Design, Synthesis and Exploration of Amphiphilic α/γ^4 Hybrid Helices as a Potent Antibacterial Agent

Thesis submitted towards the partial fulfilment of the BS-MS dual degree programme

By,

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Under the guidance of,

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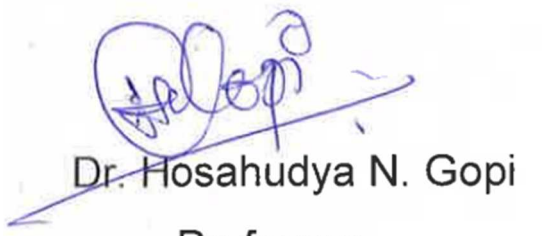
Certificate

This is to certify that this dissertation entitled "***Design, Synthesis and Exploration of Amphiphilic α/γ^4 Hybrid Helices as a Potent Antibacterial Agent***" towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents work carried out by Vivek Kumar at Indian Institute of Science Education and Research, Pune under the supervision of Dr. Hosahudya N. Gopi, Professor, department of chemistry, during the academic year 2018-2019.

20/03/2019

IISER Pune



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Declaration

I hereby declare that the matter embodied in the report entitled "***Design, Synthesis and Exploration of Amphiphilic α/γ^4 Hybrid Helices as a Potent Antibacterial Agent***" are the results of the investigations carried out by me at the Department of Chemistry, Indian Institute of Science Education and Research, Pune, under the supervision of Dr. Hosahudya N. Gopi, Professor, department of chemistry and the same has not been submitted elsewhere for any other degree.

20/03/2019

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BS-MS 5th year

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CONTENTS

Abbreviations:

Leu = Leucine

Bn = Benzyl

Boc = *tert*-Butoxycarbonyl

$(Boc)_2O$ = Di-*tert*-butyl-dicarbonate (Boc anhydride)

THF= Tetrahydrofuran

DCC = N,N'-Dicyclohexylcarbodiimide

DCM = Dichloromethane

DIPEA = Diisoproylethyl amine

DMF = N,N'-Dimethyl formamide

EDC.HCl = 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride

EtOAc = Ethyl Acetate

EtOH = Ethanol

HOBt = 1-Hydroxybenzotriazole

HBTU=(2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate

IBX = 2-iodoxybenzoic acid

MeOH = Methanol

TFA = Trifluoro acetic acid

MIC= Minimum Inhibitory Concentration

O.D.= Optical Density

TFE= Trifluoro ethanol

1,5-I-Aedans= 5-[2-(Iodoacetamido)ethylamino]naphthalene-1-sulfonic acid

DABCYL acid = 4 - ((4 - (dimethylamino) phenyl)azo) benzoic acid

FRET = Forster Resonance Energy Transfer

Fmoc-OSu = N-(9H-Fluoren-9-ylmethoxycarbonyloxy)succinimide

SEM = Scanning Electron Microscopy

CD = Circular Dichroism

Cbz = Benzyl Chloroformate

ACN= Acetonitrile

Abstract:

The emergence of drug resistant microorganisms poses a great worldwide threat and created an immediate need for the development of novel antibiotics with different mechanism of action. Microbial resistance to the antibiotics can affect any one regardless of the gender, age and the country. In addition, microbial resistance to the known antibiotics also imperil the progress in medical and health sciences. In this context, the broad spectrum antimicrobial activity shown by the cationic host-defence antimicrobial peptides (AMPs) have attracted considerable attention. Among various types of host-defence peptides, helical motifs have been an active components of a large family of cationic antimicrobial peptides. These peptides have shown excellent antibacterial properties, however they suffer from non-specificity, higher hemolytic activity and poor bioavailability. In this project, we have designed proteolytically stable hybrid peptide 12-helical foldamers and investigated their antimicrobial activities against various bacterial strains and also examine the haemolytic activity and their proteolytic stability against serine protease trypsin. In comparison to α-peptide counterpart, the hybrid peptide foldamers showed potent antibacterial activity and showed increased stability against the protease trypsin.

Introduction:

Every day we are exposed to thousands of bacterial pathogens by dermal contact, inhalation and ingestion. Our innate immunity responses protects us from growing and colonizing of bacterial pathogens by expressing cationic antimicrobial peptides in our body also called host defence peptides. As the complexity in the evolution increased over the past decades, an enormous number of fungal and bacterial strains have developed the resistance against the conventional antibiotics. This led to the increased demand in the development of new generation of antibiotics with new modes of action. The major difference between most conventional antibiotics and cationic antimicrobial peptide is that conventional antibiotics do not damage bacterial cell membrane but rather penetrate into the microorganisms and target the specific organelle in the bacterium including inhibition of DNA gyrase, inhibition of DNA synthesis, inhibition of folic acid synthesis, inhibition of biosynthesis of the cell wall by peptide-glycosylation,

etc. Under these circumstances, the bacteria can develop resistance to the specific drugs. The most important advantage of natural cationic peptides (AMP's) is that they adsorb in the cell membrane and disrupt the cell membranes of bacteria which are hard to fix. Over the last few years, a number of cationic peptides with different designs including β-hairpins, cyclic peptides, β sheets containing multiple disulphide linkages, extended loops and helical peptides of various lengths have been explored as antimicrobial agents. Hancock and his co-workers have explored a large number of cationic α helical peptides in the design of peptide based antibiotics against multi-drug resistant bacteria. The major drawback of α-helices is that they are not stable against various proteases such as serine and cysteine proteases. In the past decades, many groups had incorporated modified side chains and highly aromatic residues in alpha helices for antibacterial activities and to overcome protease instability, but these designs are far more deviated from natural systems and hence cannot be implemented for clinical trials. In this project, we have designed and investigated the potent antimicrobial activities, haemolytic activity and proteolytic stability of α/γ C_{12} hybrid helices along with alpha peptide counterpart. The preliminary investigation revealed that α/γ helix showed the excellent antimicrobial activity compared to the alpha peptide counterpart. In addition hybrid helices displayed excellent protease stability as compared to the alpha helix. Overall these studies revealed that α/γ^4 hybrid peptides can be used as potent antimicrobial agents. In addition to the antimicrobial activities, we had also investigated the stability of these peptides against serine protease trypsin.

The rational for the designing the α,γ-hybrid peptides as antimicrobials is that these peptides spontaneously fold into stable C_{12} helices in both solution and solid state. Our group has working on the design of hybrid peptide foldamers composed of alpha and γ-amino acids. Based on the single crystal conformations of various α,γ-hybrid peptides we have outlined the basic folding rules. Our previous investigations suggested that the helical structures of these peptides stabilized by consecutive 12-membered intramolecular H-bonds between the residues *i→i+3* [C=O(i) - - - H-N(i+3)]). In all helical structures, the γ-residues adopted gauche+, gauche+ conformation along the Cγ-Cβ and Cβ-Cα bonds, respectively to accommodate into the helix. Our group had developed numerous strategies to mimic α helical secondary structure by synthesizing hybrid peptides containing γ amino acids with proteolytic side chain. More importantly, the amino acid side-chains accommodated four corners of the helical

cylinder. The unique projection of the amino acid side-chains along the helical cylinder provided an excellent opportunity to design cationic amphiphilic helices, which are important for the antimicrobial activities. The crystal conformations 12-helices reported from our group is shown in Fig.1. The top view of the helices showing the projection of the amino acid sides chains along the helical cylinder.

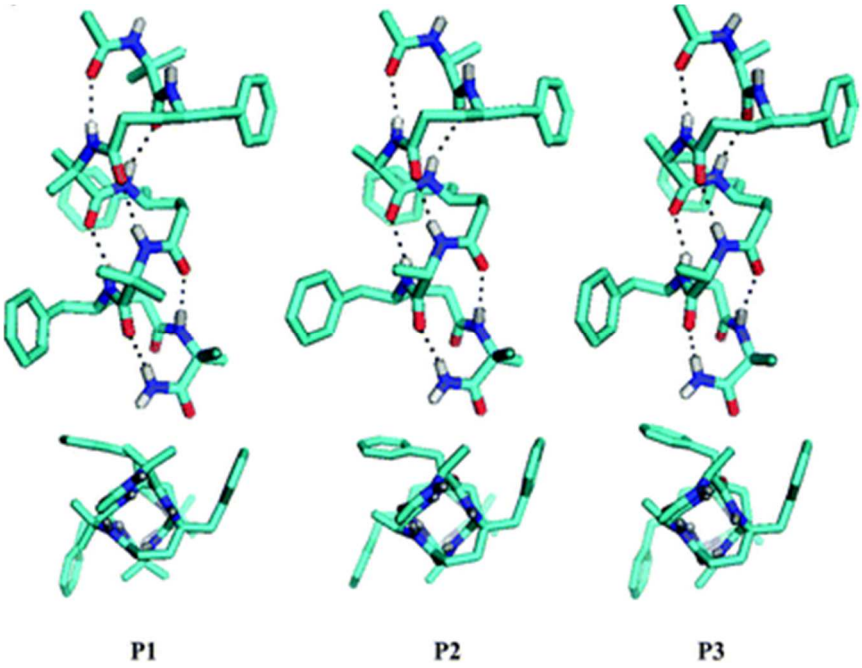
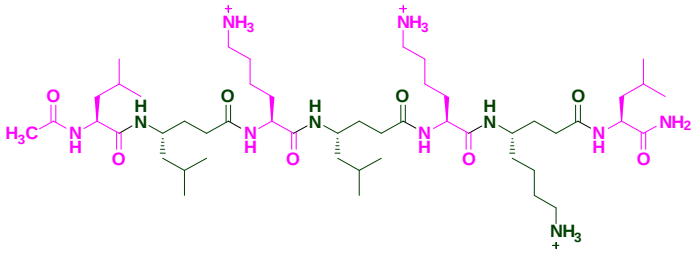


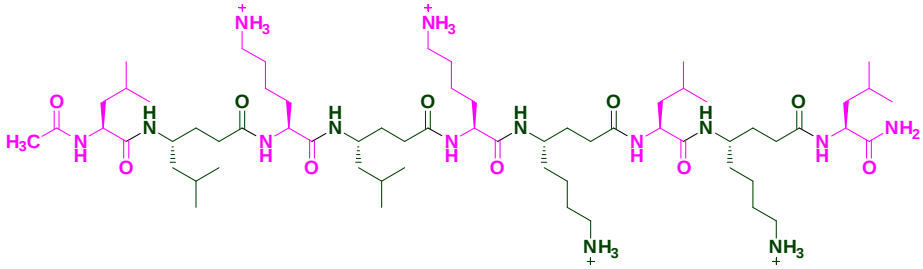
Figure 1: X-ray structures of 3 hybrid α/γ hybrid peptides. **(P1)** Ac-(Aib**-** γPhe)$_3$-Aib-NH_2. **(P2)** Ac-(Ala**-** γPhe)$_3$-Aib-NH_2.**(P3)** Ac-(Ala**-** γPhe)$_3$-Ala-NH_2.. ***Org. Biomol. Chem.***, 2013, **11**, 509-514.

Based on the crystal structures, we also predicted the helical wheel diagram of the hybrid peptide 12-helices. The helical wheel diagram is shown in the Figure 2. We utilized the 12-helix helical wheel to design amphiphilic helical peptide foldamers. In this project, we have designed several α/γ^4 hybrid peptides (**P1-P4**) of various lengths containing leucine and lysine. The sequences of hybrid peptides **P1**-**P4** along with **P5** is shown in the **Scheme 1.** Peptide **P5** is alpha-peptide analogue of peptides **P3** (12-mer) with same amino acid side-chains.

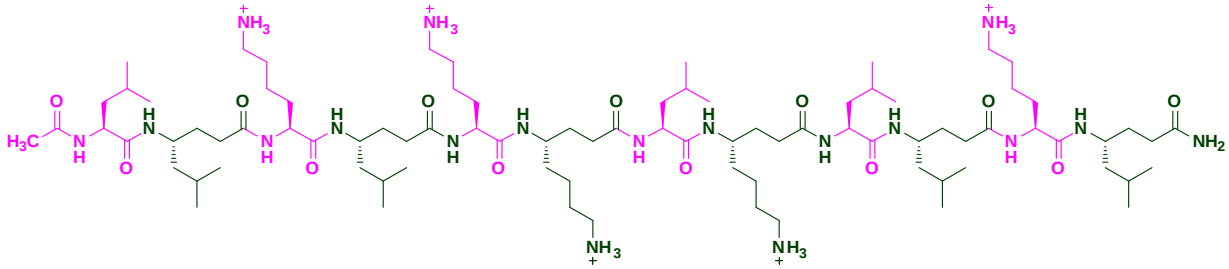
P1



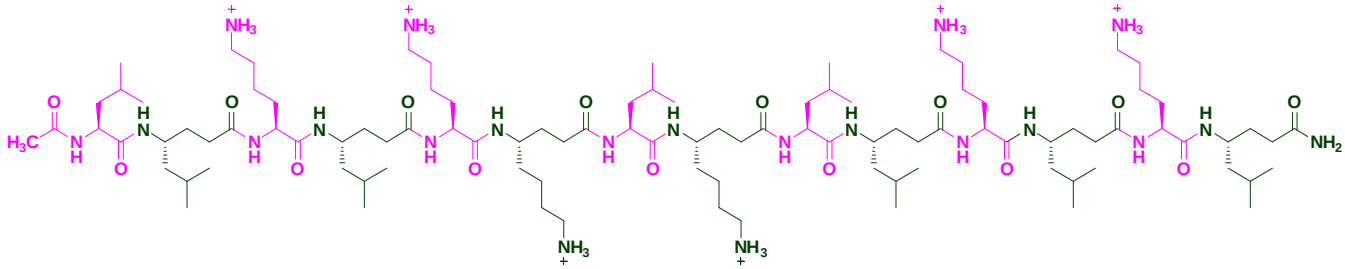
P2



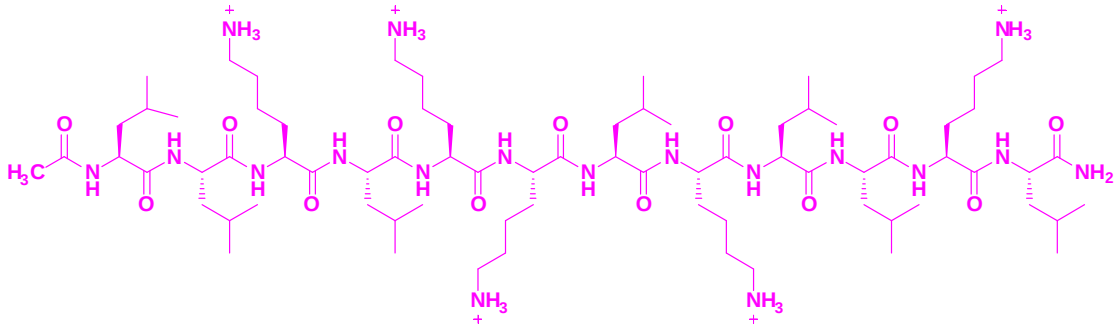
P3

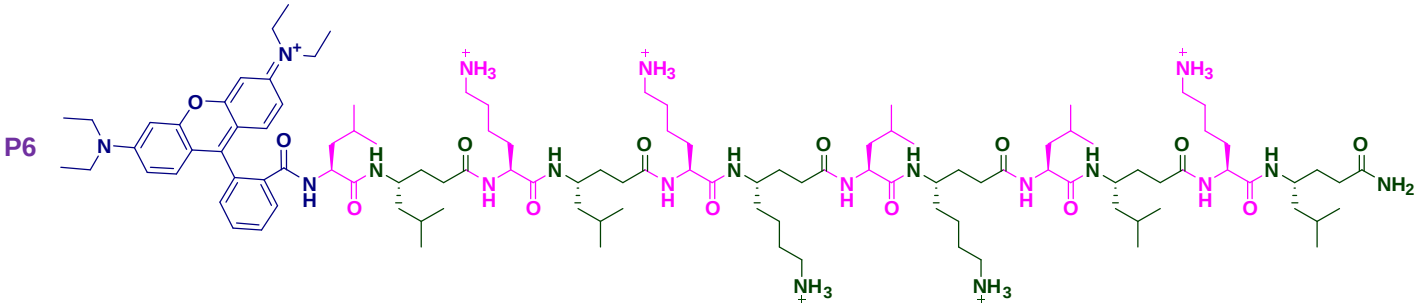


P4

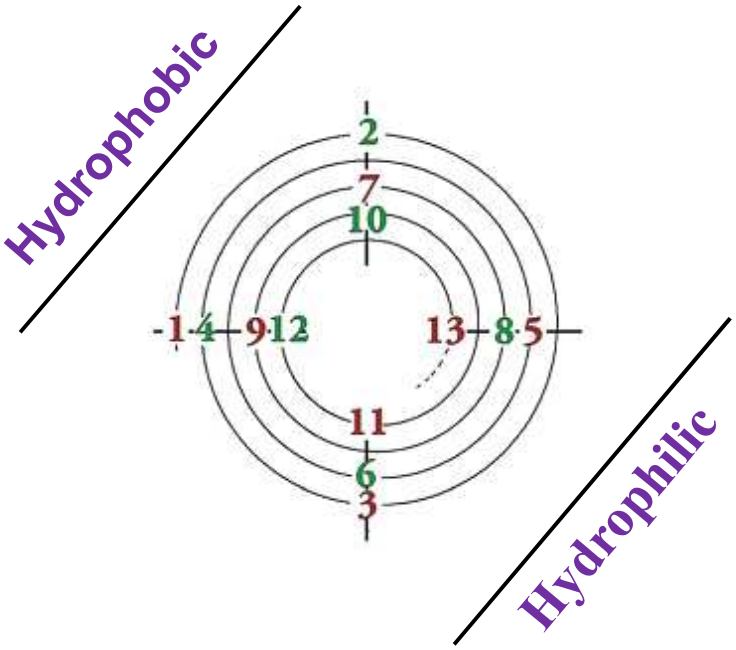


P5





Scheme 1: Sequence of designed C_{12} α/γ^4 hybrid peptides (**P1**-**P4**) and **P6** and alpha peptide **P5** for control.



Ac-Leu-γLeu-Lys- γLeu-Lys- γLys-Leu- γLys-Leu- γLeu-Lys-γLeu-Lys-NH_2

Figure 2: Helical wheel projection of 13 membered α/γ^4 hybrid peptides projecting leucine towards one face and lysine towards another face.

In this project, we have also examined the proteolytic stability of these hybrid peptides against the serine protease trypsin using FRET to understand their proteolytic stability, which is one of the major drawback in natural AMPs. The schematic representation of the peptide consisting of FRET pair is shown in the Figure 3.

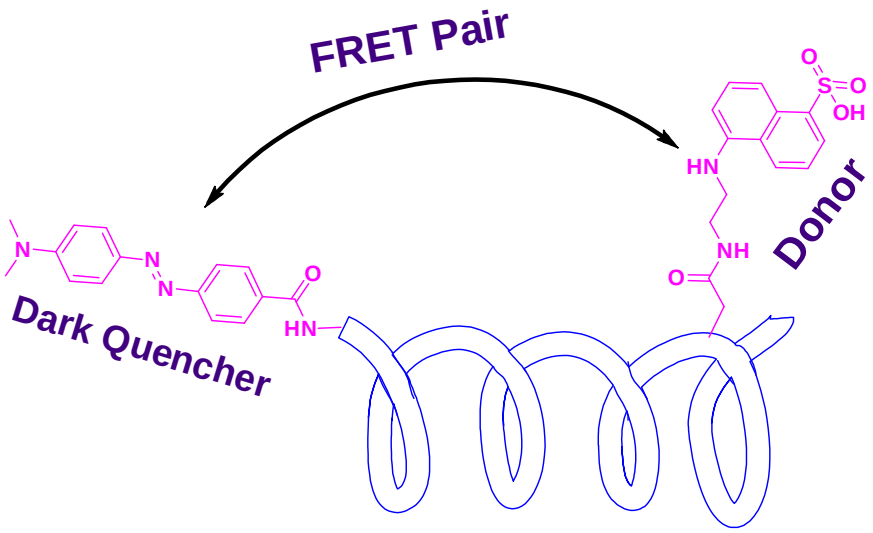


Figure 3: Model system to investigate Trypsin activity

The strategy applied to study trypsin activity by introduction of internally quenched fluorescent substrates, with DABCYL group at N-terminal of peptide, AEDANS group at C-terminal attached to cysteine side chain. The γ^4-lysine was introduced at the cleavage site in the peptide. In the other peptide, on both side of cleavage site lysine, γ^4-leucine was introduced on both sides of lysine. AEDANS serves as a fluorescent chromophore whose fluorescence is quenched by DABCYL which acts as a dark quencher. The amount of cleaving of peptides by trypsin is monitored by increase in fluorescence of AEDANS.

