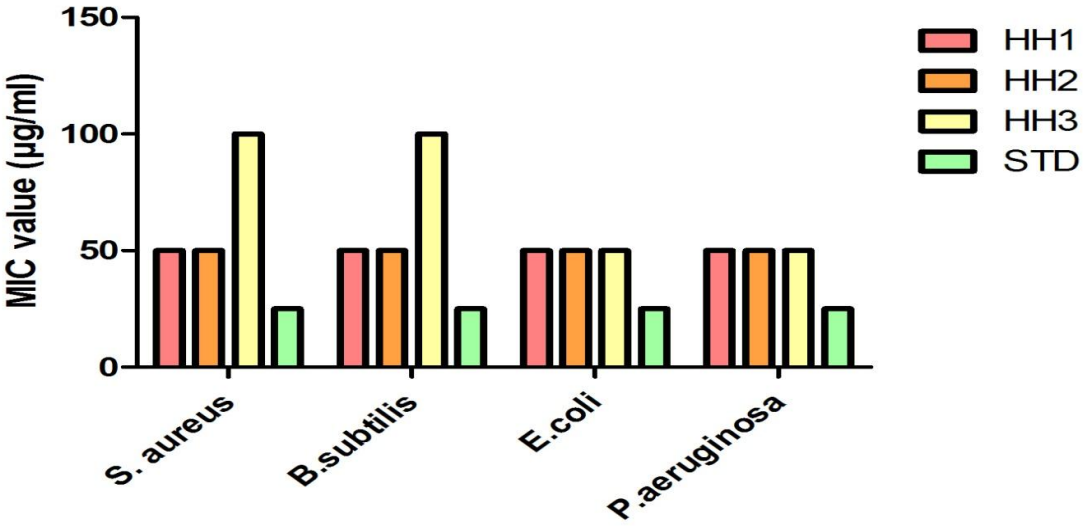


Figure No 6: **Antifungal activity of the octapeptides related Human histatin 8.**

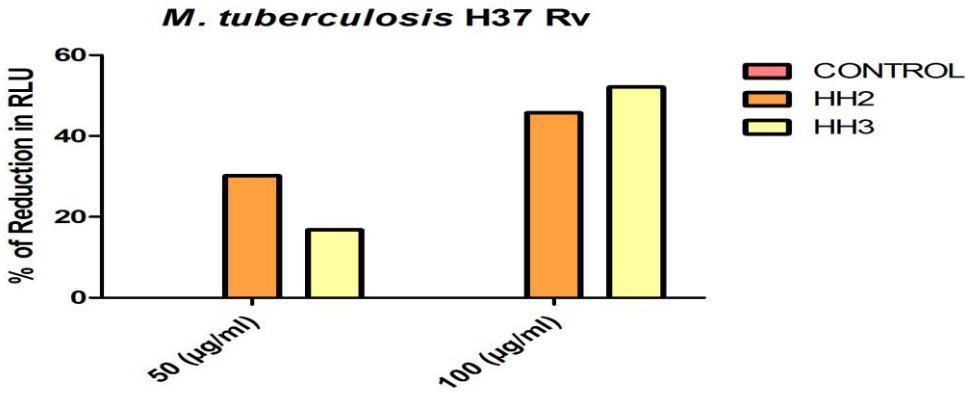


Figure No 7: Antimycobacterial activity of the octapeptides related Human histatin 8 against *M. tuberculosis* H37 Rv.

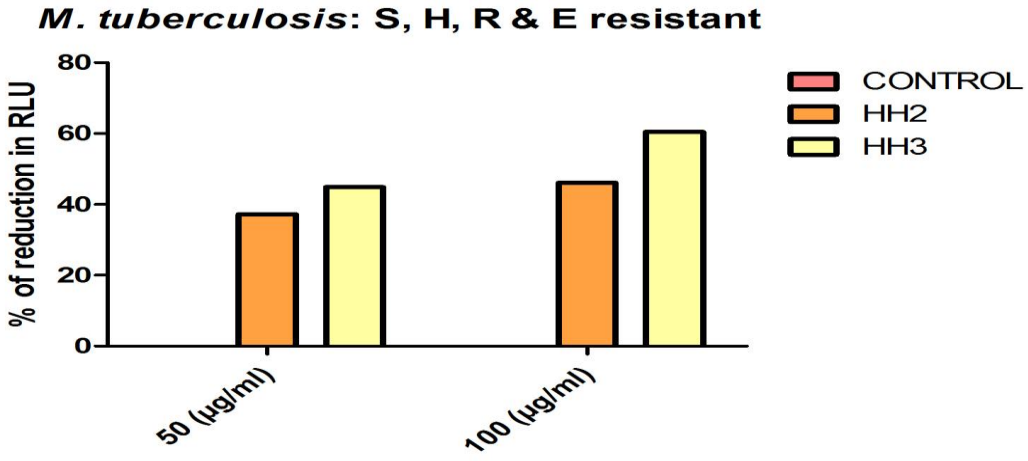


Figure No 8: Antimycobacterial activity of the octapeptides related Human histatin 8 Against Clinical isolate of *M. tuberculosis*: S, H, R & E resistant.

5. CONCLUSION

The present study was concluded that the antimicrobial octapeptides related to human histatin 8 were designed by using splicing methods from human histatin 8, synthesized by solid phase peptide synthesis using Fmoc chemistry protocol, characterized by mass spectroscopy and amino acid sequence analysis using LC-MS-MS. These peptides were evaluated for antibacterial, antifungal and anti-mycobacterial activity**.** The antibacterial activity and antifungal activity of the octa-peptide (HH2) was found to be more potent than HH1 and HH3. The antimycobacterial activity of the compound HH3 was found to be highly potent than HH2 against *M. Tuberculosis* H_{37} RV and Clinical isolates of *M. Tuberculosis* S, H, R & E resistant as compared with control.

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CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

FUNDING

None.

ETHICAL APPROVAL

Not required.

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