Experimental:

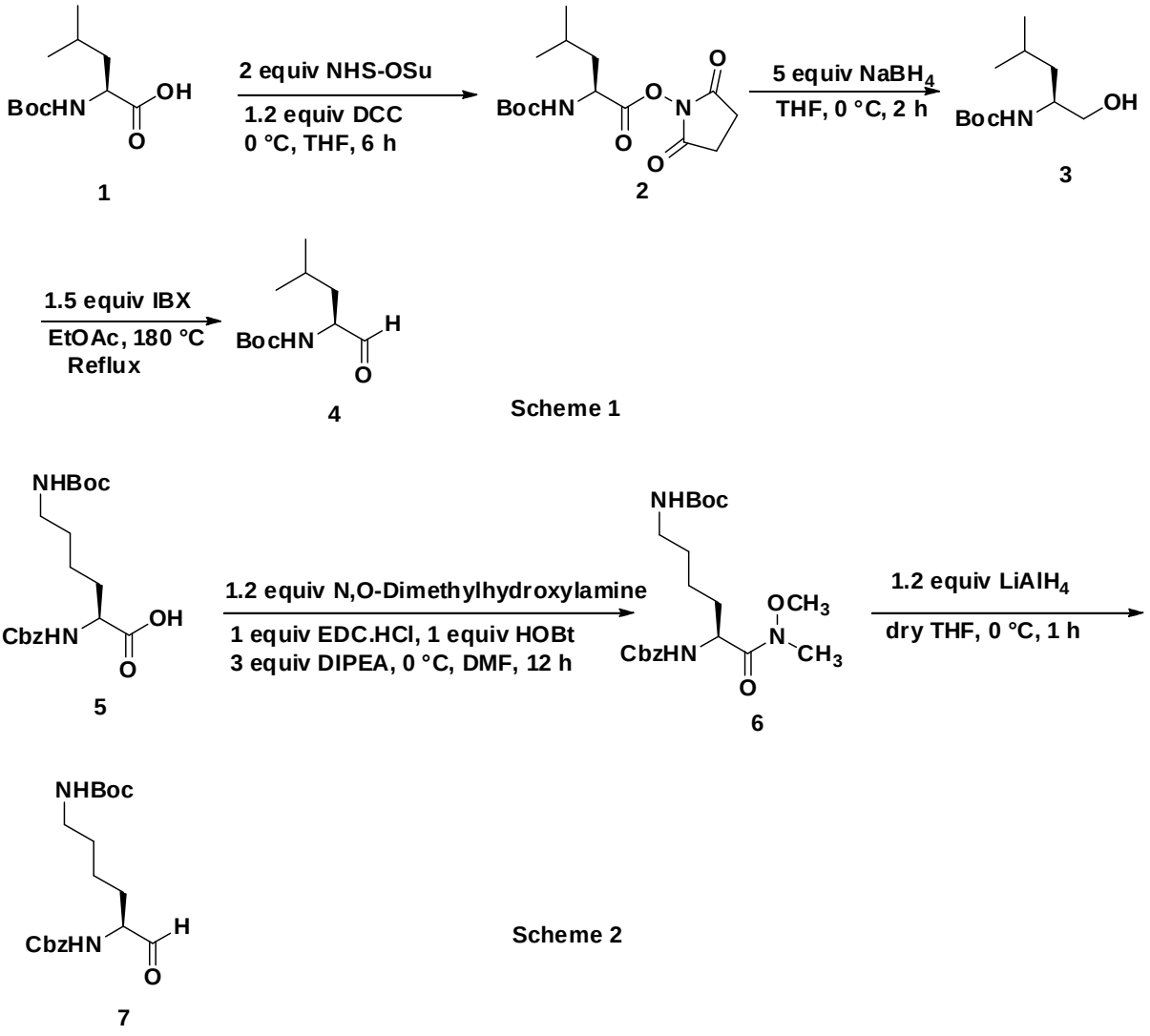
Synthesis of Boc-Leu-Aldehyde:

Briefly, Boc-Leu (50 mmol) was converted to Boc-Leu NHS active ester by treating it with 2 equiv of NHS-OSu and 1.2 equiv of DCC in THF at 0 °C for 6 h. After reaction completion which was monitored by TLC, the solid insoluble by-product DCU urea was filtered through Sintered glass funnel. The active ester of Boc-Leu was reduced with 5 equiv Of $NaBH_4$ for 2 h at 0 °C. After reaction completion monitored by TLC, reaction mixture was acidified with 10% HCl slowly under cold condition. THF was evaporated under *vacuum* to concentrate the reaction mixture. Using EtOAc (3×50 mL), the product was extracted which was then washed with 10% HCl (3×50 mL), 10% Na_2CO_3 (3×50 mL) and with brine (3×50 mL) and finally dried over anhydrous Na_2SO_4. The

white product was obtained by evaporating EtOAc under *vacuum*. The Boc-Leu alcohol was converted to Boc-Leu aldehyde by oxidizing it with 1.5 equiv of IBX in EtOAc by heating it at 180 °C in reflux condition for 6 h. After reaction completion, monitored by TLC, the insoluble unreacted IBX was filtered through sintered glass funnel and EtOAc was evaporated to give white gummy product 4. The overall conversion yield was 80%. (Scheme 1)

Synthesis of Cbz-Lys(Boc)-aldehyde:

Briefly, Boc protected Cbz-Lys (20 mmol) was converted to weinreb amide (6) by treating with 1.2 equiv of N,O-Dimethylhydroxylamine, 1 equiv of EDC.HCl and 1 equiv of HOBt in presence of 3 equiv of DIPEA as base in DMF for 12 h at 0 °C. After reaction completion monitored by TLC, the reaction mixture was neutralized with 10% HCl and the compound 6 was extracted with EtOAc (3×50 mL) and washed with 10% HCl (3×50 mL), 10% Na_2CO_3 (3×50 mL), and finally washed with brine (3×50 mL) and dried over anhydrous Na_2SO_4. The EtOAc was evaporated under *vacuum* to get compound 6. At 0 °C, the amide (6) was dissolved in dry THF at inert atmosphere and cooled for 10 to 20 min. To this cooled mixture, $LiAlH_4$ (1.2 equiv) powder was added to the reaction mixture slowly and kept stirring for 1 h. After reaction completion monitored by TLC, the reaction mixture at 0 °C was quenched slowly with 10% HCl under cold condition. The THF was evaporated under *vacuum* to concentrate reaction mixture. Using EtOAc (3×50 mL), the product was isolated which was then washed with brine (3×50 mL) and dried by adding Na_2SO_4. The EtOAc was evaporated under *vacuum* to get oily product 7. The overall conversion yield was 85% (Scheme 2).

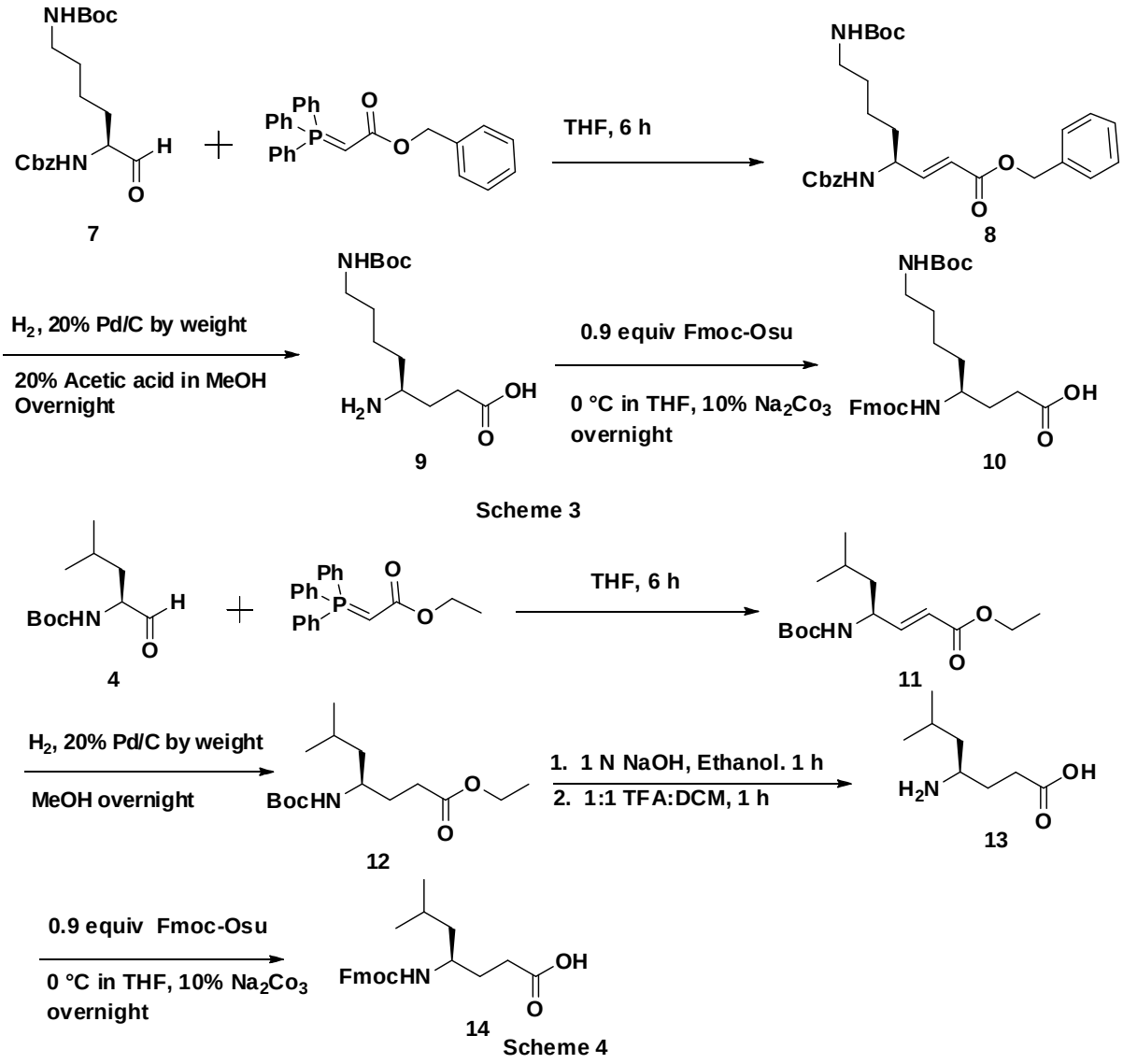


Scheme 1 & 2: Synthesis Scheme of Boc-leu-aldehyde and Cbz-lys(Boc)-aldehyde.

Synthesis of Fmoc-γ-Leu and Fmoc-γ-Lys(Boc):

Briefly, the Boc-leu-aldehyde (20 mmol) was converted into Boc-α,β-dehydro-γ-leu-OEt (compound 11) by stirring it with wittig ylide (25 mmol, 8.7 g) in 50 mL of dry THF for 6 h at room temperature. After reaction completion monitored by TLC, The THF was evaporated under *vacuum* to obtain white gummy product. Since we are not interested in isolating E and Z isomers of compound 11, both isomers of ethyl ester of the Boc-protected α,β-dehydro γ-leucine were isolated with EtOAc/petroleum ether (1:9) as a solvent system through silica gel column chromatography and proceeded for further reaction. The solvent was evaporated under *vacuum* to obtain white gummy product containing both isomers of Boc-α,β-dehydro γ-leucine in excellent yield. Both isomers of Boc-α,β-dehydro γ-leu-OEt (compound 11) (5 mmol) was reduced by hydrogenating it with 20% Pd/C by weight for overnight in methanol. After reaction

completion monitored with TLC, the Pd/C was removed by filtering it in sintered glass funnel. Methanol was evaporated under *vacuum* to obtain white gummy product. Boc-γ-leu ethyl ester (Compund 12) (10 mmol) was hydrolysed by stirring it with 50 mL of 1 N of NaOH in 50 mL of ethanol at room temperature for 1 h. After reaction completion monitored by TLC, ethanol was evaporated under *vacuum* and the reaction mixture was acidified using 10% HCl slowly. Using ethyl acetate (3× 50 mL), the product was isolated which was then washed with brine (3×50 mL) and finally dried by adding anhydrous Na_2SO_4. The EtOAc was evaporated under *vacuum*. The product was then dissolved in 10 mL of DCM and 10 mL of TFA was added slowly to the reaction mixture at 0 °C and kept for stirring for 1 h to obtain H_2N-γ-leu-OH (compound 13). After 1 h, TFA and DCM was evaporated under *vacuum* and the crude compound 13 (5 mmol) was dissolved in 10% Na_2CO_3 at 0 °C until pH of the reaction mixture reach to 9. To the cooled reaction mixture, a solution of 10 mL of THF containing Fmoc-OSu (4.5 mmol) was added slowly and kept for stirring overnight. After reaction completion monitored by TLC, 10% HCl was added dropwise to neutralize the reaction mixture under cold conditions. The THF was evaporated under *vacuum* to concentrate reaction mixture. The product was isolated using ethyl acetate (3×50 mL) and then organic layer was washed with brine (3×50 mL) and finally dried by adding anhydrous Na_2SO_4. The EtOAc solvent was evaporated under *vacuum* to give a gummy product. The crude product was further purified using EtOAc/petroleum ether solvent system through column chromatography to obtain compound 14. The overall conversion yield obtained was 70% (Scheme 4). Similarly, Fmoc-γ-lys (Boc) (compound 10) was synthesized according to the scheme 3 with overall conversion yield 75%. The pure solid Fmoc-γ-leu and Fmoc-γ-lys (Boc) were further used for solid-phase peptide synthesis.



Scheme 3 & 4: Synthesis Scheme of Fmoc-γ-leu and Fmoc-γ-lys(Boc).

Synthesis protocol for peptides (P1-P6):

Synthesis of peptides (**P1**-**P6**) were done on solid support of Rink amide resin on a 0.2 mmol scale using standard Fmoc based chemistry. 4:4 equiv of HBTU/HOBt as coupling reagents, 8 equiv of DIPEA as base and 2.5 mL of NMP as solvent were used in each coupling of 4 equiv of amino acid. After each coupling, Fmoc group was deprotected by using 5 mL of 20% piperidine in DMF for 20 min. Peptides were acylated at the Fmoc-deprotected N-terminal by using 8 equiv of acetic anhydride in presence of 10 equiv of base pyridine. After synthesis completion, peptides were cleaved from the resin using mixture of 9 mL of TFA, 600 µL of water, 200 µL of Tri-isopropyl silane and 200 µL of phenol in the ratio 90:6:2:2 by volume. The reaction mixture was kept for stirring for 2 h. After reaction completion, the resin was filtered through Sintered glass funnel. The crude reaction mixture was then evaporated under

vacuum and precipitated slowly by adding diethyl ether at 0 °C to give a white gummy product. The precipitated crude peptides were dissolved in ACN/H_2O and were purified by injecting them in reverse phase HPLC on a C_{18} column using ACN/H_2O gradient as a solvent system containing 1 percent of TFA. ACN was evaporated under *vacuum* and water was lyophilized under freeze dryer to obtain pure white powder of peptides. To check the purity of the peptides, they were further injected in a C_{18} analytical column and trace of each peptides were recorded using the same solvent gradient system.

Antibacterial Assay:

The bacterial strains used in this project for antibacterial assay were collected from National Collection of Industrial Microorganisms (NCIM) which are *Escherichia Coli* (NCIM 2065), *klebsiella Pneumoniae* (NCIM 2563), *Salmonella Typhimurium* (NCIM 2501), *Pseudomonas aeruginosa* (NCIM 5029) and *Staphylococcus aureus* (NCIM 5021).

Stock solution of 1 mg/mL of peptides (**P1**-**P5**) in water was prepared. Before starting antibacterial assay microliter tips, water, measuring cylinder, test tubes, 200 mL water containing 2.5 gm of Lysogeny broth (LB), 200 mL water containing 2.5 gm of Mueller Hinton Broth (MHB), 1.5 mL Eppendorf, 15 mL Falcon tubes, 100 mL of water containing 2.5 gm of LB Agar, and other required materials for antibacterial assay were autoclaved at 121 °C at pressure 100 kPa (15 psi) for 15 to 20 min. to make them sterile. An aliquot of 1 µL of cryo-freezed bacteria kept at -80 °C was inoculated in 5 mL of LB broth and kept for incubation at 37 °C in shaking for 6 to 8 h. After incubation, an aliquot of 10 µL of bacterial culture grown in LB broth was again inoculated in 5 mL of MHB broth and kept for incubation at 37 °C in shaking condition for the time required to reach the specific Optical Density (O.D) for each bacteria to reach ~ 1-2×10^8 CFU/mL.

Bacteria	***E.coli***	***S. Typhimurium***	***K. Pneumoniae***	***P. aeruginosa***	***S. aureus***
Culture	**~10^8 CFU/mL**	**~10^8 CFU/mL**	**~10^8 CFU/mL**	**~10^8 CFU/mL**	**~10^8 CFU/mL**
O.D	**0.15**	**0.18**	**0.10**	**0.17**	**0.10**

Peptides were diluted from stock solution in MHB broth and different concentrations ranging from 0.390 µg/mL to 200 µg/mL were prepared. A 50 µL aliquot of each

concentration of each peptide was added in the 96-well microliter plate. When the specific O.D of bacteria was obtained to reach the desired concentration of bacteria, it was diluted to 100 times in MHB broth to make final dilution of ~10^6 CFU/mL. A 50 µL aliquot of bacterial culture was added in each well of 96-well plate containing 50 µL of peptide solution, such that final dilution of peptide became **1:2** ratio of original concentrations prepared, such that final range of peptide concentration screened from 0.195 µg/mL to 100 µg/mL, and final bacterial culture suspension reached to ~ **5×10^5 CFU/mL**. The plate was kept for incubation at 37 °C in shaking condition for 16 to 18 h. After incubation, O.D reading was recorded in plate reader at wavelength 600 nm. Two columns, one containing only MHB broth for sterility control and other containing broth with bacterial inoculum without antibiotics as growth control were fixed in each experiment. The minimum concentration to eradicate complete culture of bacteria was described as Minimum Inhibitory Concentration. The experiments were repeated in triplicates and the MIC values calculated for each peptide (**P1**-**P5**) were found to be reproducible between three independent experiments.

Haemolysis Assay:

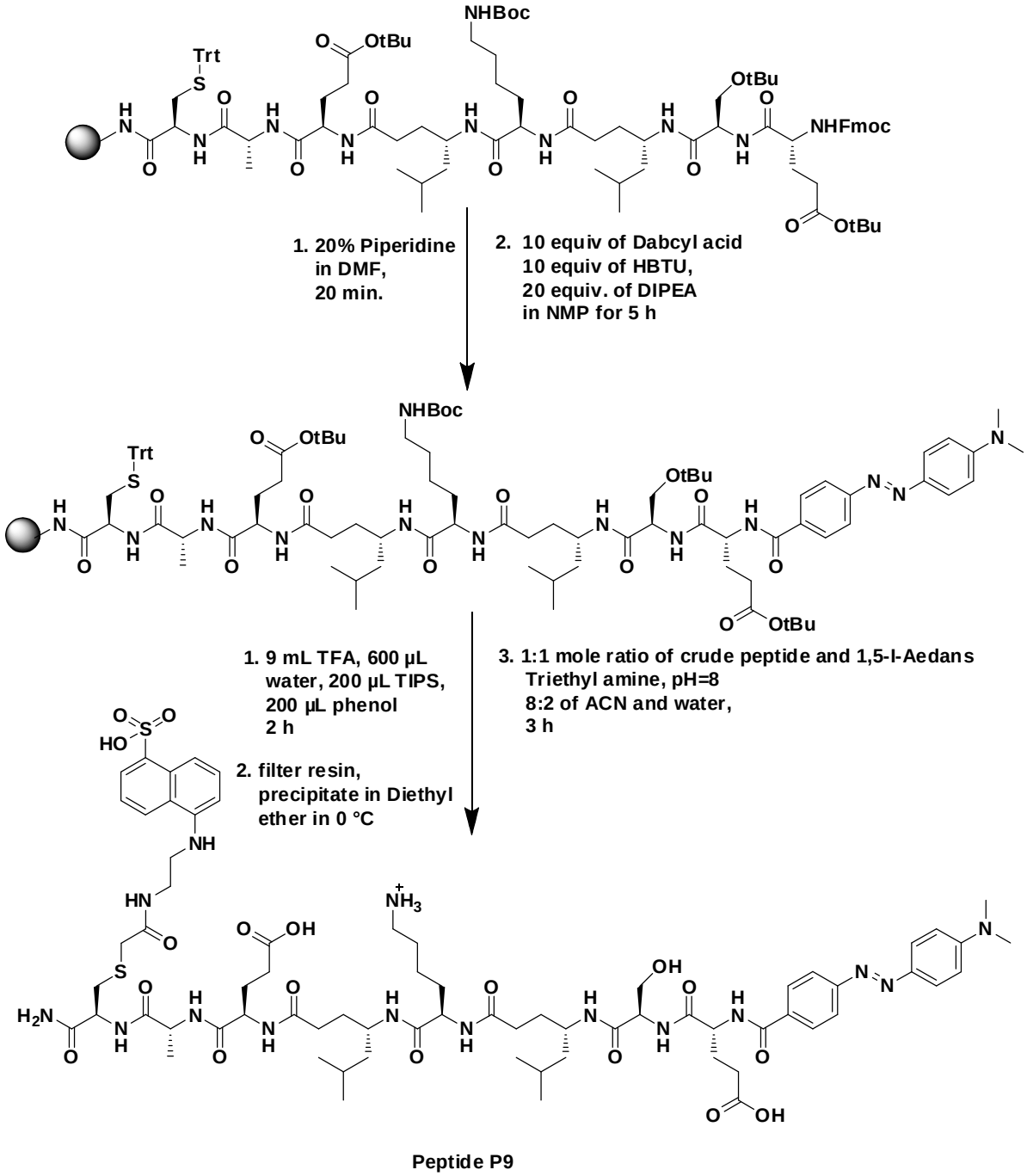
Fresh human blood sample containing red blood cells (hRBC's) were collected from nearest pathology center containing anti-coagulating agent EDTA. 4 mL of fresh blood sample containing hRBC's were diluted by adding 36 mL of Tris-buffered saline (10 mM Tris, 150 mM NaCl, pH 7.2) in the ratio of 1:9. The diluted blood sample in Tris-buffer was centrifuged at 1000 rpm for 15 min. After centrifugation, supernatant was discarded very carefully without harming red blood cells pellets. This step was repeated multiple times till the optical density of supernatant matches with the Tris-buffered saline. The hRBC's pellets collected after final centrifugation were diluted in Tris-buffer to 4% v/v. In a sterile 96 well plate containing desired peptides with varying concentration ranging from 0.488 µg/mL to 1000 µg/mL in Tris buffer, a 50 µL aliquot of hRBC suspension was added in every well containing peptide solution. The final dilution of peptide became **1:2** ratio of original concentration prepared, such that final range of concentration screened were from 0.244 µg/mL to 500 µg/mL. The plate was kept for incubation in shaking condition at 37 °C for 1 h. After incubation, the plate was centrifuged for 20 min. at 3000 rpm. 50 µL aliquot of supernatant from each well without harming healthy red blood cells settled at the bottom, was transferred to different fresh 96 well plate containing 50 µL of fresh water. The release of

haemoglobin as result of lysis of hRBC's by peptides, was monitored by measuring absorption of every well at 540 nm in plate reader. The assay was performed in triplicate, and the average of all absorption value was considered. The 1% Tritron-X treated hRBC's in Tris buffer and hRBC's suspension in Tris buffer was considered as the positive and negative controls respectively. The percentage of haemolysis was calculated from the formula, $\mathbf{(A - A_N) / (A_P - A_N) \times 100}$, and plotted with respect to varying concentration of each peptide, where A representing absorbance of test well, A_P representing absorbance of positive control as a result of 100% haemolysis and A_N representing absorbance of negative control.

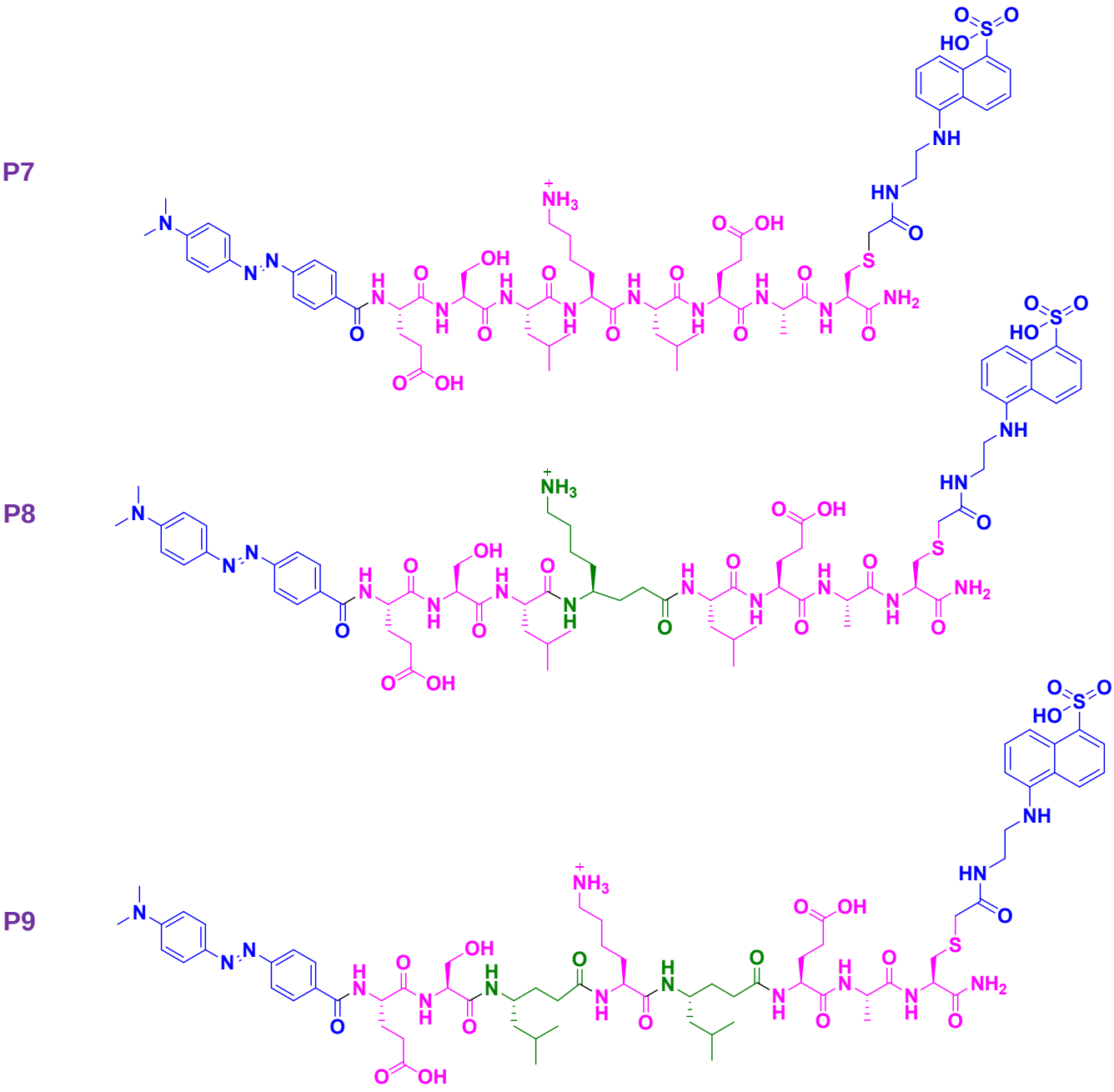
Synthesis protocol for peptides (P7-P9):

Synthesis of peptides (**P7**-**P9**) were done on solid support of Rink amide resin on a 0.2 mmol scale using standard Fmoc based chemistry. 4:4 equiv of HBTU/HOBt as coupling reagents, 8 equiv of DIPEA as base and 2.5 mL of NMP as solvent were used in each coupling of 4 equiv of amino acid. After each coupling, Fmoc group was deprotected by using 5 mL of 20% piperidine in DMF for 20 min. After the completion of all desired coupling of amino acid, the Fmoc- deprotected N terminal of peptide was coupled with 10 equiv of Dabcyl acid using 10 equiv of HBTU as a coupling reagent, 20 equiv of DIPEA as a base in 4 mL of NMP for 5 h. After completion of the synthesis, resin was washed with DMF and DCM several times to remove unreacted colour impurity of Dabcyl acid. Finally, peptides were cleaved from the resin using mixture of 9 mL of TFA, 600 µL of water, 200 µL of Tri-isopropyl silane and 200 µL of phenol in the ratio 90:6:2:2 by volume. The reaction mixture was kept for stirring for 2 h. After reaction completion, the resin was filtered through the Sintered glass funnel. The crude mixture containing peptides was then evaporated under *vacuum* and precipitated slowly by adding diethyl ether at 0 °C to give a white product. To the solution containing 8 mL of ACN and 2 mL of water, 10 mg of crude precipitated peptide was dissolved and sonicated for 10 min. Then, 10 mg of 1,5-I-Aedans was added to the solution mixture containing peptide and sonicated for 5 to 10 min. Finally, triethyl amine was added slowly to make pH approximately 8. The reaction mixture was kept for stirring for 3 h. After reaction completion, the reaction mixture was concentrated under *vacuum* and dissolved in methanol and were purified by injecting them in reverse phase HPLC on a C_{18} column using ACN/H_2O gradient as a solvent system containing 1 percent of TFA. ACN was evaporated under *vacuum* and water was lyophilized

under freeze dryer to obtain pure red colored powder of peptides. To check the purity of the peptides, they were further injected in a C_{18} analytical column and trace of each peptides (**P7**-**P9**) were recorded using the same solvent gradient system.



Scheme 5: Synthesis protocol for synthesis of peptide **P9**



Scheme 6: Sequences of peptides (**P7-P9**) for study of proteolytic stability against trypsin

Circular Dichroism (CD) spectroscopy of peptides:

CD experiment was performed using 1 mm path length cylindrical, jacketed quartz cell with wavelength ranging from 190 nm to 300 nm. CD spectra were recorded with a spectral resolution of 0.05 nm, with scan speed of 50 nm/min at 1 nm of band width

and 1 sec. of response time. CD spectra were recorded in the aqueous medium of sodium phosphate buffer with pH 7.4 and in 50 percent trifluoro ethanol in sodium phosphate buffer with pH 7.4.

FRET assay of Peptides (P7-P9):

100 mL of Tris-buffer of concentration 50 mM of pH 8.2 was prepared. Stock solution of peptides (**P7**-**P9**) was prepared by dissolving peptides in Tris-buffer to a concentration of 500 µM. The peptide solution was further diluted to final concentration of 135 µM which was utilized for FRET assay. Fluorescence reading was recorded in 400 µL Hellma® fluorescent cuvette of path length 10×2 mm, having slit width 10 nm for both excitation and emission. The excitation wavelength was fixed at 335 nm and emission spectra was recorded from the range 350 to 700 nm. Total volume of 400 µL of peptide solution of concentration 135 µM containing 0.54 µM of trypsin in Tris buffer was utilized for FRET experiment. Fluorescent reading without adding trypsin was used as a negative control. Fluorescent reading was recorded after every two min. for 75 min. Fluorescence intensity increment with time was monitored and compared for all three peptides. Intensity of fluorescence maxima of AEDANS group at 535 nm was calculated at different time interval and plotted with respect to time axis and comparison was made for all three peptides (**P7**-**P9**).

To check the amount of degradation for all 3 peptides by trypsin with varying concentration of peptides ranging from 0.5 µM to 35 µM with fixed concentration of trypsin of 2 µM, FRET assay was performed in 96-well black-walled, clear bottomed microliter plate in PerkinElmer microplate reader. An aliquots of 90 µL of each concentration of all peptides were added in 96-well plate containing 10 µL of trypsin to make trypsin concentration which was fixed at 2 µM. Excitation wavelength was fixed at 335 nm and emission reading at 535 nm was recorded after every two min for 1 h. The plate was kept at incubation at 37 °C with shaking for 10 sec. before taking every emission reading. The experiment was done in triplicate and the readings was reproducible between three independent experiments. The concentration of the product formation at different time interval was calculated and plotted with respect to time. The concentration of product formation with different interval was calculated using the equation: $\mathbf{[P]_t = (I_t - I_0) / (I_m - I_0) \times [S]_0}$ where $\mathbf{[P]_t}$ is the concentration of the

product at time **t**, I_t is the Intensity at time **t**, I_0 is the Intensity of the substrate without addition of trypsin, I_m is the maximum Intensity at saturation where complete degradation of peptide **P7** by trypsin is done and taken as a reference for all peptides, $[S]_0$ is the initial substrate concentration.

Results and Discussions:

MIC calculations for peptides (**P1**-**P5**):

We have synthesized and purified four hybrid α/γ^4 peptides **P1-P4** (Scheme 1) and alpha peptide **P5** of varying lengths and examined its antibacterial activity. The bacterial culture of final suspension **~5×10^5 CFU/mL** were treated with varying concentration of peptides ranging from 0.195 µg/mL to 100 µg/mL and incubated at 37 °C for 16 to 18 h in shaking condition. O.D. reading of 96-well plate containing peptide treated bacterial culture was taken after incubation at wavelength 600 nm. Based on the O.D. reading of different wells, MIC values was calculated. The wells containing only MHB media was considered as the negative control and the wells containing bacterial culture treated with antibiotic levofloxacin was considered as the positive control. The minimum concentration of peptide at which it completely eradicate the bacteria culture was considered as the Minimum Inhibitory Concentration for that particular peptide. For each peptide, the experiment was done in triplicate and the MIC values obtained was reproducible between three independent experiments. The MIC values for peptides (**P1**-**P5**) was reported in Table 1.

Table 1: MIC values of peptides (**P1**-**P5**) in **µg/mL.**

Microorganism	Peptide P1	Peptide P2	Peptide P3	Peptide P4	Peptide P5	Levofloxacin
Escherichia Coli	**>100**	**50**	**1.563**	**3.125**	**>100**	**0.390**
Klebsiella pneumoniae	**>100**	**25**	**3.125**	**3.125**	**>100**	**0.095**

Pseudomonas aeruginosa	**>100**	**12.5**	**3.125**	**3.125**	**>100**	**0.195**
Staphylococcus aureus	**>100**	**12.5**	**3.125**	**3.125**	**100**	**0.095**
Salmonella Typhimurium	**>100**	**50**	**6.25**	**6.25**	**>100**	**0.195**

Peptides **P3** and **P4** showed excellent antibacterial activity against both gram-positive and gram-negative bacteria. Peptide **P3** showed minimum MIC value of 1.56 µg/mL against *Escherichia Coli*. It is worth mention that the α-peptide **P5** with same number of residues and same amino acid side-chains did not showed the antibacterial activity even up to 100 µg/mL. These results revealed that the structure of the peptide is important for the activity of the peptides, not the charge on the peptides.

The encouraging MIC values of peptides (**P1**-**P5**) further motivated us to examine their haemolytic activity. 4% v/v suspension of hRBC's in Tris-buffered saline was treated with varying peptide concentration and incubated for 1 h at 37 °C in shaking condition. After incubation, the release of haemoglobin as a result of red blood cells lysis was monitored by checking absorption of 96-well plate at 540 nm containing red blood cells treated with peptides. The absorption of well containing 1% Tritron-X treated hRBC's in tris-buffered saline was considered as the positive control and the well containing only red blood cells suspension in tris-buffered saline was considered as the negative control. From the formula, **Percentage of hemolysis = $(A - A_N) / (A_P - A_N) \times 100$,** The percentage of hemolysis was calculated for every concentration of peptide ranging from 0.244 µg/mL to 500 µg/mL and plotted against varying concentration of each peptide (**P1**-**P5**). The graph has been reported in Figure 4 and 50 percent haemolytic concentration was calculated and reported in table 2. The peptides **P1**, **P2** and **P5** showed negligible haemolytic activity even in their higher concentration. The peptide **P4** showed maximum haemolytic activity at higher concentration compared to all designed peptides, and was found to be 500 µg/mL as it's 50 percent haemolytic

concentration. For all other peptides **P1**, **P2**, **P3** and **P5,** 50% haemolytic concentration was found to be above 500 µg/mL. Overall,

Table 2: 50% Haemolytic concentration reported in **µg/mL.**

HC_{50}	Peptide P1	Peptide P2	Peptide P3	Peptide P4	Peptide P5
Conc. in µg / mL	**> 500**	**> 500**	**> 500**	**500**	**> 500**

peptides **P3** and **P4** which were found to highly active against pathogenic bacteria, had almost negligible haemolytic activity at their MIC concentration.

Though CD is not a sole tool to understand the conformation of peptides, however we have examined their conformations using CD (**P1**, **P2**, **P3** and **P5**) in sodium phosphate buffer could confirm that peptides are forming stable C_{12} helices in sodium phosphate buffer at pH 7.4 which we could confirm through CD signal minima in the range between 200 nm to 220 nm. Additionally, in the 50% trifluoro ethanol in sodium phosphate buffer at pH 7.4, we obtained intensified CD signal minima for the same concentration of peptides (**P1**, **P2**, **P3** and **P5**) at same wavelength we obtained in 100% sodium phosphate buffer. In addition to the CD, we are examining the conformations of peptides using 2D NMR in PBS buffer as well as in SDS. The ^1H NMR for peptides **P5** in methanol-d_3 is shown in Figure 20. Presently, we are examining the solution conformation of **P1**, **P2**, **P3** and **P4** using 2D NMR techniques and NMR modelling.

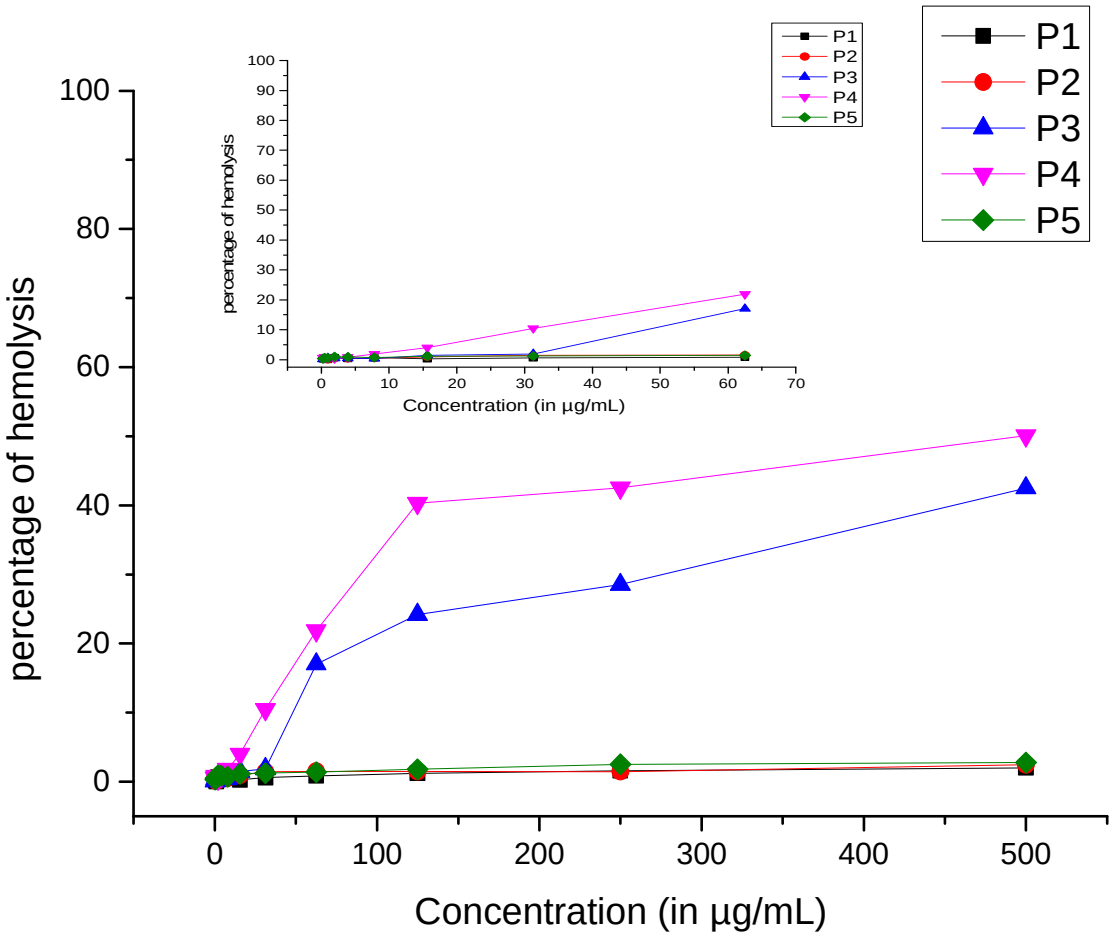


Figure 4: Haemolysis plot representing percentage of haemolysis with varying concentration of peptides (**P1**-**P5**).

CD spectra of peptides P1, P2, P3 and P5:

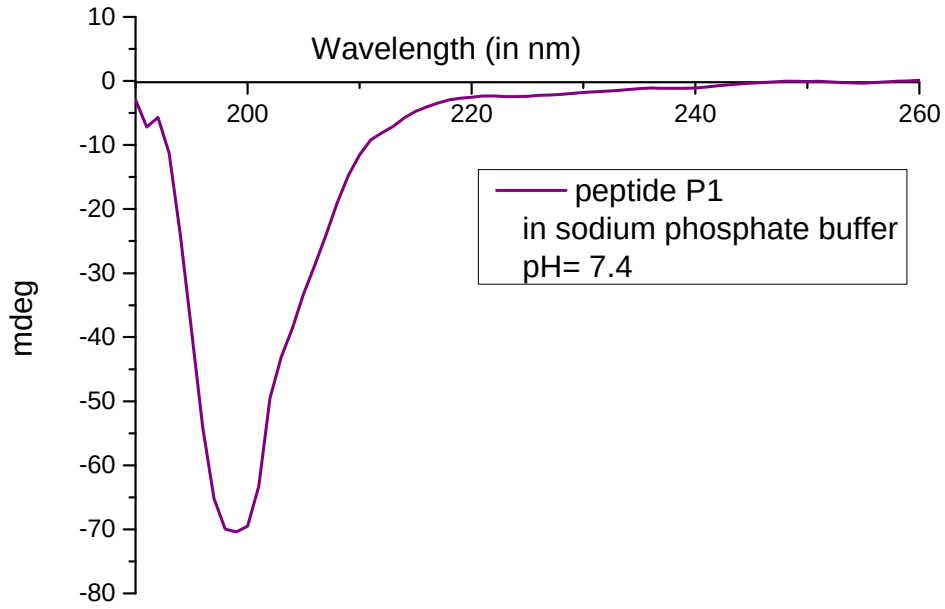


Figure 5: CD spectra of peptide **P1** in sodium phosphate buffer pH 7.4.

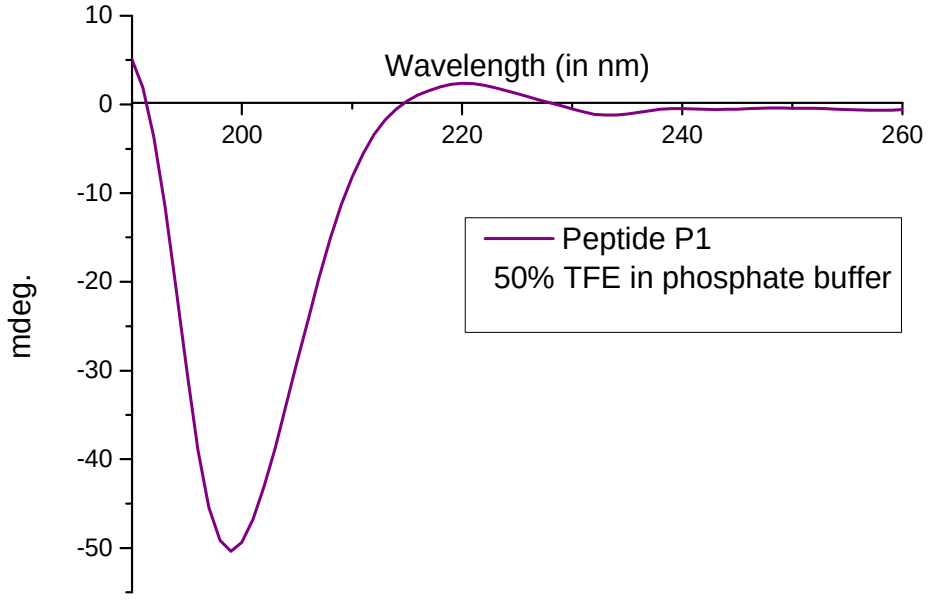


Figure 6: CD spectra of peptide **P1** in 50% TFE in phosphate buffer.

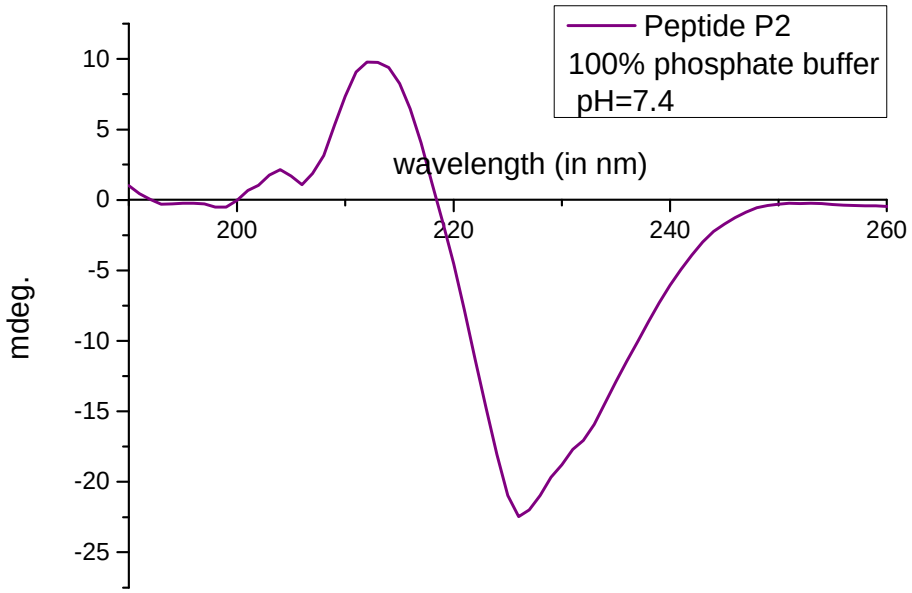


Figure 7: CD spectra of peptide **P2** in sodium phosphate buffer pH 7.4

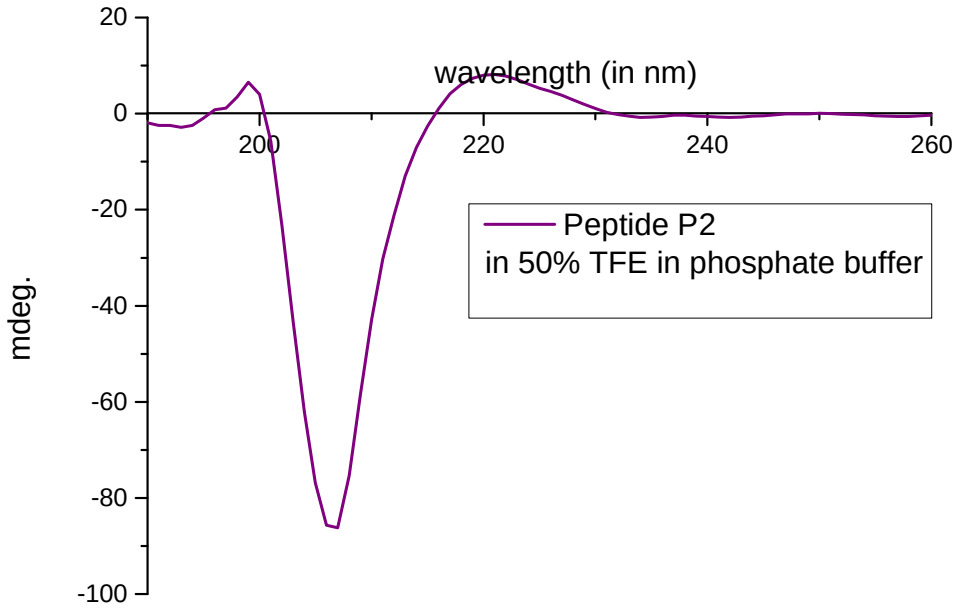


Figure 8: CD spectra of peptide **P2** in 50% TFE in phosphate buffer.

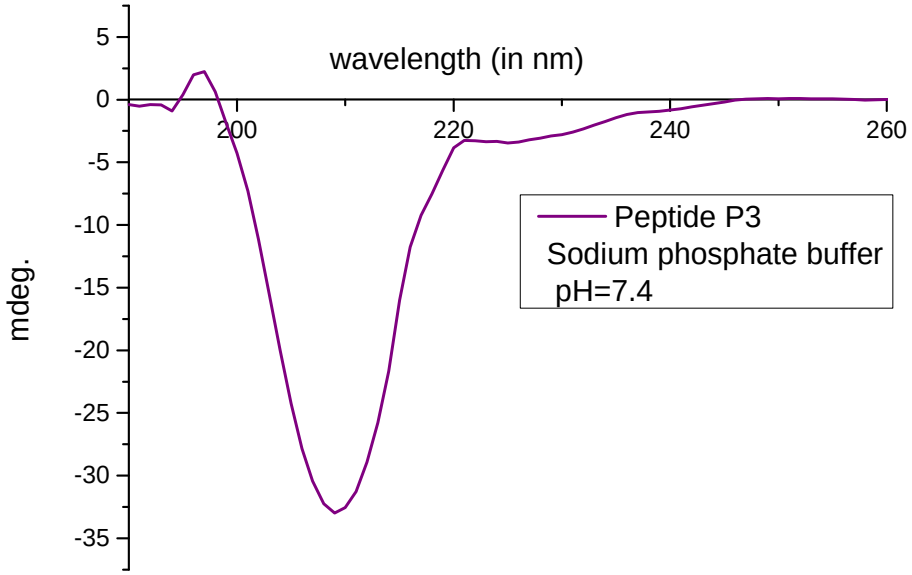


Figure 9: CD spectra of peptide **P3** in sodium phosphate buffer pH 7.4

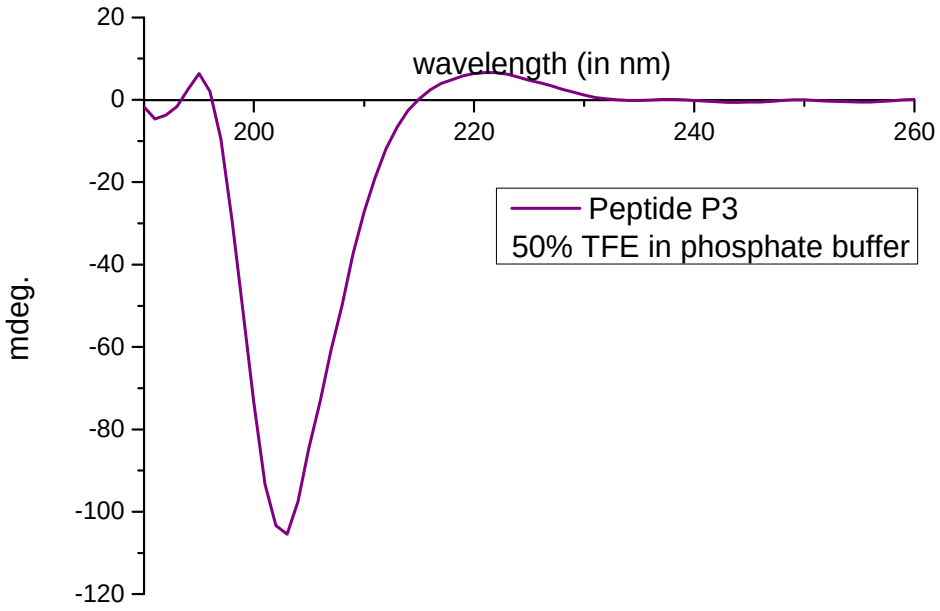


Figure 10: CD spectra of peptide **P3** in 50% TFE in sodium phosphate buffer

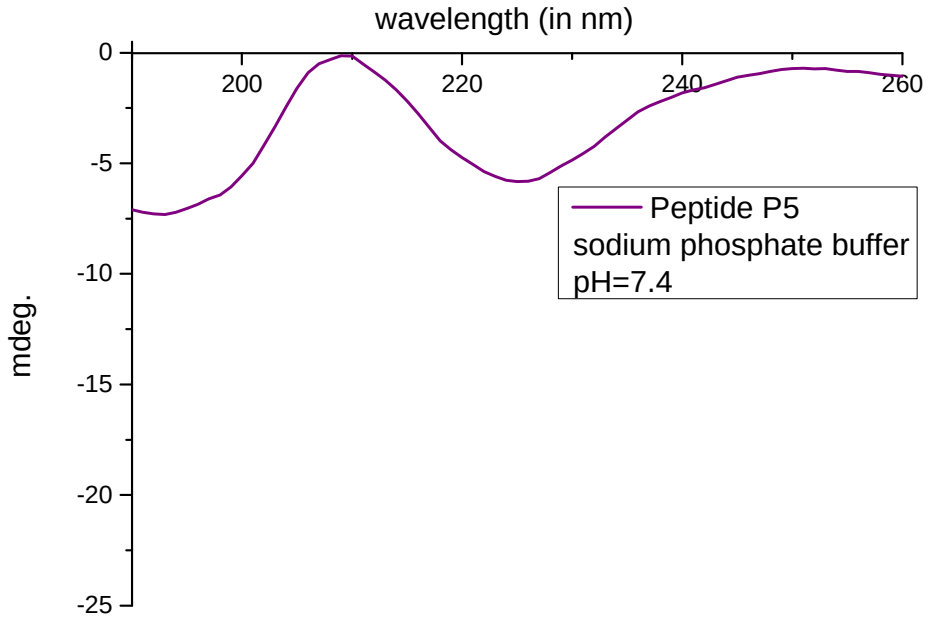


Figure 11: CD spectra of peptide **P5** in sodium phosphate buffer pH 7.4.

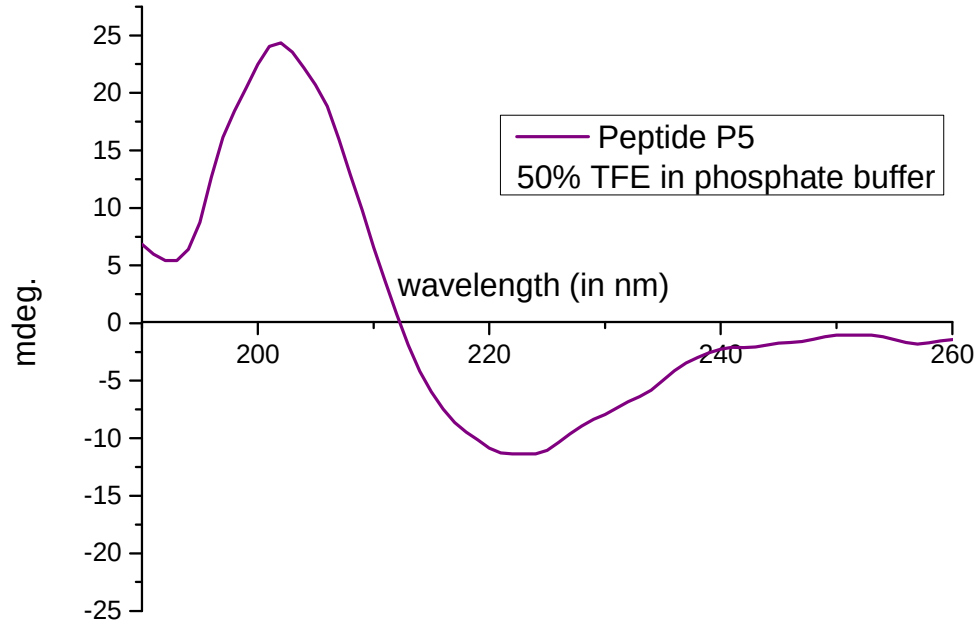
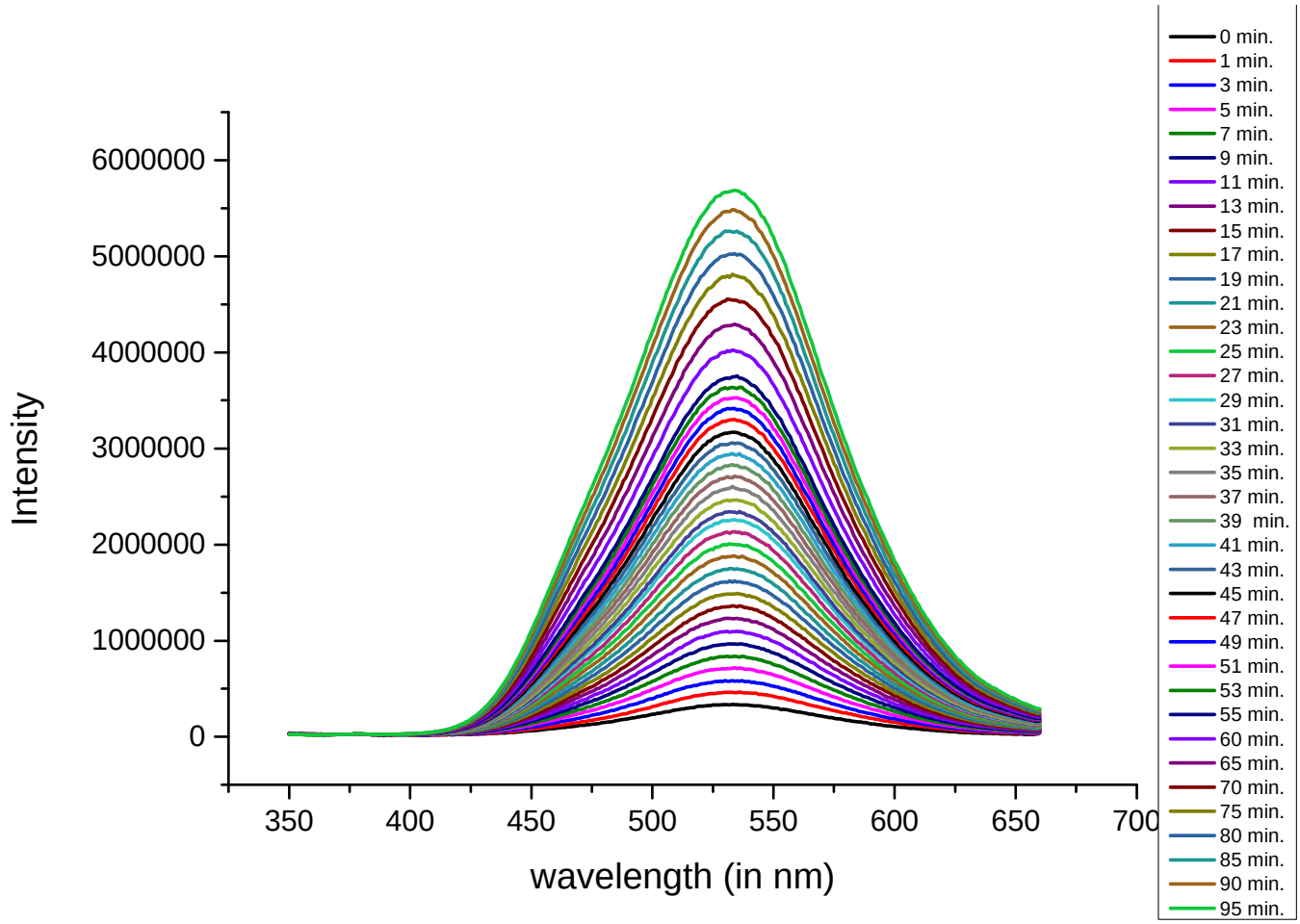


Figure 12: CD spectra of peptide **P5** in 50% TFE in sodium phosphate buffer.

Trypsin degradation analysis of peptides (**P7**-**P9**):

Fig. 13 clearly tells that peptide **P7** is rapidly cleaved by trypsin, with the rapid increase in fluorescence at 535 nm. On the other hand, peptides **P8** and **P9** show minute to negligible increase in fluorescence of AEDANS, as it become difficult for trypsin to recognize γ4-lysine and lysine surrounded by γ^4-amino acids and hence it cannot able to cleave them efficiently. Then we were interested to find out, how different concentration of each of these peptides (**P7**-**P9**) from lower to higher respond to fixed concentration of trypsin of 2 µM. In case of peptide **P7**, within few minutes every concentration of peptide **P7** reached to saturation, with the drastic increase in the initial velocity $\frac{d[P]}{dt}$ at time t=0, with increasing concentration from 0.5 µM to 35 µM. But in case of peptide **P9**, there is very slow formation of product with time. As compared to alpha peptide **P7**, increase in initial velocity in peptide **P9** is very slow with increasing concentration of peptide **P9**. As compared to peptide **P7**, there is insignificant increase in the formation of product with time in the case of peptide **P8**. Additionally, in the case of peptide **P8**, increase in the initial velocity with increasing concentration is insignificant as compared to alpha peptide **P7** counterpart.



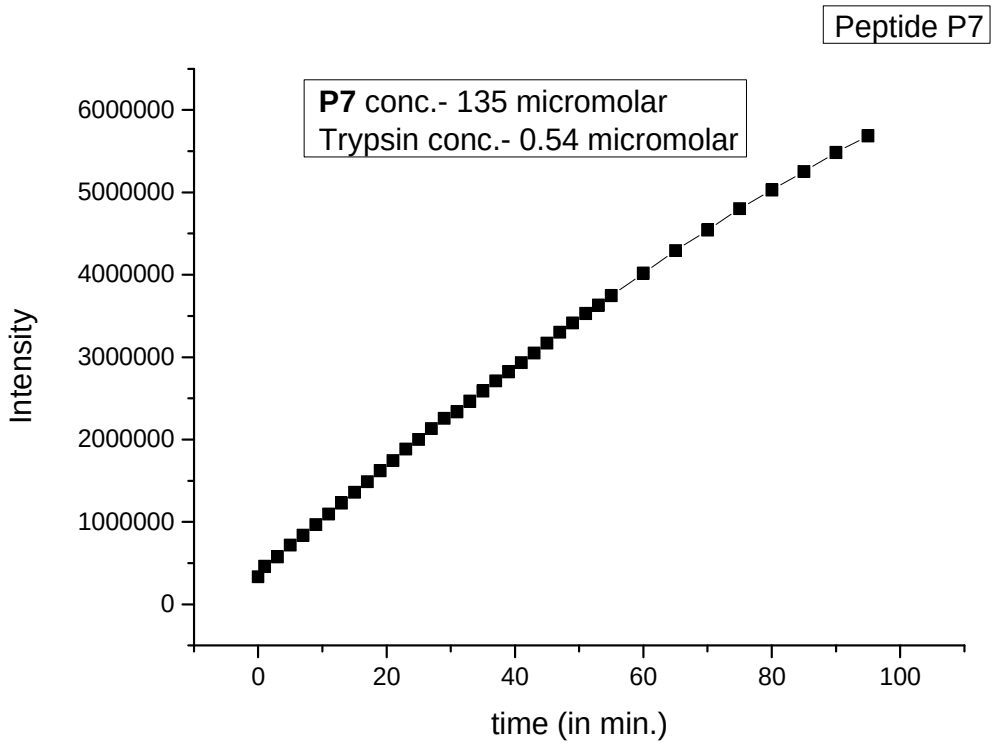
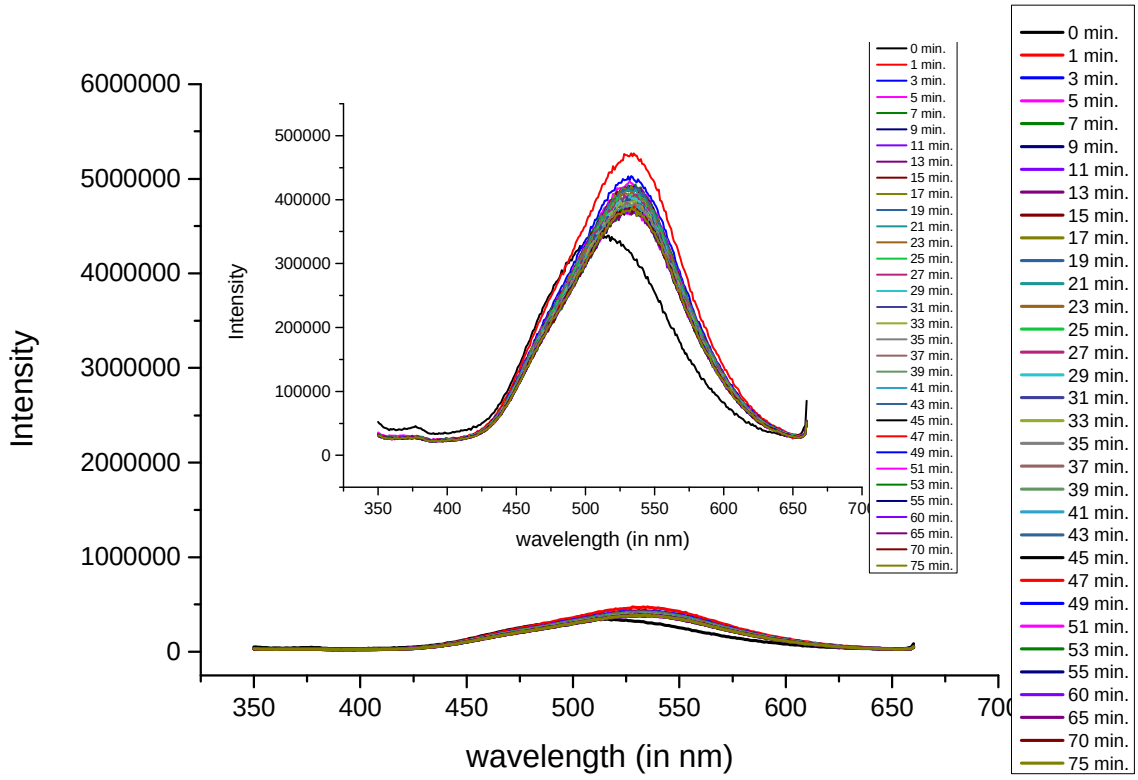


Figure 13: Fluorescent scan for peptide **P7** in different time interval and Intensity at emission maxima at 535 nm was calculated and plotted against time axis with Intensity at time t=0 min. representing fluorescent Intensity of peptide **P7** without trypsin.



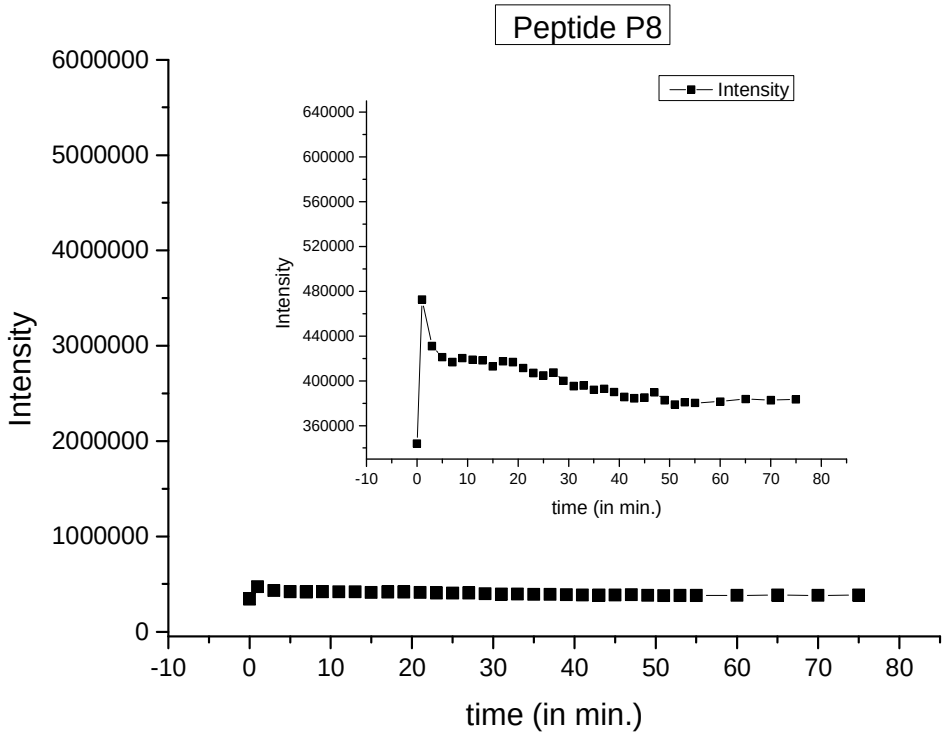
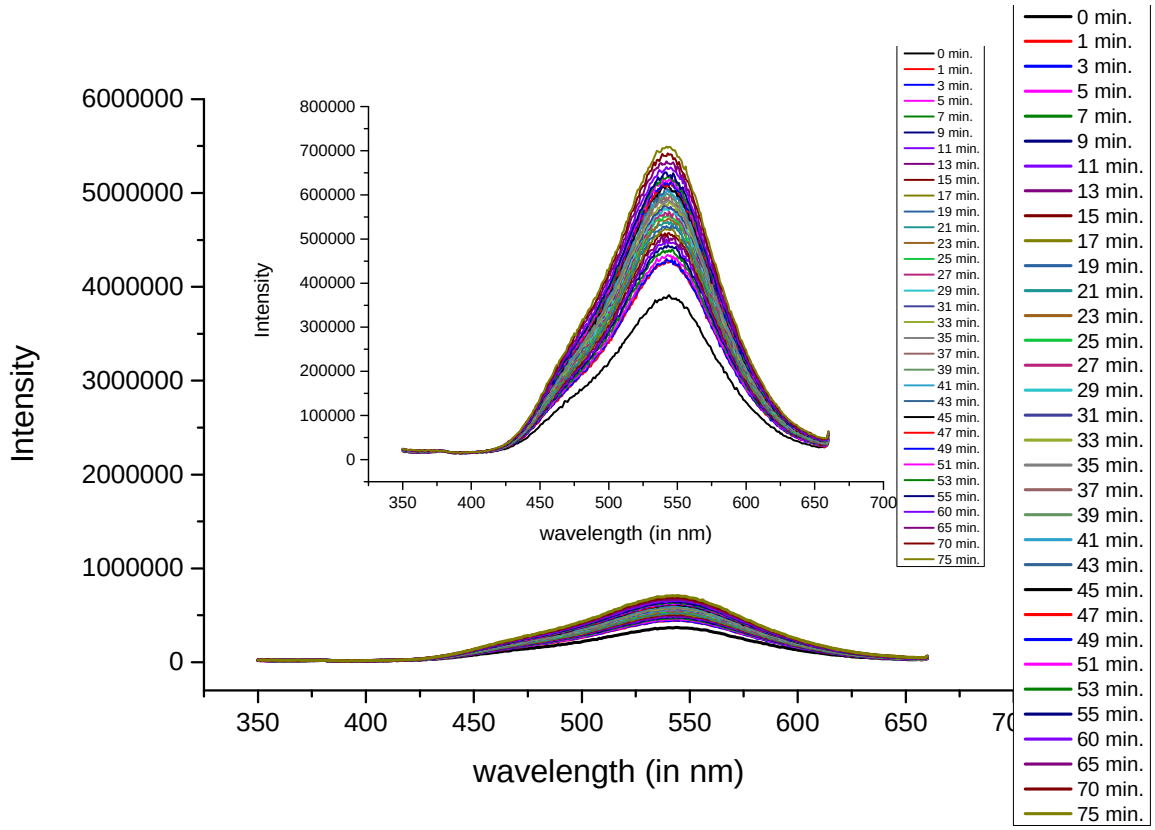


Figure 14: Fluorescent scan for peptide **P8** in different time interval and Intensity at emission maxima at 535 nm was calculated and plotted against time axis with Intensity at time t=0 min. representing fluorescent Intensity of peptide **P8** without trypsin.



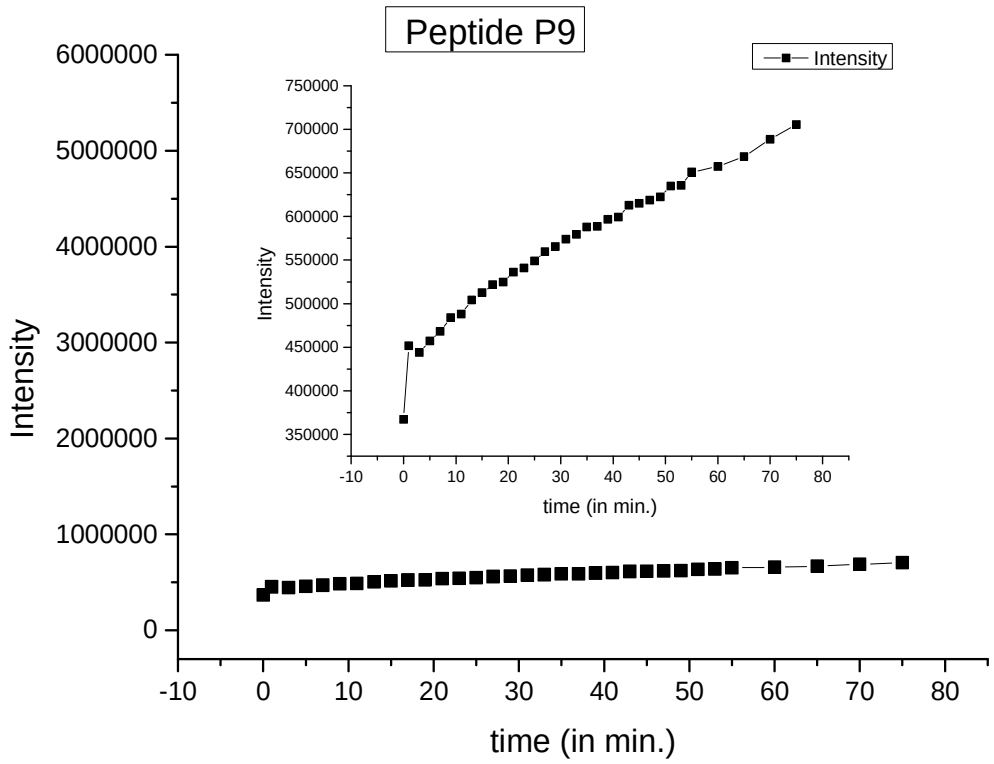
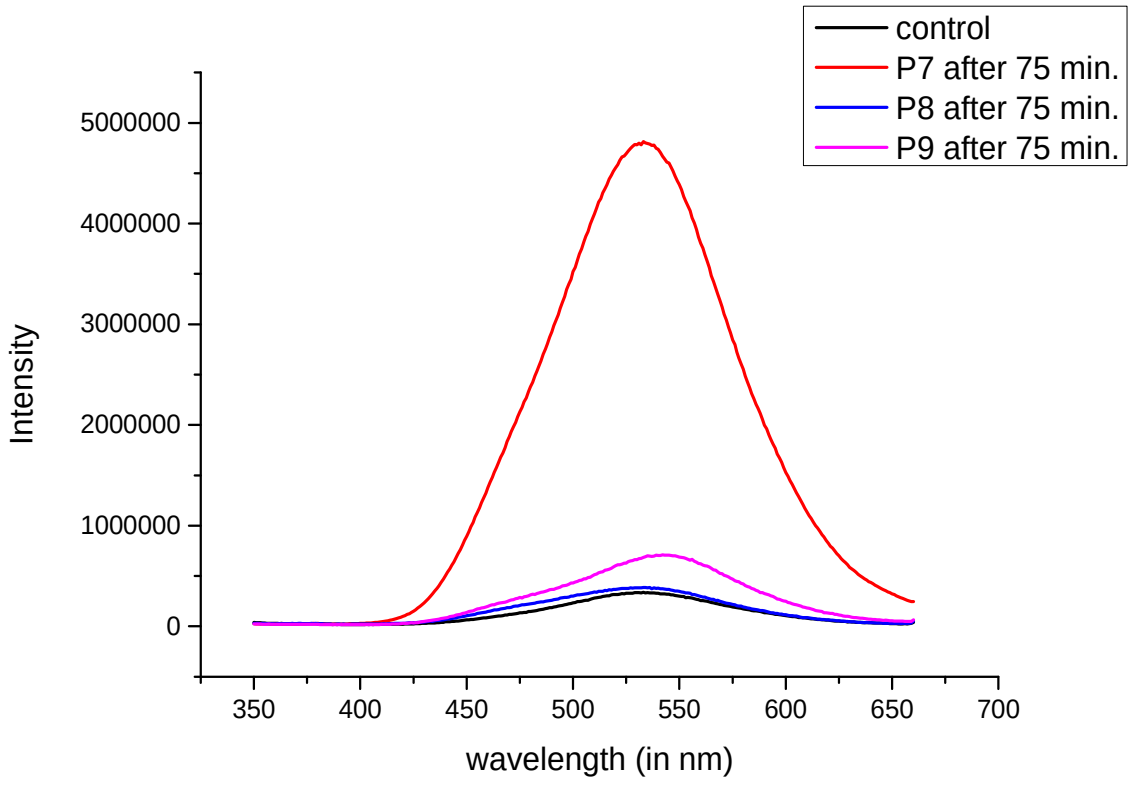


Figure 15: Fluorescent scan for peptide **P9** in different time interval and Intensity at emission maxima at 535 nm was calculated and plotted against time axis with Intensity at time t=0 min. representing fluorescent Intensity of peptide **P9** without trypsin.



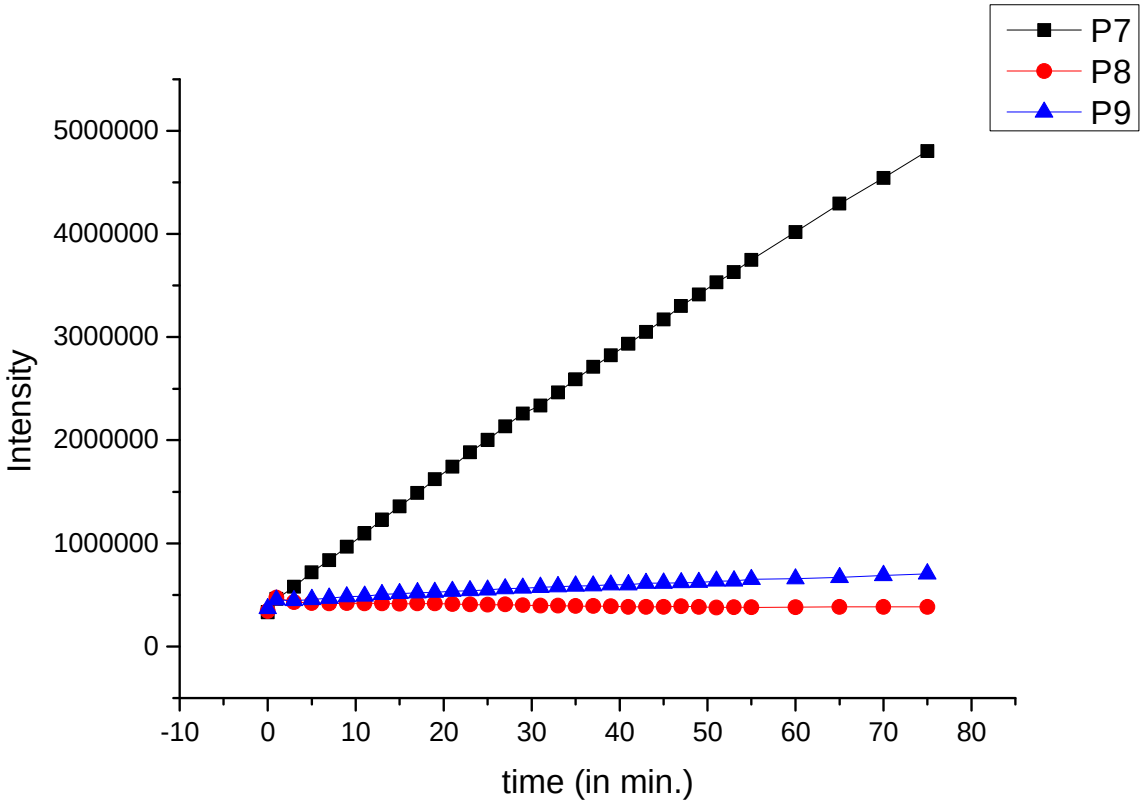


Figure 16: Comparison for Intensity variation with time for all 3 peptides (**P7**-**P9**) and Intensity of alpha peptide **P7** after 75 min. without trypsin was selected as control.

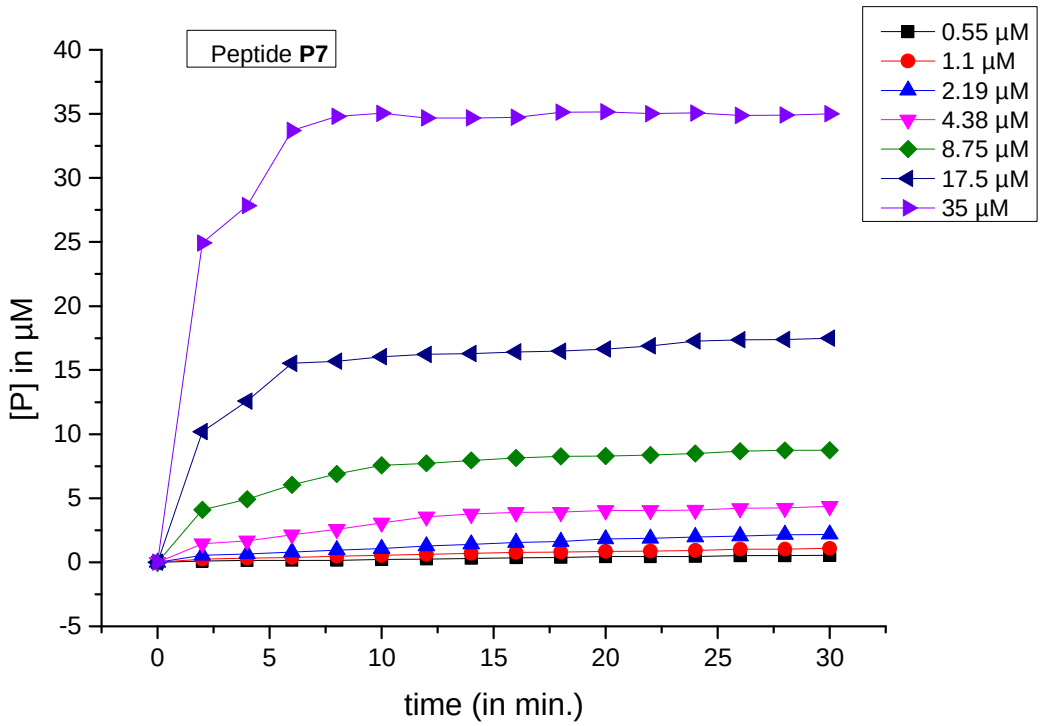


Figure 17: Trypsin degradation analysis of peptide **P7** with varying concentration.

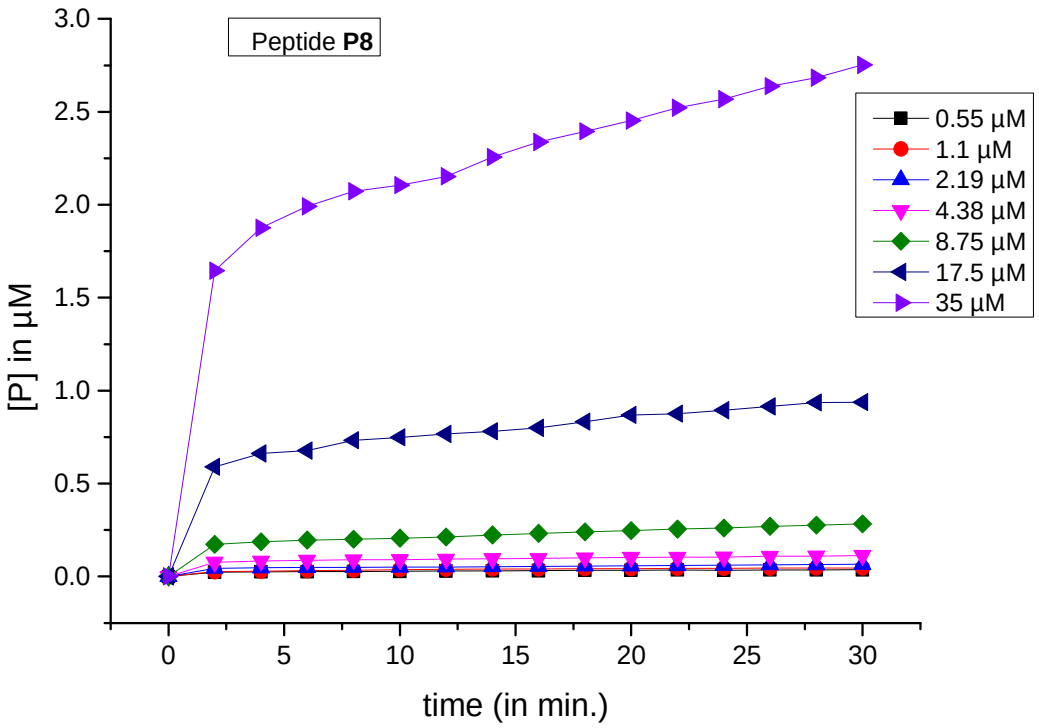


Figure 18: Trypsin degradation analysis of peptide **P8** with varying concentration.

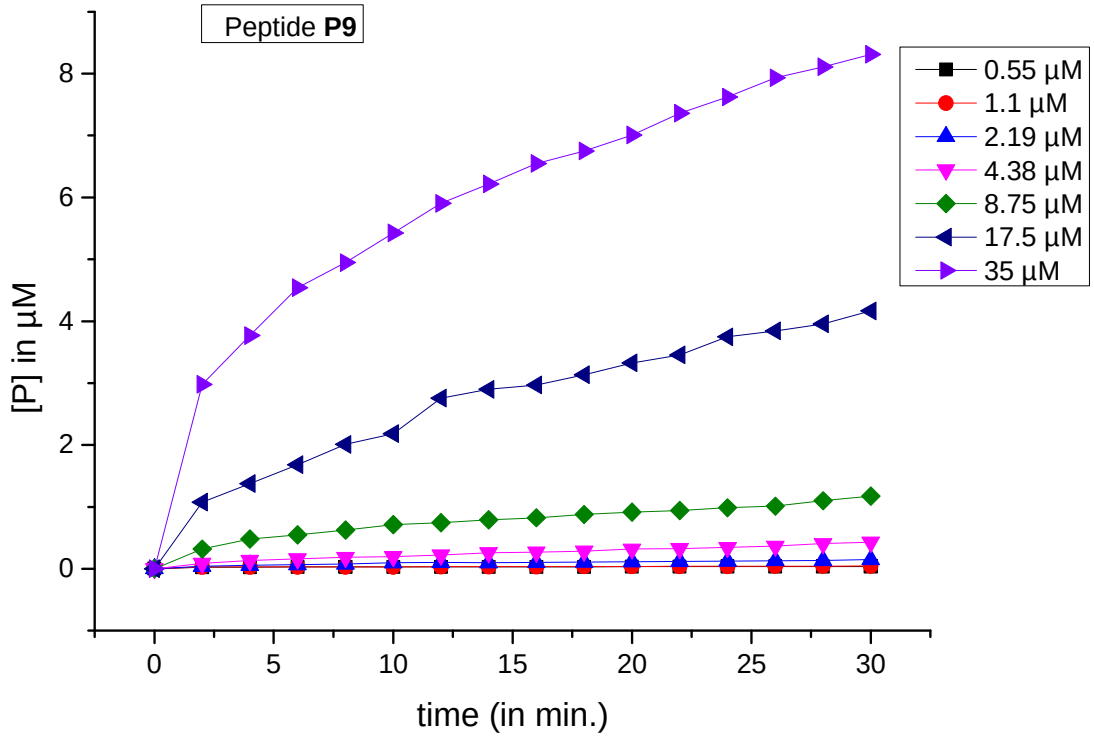
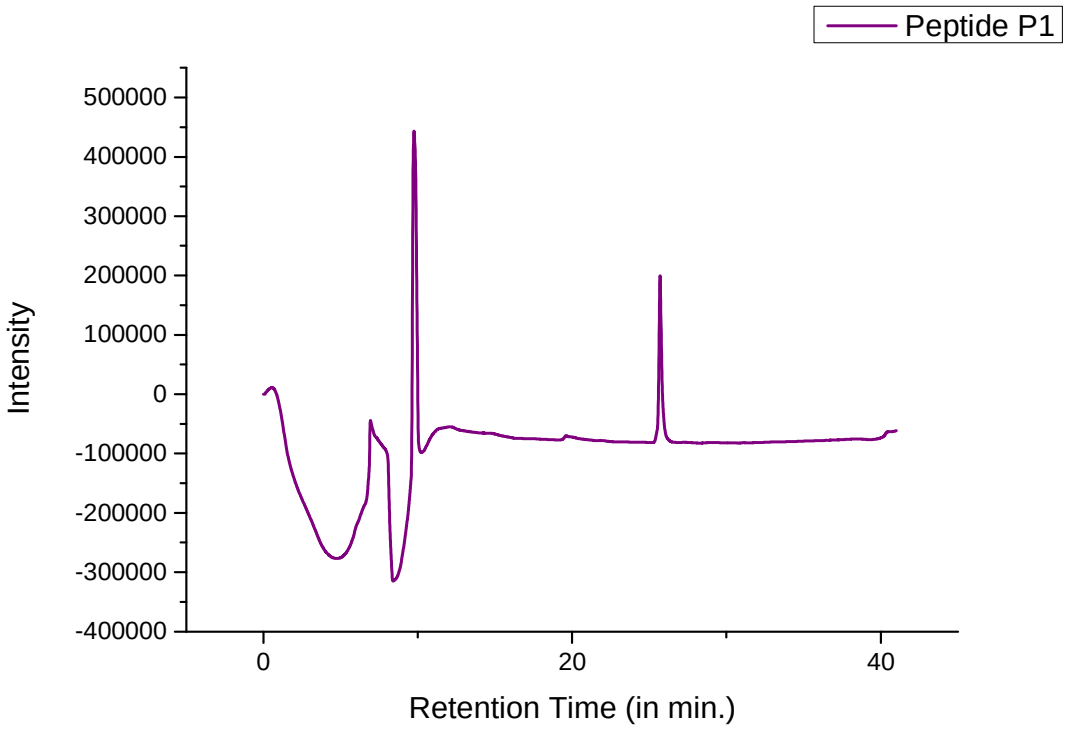
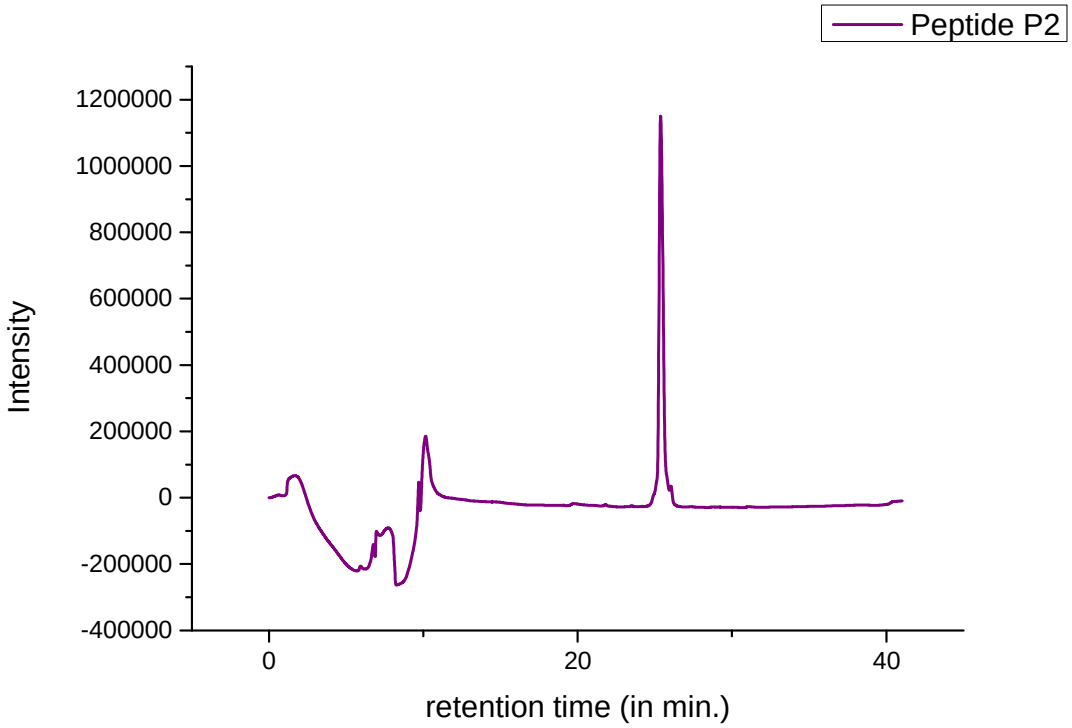


Figure 19: Trypsin degradation analysis of peptide **P9** with varying concentration.

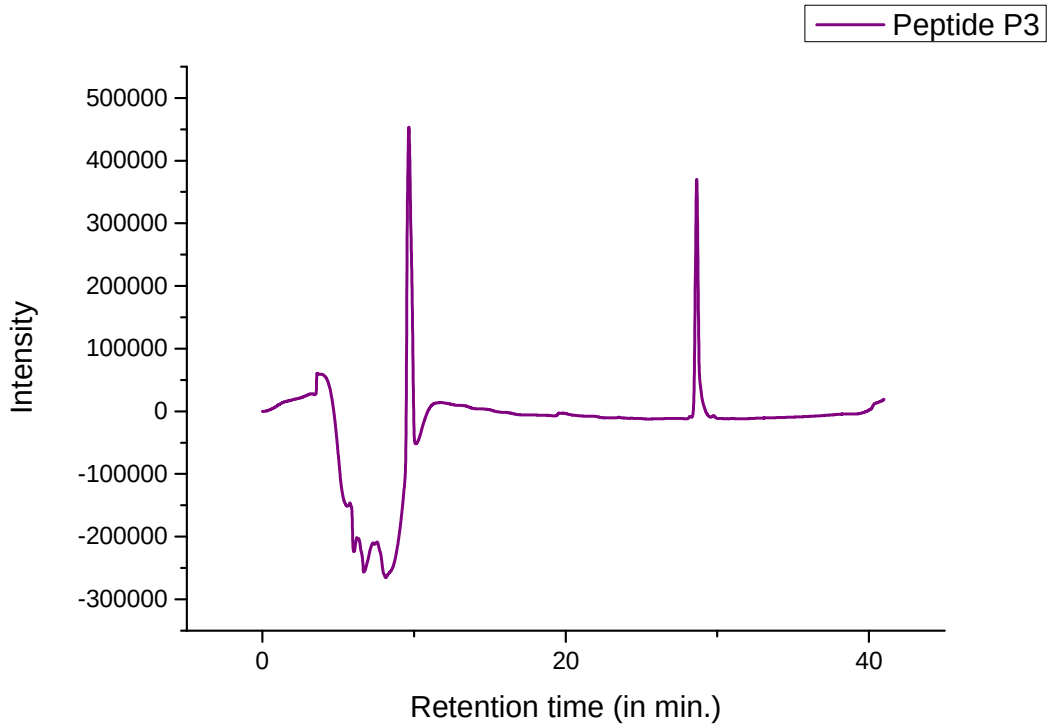
Characterization:



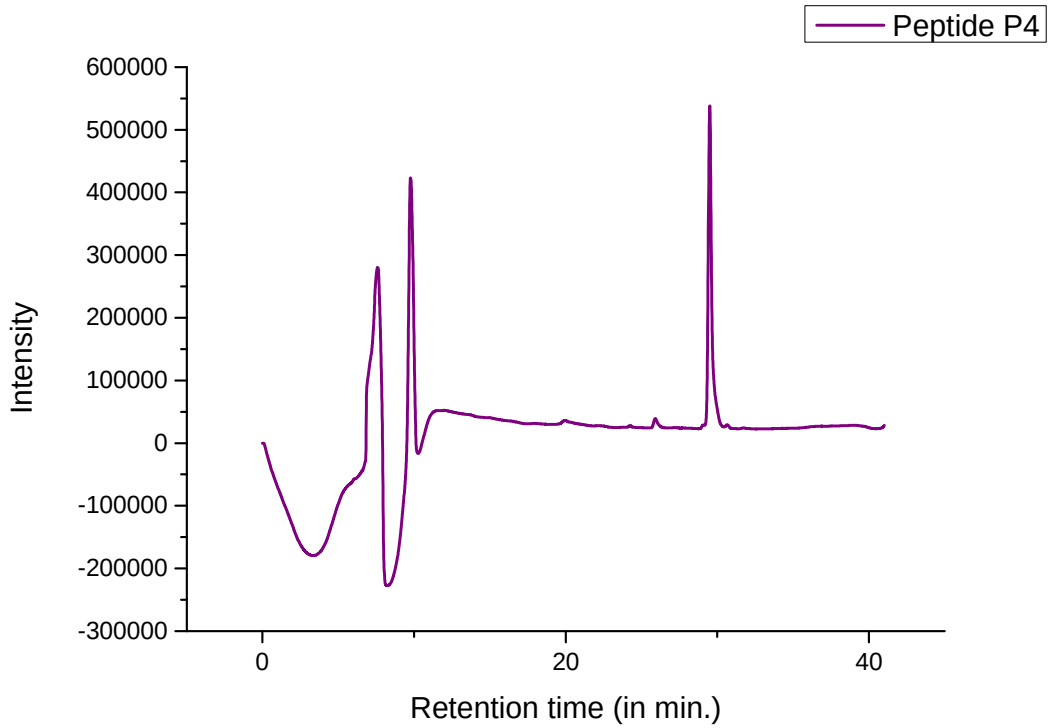
HPLC trace of Peptide **P1**.



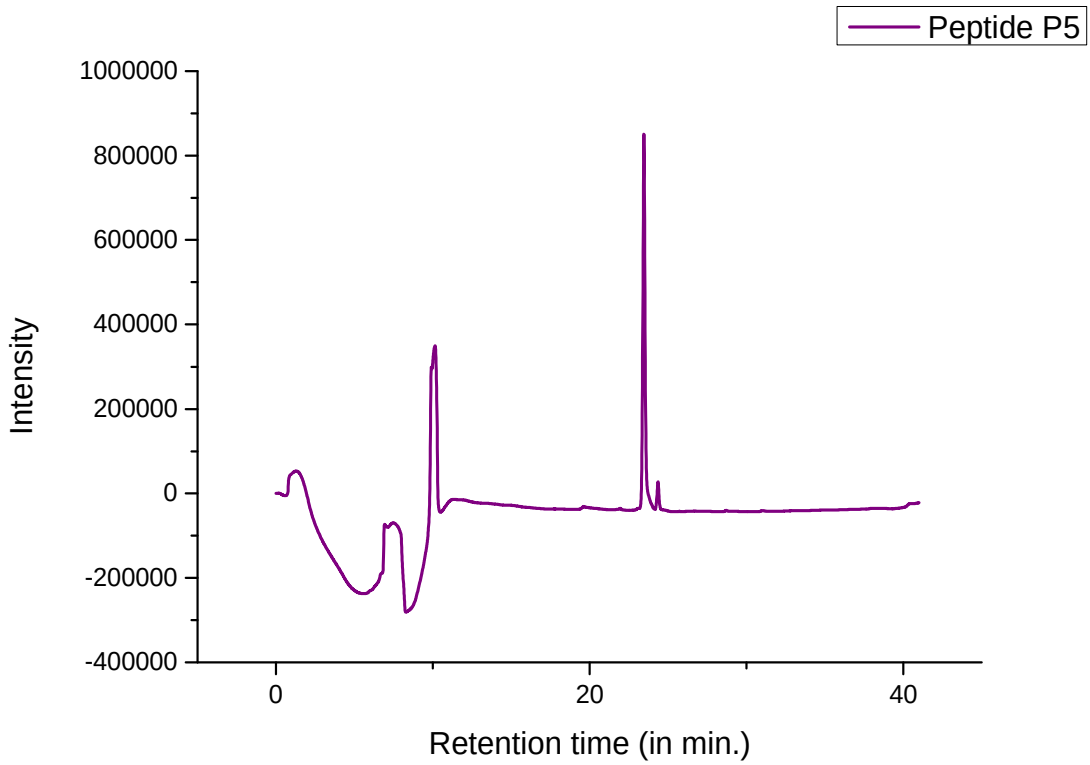
HPLC trace of Peptide **P2**.



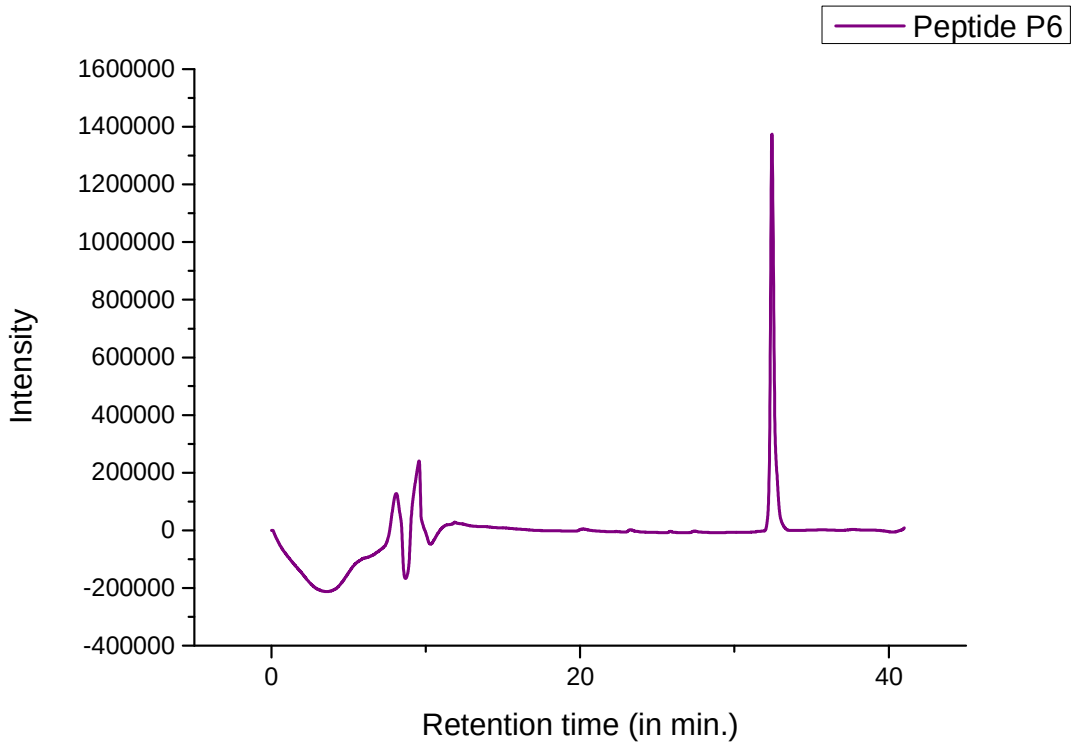
HPLC trace of Peptide **P3.**



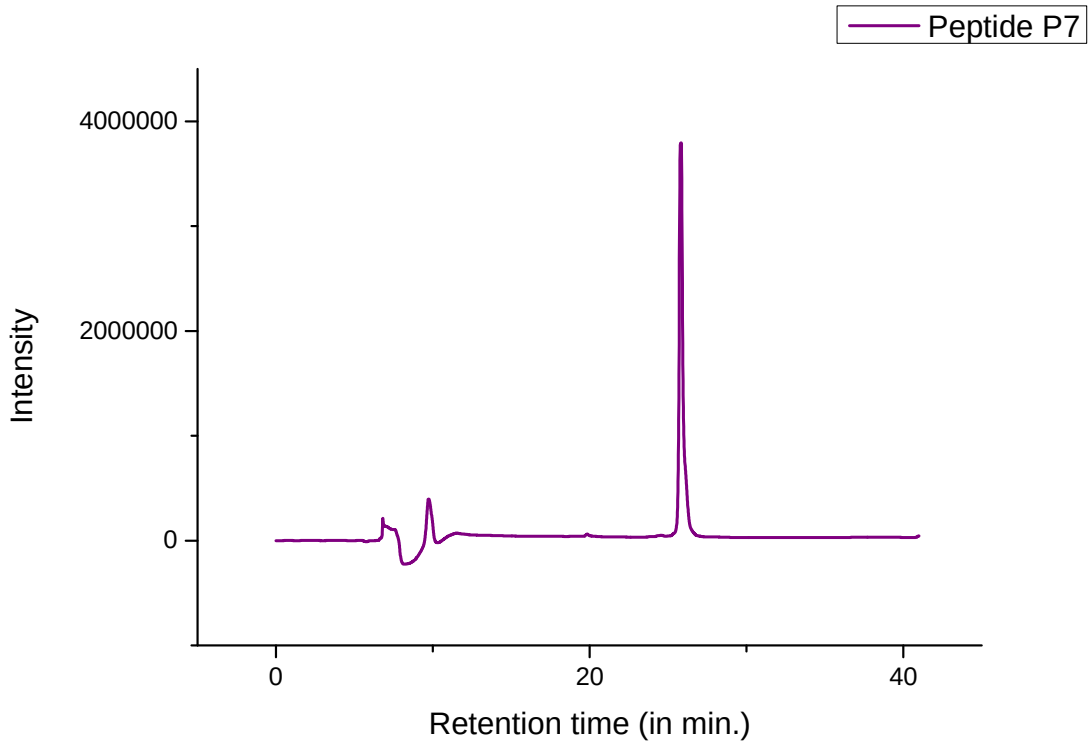
HPLC trace of Peptide **P4**.



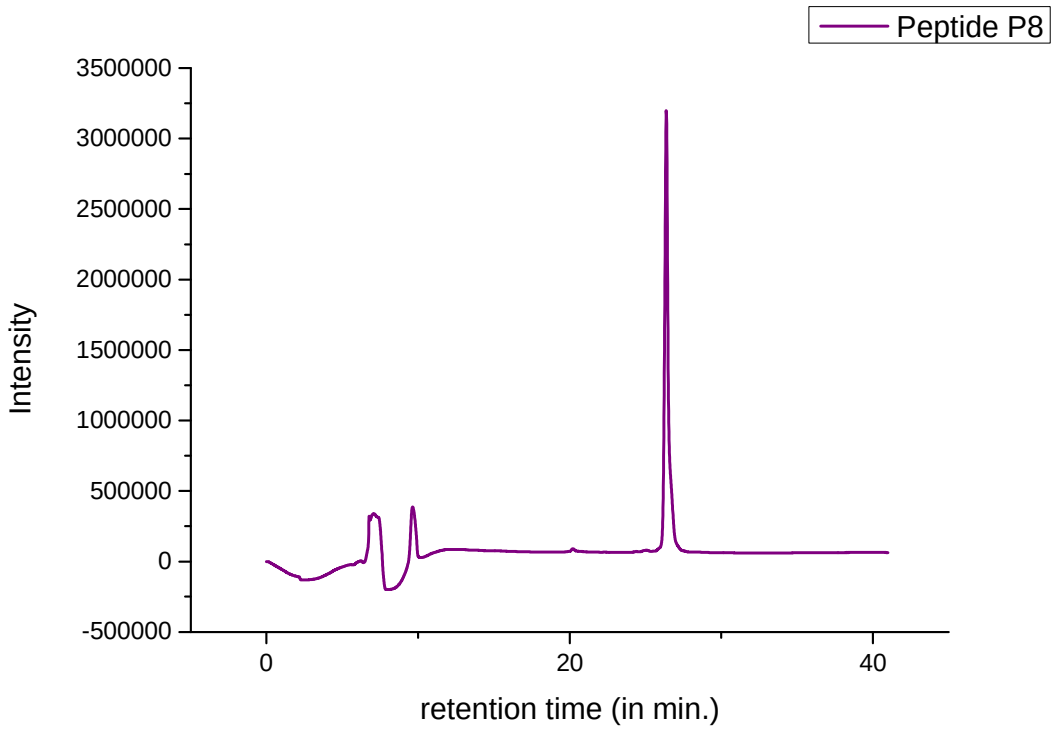
HPLC trace of Peptide **P5**.



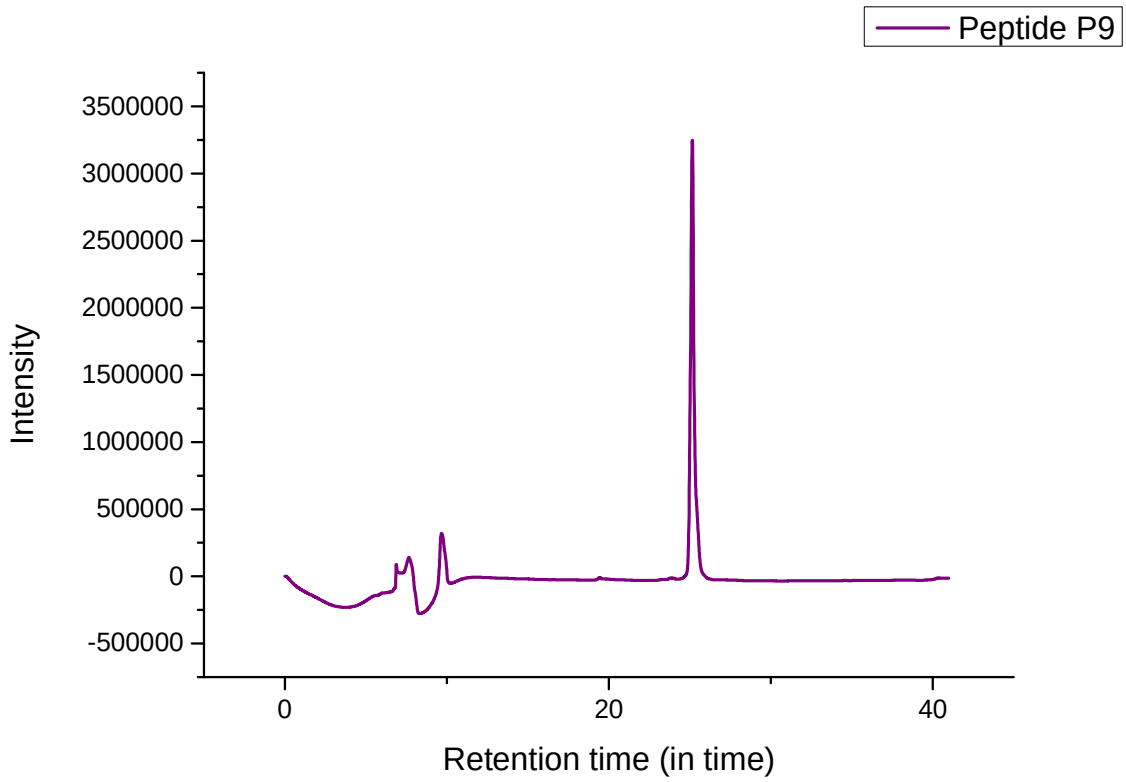
HPLC trace of Peptide **P6**.



HPLC trace of Peptide **P7**.

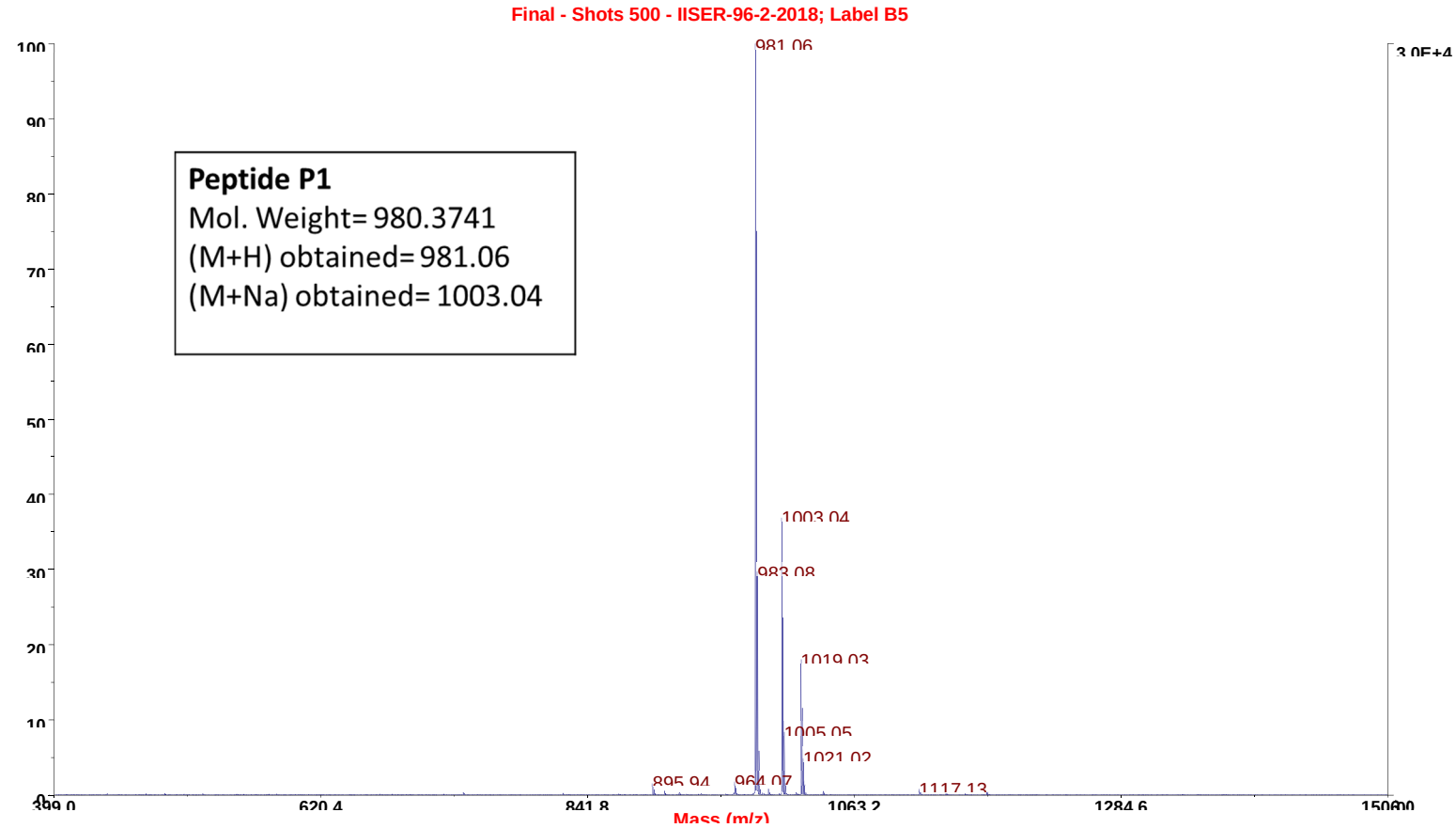


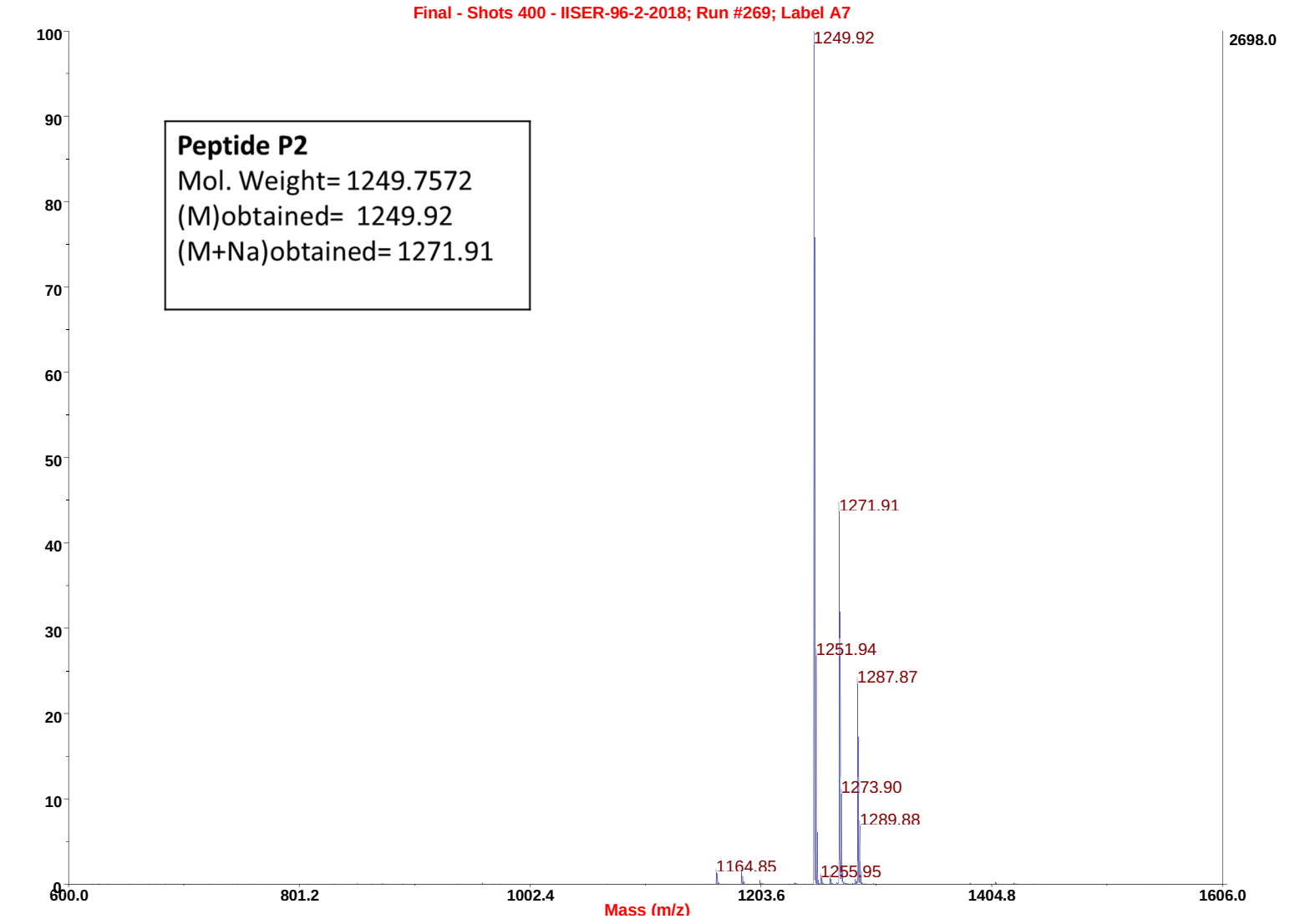
HPLC trace of Peptide **P8**.



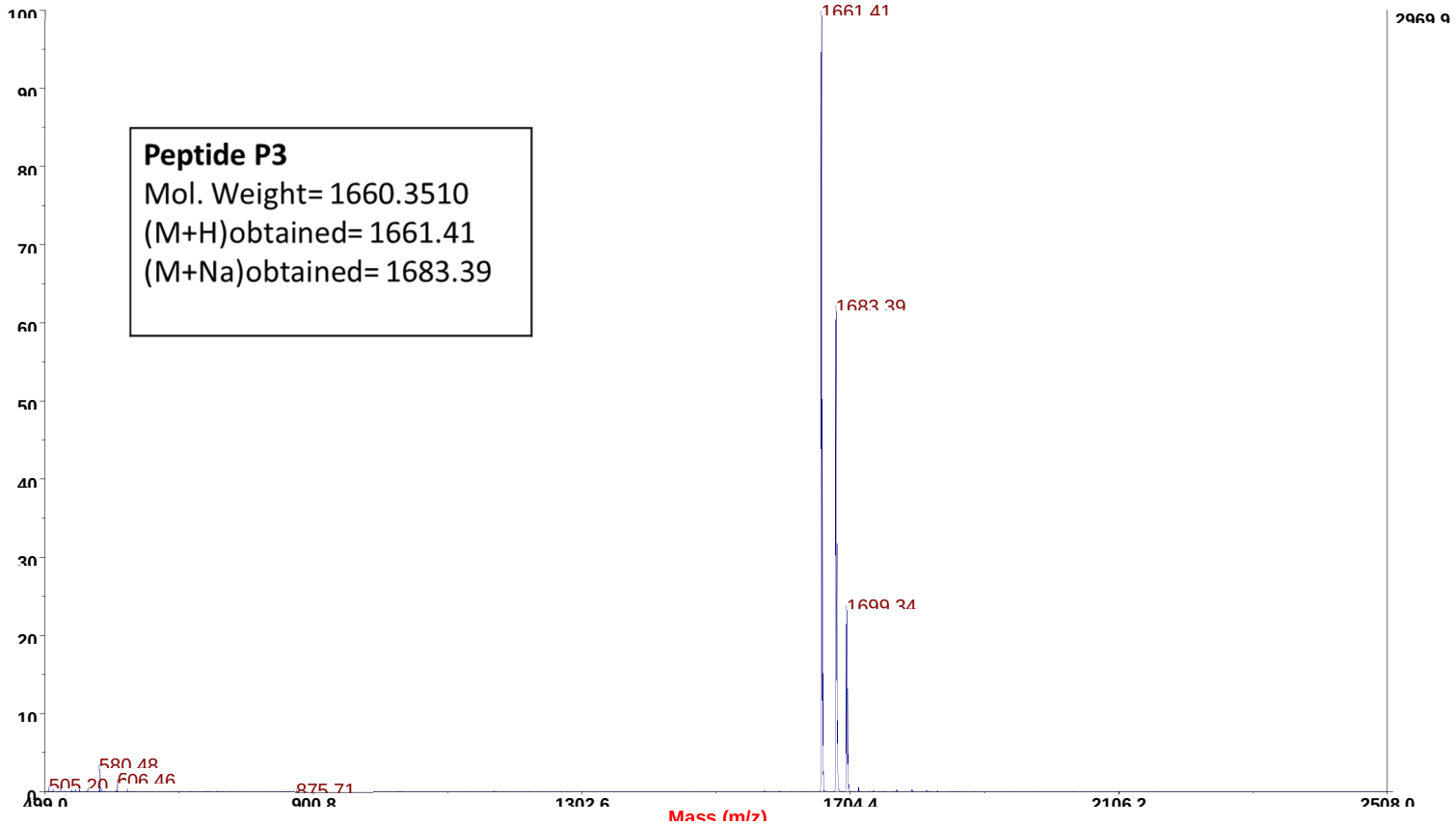
HPLC trace of Peptide **P9**.

MALDI-TOF spectra of peptides (P1-P9):

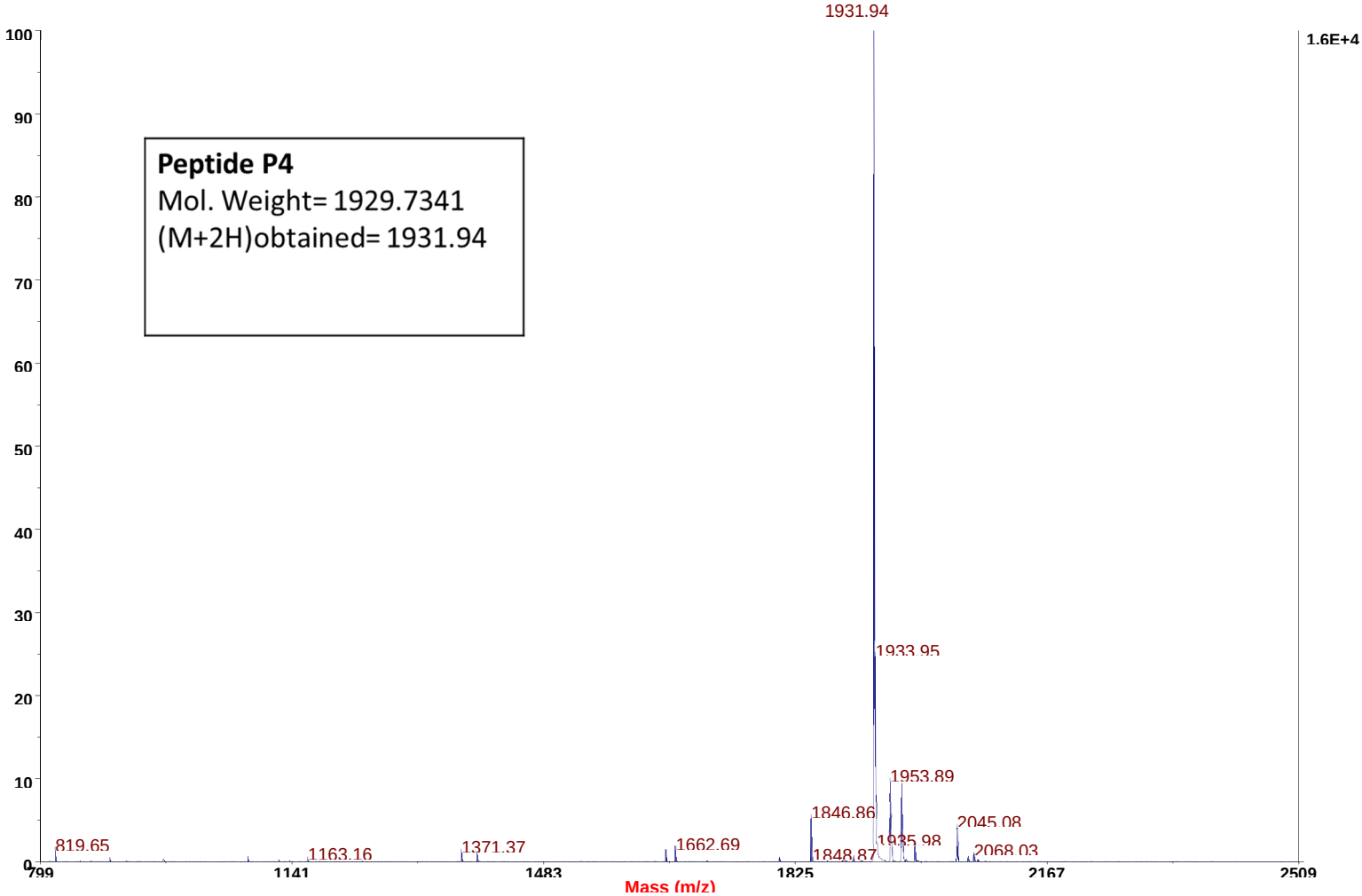

Final - Shots 500 - IISER-96-2-2018; Label B5
Peptide P1
Mol. Weight= 980.3741
(M+H) obtained= 981.06
(M+Na) obtained= 1003.04
981.06
1003.04
983.08
1019.03
1005.05
1021.02
895.94
964.07
1117.13
Mass (m/z)

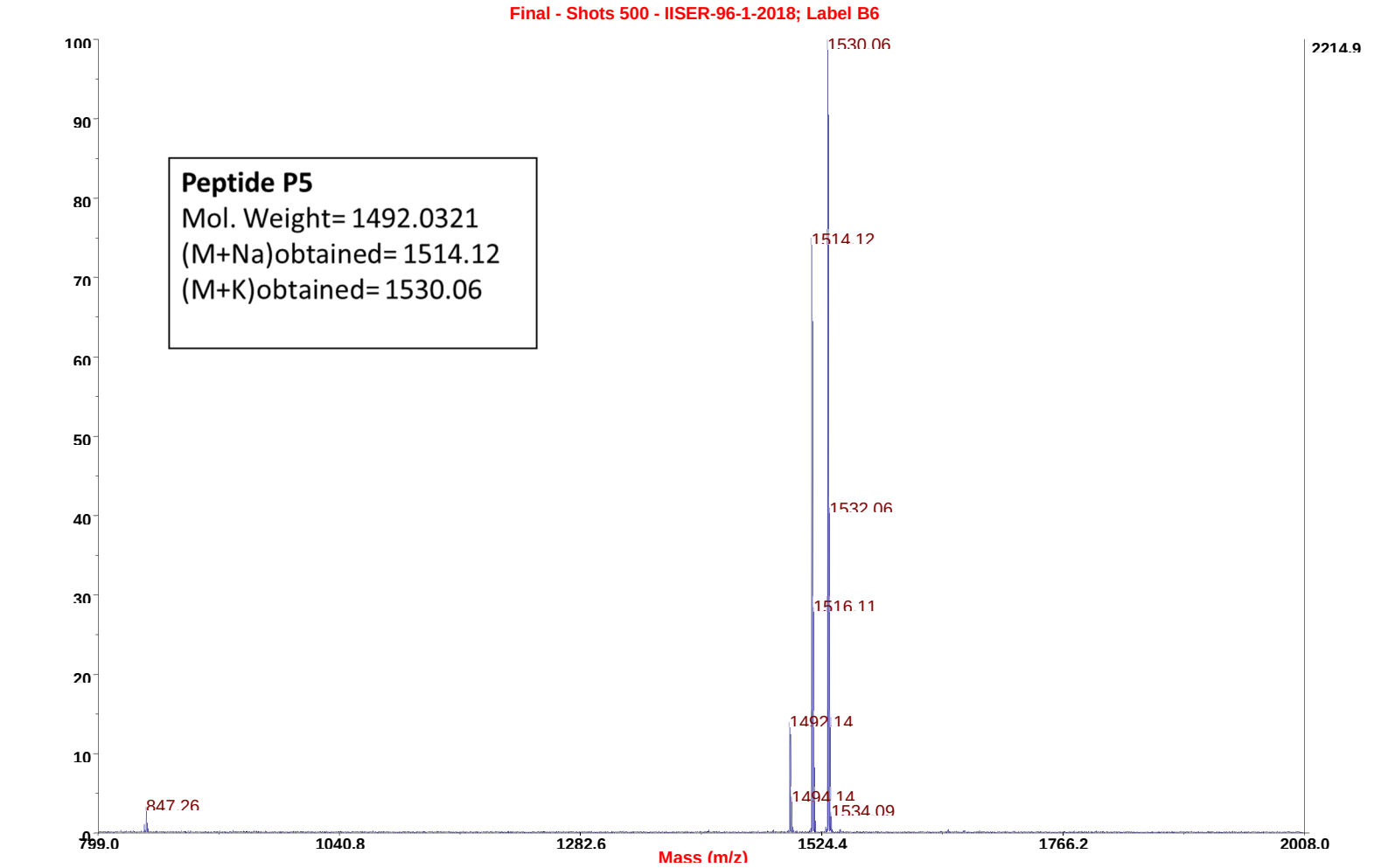

Final - Shots 400 - IISER-96-2-2018; Run #269; Label A7
Peptide P2
Mol. Weight= 1249.7572
(M)obtained= 1249.92
(M+Na)obtained= 1271.91
1249.92
2698.0
1271.91
1251.94
1287.87
1273.90
1289.88
1164.85
1255.95
Mass (m/z)

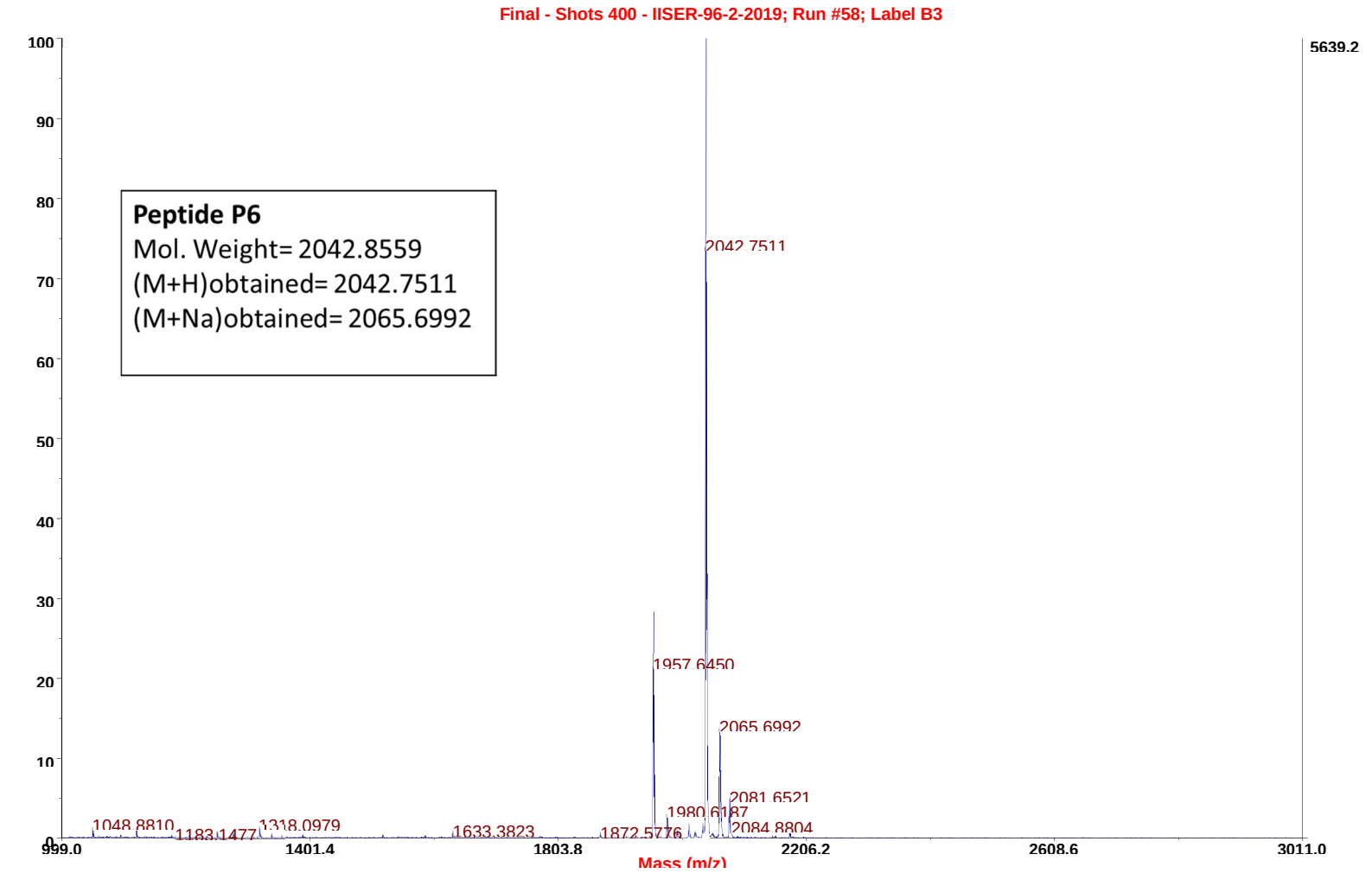
Final - Shots 600 - IISER-96-2-2018; Run #271; Label B1



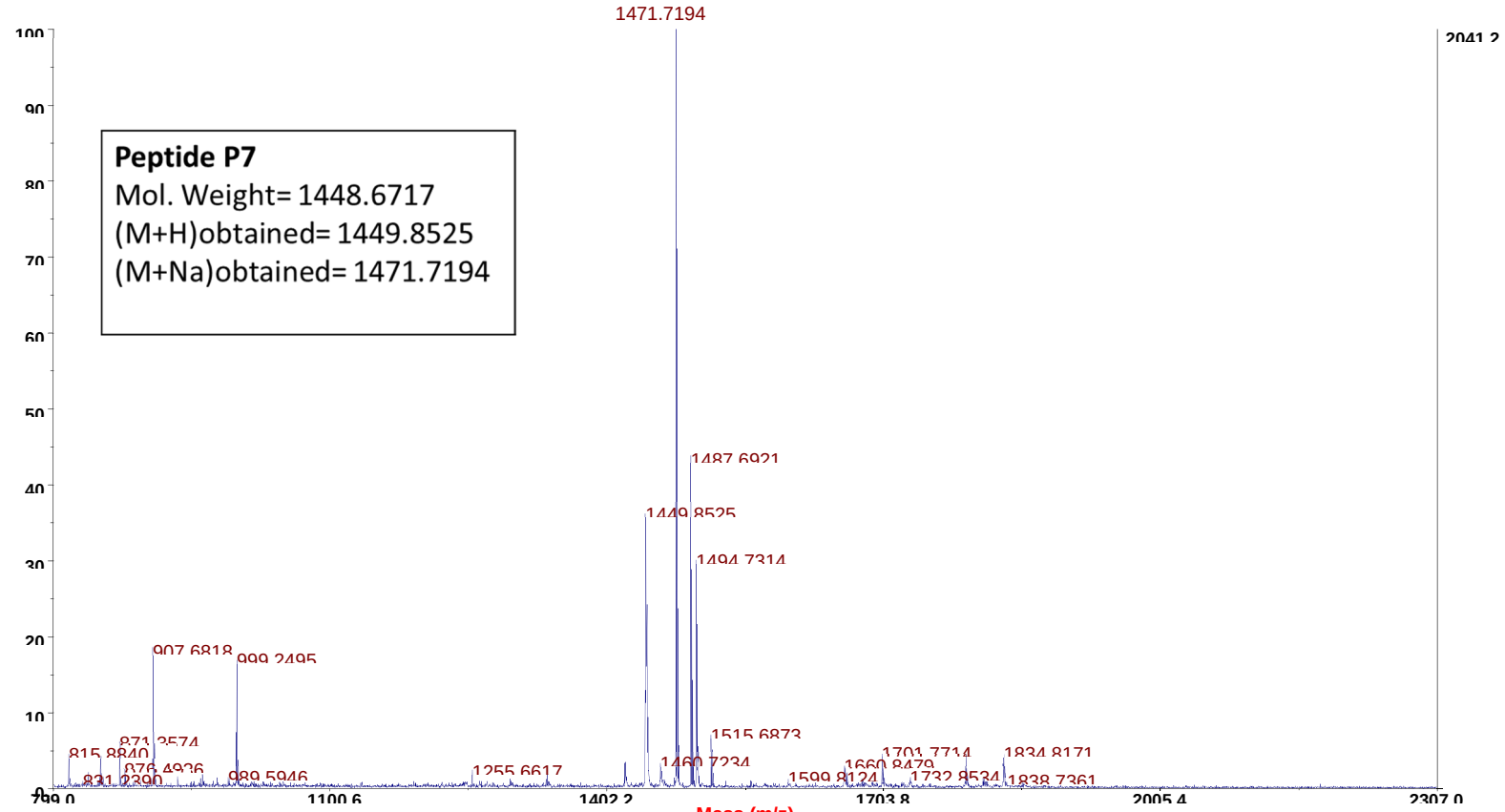
Final - Shots 600 - IISER-96-1-2018; Run #301; Label A11



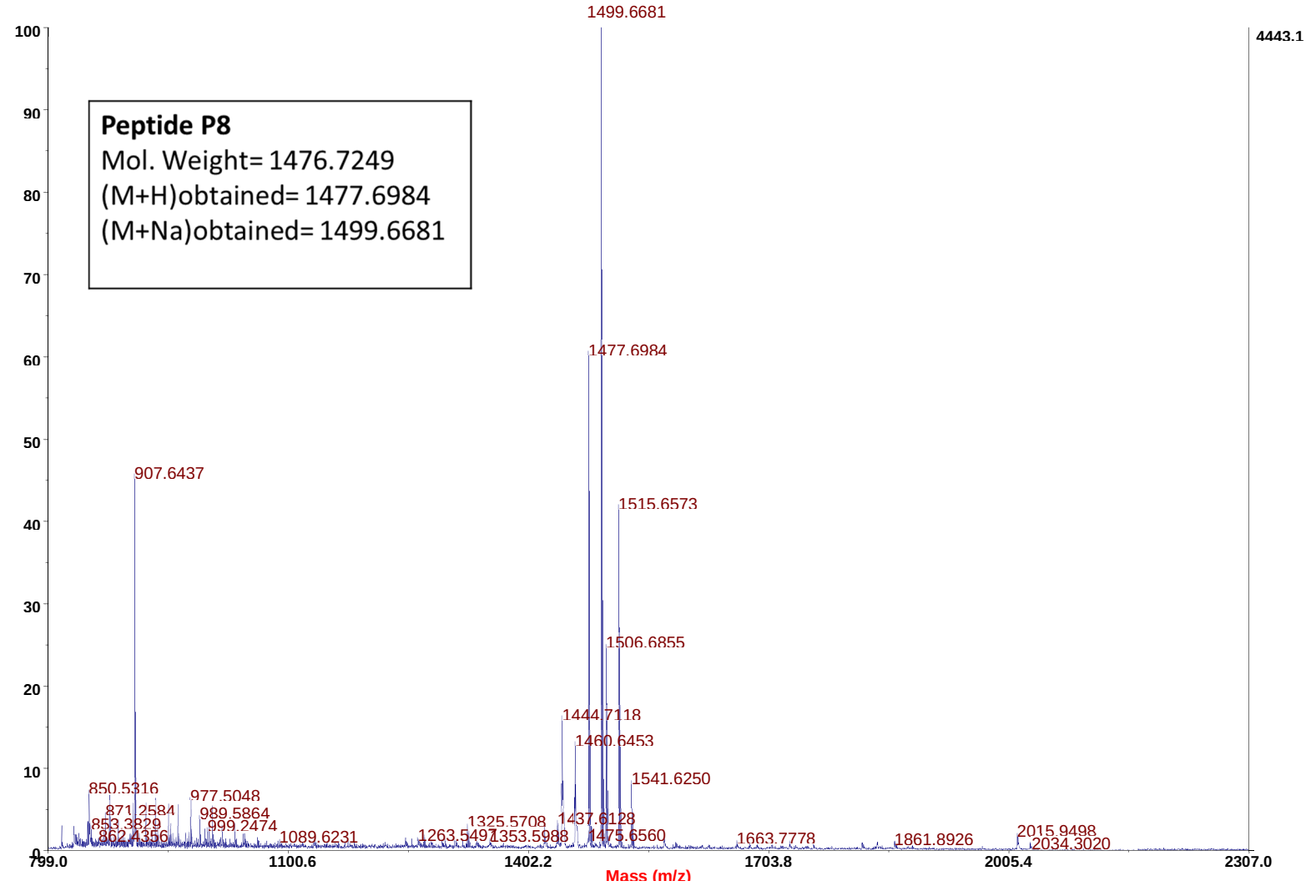

Final - Shots 500 - IISER-96-1-2018; Label B6
Peptide P5
Mol. Weight= 1492.0321
(M+Na)obtained= 1514.12
(M+K)obtained= 1530.06
1530.06
2214.9
1514.12
1532.06
1516.11
1492.14
1494.14
1534.09
847.26
Mass (m/z)

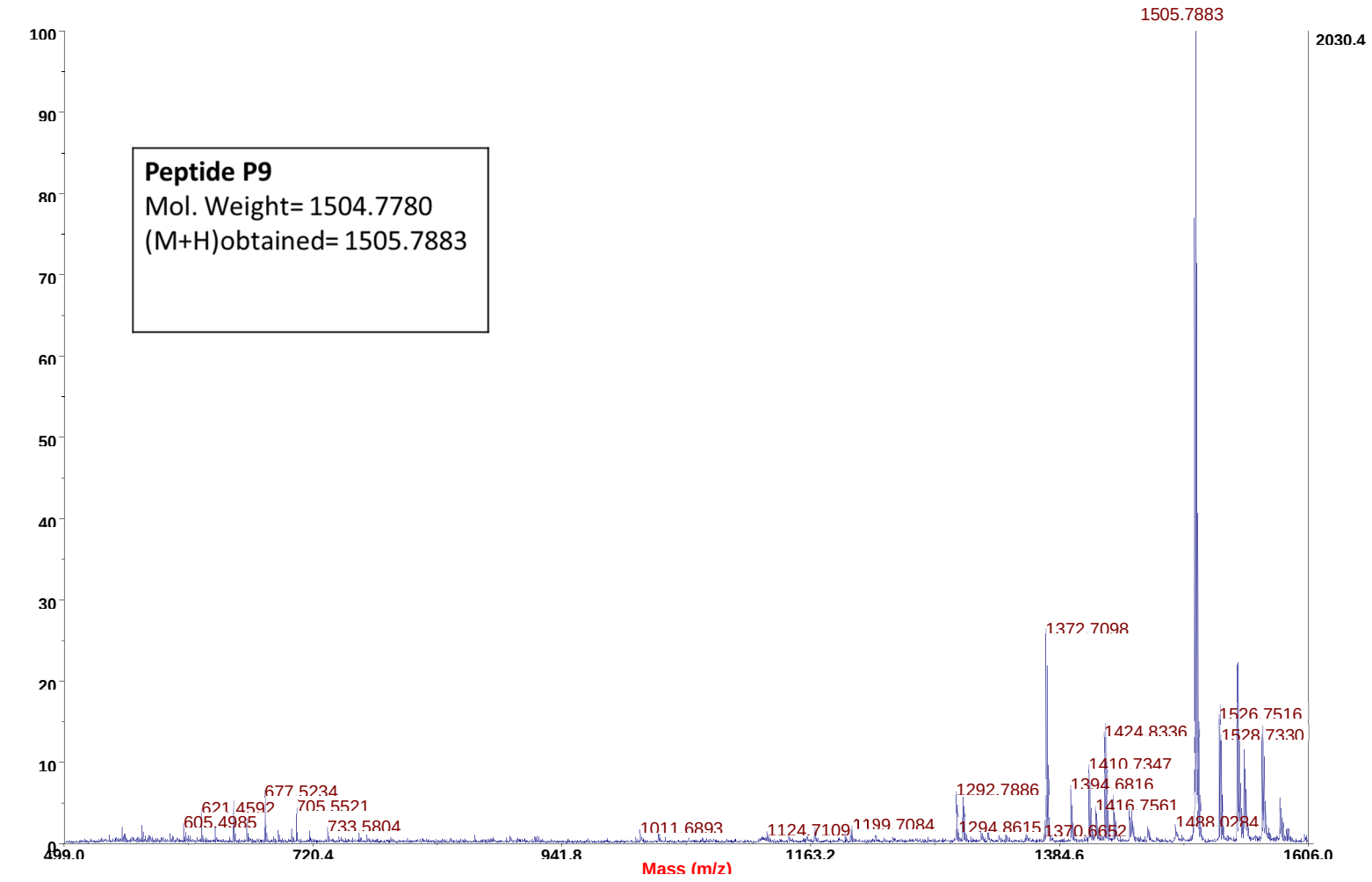

Final - Shots 400 - IISER-96-2-2019; Run #58; Label B3
Peptide P6
Mol. Weight= 2042.8559
(M+H)obtained= 2042.7511
(M+Na)obtained= 2065.6992
5639.2
2042.7511
1957.6450
2065.6992
2081.6521
1980.6187
2084.8804
1048.8810
1183.1477
1318.0979
1633.3823
1872.5776
Mass (m/z)

Final - Shots 400 - IISER-96-1-2019; Run #32; Label A1



Final - Shots 400 - IISER-96-1-2019; Run #32; Label A2





^{1}H spectra of peptides P5 in methanol-d_3:

Peptide P5:

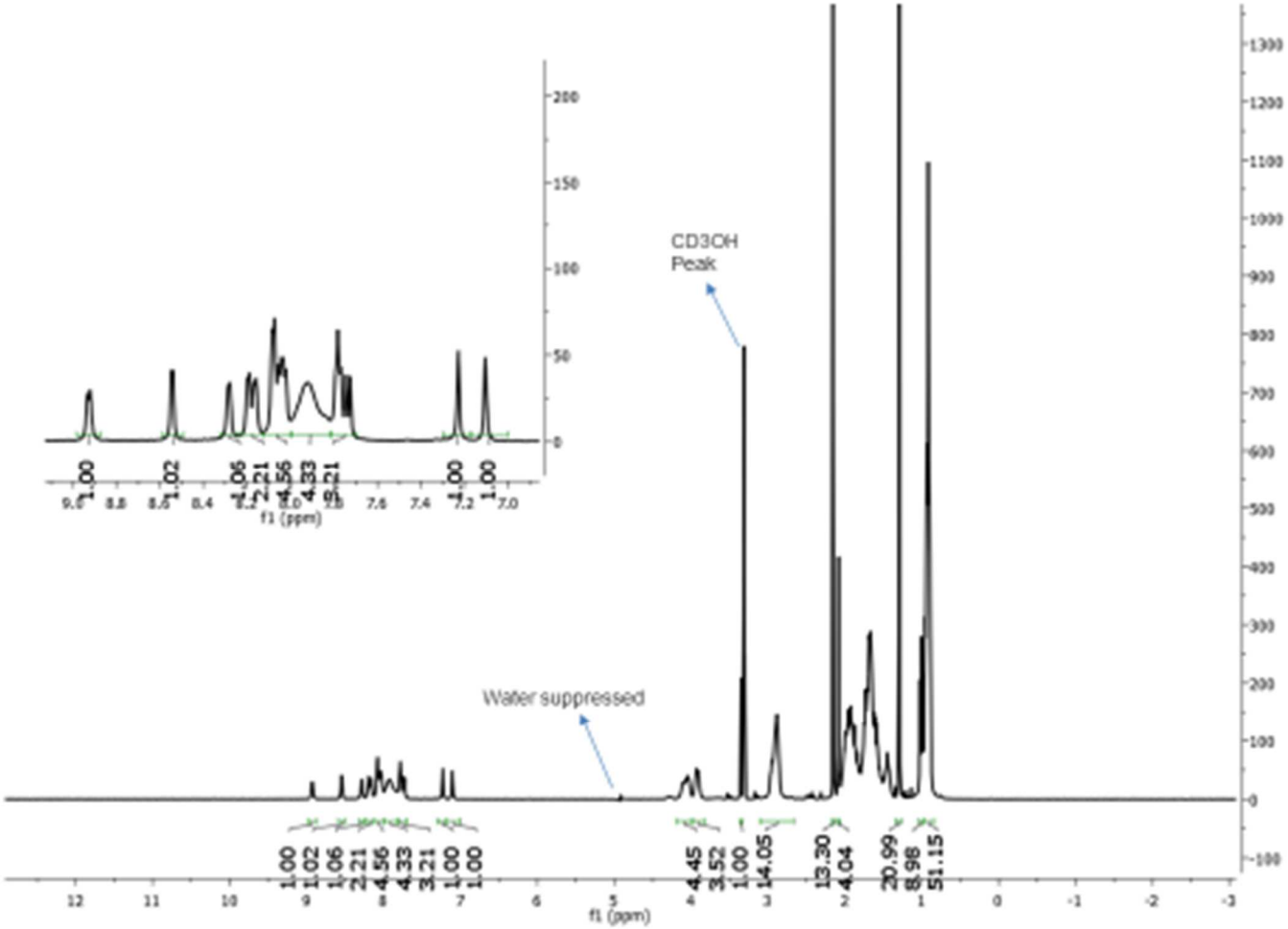


Figure 20: ^{1}H NMR spectra of alpha peptide **P5** in methanol-d_3 at 400 MHz.

Conclusion:

We have successfully demonstrated the potent antibacterial activities of designed α/γ^4 hybrid peptides. The investigation revealed excellent the antimicrobial activity of α/γ helix compared to the alpha peptide counterpart. These results suggested that the projection of side-chains of the amino-acids were important for the activity of the peptides than the net charge of the peptides. In addition, hybrid peptides displayed excellent protease stability as compared to the alpha helix. Further, the haemolytic activities indicate that these peptides are not haemolytic at their MIC values. In addition, we had successfully proved that on incorporation of γ^4 lysine in place of lysine and incorporation of γ^4 amino acids on both sides covering lysine in the alpha peptide backbone make extremely difficult for the serine protease trypsin to recognize the lysine from the C-terminal of peptide and hence the degradation of peptides by trypsin could be avoided or minimized. On incorporation of γ^4 amino acids, not only proteolytic stability can be improved but flexibility of the peptide backbone and side chain projections can also be improved as compared to the alpha peptide counterpart. The major advantage of flexible helices is they can adsorb in the bacterial cell membrane very efficiently as they are flexible enough to adjust in minor perturbations. The solution structures of these peptides are under investigation. Overall these studies revealed that α/γ^4 hybrid peptides can be effectively used to develop protease stable potent antimicrobial agents.

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